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# Immuno-Modulating Properties of Tulathromycin in Porcine Reproductive and Respiratory Syndrome Virus-Infected Macrophages In Vitro.

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UNIVERSITY OF CALGARY

# Immuno-Modulating Properties of Tulathromycin in Porcine Reproductive and Respiratory Syndrome Virus-Infected Macrophages *In Vitro*.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Immuno-Modulating Properties of Tulathromycin in Porcine Reproductive and Respiratory Syndrome Virus-Infected Macrophages *In Vitro*", submitted by Dimitri Desmonts de Lamache in partial fulfilment of the requirements for the degree of Master of Science.

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Date

# ABSTRACT

With a total cost of productivity losses estimated at $600 million annually in the U.S alone, porcine reproductive and respiratory syndrome (PRRS) is a major concern in the swine industry. PRRS etiological agent, the porcine reproductive and respiratory syndrome virus (PRRSV) is a small positive-strand RNA virus that primarily grows in alveolar macrophages. Due to its high antigenic variability, and poorly understood immunopathogenesis, there is currently no treatment to control PRRSV infection. Commercially available vaccines are inefficient and cannot meet practical needs encouraging more researchers to explore different approaches to treat PRRSV infections. The common occurrence of PRRSV infection with bacterial infections as well as its inflammatory-driven pathobiology raises the question of the value of antibiotics for the treatment of the disease it causes. Tulathromycin (TUL), a macrolide antibiotic previously studied in our laboratory has been shown to exhibit potent anti-inflammatory and immunomodulatory actions in cattle and pigs. The aim of this study was to identify and characterize anti-viral and immunomodulating properties of TUL in PRRSV-infected porcine macrophages. Our findings indicate that blood monocyte-derived macrophages are readily infected by PRRSV and can be used as a cellular model to study PRRSV pathogenesis. TUL was found to not change viral titers and viral receptors (CD163 and CD169) expression suggesting that the drug does not possess direct antiviral effects against PRRSV. In addition, we showed that TUL acts synergistically with PRRSV to induce apoptosis but prevents virus-induced early necrosis. TUL was also found to attenuate PRRSV-induced macrophage pro-inflammatory signaling (CXCL-8 and mitochondrial ROS production) and prevent phagocytosis inhibition. Together, these data demonstrate that tulathromycin downregulates PRRSV-induced inflammatory response in macrophages which may in turn reduce

virus-related tissue injury. More importantly, this study sheds the light on the potential clinical benefits of an antibiotic in the context of a virus-induced inflammation.

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&

To my friends and extended family.

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# List of Abbreviations

| | |
|---|---|
| AEC | Alveolar epithelial cell |
| AM | Alveolar macrophage |
| APAF-1 | Apoptotic activating factor-1 |
| APC | Antigen presenting cell |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluids |
| Bcl-2 | B-cell lymphoma |
| BMDMΦ | Blood monocyte derived macrophages |
| BSA | Bovine serum albumin |
| CFU | Colony forming unit |
| CMI | Cell mediated immune response |
| COPD | Chronic obstructive pulmonary disease |
| CXCL-8 | Chemokine (C-X-C motif) ligand 8 |
| Cyt c | cytochrome c |
| DAMPs | Danger-associated molecular pattern |
| DAPI | 4’,6-diamidino-2-phenylindole |
| DC | Dendritic cell |
| DCF | Dichlorodihydrofluorescein |
| DCFH-DA | 2’,7’-Dichlorodihydrofluorescein diacetate |
| DISC | Death-inducing signaling complex |
| DMEM | Dulbecco’s modified eagle medium |
| EAV | Equine arteritis virus |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| FcR | Fc receptor |
| FITC | Fluorescein isothiocyanate |
| $H_20_2$ | Hydrogen Peroxide |
| HBSS | Hank’s balanced salt solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HI | Heat inactivated |
| HIV-1 | Human immunodeficiency virus |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IMDM | Iscove’s modified dulbecco’s medium |
| ITAMs | Immunoreceptor tyrosine-based activation motif |
| ITIMs | Immunoreceptor tyrosine-based inhibition motif |
| IU | International unit |
| JNK | c-Jun N-terminal kinase |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| LTB4 | Leukotriene B4 |
| LDV | Lactate dehydrogenase elevating virus |

| | |
|---|---|
| m.o.i | Multiplicity of infection |
| M-CSF | Macrophage colony stimulating factor |
| MEM | Modified eagle medium |
| MHC-1 | Major histocompatibility complex |
| MR | Mannose Receptor |
| Nab | Neutralizing antibody |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa B |
| NK | Natural killer |
| NLRs | Nod-like receptor |
| NOX2 | NADPH oxidase 2 |
| NSE | Non-specific esterase |
| $O_2^{-\bullet}$ | Superoxide anion |
| p.i. | Post infection |
| PAM | Porcine alveolar macrophage |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate buffer saline |
| PCV2 | Porcine circovirus 2 |
| pDC | Plasmacytoid dendritic cell |
| PGE2 | Prostaglandin E2 |
| PI3K | Phosphoinositide 3-kinase |
| PIM | Pulmonary intravascular macrophage |
| PLC | Phospholipase C |
| PRR | Pattern recognition receptor |
| PRRS | Porcine reproductive and respiratory syndrome |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PS | Phosphatidylserine |
| RLRs | RIG-I-like receptor |
| ROS | Reactive oxygen species |
| RPE | R-phycoerythrin |
| RSV | Respiratory syncitial virus |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RV | Rhinovirus |
| SEM | Standard error of the means |
| SHFV | Simian hemorrhagic fever virus |
| Sn | Sialoadhesin |
| STS | Staurosporine |
| TGF | Transforming growth factor |
| TLRs | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TNF | Tumor necrosis factor |
| TRM | Tissue resident memory T cell |
| TUL | Tulathromycin |
| Trit-X | Triton-X |

# 1 INTRODUCTION

## 1.1 The role of mononuclear leukocytes in pulmonary inflammation.

Discovery of inflammation can be traced back to the first century A.D. and was already associated with the "cardinal signs" of inflammation – redness, swelling, heat, pain and loss of function [1], [2]. Initially viewed as detrimental to the host, hundred of years of studies demonstrated that inflammation is an essential response to injury and invading pathogens [1], [2]. Inflammation consists of a series of complex and coordinated cellular and biochemicals events aiming to restore homeostasis [1]–[3]. When self-limited, inflammation is protective and beneficial to the host, however, if dysregulated it can be highly detrimental [2], [3]. Excessive recruitment and activation of inflammatory cells, which cause self-perpetuating tissue damage are hallmarks of any inflammatory diseases [2], [3].

### 1.1.1 Pulmonary inflammation.

Breathing. This basic physiological function of any multicellular organism is a perfect manifestation of the paradox of life. Necessary to sustain life, it also contributes to mortality. With every breath comes dioxygen but also allergens, pollutant and other particles. Lungs are constantly exposed to an innumerable quantity of foreign materials, the majority of which is ignored or eliminated with minimal inflammation in order to keep the airways opened at all times and not compromise vital gas exchange. As such, multicellular organisms developed many different mechanisms to protect the respiratory tract from environmental particulates and pathogens. The majority of large particles are removed from the upper airways through coughing, sneezing and the mucociliary movements [4]. In the lower airways particles are exposed to non-specific components of the immune system including antimicrobial peptides and surfactant proteins [4]. In healthy conditions, overreaching antigens are detected by the airway epithelium and tissue resident

leukocytes which initiate the immune response. Subsequently, in an ordered, stepwise fashion, immediate and adaptive defenses of the tissue are engaged to eliminate pathogens with the minimal necessary response. In this section we will describe the inflammatory response to respiratory infections.

#### *1.1.1.1 Pathogen detection*

The pulmonary immune response involves a variety of immune cells closely interacting with each other to contain the infection. Initial detection of foreign particles is carried out by local sensor cells which express a wide array of pattern recognition receptors (PRRs) including Toll like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs) and nucleotide-binding oligomerization-domain protein-like receptors (NLRs) [5], [6]. PRRs can be classified based on their structure, specificity, cellular localisation and tissue-specific expression [6]. PRRs do not specifically target a particular pathogen, rather they recognize broadly conserved molecular patterns in pathogens – also referred as pathogen associated molecular patterns (PAMPs) – such as lipopolysaccharides (LPS), flagellin or single stranded RNA [5], [6]. This maximizes the detection of any kind of pathogens (bacteria, fungi, virus, protozoa) without exponentially increasing the number of PRRs coding sequences. Although PRRs are expressed by most cells, in the lungs, they are predominantly expressed by epithelial cells and tissue resident macrophages called alveolar macrophages (AMs) [5], [6].

#### *1.1.1.2 Pathogen phagocytosis.*

Phagocytosis is one of the main function of macrophages. This extraordinarily complex process involves numerous receptors and signaling pathways to induce major cytoskeletal reorganisation and drive macrophage response to the internalized particle [7]. Phagocytosis can be divided into a series of defined steps: (1) particle recognition, (2) particle internalization and (3)

particle degradation [7] (figure 1). Given phagocytes can ingest foreign materials of different nature, it is not surprising that numerous phagocytic receptors have been identified [7], [8]. For simplicity, here we will only discuss Fcγ receptor (FcγR) and mannose receptor (MR) mediated phagocytosis. Fcγ receptor mediated phagocytosis, which requires the interaction between Fcγ receptors and the Fc portion of immunoglobulin G (IgG), is by far the most characterized model of phagocytosis. Importantly, FcγR contain either an immunoreceptor tyrosine-based activation or inhibition motifs (ITAMs and ITIMs respectively) [7], [8]. Multivalent ligand binding to Fcγ receptors is mandatory to initiate the signaling cascade. Following the clustering of activating FcγR, ITAMs motifs are phosphorylated by Src-family kinases which allow Syk binding. Downstream signaling lead to activation of GTPases, phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) all of which are necessary to stimulate actin polymerization and induce phagosome formation [7], [8]. The mannose receptor is a single chain receptor containing 8 lectin-like carbohydrate binding domains that recognize and binds to mannans motifs found in yeasts cell wall [8]. Little is known about signaling pathways involved in mannose-dependent phagocytosis but numerous proteins including F-actin, myosin and talin are recruited around the nascent phagosome [8]. It is worth noting that some important proteins involved in Fcγ mediated phagocytosis are not found in MR phagocytosis. This underlines the diversity in cell signaling to initiate phagocytosis.

Although receptors and signaling pathway vary, particle internalisation always result in the formation of a vacuole called phagosome [9], [10]. While early phagosomes are totally inoffensive, a series of membrane fusion and fission transform this vacuole into a potent microbicidal organelle [9], [10]. Upon fusion with preformed lysosomes, the early phagosome changes its membrane composition and its content to turn into a phagolysosome. To eliminate the ingested particle

phagolysosomes possess a wide arsenal of microbicidal effectors including hydrolytic enzymes, scavenger molecules and oxygen radicals [9], [10]. During infections, reactive oxygen species (ROS) are of crucial importance. Any defect in their synthesis strongly impede the ability to eliminate invading pathogens [9]. Within the phagosome ROS are generated by an isoform of the nictotinamide adenine dinucleotide phosphate-dependent (NADPH) oxidase, the NADPH oxidase 2 (NOX2) [11]–[13]. Superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide ($H_20_2$) and other oxygen intermediates are highly reactive molecules capable of interacting with DNA, proteins, lipids and metal centers conferring them potent cytotoxic properties not only against pathogens but also against the host's cells. Oxygen radical differ by their reactivity, stability and biological activities [11], [14]. For instance, $H_20_2$ interaction with the phagosome content can induce the formation of hydroxyl radicals via Fenton reaction or hypochlorous acid [11], [14]. Despite their crucial role in pathogen killing, ROS overproduction and release in the environment promote tissue injury and cell death which can amplify the inflammatory response [15].

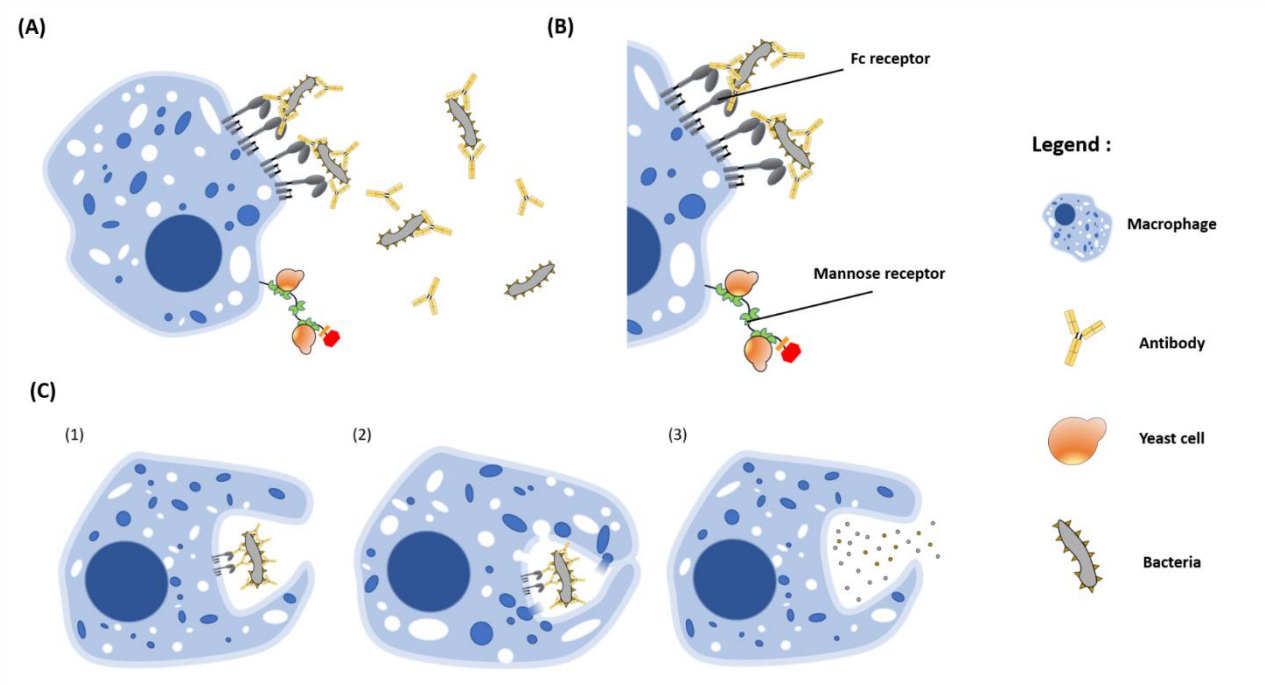



**Figure 1. Pathogen phagocytosis**. Antigens are engulfed and eliminated in a process called phagocytosis. (A-B) Phagocytes express numerous receptors allowing them to detect foreign particles directly or indirectly through the intermediate of opsonins including antibodies. Direct recognition involves pattern recognition receptors (PRRs) - such as the mannose receptor - that recognize conserved motifs on pathogens (also referred as pathogen-associated molecular patterns or PAMPs) such as mannans motifs found in yeast cell walls or bacterial lipopolysaccharides. Soluble molecules or opsonins can also detect and "coat" antigens thereby facilitating their uptake by phagocytes in a process call opsonization. (C) Following recognition, particles are (1) internalized and (2) digested in the phagolysosome, (3) finally digested products are neutralized and released in residual bodies. Figure adapted from [7] and [9].

#### *1.1.1.3 Acute inflammation*

The term "pro-inflammatory mediators" regroups all the chemicals (cytokines, chemokines and lipid mediators) secreted by cells present in the injured tissue that participate in the inflammatory process. The most studied pro-inflammatory mediators include interleukin- (IL-6), Chemokine (C-X-C motif) ligand 8 (CXCL-8), tumor necrosis factor (TNF) or interferon-γ (IFN-γ). Pro-inflammatory mediators' functions are diverse but together synergize to promote leukocyte recruitment, proliferation and activation.

In the lungs, epithelial cells and AMs secreted products firstly reach tissue resident lymphoid cells. Interleukin-12 (IL-12) and IL-23 activate natural killer (NK) epithelial γδ T cells, and tissue-resident memory T ($T_{RM}$) cells and enhance killing of infected cells to contain pathogen spread [16]–[18]. In turn, these cells secrete IFN-γ, IL-17 and IL-22, which further activate epithelial cells and macrophages in a positive feedback loop. This loop results in an increase in microbicidal capacities of AMs concomitant with an increase in AECs proliferation as well as the induction of chemokines [16]–[18]. In most cases, local defenses are sufficient, but when overwhelmed, circulating leukocytes are called upon.

Leukocyte migration to the site of inflammation is dependent on pro-inflammatory cytokines release such as IL-1β and TNF-α. These cytokines activate the endothelium which result in the upregulation of cell adhesion molecules (selectins, integrins and immunoglobulins) [19], [20]. Concurrently, chemoattracting molecules such as CXCL-8 and platelet activation factor (PAF) create a chemotactic gradient that "guide" leukocytes to the inflamed tissue. On site leukocytes seek for PAMPs and danger-associated molecular patterns (DAMPs). These signals are necessary to activate the cell, tether its interaction with the endothelium and initiate extravasation [19], [20].

Polymorphonuclear (PMN) cells (or neutrophils) make up the majority of transmigrating leukocytes during the onset of inflammation. Neutrophils can migrate to the lungs interstitium within minutes [21], [22]. These cells are considered professional bacterial killers and have been shown to possess many antimicrobial actions including phagocytosis and the production and release of inflammatory mediators, ROS and reactive nitrogen species (NOS) and extracellular traps [21], [22]. Neutrophils are particularly efficient in pathogen phagocytosis and degradation via oxygen-dependent and/or independent mechanisms. While the NADPH oxidase carry out the oxidative metabolism, oxygen-independent microbicidal functions rely on the release of intracellular granules [23]–[27]. Accordingly, neutrophils contain an impressive number of granules, each of which are capable of storing many different proteolytic enzymes [26], [27]. During pulmonary inflammation neutrophil activation and death can result in the release of their cytotoxic contents which exacerbate tissue damage and participate to the inflammatory process [28], [29]. Therefore, their inactivation and removal from the tissue is essential for the resolution to occur.

#### *1.1.1.4 Resolution of inflammation*

Complete resolution of acute inflammation is essential to restore homeostasis. Once thought to be a passive process, the identification of new pathways and mediators revealed that this active process is tightly regulated. The resolution phase of inflammation requires the inhibition of pro-inflammatory components concurrent with the activation of many anti-inflammatory and pro-resolving cellular pathways [3], [30].

Among all the anti-inflammatory mediators, IL-10 possesses many potent anti-inflammatory actions. Firstly, IL-10 downregulates the synthesis and secretion of many pro-inflammatory cytokines secretion including TNF-α, IL-1β, IL-6 and CXCL-8 in monocytes,

macrophages, neutrophil, eosinophils and lymphocytes [31]–[37]. This pleiotropic cytokine has been shown to inhibit antigen-presenting cells and lymphocytes functions and proliferation [31], [36], [37]. It can also control macrophage influx and efflux from an injured tissue [38]. Furthermore, IL-10 enhances the synthesis of anti-inflammatory mediators such as IL-1 receptor antagonist and soluble TNF-α receptor [39], [40]. The primary IL-10 secreting cells are monocytes, macrophages and T lymphocytes [41], [42].

Inflammatory cells clearance from the inflamed tissue and leukocyte recruitment inhibition is integral to the resolution of phase of inflammation. Apoptosis is the most commonly studied form of programmed cell death. This process involves many pro- and anti-apoptotic signals [43], [44]. Once they undergo apoptosis, cells exhibit distinct morphological and biochemical features which allow the differentiation from other types of cell death such as pyroptosis and necrosis. To date, two distinct pathways of apoptosis have been identified, the intrinsic and the extrinsic pathways [43], [44]. Initiation of the intrinsic pathway of apoptosis is mediated by a loss in mitochondrial integrity. In response to cellular stress, the Bcl-2 protein dissociates from the mitochondrial membrane which induces the release of the hemeprotein cytochrome c (cyt c) into the cytoplasm. Realeased cyt c molecules interact with the activating factor-1 (APAF-1) to form the apoptosome which then triggers apoptosis through caspase 9 and 3 cleavage [43]–[45]. The extrinsic pathway is receptor-mediated. The ligation of a soluble ligand such as FasL or TNF, to its cognate receptor on the cell surface initiate death inducing signaling complex (DISC)-mediated apoptotic pathway [46], [47]. Apoptosis results in the formation of apoptotic bodies which contain the former cell's intact organelles and cytoplasmic content and therefore does not induce inflammation. In contrast, cell lysis during necrosis leads of the release of the cytoplasmic contents, including cytotoxic compounds and pro-inflammatory mediators [43], [44].

Following apoptosis, apoptotic bodies are removed by phagocytes like macrophages in a process called efferocytosis [48]. To this extent, apoptotic cells release “find me” signals and upregulate the expression of specific markers to attract phagocytes and facilitate engulfment [48]. Failure to remove apoptotic cells result in secondary necrosis which exacerbate inflammation through the release of cytotoxic and pro-inflammatory molecules (CXCL-8, TNF-α, Il-1β) [49], [50]. Therefore, efferocytosis is imperative to the resolution of inflammation. In fact, phagocytosis of apoptotic cells inhibits the synthesis of pro-inflammatory mediators such as TNF-α and promotes anti-inflammatory cytokine release including IL-10 and TGF-β [51], [52].

### 1.1.2 Pulmonary mononuclear leukocyte populations

Mononuclear cells are a heterogenous population of cells that consist of lymphocytes (T cells, B cells and NK cells) and monocytes. These cells derive from committed haematopoietic stem cells located in the bone marrow or long-lived yolk sac-derived fetal mononuclear precursors. Monocytes are released into the circulation where they stay for a few days before migrating to tissues and differentiate into macrophages. Depending on the tissue they are migrating in, macrophages will have completely distinct roles [6], [53]–[55]. Interstitial macrophages, microglial cells and Kupffer cells will not be exposed to the same stimulus and thus will not perform the same functions. As such, macrophages role needs to be specifically adapted to the requirement of the tissue in which they reside. At baseline, lungs are constantly exposed to a remarkable diversity of pathogens. Moreover, this tissue compartment is characterized by constant environmental fluctuation. Therefore, alveolar macrophage must change their physiology depending on environmental cues and be able to induce an effective immune response to antigens while limiting pro-inflammatory responses to tissue debris and antigens within the lungs [6], [56]. To mimic T helper cell nomenclature, macrophages are usually divided into two functionally

distinct subtypes: M1 and M2 macrophages, also referred as classic and alternative macrophages, respectively [57] .However, it is worth noting that the current M1/M2 classification is bipolar and does not reflect macrophage heterogeneity found *in vivo* [57]. Whereas M1 macrophages inhibit cell proliferation, cause tissue damage and are pro-efficient in phagocytic and antimicrobial activities [55], [57]. M2 macrophages are anti-inflammatory cells promoting cell proliferation, tissue remodeling, angiogenesis and immune-regulation [55], [57]. Macrophage polarization towards the M1 or M2 phenotype is dependent on microenvironmental signals. For instance, *in vitro* stimulation of macrophages with LPS or inflammatory cytokines such as IFN-γ or TNF- α result in M1 polarization. On the other hand, Th2-related cytokines (IL-4 and IL-13) drive macrophage polarization towards a M2 phenotype [55], [57], [58] M1 macrophages are characterized by a strong secretion of pro-inflammatory cytokines (IL-1β, TNF, IL- 6) and an efficient production of reactive oxygen species (ROS) and nitric oxide (NO) molecules [55], [57], [58]. Conversely, M2 macrophages have been shown to secrete anti-inflammatory molecules such as IL-10, TGF-β, and glucocorticoids [55], [57], [58]. As seen in the previous section, inflammation requires the interplay between many different cell types, however in the context of lung infections, macrophages are central to induce an efficient immune response.

#### *1.1.2.1 Alveolar Macrophages*

In most tissues, resident macrophages account for 10 to 15% of the leukocyte population. However, in a healthy lung, 95% of leukocytes are alveolar macrophages [59], [60]. This number highlights their critical role in pulmonary defenses during homeostasis. Under steady state, alveolar macrophages are in close contact with the epithelium and held a quiescent state [6], [56]. This suppressed phenotype is controlled by cell-cell interactions and soluble mediators. Over the past decades, many receptors regulating alveolar macrophages activation have been identified.

Among those, the CD200 receptor (CD200R) and the TGFβ receptor (TGFβR) are of particular interest. These receptors expressed by AMs tether the cells to the epithelium and inhibit any pro-inflammatory behavior through p38 mitogen-activated protein kinase (MAPK) and SMAD pathways respectively [6], [61], [62]. In addition, the lumen of the airways contains elevated levels of IL-10 which provide another control level [6], [56]. AMs main roles during homeostasis is to clear cellular debris and eliminate innocuous antigens through phagocytosis without inducing inflammation. In addition, they suppress T cells activation and dendritic cells (DCs) function to prevent a strong inflammatory reaction against innocuous agents and self-proteins. However, even in a suppressed state, AMs express high levels of PAMPs and DAMPs to initiate a robust immune response [6], [56] (figure 2).

The mechanisms leading to AMs activation upon infection are still poorly understood but the integrity of the epithelium seems to be of critical importance. The loss of interaction with regulatory ligands expressed on the epithelium is thought to induce a pro-inflammatory behavior [6], [56]. Moreover, whether infected cells undergo apoptosis or necrosis will have a strong influence on macrophage activation. While apoptotic cells are readily efferocytosed by AMs and elicit an anti-inflammatory response, necrotic cells liberate pro-inflammatory molecules (see section 1.1.2.1). Following activation, first macrophage responders secrete various pro-inflammatory mediators such as IFN-γ, IL-1β, Il-6, CXCL-8, LTB4 necessary to induce local activation of epithelial cells and resident leukocyte but also to recruit neutrophils, blood monocytes and lymphocytes [63], [64] (figure 2). Moreover, AMs stimulate and potentiate the adaptive immune response through the secretion of IL-12, IL-23 and IL-4 which promote a Th1, Th17 and a Th2 response respectively [65]–[67]. Activated AMs also exhibit increased phagocytic and oxidative burst compared to their suppressed counterpart allowing them to eliminate efficiently

infectious agents [6], [56]. Beside their essential role in the induction of the inflammatory response, AMs are also crucial in the resolution of inflammation and the regulation of tissue repair. Firstly, AMs-derived matrix metalloproteinases can block leukocyte recruitment, especially neutrophil through MIP-2 and ICAM-1 downregulation [68], [69]. Concurrently, AMs promote neutrophil apoptosis by increasing TNF-α secretion and upregulating TNF-related apoptosis-inducing ligand (TRAIL) expression [70], [71]. Apoptotic neutrophils are then efferocytosed by macrophages resulting in phenotypic changes. Neutrophil efferocytosis inhibits macrophages pro-inflammatory signaling and promotes the release of anti-inflammatory and pro-resolving cytokines such as IL-10 and TGF-β [51], [52]. Efferocytosis also promote 15-lipoxygenase signaling which result in the secretion of many pro-resolving lipid mediators including lipoxins, resolvins and protectins. However, when the injury is severe, circulating monocytes are recruited to the lungs [55]

#### *1.1.2.2 Monocyte-derived macrophages*

During the first phases of the pulmonary inflammation most alveolar macrophages are lost due to tissue adherence, emigration, apoptosis and necrosis [55], [72]. To face this drastic loss, blood monocytes are heavily recruited to the site of inflammation. However, resident AMs reside tend to inhibit monocyte recruitment from the blood via mechanisms that remain largely unknown [55]. The pulmonary microenvironment contributes to the differentiation of blood monocyte-derived macrophages [6], [55], [73]. During the acute phase of inflammation recruited monocytes are source of inflammatory macrophages (M1 polarized). These inflammatory blood monocyte-derived macrophages (BMDMΦs) upregulate a wide array of pro-inflammatory and pro-fibrotic genes as well as major histocompatibility complex (MHC) class II molecules allowing them to activate effector T cells [74] . Inflammatory BMDMΦs) cells are considered “end type” killer and

are thought to die during the inflammatory response, killed by their own nitric oxide production [75], [76]. Following the acute phase of inflammation, surviving inflammatory macrophages mature into M2 macrophages and actively participate to the resolution phase of inflammation by inhibiting pro-inflammatory signaling and promoting tissue repair [55].

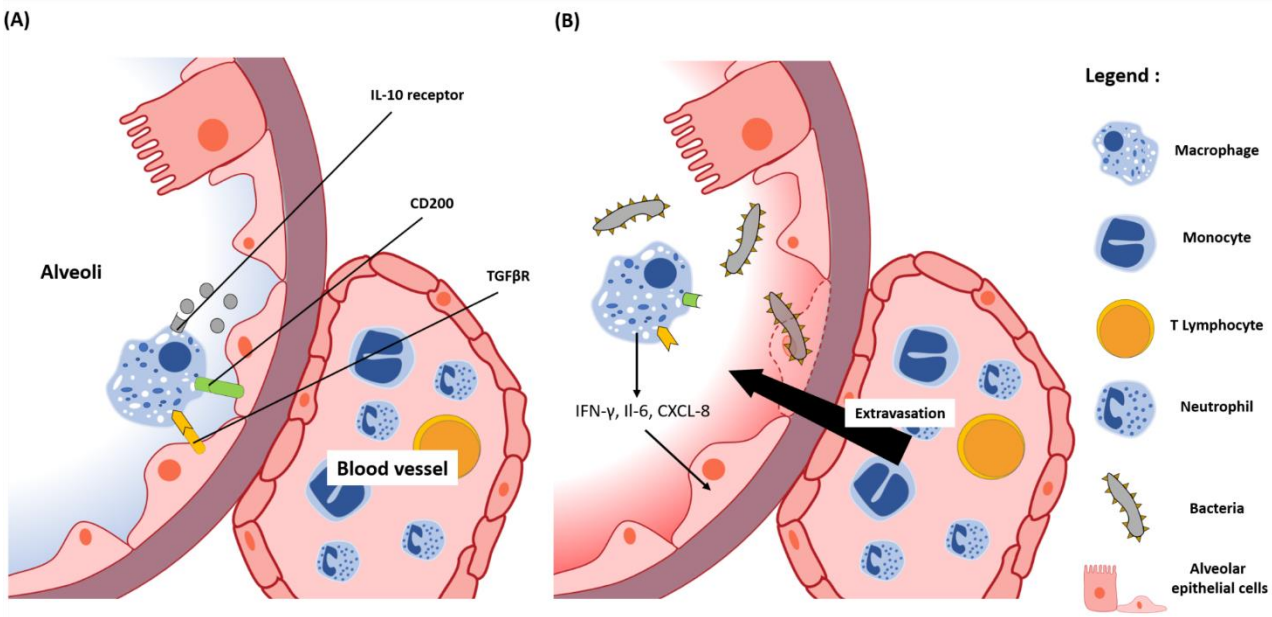



**Figure 2**. **Alveolar macrophages activation during injury.** (A) During homeostasis, alveolar macrophages are maintained in a quiescent state by the alveolar epithelium. The CD200 receptor (CD200R) and the TGFβ receptor (TGFβR) expressed by alveolar macrophages tether the cell to the epithelium and inhibit any pro-inflammatory behavior. The alveolar lumen also contains elevated levels of IL-10 to prevent unnecessary or uncontrolled activation. (B) Following injury, interaction with the epithelium is lost resulting in macrophage activation. Activated macrophages secrete various pro-inflammatory mediators such as IFN-γ, IL-6 and CXCL-8 necessary to initiate the immune response. Figure adapted from [6]

## 1.2 Porcine Reproductive and Respiratory Syndrome

With an estimated economic impact exceeding $600 million in the USA alone, Porcine Reproductive and Respiratory Syndrome (PRRS) is one the most economically impacting swine disease [77]. First identified in Europe and North America in the late 1980s this syndrome is currently prevalent in most swine-producing countries [78]–[80]. To date, and despite 30 years of research, there is no efficient treatment to this disease. This can be explained by the high antigenic variability, and the largely unknown immunopathogenesis of PRRS etiologic agent. PRRS clinical symptoms mainly manifest through reproductive failure in sows and gilts, including mummified and aborted fetuses, and respiratory distress in growing pigs [81]–[83]. Interestingly, the pathobiology of the reproductive form and respiratory form of the disease are totally different [81]. Herein we will only focus on the respiratory component of the syndrome, which is characterized by a non-specific lymphomononuclear interstitial pneumonia [83]–[85].

### 1.2.1 Etiology

PRRS causative agent is a small enveloped, single-stranded positive sense RNA virus called Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) or “Lelystad virus” [79], [80]. PRRSV is classified in the *Arteriviridae* family within the *Aterivirus* genus along with the simian hemorrhagic fever virus (SHFV), the equine arteritis virus (EAV) and the mouse lactate dehydrogenase elevating virus (LDV). As a member of the *Arterivirus* genus, PRRSV possesses several cytopathogenic properties including persistent asymptomatic infections and a very narrow cell tropism [86], [87].

PRRSV genome is 15 kilobase in length and encodes for at least 14 non-structural proteins and 8 structural proteins [88]–[90]. Sequence comparison between viral isolates demonstrated that PRRSV exist in at least two distinct genotypes, the European genotype (EU type or type I) and the

North American genotype (NA type or type II). Type I and type II strains differ genetically – they share only 63% of nucleotide identity suggesting that despite emerging concurrently, the two isolates emerged independently [91], [92]. In addition, many studies demonstrated a high degree of genetic variability within both types which strongly minimize the impact and efficacy of most vaccines [93], [94]. This remarkable genetic diversity which results from the lack of RNA dependent RNA polymerase proofreading function and numerous viral recombination spurs PRRSV rapid evolution [95]. The emergence of the devastating Chinese highly pathogenic PRRSV (HP-PRRSV) in 2006 is a good example of how RNA-viruses adaptation to selective pressure in the field significantly impedes the development of effective control strategies [96].

### 1.2.2 PRRSV infectivity

In its natural host (Swine), PRRSV preferentially targets fully differentiated alveolar macrophages; however, *in vivo* and *in vitro* experiments demonstrated that PRRSV is able to infect most cells of the monocyte-macrophage lineage such as intravascular and lymph node macrophages and DCs [97]–[100]. Very few immortalized cell lines were found to be fully permissive to PRRSV; the African green monkey kidney cell line MA-104 its derivative MARC-145 and the St-Jude porcine lung cells (SJPL) [101], [102]. As any other enveloped virus, PRRSV entry is dependent on the expression of specific receptors and or protein at the cell surface. The interaction between the virus particle and its receptors dictate the mechanisms by which the virus will be able attach to the host cell and penetrate the cytosol and, ultimately the virus cell tropism [103]. Thus, understanding PRRSV entry mechanisms and its receptors is crucial to understand its infectivity.

### 1.2.3 PRRSV entry

Based on transfections of PRRSV RNA in several non-permissive cell lines and on the absence of direct fusion of the PRRSV envelope with the cellular membrane, it was hypothesized that PRRSV entry is dependent on the expression of specific receptors and/or proteins at the cell surface [104]–[107]. Since, it was demonstrated that PRRSV entry into its target cells occurs through clathrin-mediated endocytosis followed by membrane fusion with the endosome membrane [108]. At least six cellular receptors potentially involved in PRRSV entry pathway have been identified [90]. Among these receptors, three have been extensively studied and incorporated in a model for PRRSV attachment and internalization; heparan sulfate, sialoadhesin (CD169) and CD163 (cystein-rich scavenger receptor). In this model, heparan sulfate molecules serve as attachment factors that concentrate virions at the membrane and facilitate their interaction with sialoadhesin receptors which in turn initiate internalization to early endosome [109]–[111] (figure 3). Once internalized, viral particles are transported to the late endosome where uncoating takes place [90]. The mechanisms by which the viral envelope fuses with the endosomal membrane remain largely unknown but it may involve a drop in pH to induce structural changes of the nucleocapsid [112]. CD163 was found to co-localize with PRRSV in early endosomes but not at the plasma membrane indicating that this receptor is necessary for virus uncoating [110], [113] (figure 3). To date, the necessity of each of these receptors is still debated but only CD163 has been shown capable of conferring PRRSV-permissivity to non-susceptible cell lines [114]–[116].

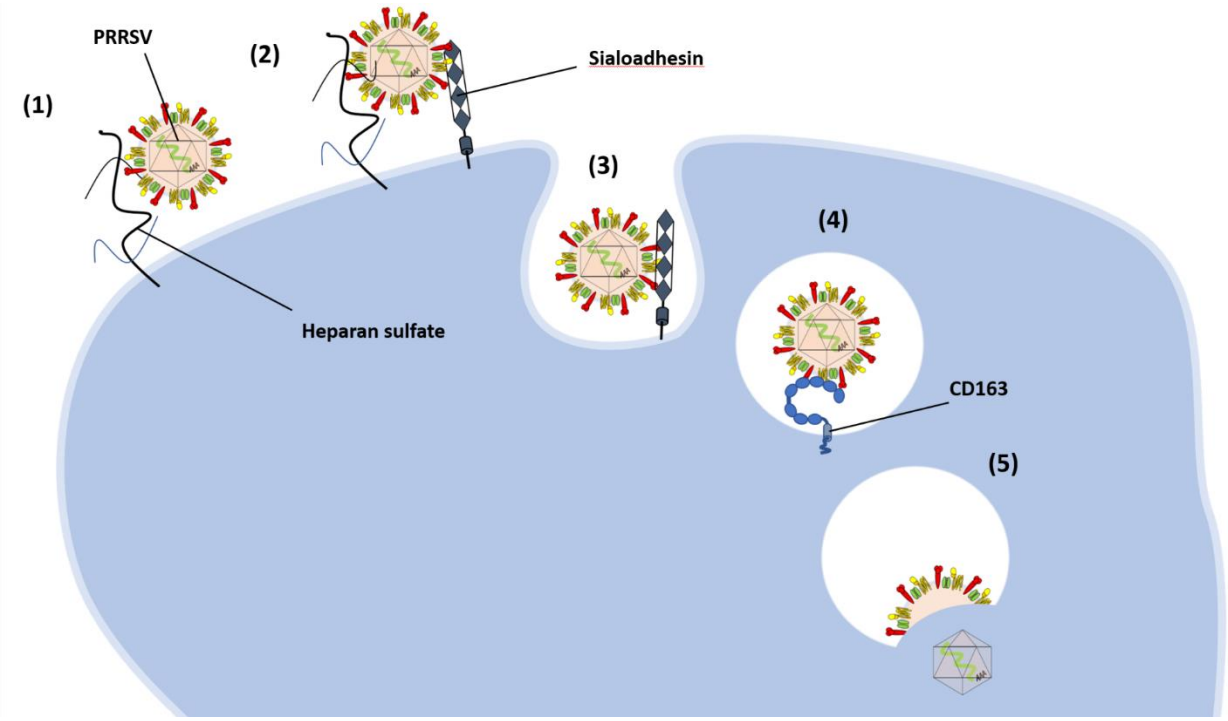



**Figure 3. Porcine reproductive and respiratory syndrome virus entry in target cells**. PRRSV entry is mediated by clathrin-dependent endocytosis followed by fusion at the endosome membrane. (1) PRRSV interacts with heparan sulfate molecules present at the cell surface. (2) PRRSV binds to siloadhesin molecules stabilizing its interaction with the cell (3) initiating its internalization. (4) Within the early endosome, the virus interacts with CD163. (5) pH acidification induces conformational changes of the PRRSV nucleocapsid resulting in the viral envelop fusion with the endosome membrane. Adapted from [90].

### 1.2.4 PRRSV viremia

PRRSV infections are often defined as “life-long” which is not accurate. In fact, viremia analyses of infected pigs demonstrate that PRRSV levels slowly decay over time until the virus eventually becomes extinct [81], [117]–[119]. However, viral particles can be detected in the serum for as long as 250 days after exposure, a period covering the average lifetime of a production pig [120]. PRRSV infections can therefore be considered as “life-long” even though the virus is not “persistent”. Based on the literature, PRRSV infection can be divided in at least three phases: acute infection, persistence and extinction [81]. During the acute phase of viremia, which usually last 28 days, PRRSV primarily targets alveolar macrophages and dendritic cells of the lungs and the upper respiratory tract [97]–[100]. During this phase, uncontrolled leukocyte death and pro-inflammatory mediators release, cause local inflammation that results in respiratory distress. Subsequent to the production of neutralizing antibodies (Nabs), PRRSV titers decrease to the point its not detected in the lungs or in the blood [121]. Low levels of viral replication persist in the lymphoid tissues, including tonsils and lymph nodes up to 100 days after infection [119], [122]. These replication sites likely facilitate viral transmission to naïve pigs via shedding from tonsils [119]. The eventual disappearance of the virus from the host marks the last stage of the infection. The mechanisms leading to PRRSV extinction are not fully understood but are thought to be due to the reduction of PRRSV-permissive cell populations and the development of a partially effective immune response [81], [118].

### 1.2.5 PRRSV pathogenicity

#### *1.2.5.1 Innate immune response*

Due to significant genetic and virulence differences among PRRSV isolates, clinical signs of PRRS varies considerably between herds and even between pigs [85], [123]. Symptoms range

from subclinical to severe depending on the PRRSV isolate, host immune status and susceptibility as well as concurrent infections. Interestingly, bronchoalveolar lavage fluid analysis of infected pigs showed that only 2% of alveolar macrophages are infected during the acute phase of the infection [98]. Similarly an *in vitro* study observed around 5 to 10% positive AMs throughout the course of infection [124]. As we described previously, cells of the monocyte lineage are crucial to trigger and maintain efficient innate and adaptive immune responses in the lungs, therefore, any suppression of macrophage functions and/or populations can have huge consequences for the host immunity. In this section we will investigate on the mechanisms by which PRRSV modulate and evade the antiviral defense of the host.

#### *1.2.5.2 Cytokine secretion during PRRSV infection*

Following any viral infection, the induction of cytokines is critical to initiate an antiviral state, control the infection at early stages and stimulate the adaptive immune response [125], [126]. PRRSV ability to escape the immune system and establish long term infections is thought to be directly linked to its proficiency to modulate cytokine secretion. In fact, PRRSV-induced secretion of TNF-α and or IL-10 has been used to classify PRRSV isolates [127].

##### 1.2.5.2.1 Anti-viral cytokines

Aside from HP-PRRSV isolates, it is generally accepted that PRRSV weakly induces or even suppresses pro-inflammatory cytokines. This paradigm arises from early *in vitro* and *in vivo* experiments that focused on the two most potent antiviral cytokines: TNF-α and IFN-α [128]–[130]. However, the acute phase of PRRSV infection is often characterized by respiratory distress indicating pro-inflammatory cytokine release in the lungs [118]. In fact, extensive studies demonstrated that pro-inflammatory cytokine release is dependent on the viral strain, the cell type and the host condition [130]–[135].

While most cell types are able to produce type I interferons, these cytokines are predominantly produced by alveolar macrophages during pulmonary viral infections [136]. Many *in vitro* and *in vivo* studies demonstrated that throughout the course of PRRSV infection IFN-α/β levels remain unusually low [128], [129], [137]. These inhibitory effects were observed in infected macrophages but also in non-permissive cells such as plasmacytoid dendritic cells (pDCs) [128], [131], [137], [138]. Multiple PRRSV proteins (structural and non-structural proteins) have been shown to suppress type I interferon which can explain PRRSV high efficiency in IFNs inhibition [139]–[142]. Consistently, PRRSV significantly reduces TNF-α both *in vivo* and *in vitro*, in alveolar macrophages and in pDCs [130], [143]. These two cytokines have proven to play critical roles during early stages of viral infections even exhibiting synergetic effects in promoting antiviral activities [139], [144]–[147]. Accordingly, exogenous addition of TNF-α and IFN-α were found to reduce PRRSV titers [128], [130]. Therefore, PRRSV inhibition of these antiviral cytokines seems crucial in its ability to evade the immune system and replicate efficiently (figure 4).

1.2.5.2.2 Anti-inflammatory cytokines

Poor TNF-α and IFN-α induction is crucial in PRRSV immune evasion, however it only partially accounts for this characteristic. Many viruses targeting macrophages including the porcine circovirus type 2 (PCV-2) and the human immunodeficiency virus-1 (HIV-1) have been shown to modulate the host immune response through IL-10 suggesting for a potential role in PRRSV pathogenesis [148], [149].

Different reports demonstrated PRRSV ability to up-regulate IL-10; however, this property seems to be strain-dependent and correlates with the virulence of the strain [133], [150]–[154]. Functional analysis of infected cells showed that IL-10 upregulation coincides with a down-

regulation of MHC I molecules expression and supressed T cell responses [132], [155]. More interestingly, exogenous IL-10 increases PRRSV infectivity. At least three different groups found that differentiating or fully differentiated cells of the monocyte lineage exposed to IL-10 were more susceptible to PRRSV – by 10 to 20% [58], [115], [156]. This increased susceptibility was associated with an increase in CD163 expression [58], [115], [156]. These results are consistent with other studies indicating that IL-10 upregulates CD163 in humans [157], [158]. Therefore PRRSV-induced IL-10 might not only impede the immune response but also to facilitate viral replication [58].

Even though it is strain dependent, modulation of crucial antiviral and anti-inflammatory cytokines seems to be highly conserved among strains. TNF-α and IFN inhibition concurrent with IL-10 increase is thought to induce an immunosuppressive state in the lungs through a Th2 mediated response which promote virus infectivity, persistence and secondary infection [58], [159]–[161] (figure 4).

1.2.5.2.3 Pro-inflammatory cytokines

Despite PRRSV anti-inflammatory and immunosuppressive properties, infection can cause high fever, lymphocytic encephalitis and interstitial pneumonia indicating that inflammation is a crucial component in PRRSV pathogenesis. BALF analyses and *in vitro* infections of MARC-145, PAMs and bone marrow-derived immature dendritic cells demonstrated that PRRSV induces the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α likely through NF-κB activation [153], [162]–[166]. Consistently, transcriptomic analyses of lung samples of infected pigs showed that PRRSV upregulates the expression of pro-inflammatory genes [167]. PRRSV-induced pneumonia has been shown to induce the remodelling of lung structures and leukocyte infiltration [168], [169]. Given CXCL-8 role in neutrophil chemotaxis, researchers sought to

investigate its role in PRRSV pathogenesis. Several studies measured a CXCL-8 increase in the serum and BALF of infected pigs as well as in cultured cells (bone-marrow derived DCs and PAMs) but not in pDCs [120], [132], [135], [170]–[172]. In contrast, another group found that PRRS infection resulted in a CXCL-8 mRNA increase in PAMs but its secretion was downregulated [173]. As for anti-viral and anti-inflammatory cytokines, pro-inflammatory cytokines upregulation by PRRSV seems to be highly dependent on the cell type, the virus strain and the host conditions [130]–[132], [135], [170].

*1.2.5.3 PRRSV-induced cell death*

Evasion of the host immune response is not the only strategy used by viruses to ensure a successful infection. Efficient viral replication requires the survival of the host cell until enough virions are produced [174], [175]. Thereby, many viruses developed mechanisms to block or delay apoptosis during replication. On the other hand, other viruses use apoptosis to enhance viral transmission and avoid the immune system [174], [175]. Interestingly, a study conducted in 2008 found that PRRSV can stimulate anti- or pro-apoptotic pathways depending on the infection stage [176]. During the early stages of the infection

, PRRSV prevents staurosporine-induced apoptosis of AMs, perhaps to complete its replication cycle, while it promotes cell death at the late stages of the infection [176] (figure 4).

Although it is clear that apoptosis contributes to the pathobiology of the disease , whether PRRSV induces cell death directly (in infected cells) or indirectly (in bystander cells) remains to be fully ascertained [177]–[179]. The first paper to provide evidence of direct apoptosis by PRRSV was published in 1996. Using viral vector expressing the viral protein GP5, the authors were able to induce apoptosis in cell monolayers [180]. Further characterisation of GP5 activity demonstrated that GP5 induction of apoptosis is caspase 3 dependent [177]. Since, PRRSV has

been reported to trigger apoptosis in infected cells through multiple signaling pathways including the intrinsic and extrinsic apoptotic pathways (via caspase-8 and caspase 9 activation respectively) and c-Jun N-terminal kinase (JNK) pathway [178], [181]. However, thorough analyses of BALF, lungs and lymphoid tissues showed that, *in vivo*, the majority of apoptotic cells are not infected with PRRSV [124], [153], [182]. For example, Chang and collaborators observed that although only 5-10% AMs were positive for PRRSV, 22 to 34% were apoptotic [124]. Collectively, these data indicate that despite being able to induce apoptosis in infected cells, PRRSV primarily do so in bystander cells [161], [183], [184] (figure 4).

Apoptosis is not the only type of cell death resulting from PRRSV infections. Necrosis is detected *in vivo* and *in vitro*, sometimes at much higher levels than apoptosis [133], [176], [178], [179]. However, it is necessary to precise that many studies investigating on PRRSV-induced cell death used techniques that does not allow to differentiate efficiently between apoptosis and necrosis [178], [179]. Therefore, in these studies it is not clear whether infected cells undergo first hand necrosis or secondary necrosis.

*1.2.5.4 Modulation of microbicidal functions*

Surprisingly, even though PRRSV targets professional phagocytes and secondary infections are often associated with PRRS, modulation of microbicidal functions by the virus is poorly documented. In addition, the few papers reporting on the effects of PRRSV exposure on bacterial uptake and killing show conflicting results. Pathogen removal by antigen presenting cells (APCs) can be broken down into series of defined steps upon which PRRSV could act on to compromise macrophage microbicidal functions.

#### 1.2.5.4.1 Phagocytosis

Pathogen internalization (or phagocytosis) is mandatory to sequester pathogens within the cytosol and initiate the degradative phase. Some viruses including HIV-1 and influenza have been shown to increase secondary infections through phagocytosis inhibition hinting towards potential shared mechanisms with PRRSV [185], [186].

To investigate on this hypothesis, PRRSV-infected cells phagocytic functions were tested. Whereas PRRSV exposure did not affect *Staphylococcus aureus* and *Escherichia coli* uptake, decreased phagocytosis against *Salmonella typhimurium*, *Streptococcus suis* and *Candida albicans* has been reported [187]–[191]. A hypothesis to explain the discrepancies between the papers might be that each pathogen reacts with macrophages in a different way, stimulating different signaling pathways that might or might not be downregulated by the virus. Despite the use of opsonised latex beads to circumvent this limitation, contradictory results are still observed [192], [193]. In 2012, a study demonstrated that PRRSV significantly inhibits opsonized latex beads phagocytosis [192]. In contrast, a more recent paper did not observe any change in latex beads uptake following PRRSV infection [193]. These results suggest that the previous hypothesis cannot fully account for the differences in phagocytosis modulation and that the virus isolate and/or the cell type influence the outcome of the experiments.

The mechanisms by which PRRSV inhibits phagocytosis are largely unknown but might be dependent on sialoadhesin (Sn) expression [192]. Considering that antibody binding to Sn was sufficient to inhibit macrophage phagocytosis and that PRRSV interacts with Sn, De Baere and colleagues wondered whether PRRSV binding to its receptors would downregulate phagocytosis [192], [194]. PRRSV infection resulted in a significant decrease in opsonized latex beads

phagocytosis. In addition, their results demonstrated that PRRSV binding to Sn is sufficient to downregulate phagocytosis and that viral internalization is not necessary in this process [192] .

1.2.5.4.2 Reactive Oxygen species production

Following internalization, pathogen killing requires the recruitment and activation of numerous antimicrobial components including ROS [10]. However, deregulated ROS production is thought to be involved in sustained inflammatory processes [195]. Therefore, while a decrease in ROS production by PRRSV could significantly hinder macrophage capacity to eliminate invading pathogens, unrestrained production would contribute to the viral pathogenesis. Such dual role has been reported in the literature. Indeed, PRRSV was found to decrease NADPH oxidase-dependent respiratory burst in PIMs, PAMs, and in porcine bone marrow derived macrophages [189], [191], [193]. On the contrary mitochondrial ROS production is increased upon PRRSV infection likely contributing to PRRSV induced apoptosis [166], [178]. Analyses of macrophage microbicidal activity using live extracellular bacteria including *Haemophilus parasuis* and *Streptococcus suis* showed either a decreased activity or no change adding a layer of complexity regarding the potential influence of PRRSV on ROS synthesis [190], [196], [197].

*1.2.5.5 Adaptive immunity*

An efficient adaptive antiviral response relies on strong neutralizing antibody production as well as cytotoxic T cells activation. PRRSV elicits a weak cell-mediated immune response (CMI) and even though an antibody response is detected as early as 5 days post-infection, neutralizing antibodies titers remain low throughout the course of the infection [198]–[203]. The development of such weak adaptive responses is likely the consequence of PRRSV modulation of the innate immunity. For instance, type I IFNs enhance antigen presentation, antibody production and CD8+ T cell differentiation [139], [202]. Usually, upon infection, infected cells secrete type I

IFN which stimulate the proliferation of virus specific IFN-γ secreting T cells [204], [205]. As stated previously, PRRSV does not elicit and even suppresses type I IFNs hence significantly hindering the development of a sufficient IFN-γ secreting T cells repertoire. Moreover, PRRSV-induced IL-10 secretion likely bolster CMI suppression through regulatory T cell stimulation [151], [154], [206]. Together, IFNs inhibition and IL-10 secretion favor a Th2 response that results in a strong increase in IL-4 production. Interestingly, the role of IL-4 in pigs is different when compared with human and mice. In fact, while IL-4 promotes antibody production in human and mice, it blocks it in pigs [207], [208]. Thus, PRRSV-induced IL-4 is thought to delay NAbs production, which significantly contributes to the establishment of "persistent" infections since non-NAbs enhance viral replication in AMs [209] (figure 4)

Importantly, PRRSV-induced modulation of the immune response does not cause the death of infected animals. PRRSV rarely kill its host on its own, rather it promotes secondary bacterial infections that are more likely to kill PRRSV-infected pigs.

*1.2.5.6* Opportunistic pathogens.

Field reports and clinical evidence demonstrate that PRRSV infections are almost always associated with secondary infections [137], [210]. Consequently, attributing clinical symptoms to PRRSV is inherently difficult. Nonetheless, it is undeniable that PRRSV primes the host for secondary infections and increases the severity of those infections. For instance, a study using a PRRSV/LPS co-infection model showed that TNF-α, IL-1, and IL-6 levels were 10 to 100 times lower in PRRSV-LPS-inoculated pigs compared to pigs inoculated with PRRSV or LPS alone. Moreover, only co-infected pigs exhibited severe respiratory distress [163]. Other papers report that mortality rates of PRRSV and *Streptococcus suis* co-infected piglets were significantly higher than those infected with PRRSV and *S.suis* alone [211], [212]. Transcriptional analyses of

PRRSV-infected cells demonstrate a clear synergism between PRRSV and *S.suis* leading to the up-regulation of pro-inflammatory related genes hence explaining increased mortality rates in co-infected animals [190]. Similar findings were reported, during *Mycoplasma hyopneumoniae* /PRSSV co-infections [213].

In summary PRRSV interferes with the innate immunity at multiple levels, from cytokine secretion to apoptosis and phagocytosis modulation, which significantly weakens and delays the adaptive immune response. Consequently, opportunistic pathogens infect immunosuppressed pigs and synergize with PRRSV to induce a strong inflammatory response with result in severe pneumonia and death of the animals (figure 4).

Given the viral nature of its etiological agent, most PRRS control strategies aimed to develop a vaccine. However, mainly due to PRRSV rapid evolution and safety issues, vaccines have not succeeded to reduce the prevalence of the disease [93]. During viral infections, remodeling of the pulmonary architecture is primarily caused by the host immune response rather than the virus itself [214]. Therefore, another strategy to control viral respiratory infections might be to harness and reduce the inflammatory response occurring in the lungs throughout the course of the infection.

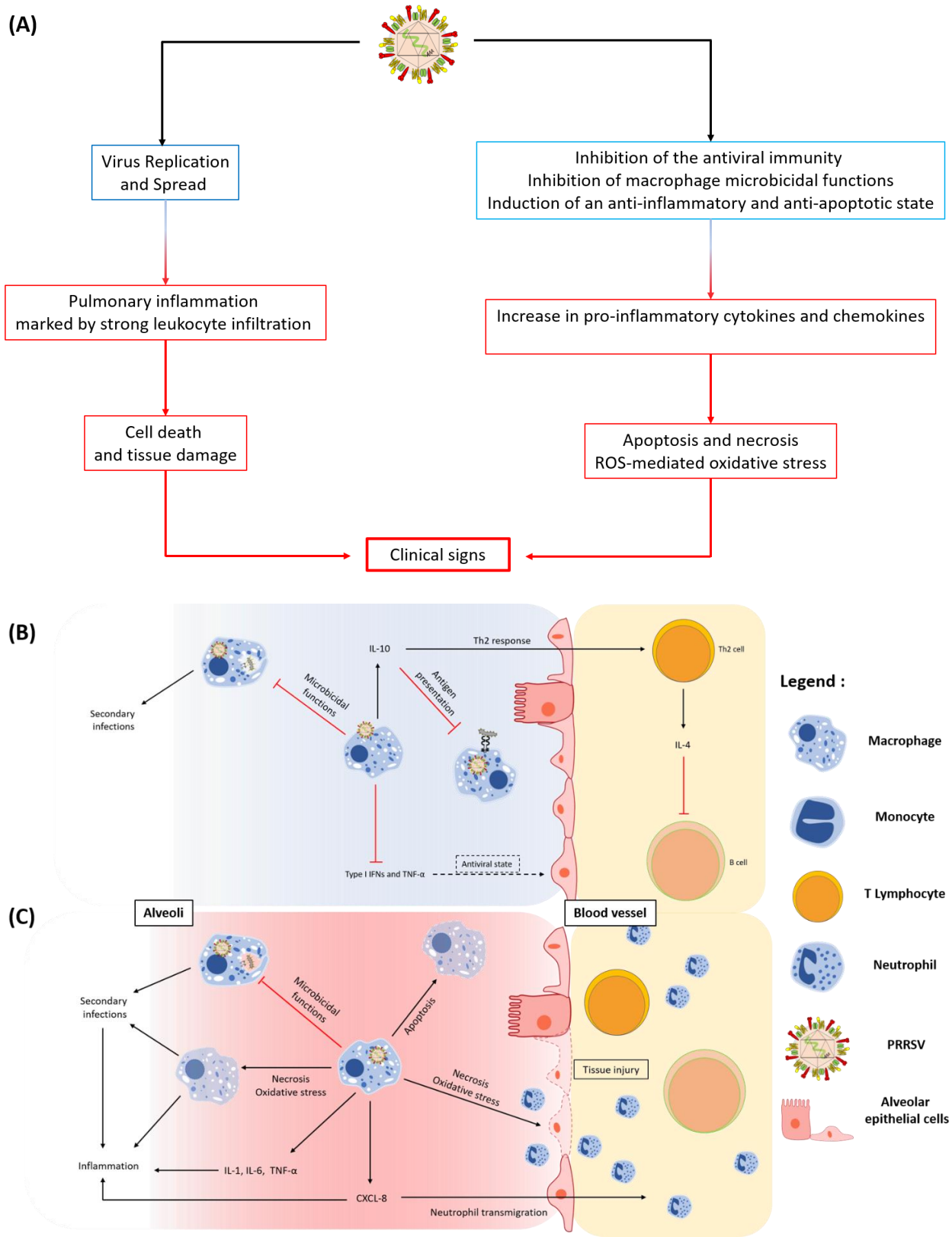

(A)
Virus Replication and Spread
Inhibition of the antiviral immunity
Inhibition of macrophage microbicidal functions
Induction of an anti-inflammatory and anti-apoptotic state
Pulmonary inflammation marked by strong leukocyte infiltration
Increase in pro-inflammatory cytokines and chemokines
Cell death and tissue damage
Apoptosis and necrosis
ROS-mediated oxidative stress
Clinical signs
(B)
IL-10
Th2 response
Th2 cell
Secondary infections
Microbicidal functions
Antigen presentation
IL-4
B cell
Type I IFNs and TNF-α
Antiviral state
(C)
Alveoli
Blood vessel
Secondary infections
Microbicidal functions
Apoptosis
Necrosis
Oxidative stress
Necrosis
Oxidative stress
Tissue injury
Inflammation
IL-1, IL-6, TNF-α
CXCL-8
Neutrophil transmigration
Legend :
Macrophage
Monocyte
T Lymphocyte
Neutrophil
PRRSV
Alveolar epithelial cells

**Figure 4. Porcine reproductive and respiratory syndrome virus pathogenesis**. (A) PRRSV modulation of the immune response is sequential. (A and B) During the first phases of the infection, concurrent inhibition of antiviral cytokines (IFNs and TNF-α) with IL-10 increase induce an immunosuppressive state. Il-10 inhibits antigen presentation and induces a Th2 mediated response which in turn inhibits neutralizing antibodies production through IL-4. Together these factors promote PRRSV infectivity and persistence. Viral inhibition of macrophages microbicidal functions (phagocytosis and NADPH oxidase ROS production) increases host susceptibility to secondary infections. (A and C) During the later stages of the infection, PRRSV upregulates the expression and the secretion of pro-inflammatory mediators such as CXCL-8 resulting in pulmonary inflammation marked by the thickening of the alveolar septa associated with strong leukocyte infiltration. Sustained cell apoptosis and necrosis combined with ROS-mediated oxidative stress result in tissue damage and perpetuate the inflammatory response. Upon secondary infections, opportunistic pathogens synergize with PRRSV to induce an exacerbated inflammatory response. (B and C) Red arrows indicate an inhibition whereas black arrows denote an increase. Figure A adapted from [167].

## 1.3 Immunomodulatory antibiotics

The common occurrence of PRRSV infection with bacterial infections as well as its inflammatory-driven pathobiology raises the question of the value of antibiotics for the treatment of the disease it causes. A particular class of antibiotics, macrolides have been of particular interest during the past decade. Many studies demonstrated that these antibiotics possess potent anti-inflammatory and immunomodulatory properties beyond their antimicrobial effects [215], [216]. Macrolides' unique properties allow them to target both the invading pathogen and the host inflammatory response. These drugs were found to be highly effective in treating multifactorial pulmonary diseases such as chronic obstructive pulmonary disease (COPD) and asthma [217], [218]. In this section, we will review the non-microbial and immunomodulatory actions of macrolides that suggest that they might be considered as promising treatment options following respiratory viral infections.

### 1.3.1 Macrolides

Macrolides constitute a class of antimicrobial compounds characterized by the presence of a macrocyclic lactone ring. This ever-expanding group of natural and synthetic compounds are isolated from fungi (*Streptomyces* genus) and from bacteria (*Arthrobacter* genus). To date, the most commonly used macrolides are semi-synthetic derivatives from erythromycin A such as clarithromycin, azithromycin. Macrolides are powerful biostatic molecules that are known to inhibit protein synthesis of numerous Gram-positive and Gram-negative bacteria through the binding of the 50S ribosomal subunit [215], [216]. These antibiotics accumulate within leukocytes at high concentration, reaching 500 times systemic levels which allow them to be transported directly to the inflammation site, conferring them superior pharmacodynamics [219]–[221]. By accumulating with the cytosol, macrolides may interact with receptors or second messengers which

may ,in turn, alter leukocyte survival and functions; Moreover, this property provides increased activity against intracellular and cytotoxic pathogens [222].

Traditionally, antibiotic efficacy is evaluated solely on their antimicrobial properties. However, macrolides have been shown to possess immunomodulatory actions extending beyond their antimicrobial activities [216], [223]–[229]. In fact, *in vivo* and *in vitro* reports demonstrated that some macrolides are capable of reducing inflammation through the modulation of immune cell functions. These effects include a reduction of ROS and pro-inflammatory cytokines production as well as the inhibition of neutrophil migration and activation concurrent with an acceleration of their apoptosis. Macrolides efficiency to downregulate inflammation resides in their ability to affect several pro-inflammatory pathways at the same time. For instance, erythromycin inhibits the production of pro-inflammatory mediators such as TNF-α, IL-6, CXCL8 and LTB4 but also downregulate the expression of adhesion molecules (ICAM-1, β2 integrins), promotes neutrophil apoptosis, macrophages efferocytosis and IL-10 production [216], [223]–[227]. Similar effects have been observed with other macrolides including azithromycin, tilmicosin and tulathromycin [228]–[232]. As we extensively described in the previous section, PRRSV pathobiology as a strong inflammatory basis and the severity of infection is closely related with the capacity of the strain to dysregulate macrophage functions and induce an inflammatory response. Therefore, macrolides immunomodulatory actions may attenuate PRRSV-related exacerbation of inflammation.

Despite their immunomodulatory properties, the use of macrolides to treat viral infections is still highly controversial [233]. Most studies investigating on the potential use of these drugs in respiratory viral infections focused on human viruses such as rhinovirus (RV), respiratory syncytial virus (RSV) and influenza virus [234]–[236]. The results of these studies have been

reviewed in [233]. Although numerous *in vitro* studies demonstrated macrolides ability to reduce viral titers and viral-induced inflammation, *in vivo* and clinical data are very rare and show limited clinical benefits [233].

Interestingly, several lines of evidence suggest that pigs could be used as a model system to study respiratory diseases and improve our knowledge on human health. Indeed, pigs are really close to humans genetically, anatomically and immunologically. In addition, respiratory infections in pigs and humans show similar pathogenesis and are caused by the same pathogens [237] [238].

At present, very few groups explored the potential clinical benefits of macrolides in PRRSV infections. So far only erythromycin, tilmicosin and tylvalosin have been used for *in vitro* and *in vivo* experiments [239]–[241]. While erythromycin failed to inhibit virus replication, tylvalosin and tilmicosin challenge did reduce PRRSV viral load. In addition, tylvalosin and tilmicosin significantly reduced PRRSV-induced ROS production and pro-inflammatory cytokines secretion including TNF-α, IL-6, IL-1β and CXCL-8 [239]–[241].

### 1.3.2 Tulathromycin.

Tulathromycin, is a new trialimide macrolide derived from erythromycin which is used for the treatment and the prevention of bacterial respiratory diseases in swine and cattle [238], [242]. This antimicrobial has low inhibitory concentrations against different bacterial pathogens such as *Actinobacillus pleuropneumoniae*, *Pasteurella multicoda* and *Mycoplasma bovis* [238], [242]. As other macrolides, tulathromycin accumulates in leukocytes and it can be found in high, prolonged concentrations in tissues following a single-injection [243]. Moreover, clinical studies demonstrated superior clinical efficacy of tulathromycin compared to other antibiotics including macrolides such as tilmicosin and tildipirosin [244], [245]. However, until recently, tulathromycin immunomodulatory properties were uncharacterized.

*In vitro* and *in vivo* studies conducted in our laboratory showed that tulathromycin possesses immunomodulating properties that may dampen microbial-induced inflammatory responses and promote the resolution of inflammation [228]–[230], [246]. *In vitro*, tulathromycin induced porcine and bovine neutrophil and monocyte-derived macrophage apoptosis in a time- and dose-dependent manner. Moreover, neutrophil apoptosis was accompanied by increased efferocytosis. The drug was also able to inhibit the production of CXCL-8, and $LTB_4$ whereas it increased the secretion of lipoxin A4, a pro-resolving lipid mediator. Consistently, *in vivo*, tulathromycin promoted leukocyte apoptosis and efferocytosis, reduced the levels of pro-inflammatory $LTB_4$ and prostaglandin E2 (PGE2) in both bovine and porcine experimental models [228]–[230], [246].

Considering that tulathromycin immunomodulatory properties are independent of its antimicrobial actions and that compared to other macrolides, it exhibits superior clinical efficacy, tulathromycin seems to be one of the best macrolide candidates to treat viral-induced inflammation.

## 1.4 Hypothesis and Objectives

### 1.4.1 Hypothesis

Based on the evidence from the literature and past studies in our laboratory, we hypothesize that tulathromycin will exhibit anti-viral and immunomodulating properties that may in turn attenuate the detrimental effects of PRRSV in porcine macrophages

### 1.4.2 Objectives

The specific aims of this project were as follows:

- To develop a new cellular model of PRRSV pathogenesis *in vitro*

- To assess whether tulathromycin possesses direct anti-viral effects in PRRSV-infected porcine macrophages
- To identify immunomodulating properties of Tulathromycin in macrophages infected with PRRSV including effects on:
    - Cell death (apoptosis and necrosis)
    - Chemokine and Cytokine profiles (CXCL8, IL-10, IL-1β)
    - Phagocytic potential

# 2 MATERIAL AND METHODS

## 2.1 Cell line and virus strain.

The African green monkey kidney cell line highly permissive to PRRSV, MARC-145, was used for plaque titration assay. MARC-145 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fischer Scientific) supplemented with 10% heat inactivated (HI)-FBS (Invitrogen) and 100 IU/mL penicillin-streptomycin (Thermo Fischer Scientific). The cells were maintained at 37°C, 5% $CO_2$ and passaged twice weekly. PRRSV isolate NVSL 98-7895 (GenBank accession no. AY545985.1) was generously given by Dr. Robin Yates (University of Calgary, AB, Canada) and used as the only viral strain in all experiments. The L929 murine cell line was used as a source of macrophage colony stimulating factor (M-CSF) to potentiate monocyte differentiation into macrophages. As for MARC-145 cells, L929 cells were cultivated in Dulbecco's Modified Eagle's Medium (Thermo Fischer Scientific) supplemented with 10% HI-FBS (Invitrogen) and 100 IU/mL penicillin-streptomycin (Thermo Fischer Scientific) and passaged twice weekly. At 90% confluence, HI-FBS containing media was discarded and replaced by serum-free media containing penicillin and streptomycin (100 IU/mL) for 24 to 48 hours. The media was then collected and used to cultivate macrophages or stored at -80°C until use.

## 2.2 Virus titration.

Virus titration was performed via plaque assay. Briefly, MARC-145 cells were seeded in 12 well plates (Costar) and grown until confluency. PRRSV samples of unknown concentration were serially diluted and overlaid onto confluent MARC-145 for 1 hour in serum-free DMEM to allow viral particles attachment. Following attachment, MARC-145 were covered with a solution of 2X MEM diluted in 0.5% agarose (1:1). Plates were incubated at 37°C, 5%$CO_2$ to allow plaques

formation. Infection was carried out for 5 days and plaques were revealed with 0.4% neutral red (Sigma-Aldrich).

### 2.3 Blood collection from animals.

All animal experimental practices and care were conducted according to the standards of the Canadian Council of Animal Care guidelines and approved by the University of Calgary Veterinary Sciences Animal Care Committee (#AC14-0031). Peripheral blood monocytes were collected from healthy Large White and Landrace cross 10- to 22 weeks old (15- to 60 kg) castrated male and female piglets. The animals were housed at the Veterinary Science Research Station (University of Calgary) in a housing unit maintained at 22°C ± 2°C with 40% humidity, light cycles consisted in 12 hours continuous light exposure followed by 12 hours of darkness. Swine were provided with solid black rubber mats, toys and small water pools for comfort and leisure time. Sleeping and resting patterns were monitored twice daily to ensure that there were no signs of stress. Piglets were fed twice per day with the antibiotic-free feed 16% Hog Grower (Hi-Pro Feeds), water was provided ad libitum. Upon arrival, piglets were given a single injection of EXCEDE© 100 for Swine (ceftiofur crystalline free acid; Zoetis, Parsippany, NJ) as prophylactic treatment and to prevent bacterial septicemic disease following transport and relocation stress. Blood collections began after a minimum of one-week post-arrival to ensure the acclimation of the animals. During blood collections, the animals were restrained by a trained handler with hog holder. Peripheral blood was drawn from the cranial vena cava (<60 kg) or the jugular vein (>60 kg) into 1.5 mL whole blood tubes with heparin (Thermo Fischer Scientific). To provide post-procedure analgesia, pigs were orally given NSAID meloxicam (0.4 mg/kg; Boehringer Ingleheim, Burlington, Ontario) immediately following collection. With a half-life of 2.3 hours, meloxicam

was cleared from the swine system before the following blood collection. Animals were kept until restraint became too challenging and were then euthanized.

### 2.4 Monocyte Isolation and macrophage differentiation.

Monocytes were obtained as described previously [230]. Briefly, collected blood was pooled and centrifuged for 20 minutes at 1200 x g, 4°C in a Heraeus Megafuge 16R (Thermo Fischer Scientific). The plasma was removed, and the buffy coat layer was collected into a new tube and diluted 1:1 with a filter-sterilized 0.9% NaCl solution. Five mL of a sterile solution of polysucrose and sodium diatrizoate gradient (Histopaque; Sigma-Aldrich) was added into each tube before mixing and centrifugation for 40 minutes at 1200 x g, 4°C. The monocytes, located at the opaque interphase were then collected, washed with 20 mL of sterile-filtered 2X Hank's Balanced Salt Solution (HBSS; Thermo Fischer Scientific) and centrifuged for 10 minutes at 500 x g, 4°C. Contaminating erythrocytes were removed by three hypotonic lysis cycles. These cycles consisted in the addition of 10 mL of sterile ice-cold double-distilled water for 30 seconds followed by the addition of 2X HBSS to restore tonicity and centrifugation for 10 minutes at 500 x g, 4°C. Monocytes were then resuspended in serum-free Iscove's modified Dubelcco's medium (IMDM; Thermo Fischer Scientific) supplemented with 100 IU/mL penicillin-streptomycin. Cells were counted using a hemocytometer and viability was assessed by the determination of the percentage of cells that excluded 0.1% trypan blue (Flow Laboratories). Monocyte purity was determined by Diff-Quick staining on cytospined samples (CytoSpin4 cytocentrifuge, Thermo Fischer Scientific). The cells were then plated in tissue-culture treated plates 6, 12, 24 and 96 well plate (Costar) or in LabTek chamber slides (Thermo Fischer Scientific) at the optimal concentration of 1.0 x$10^6$ cell/mL for two hours to allow attachment. Non-adherent cells were washed away with warm HBSS (37°C). Adherent cells were incubated for 7 days at 37°C, 5% $CO_2$ in IMDM

supplemented with 10% HI-pig serum (GE Healthcare) and 100 IU/mL penicillin-streptomycin (serum only media) or in IMDM supplemented with 25% L929 supernatant, 10% HI-pig serum (GE Healthcare) and 100 IU/mL penicillin-streptomycin (L929-supernatant supplemented media) to allow macrophage differentiation. Culture medium was changed every 2-3 days. Macrophage differentiation was monitored by microscopic morphological changes using Diff-Quick staining and by esterase assay at day 1, 4 and 7 after plating. At day 7, more than 95% of the monocytes differentiated in macrophages.

### 2.5 Tulathromycin treatment and PRRSV infection.

Seven days old BMDMΦs were incubated with tulathromycin (Draxxin; Zoetis) diluted in IMDM + 10% HI-pig serum at a concentration of 0.5 mg/mL or 1 mg/mL or with vehicle control (IMDM + 10% pig serum). Tulathromycin treatment was performed 1 hour before PRRSV infection. Macrophages were then infected with PRRSV or not infected (uninfected controls) and incubated for 1 h at 37°C, 5% $CO_2$ to allow virus attachment and entry. This time point was considered T=0. PRRSV was diluted in serum-free DMEM to reach a multiplicity of infection (m.o.i) of 0.1 or 0.5 depending on the experiment. Following virus attachment, culture media was replaced by pre-warmed IMDM supplemented with 10% HI-pig serum for all experimental groups. PRRSV infection was performed for a period ranging from 2 to 48 hours depending on the experiment. All functional assays contained at least four experimental groups: untreated and uninfected control; tulathromycin treated and uninfected (TUL; untreated and PRRSV infected (virus); Tulathromycin treated and PRRSV infected (TUL+virus) ± appropriate controls.

### 2.6 PRRSV attachment receptors staining.

PRRSV receptors expression levels by BMDMΦs in presence and absence of tulathromycin were assessed by immunofluorescence. Seven days old BMDMΦs cultivated in serum only or

L929 conditioned medium were treated with HBSS (control) or tulathromycin (1 mg/mL for 12 hours. Cells were then fixed in 4% paraformaldehyde in PBS for 15 minutes, washed 3 times in cold PBS and stained for 1 hour with an R-phycoerythrin (RPE) conjugated anti-CD163 antibody (Bio-Rad; product #MCA2311PE) and a fluorescein isothiocyanate (FITC) conjugated anti-CD169 antibody (Bio-Rad; product #MCA2316F) at a dilution of 1 to 500 and 1 to 250 respectively. Cells were washed 3 times in cold PBS and observed under Leica DMR fluorescent microscope. Slides were then randomly chosen and observed. Fluorescence ratio was calculated using the software ImageJ. Images were taken with a Retiga 2000x (Q imaging) camera and analyzed on ImageJ. To prevent any bias, slide labeling was covered with tape prior to microscopic observations.

### 2.7 Assessment of tulathromycin direct anti-viral effects.

To assess the potential anti-viral effects of tulathromycin, extracellular and intracellular viral particles counts were monitored with plaque titration assays. For extracellular counts, macrophages or MARC-145 cells were treated with tulathromycin and/or infected with PRRSV at the concentrations and m.o.i. described above and supernatants were harvested at 2, 12, 24 and 48 hours and incubated onto confluent MARC-145 cells for 1 hour (37°C). For intracellular counts, macrophages and MARC-145 were washed twice with warm (37°C) phosphate buffer saline (PBS; Sigma-Aldrich) and lysed with double distilled water exposure and thorough mixing. Cellular debris were removed by centrifugation at 10,000 x g for 30 minutes and supernatants were harvested and incubated onto a monolayer of MARC-145 cells for 1 hour (37°C). In both instances, the MARC-145 monolayer was overlaid with a solution of 0.5% agarose and 2X MEM (1:1) and incubated for 5 days at 37°C to allow plaque formation. At day 5, plaques were revealed and counted with the help of a 4% neutral red solution (Sigma-Aldrich). Viral foci in MARC-145 were

also revealed using the anti-PRRSV nucleocapsid antibody SR-30F. Briefly, MARC-145 cells were seeded in LabTek chamber slides, grown to 90% confluence and infected with PRRSV (m.o.i. 0.1) for 24h at 37°C, 5% $CO_2$. Cells were fixed in 80% acetone and stained with SR-30F diluted in cold PBS (1:500) for 45 minutes. MARC-145 nuclei were revealed using 4',6-diamidino-2-phenylindole (DAPI). Cells were washed 3 times with cold PBS and observed under a Leica DMR fluorescent microscope. Images were taken with a Retiga 2000x (Q imaging). PRRSV foci numbers and size were revealed by the SR-30F antibody (RTI, LLC). Fluorescence ratio was calculated using ImageJ.

### 2.8 Macrophage apoptosis (Cell death ELISA and Annexin V staining).

The pro-apoptotic effects of tulathromycin and PRRSV were assessed using a cell death detection ELISA kit (Roche) according to the manufacturer's instructions. Absorbance was measured using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA) set at 405nm. Seven days old macrophages were incubated with tulathromycin (1 mg/mL) for 1 hour and infected with PRRSV (m.o.i. of 0.5) for 2, 12 or 24h at 37°C, 5% $CO_2$. For all experiments, cells incubated with IMDM containing 10% HI-pig serum or staurosporine (1µM) were used as negative and positive controls respectively. Apoptosis was expressed as the absorbance ratios of the experimental groups versus absorbance from the negative control, arbitrarily set at 1.0 (100%). Apoptosis in individual cells was determined using Annexin V FLUOS staining kit (Roche). Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine (PS), a known marker of early apoptosis. Staining was performed as per manufacturer's instructions. BMDMΦs plated in LabTek chamber slides (1 x $10^6$ cells/mL) were treated with HBSS (control) or tulathromycin (1mg/mL) for 1 hour. Cells were then infected with PRRSV (m.o.i = 0.5) for 24 hours. Following incubation cells were washed in IMDM and resuspended in a 100 µL of annexin

V staining solution for 15 minutes in the dark at room temperature. Following incubation, cells were washed 2 times in HEPES and visualized using a Leica DMR fluorescent microscope. Images were taken with a Retiga 2000x (Q imaging) camera and analyzed on ImageJ.

### 2.9 Assessment of cytotoxicity.

Cytotoxicity of PRRSV and tulathromycin on porcine macrophages, was assessed using a commercially available cytotoxicity detection kit (LDH; Roche Applied Science; 11644793001). Briefly, differentiated macrophages were treated with vehicle medium alone (control) or with tulathromycin (1mg/mL) for 1 hours. Cells were then infected with PRRSV for 2, 12 or 24h (m.o.i. 0.1) or supplemented with control medium, a 1% Triton X 100 in media group was used as positive control. Supernatants were then collected and processed following manufacturer's instructions. The SpectraMax M2e microplate reader (Molecular Devices) was used to measure LDH concentrations in each sample at 492 nm. The assay quantified LDH released into the supernatant. Released 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. Therefore, colorimetric changes are directly proportional to the concentration of LDH in the sample. Necrosis was expressed as the absorbance ratios of the experimental cell lysates versus absorbance from controls, arbitrarily set at 1.0 (100%).

### 2.10 Macrophage differentiation and activity.

The effects of tulathromycin and PRRSV on macrophage differentiation and activation was determined via microscopic observations and cytokines quantification. Monocytes were seeded in LabTek chamber slides or on coverslips at $1x10^6$ cells/mL and incubated at 37°C, 5% $CO_2$ for 7 days to allow differentiation. At day 7, macrophages were treated with tulathromycin (0.5 or 1 mg/mL) for 1 hour and infected with the PRRSV (m.o.i. of 0.1) for 2, 4, 12 or 24h at 37°C, 5%

$CO_2$. Supernatants were collected and frozen at -80°C until processed and macrophages were stained with DiffQuick (Electron Microscopy sciences) and observed with the Nikon eclipse T300 microscope to assess morphological changes. Images were taken with a Retiga 2000x (Q imaging) camera on a Leica DMR fluorescent microscope and analyzed on ImageJ. Individual macrophage morphology was assessed and based on cytoplasm size macrophages were classified as "resting" or "fibroblast-like" morphology. At least 150 macrophages per group in 3 independent experiments were observed and classified. Supernatants were processed to evaluate CXCL-8 and interleukin-10 (IL-10) concentrations using the porcine CXCL-8 Quantikine enzyme-linked immunosorbent assay (ELISA; R&D systems P8000) and the IL-10 Quantikine ELISA (R&D systems P1000) respectively. Samples were processed as per manufacturer's instructions.

### 2.11 Mitochondrial reactive Oxygen Species (ROS) production.

Intracellular ROS production following tulathromycin treatment and/or PRRSV infection was monitored with the Oxiselect intracellular ROS assay kit (Cell BioLabs). Monocytes were seeded on 96 well plate at $1x10^6$ cells/mL and incubated for 7 days to allow differentiation. Non-adherent macrophages were washed away with warm PBS and remaining adherent macrophages were treated with tulathromycin (0.5 mg/mL) for 1 hour or left untreated (vehicle control). Macrophages were then infected with PRRSV for 24h (m.o.i of 0.5). Same treatments were performed on macrophage stimulated with lipopolysaccharide (LPS; 1μg/mL from E. coli O26:B6; Sigma-Aldrich). Subsequent to infection, macrophages were exposed to 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) a cell-permeable fluorogenic probe oxidized to highly fluorescent 2',7'-Dichlorodihydrofluorescein (DCF) by ROS for 1 hour. Fluorescence intensity, proportional to ROS levels within the cytosol was measured using a SpectraMax M2e microplate reader (Molecular Devices) reading at 480 nm (excitation) and 530 nm (emission).

## 2.12 Phagocytosis Assays.

Phagocytic capacity of BMDMΦs was assessed using non-opsonized zymosan particles and opsonized latex beads. Non-opsonized phagocytosis was monitored using fluorescently labeled *Saccharomyces cerevisiae* zymosan A particles (Texas Red; Sigma-Aldrich). Briefly, monocytes were seeded in Labtek chamber slides or on coverslips at $1x10^6$ cells/mL and incubated for 7 days to allow differentiation. Mature macrophages were washed twice with warm PBS to remove non-adherent cells and then treated with tulathromycin (1 mg/mL) for 1 hour or left untreated (vehicle control; IMDM 10% HI-pig serum). Subsequently, macrophages were infected for 2 or 12 hours (m.o.i of 0.5) or uninfected (uninfected control). Following infection, experimental groups were incubated with zymosan A particles diluted in control media to a final ratio of 10:1 (zymosan:cells) for 1 hour. After exposure, extracellular zymosan A particles were washed away with warm PBS (3 times) and the cells were fixed in ice-cold 80% acetone solution. Actin was stained with the Alexa Fluor 488 phalloidin antibody (Thermo Fischer Scientific) and the nucleus was revealed with DAPI (Thermo Fischer Scientific). Enumeration of intracellular zymosan was performed using a fluorescent microscope (Leica). Fc-mediated phagocytic index was measured using carboxylate-modified 3 µm diameter latex silica beads (Kisker Biotech) covalently coated with BSA and human IgG (Sigma-Aldrich). The beads were incubated with macrophages for 45 minutes at a 10:1 ratio (beads:cells). Following phagocytosis, extracellular beads were washed away with warm PBS (3 times) and the cells were stained with DiffQuick (Electron microscopy sciences) before microscopic observations. Macrophages containing one or more zymosan particles or latex beads were considered as ‘positive cells’, the phagocytic index was calculated as the ratio of positive macrophages versus total macrophages. A minimum of 150 cells per experimental group were counted. All pictures were taken using Leica DMR fluorescent

microscope with a Retiga 2000x (Q imaging) and analyzed on ImageJ. In order to prevent any counting bias slides labelings were covered with tape prior to microscopic observations. Slides were then randomly chosen and phagocytic cells numbered.

### 2.13 Statistical analysis.

All statistical analyses were made using Prism 5 software and are expressed as means $\pm$ standard error of the mean (SEM). For all data sets normality was tested. When data sets passed normality tests, comparisons where made using student's *t*-test or one-way ANOVA where appropriate. When data sets failed to pass normality, the non-parametric Kruskal-Wallis statistical test was performed. For every assay, a minimum of 3 separate, independent experiments were conducted with all experimental groups assayed in duplicates or triplicates. Statistical significance was established at $P < 0.05$.

# 3 RESULTS

## 3.1 Tulathromycin does not change PRRSV titers.

### 3.1.1 Blood monocyte-derived macrophages (BMDMΦs) are productively infected by PRRSV.

To date, most PRRSV *in vitro* studies use two cellular models, MARC-145 cells (African green monkey kidney epithelial cells) and porcine alveolar macrophages (PAMs). Despite being highly susceptible to PRRSV these two cell lines have major drawbacks. MARC-145 cells do not originate from pigs and are very distinct from PRRSV *in vivo* cellular targets. On the other hand, PRRSV primary target cells are difficult to isolate and the collection is stressful for the animals. Moreover, PAMs are functionally dependent on the age and environment of the animal [247]. As seen in the section number 1.1.2.2, monocyte-derived macrophages numbers rapidly increase during the first phase of the pulmonary inflammation. However, their permissivity to PRRSV and role during infection is still debated. In order to determine whether blood monocytes and BMDMΦs are susceptible to PRRSV, we isolated blood monocytes from healthy pigs and cultured them for a period of 7 days in medium supplemented with pig serum to mimic biological conditions. Over the cultivation period, we microscopically observed the cells to assess any morphological change. After plating, adherent monocytes exhibited a round shape morphology and were approximatively 10µm in diameter (figure 5A). Four days after plating, some cells exhibited a significant increase in cell size (around 20µm) associated with an increase in cytoplasmic vacuoles which is characteristic of macrophage morphology (figure 5B). At day 7, most cells displayed this macrophage-like morphology (figure 5C). Monocyte differentiation was also measured using non-specific esterase (NSE) staining. Macrophage express NSE to a much greater extent than monocytes. By day 7 more than 95% of cells were esterase-positive cells.

To determine whether blood monocytes and BMDMΦs were susceptible to PRRSV infection, 1-day old monocytes and 7-days old BMDMΦs were infected at a multiplicity of infection (m.o.i) of 0.1 for 2 to 48 hours. Subsequently, supernatants were harvested, and viral titers were calculated using plaque assay on MARC-145 cells. Blood monocytes were poorly susceptible to PRRSV compared to differentiated BMDMΦs at all times of the infection (figure 6). In our experimental model, PRRSV viral particles numbers increased by log1.69 (50-fold increase) in BMDMΦs and by log1.1 (13-fold increase) in blood monocytes between 2 and 48 hours post infection (p.i). In both cell types, PRRSV infection plateaued at 24h p.i. (figure 6). However, in our experimental model, we were not able to clearly measure the percentage of PRRSV-infected cells.

### 3.1.2 L929 supernatant increases PRRSV infectivity in BMDMΦs.

To optimize our macrophage differentiation protocol, we decided to cultivate isolated monocyte in an L929-conditioned medium. The murine L929 fibroblast cell line, known to secrete macrophage-stimulating factor (M-CSF) is widely used to induce macrophage differentiation from monocytes and prevent differentiation into dendritic cells [248]. Monocytes cultivated in L929-conditioned medium showed the same morphology as those cultivated in medium devoid of L929-factors. By day 7, more than 95% of cells were macrophages.

L929 cultivated monocytes and BMDMΦs were then infected by PRRSV at an m.o.i of 0.1 for 2 to 48 hours. Supernatants were harvested and processed as described previously to determine the number of infectious viral particles. As expected, virus infection in L929-cultivated BMDMΦs and, to a lesser extent, monocytes were productive (figure 7). Monocyte exposure to L929 supernatant did not change PRRSV viral counts from 2 to 24 hours post infection compared to viral counts from monocyte cultivated without L929 supernatant (figure 7). However, viral titers

were significantly higher at 48 hours p.i. compared to viral titers in monocytes cultivated without L929 supernatant (figure 7). In sharp contrast, we measured a significant increase in PRRSV infectious particles in BMDMΦs cultivated with L929-supernatants at all time of infection compared (except from the 8 hours p.i. time point) to BMDMΦs cultivated in medium supplemented with HI-pig serum alone (figure 7). In L929-cultivated BMDMΦs, PRRSV infection peaked at 24 h p.i. and declined afterward, whereas it continued to increase at 48 hours in L929-cultivated monocytes (figure 7). Based on these experimental data, we chose to use L929-cultivated BMDMΦs for functional experiments, unless stated otherwise.

### 3.1.3 Tulathromycin does not change PRRSV receptors expression in BMDMΦs.

Some macrolides such as tilmicosin and tylvalosin have been shown to possess direct anti-viral effects against PRRSV [239]–[241]. To determine if tulathromycin had direct antiviral effects, we firstly observed viral receptors expression in BMDMΦs following TUL treatment. To date, two major PRRSV receptors have been extensively studied (CD163 and CD169) and it is not entirely clear which one of these two receptors is essential for PRRSV infection (see section 1.2.3). Since L929-conditioned medium increases viral titers, we hypothesized that it might be due to an increase in cell permissivity resulting from an increase in PRRSV receptor expression.

To test this hypothesis, we cultivated monocytes in medium containing pig serum alone or in L929-conditioned medium for 7-days and then treated them with tulathromycin at 1 mg/mL for 12 hours. Tulathromycin concentration was chosen based on previous data collected in the laboratory. At this concentration and time, the drug exhibited immunomodulating properties in bovine macrophages without inducing apoptosis [228]. Cells were then stained with Texas Red-conjugated CD163 and FITC-labelled CD169 antibodies. BMDMΦs differentiated in medium devoid of L929 supernatant expressed both receptors. Approximatively 28% of cells expressed

CD163 and 89% of cells expressed CD169. Tulathromycin treatment did not significantly change the percentage of CD163 and CD169 positive cells (respectively 29% and 83% of positive cells) (figure 8A; upper panels; figure 8B). In contrast, BMDMΦs cultivation in L929 supernatant-supplemented medium was sufficient to significantly increase the number of CD163 positive cells (more than 90 % of BMDMΦs were CD163 positive versus less than 30% in pig serum-supplemented medium alone). In addition, following L929-supernantant exposure we were not able to detect any CD169 positive cells (figure 8A; lower panels; figure 8B). Tulathromcyin treatment following L929-cultivation did not have any impact on viral receptors expression in this experimental setting either. More than 90% cells were positive for CD163 but none for CD169 (figure 8A; lower panels; figure 8B).

### 3.1.4 Tulathromycin does not alter PRRSV viral counts

To further assess potential direct-antiviral effects of tulathromycin, we calculated virus titers in the presence and absence of the drug in BMDMΦs (figure 9A) or MARC-145 cells (figure 9B). To that extent, we pre-treated BMDMΦs or MARC-145 cells with tulathromycin at 1mg/mL for 1 hour and then infected them with PRRSV (m.o.i = 0.1) for 2 to 48 hours. Subsequently, extracellular (black plain lines) and intracellular (blue dashed lines) viral infectious viral particles were numbered via plaque assay. Tulathromycin did not change intracellular or extracellular viral titers in either of these cellular models (figure 9A and B). Consistently to what was observed previously, PRRSV infection was productive in BMDMΦs and peaked at 24 h p.i. before declining. In MARC-145, virus titers increased consistently throughout the course of the experiment. To confirm these results, MARC-145 cells were stained with FITC-conjugated anti-PRRSV nucleocapsid antibody SR30F antibody. Size and numbers of viral foci were calculated in the presence or absence of tulathromycin (figure 9C). We did not observe significant changes in

either of these parameters with tulathromycin pre-treatment compared to virus infection alone (figure 9C and D). Taken together, these data indicate that any change in macrophage functions following tulathromycin treatment is not the result of a decrease in viral load but rather the result of independent immunomodulating properties of the drug.

**Figure 5. Peripheral blood monocytes can be differentiated *in vitro***. Cells were cultured for (A) 1 (B) 4 and (C) 7-days following seeding onto plastic 24 and 48 well plate or cover slides. (C) Cells took on macrophage-like morphology by day 7, forming large cells with extensive cytoplasm and dendritic projections at the cell surface. BMDMΦs cultures were fixed and stained using Diff-Quick stain to examine cytosolic and nuclear morphologies. Bar = 100 μm, bars within the insert = 20μm.

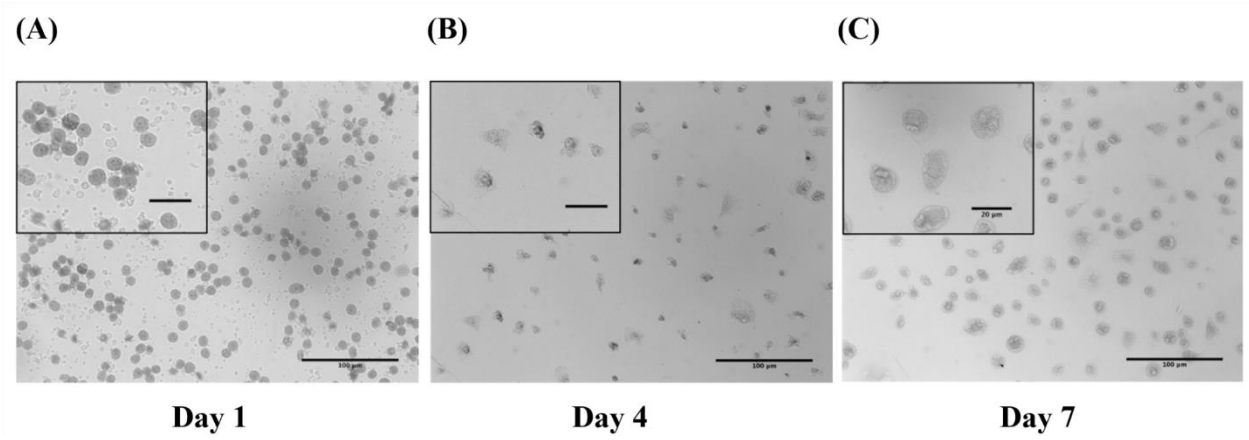

(A)
(B)
(C)
Day 1
Day 4
Day 7

**Figure 6. PRRSV efficiently infects macrophages but not monocytes**. To determine if PRRSV productively infects monocytes and BMDMΦs, monocytes and differentiated BMDMΦs were infected at an m.o.i. of 0.1 and incubated for the indicated times at 37 °C; 5% $CO_2$. Supernatants were harvested at the indicated time points and titrated by plaque assay on confluent MARC-145 cells. Data are representative of at least 3 independent experiments. For each time point, biological triplicates were titrated in technical duplicates. Data are expressed as mean ± SEM; n = 10. * denotes $p<0.05$ between monocytes and macrophages at each time point.

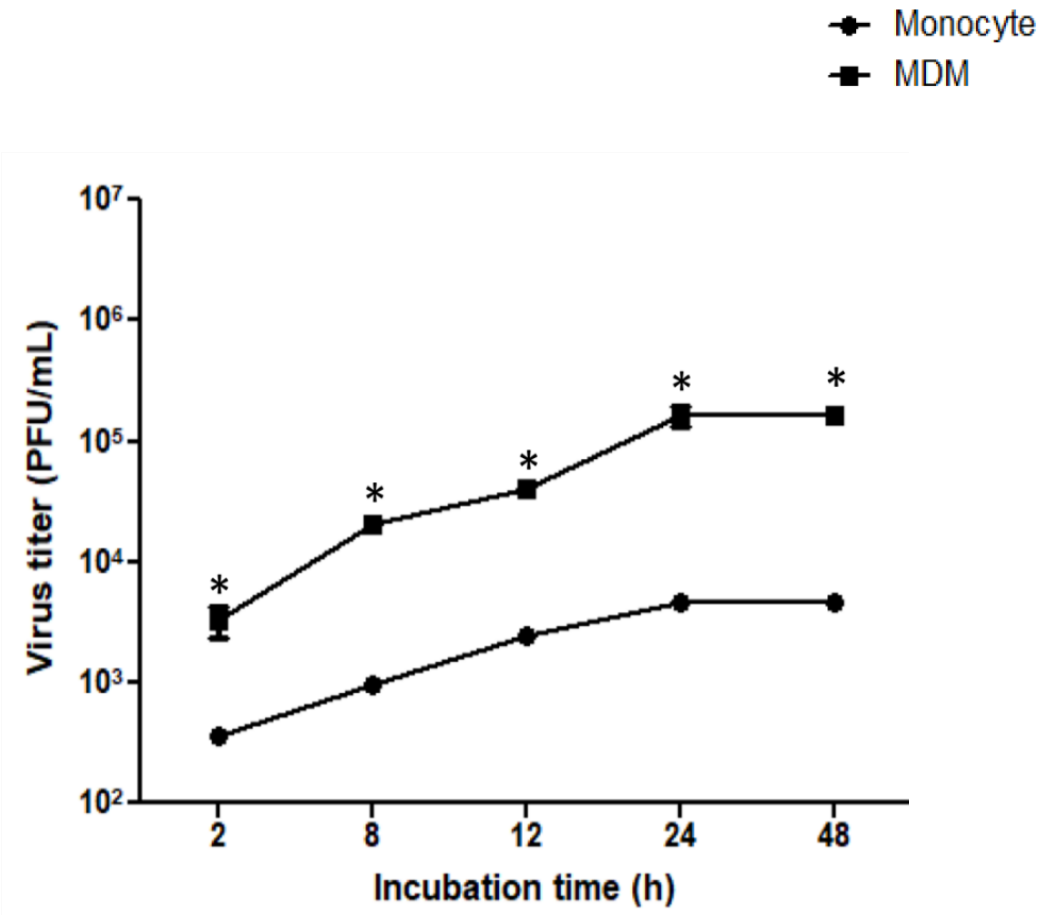

Monocyte
MDM
Virus titer (PFU/mL)
10⁷
10⁶
10⁵
10⁴
10³
10²
*
*
*
*
*
2
8
12
24
48
Incubation time (h)

**Figure 7. L929-differentiated BMDMΦs are more permissive to PRRSV than monocytes**. Monocytes were cultured for 7-days in the presence or absence of L929 supernatants and infected with PRRSV at an m.o.i. of 0.1. Supernatants were harvested at the indicated time points post-infection and titrated via plaque assay on confluent MARC-145 cells. For each time point, biological triplicates were titrated in technical duplicates. Data are expressed as mean ± SEM. n = 10. * denotes $p<0.05$ between monocytes and macrophages respectively cultivated with (blue lines) or without L929 supernatant (black lines).

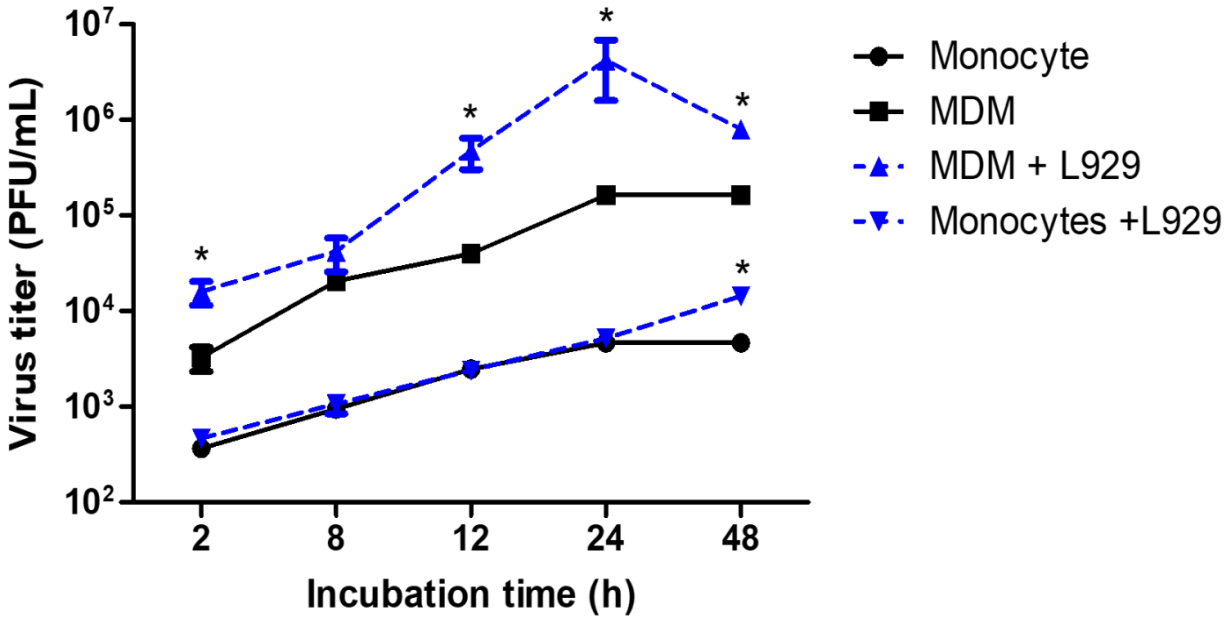
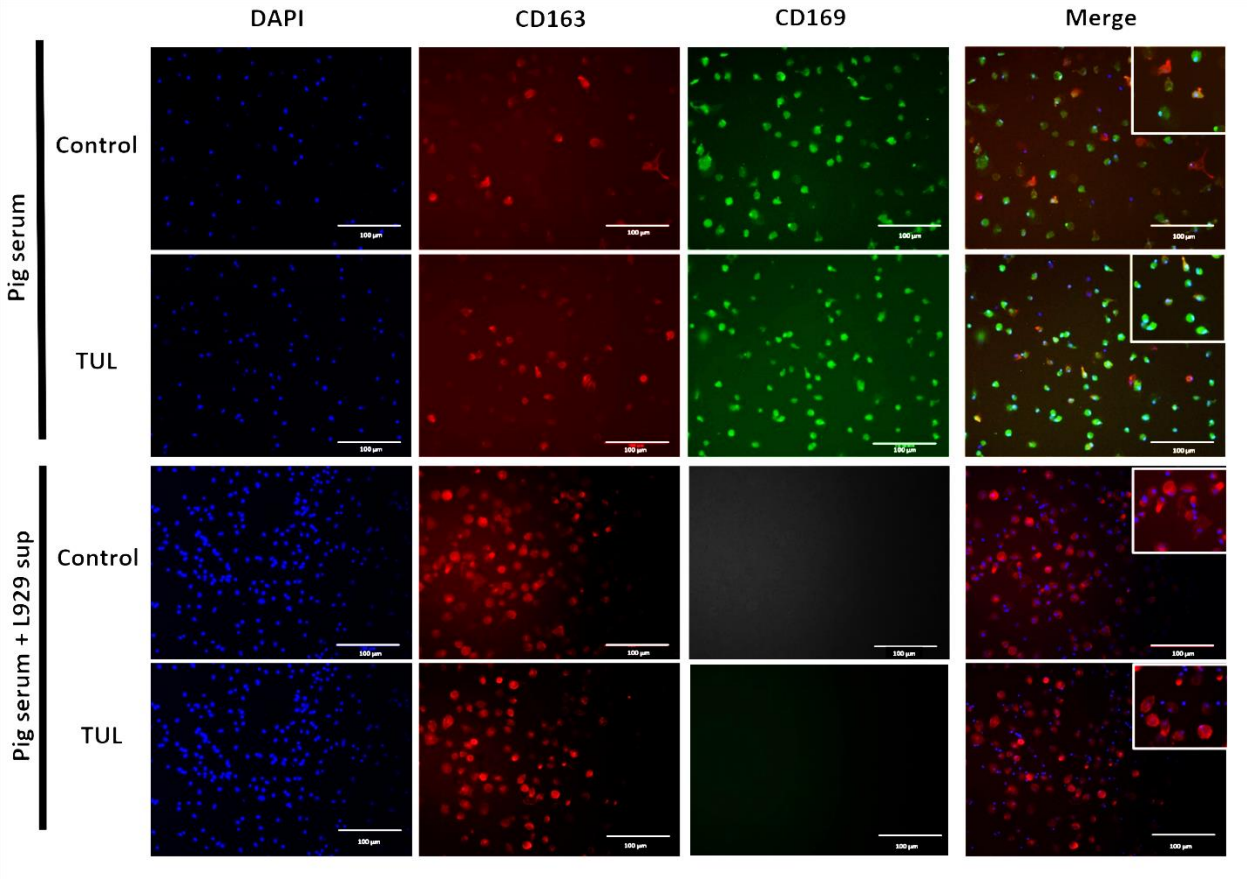

Virus titer (PFU/mL)
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
2
8
12
24
48
Incubation time (h)
*
Monocyte
MDM
MDM + L929
Monocytes +L929

**Figure 8. Tulathromycin does not change PRRSV receptor expression**. Seven days old BMDMΦs cultivated with or without L929 supernatant were treated with HBSS (control) or tulathromycin (TUL; 1mg/mL) for 12 hours at 37°C and then stained with anti-CD163 (red) and anti-CD169 (green) antibodies. (A) Microscopic fluorescent images of BMDMΦs stained for CD163 and CD169. Pictures are representative of 3 independent experiments. Bar = 100µm. (B) Percentage of BMDMΦs cultivated with or without L929 supernatant expressing CD163 and CD169. A total of 100 cells/group from 3 independent experiments were counted.

**(A)**


DAPI
CD163
CD169
Merge
Pig serum
Control
TUL
Pig serum + L929 sup
Control
TUL

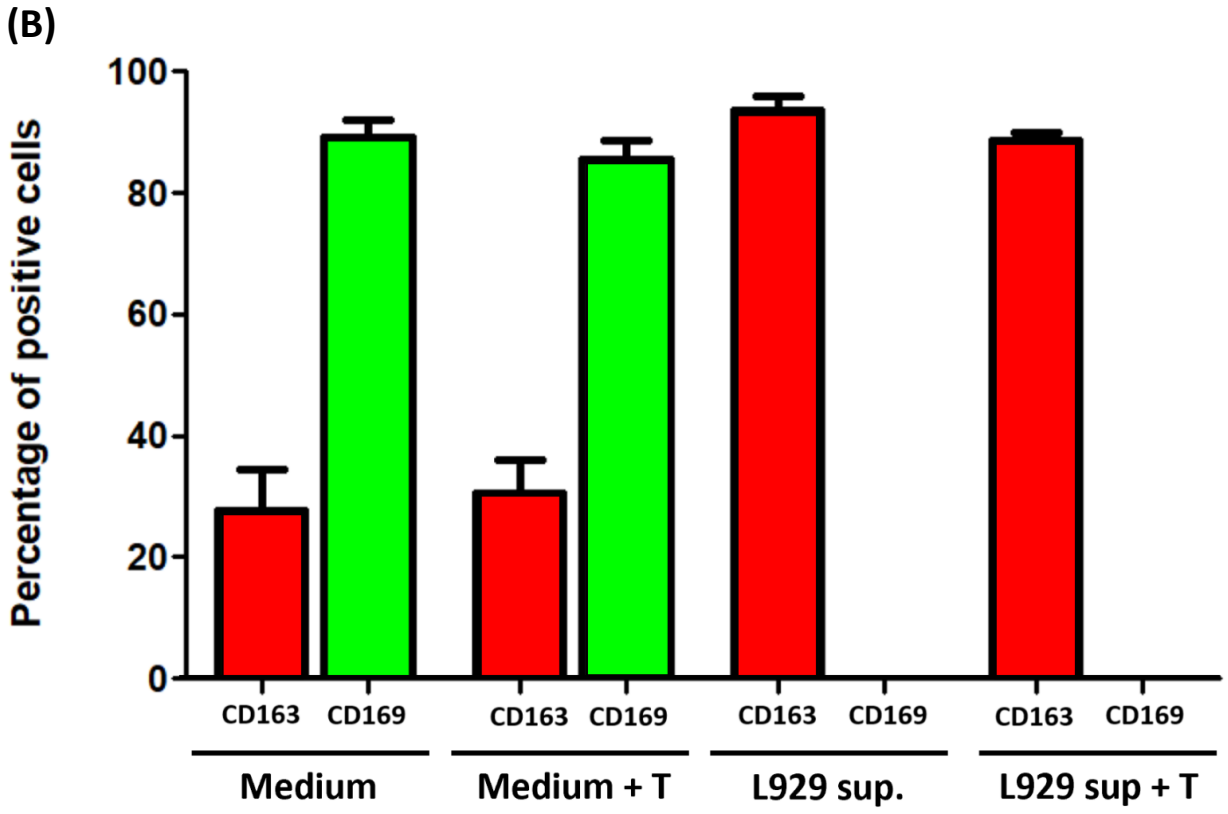
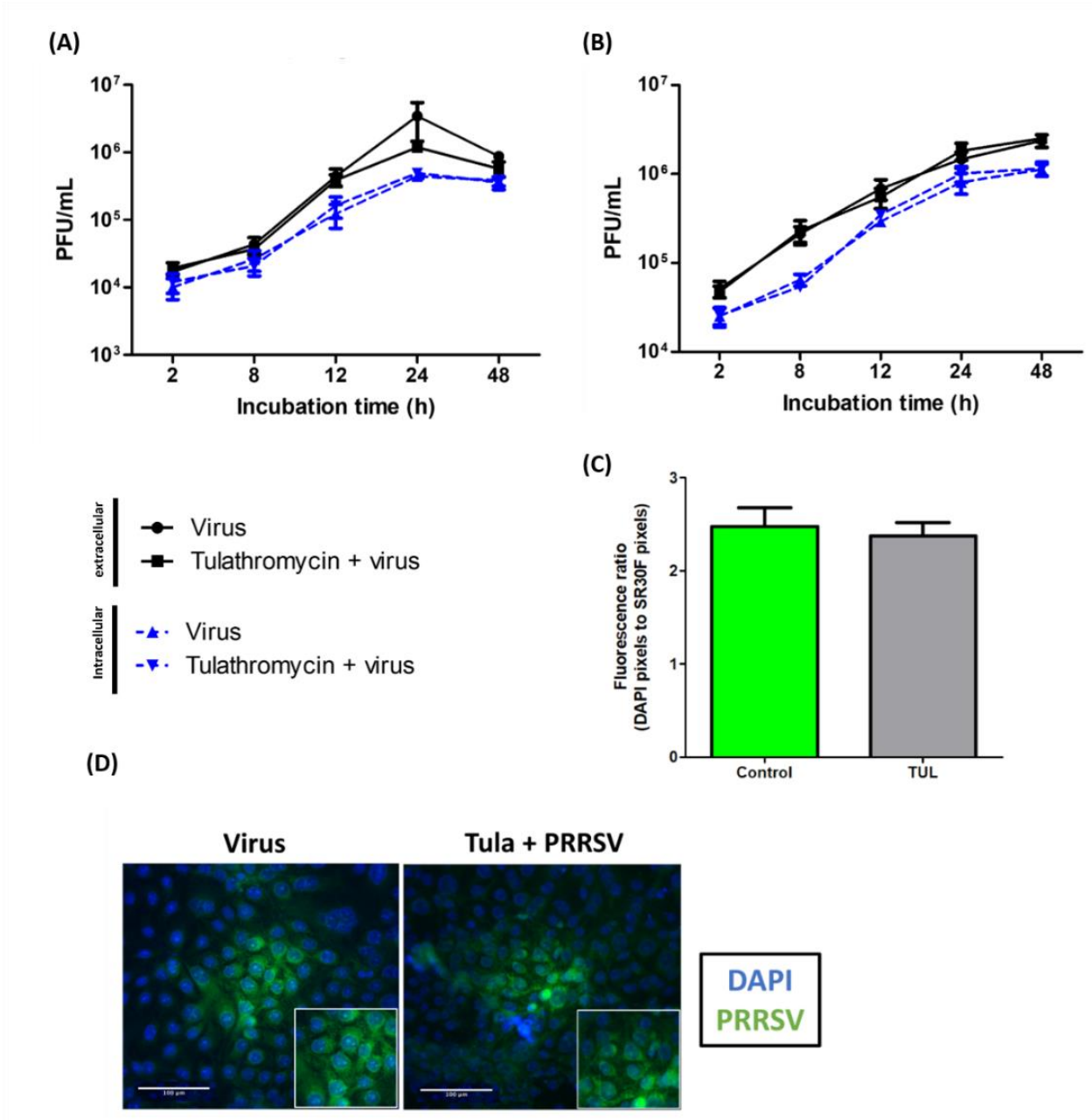

(B)
Percentage of positive cells
100
80
60
40
20
0
CD163
CD169
CD163
CD169
CD163
CD169
CD163
CD169
Medium
Medium + T
L929 sup.
L929 sup + T

**Figure 9. Tulathromycin does not alter PRRSV viral counts**. Intracellular and extracellular infectious viral particles were numbered in (A) BMDMΦs and in (B) MARC-145 using plaque assays. MARC-145 cells were incubated with cell lysates (intracellular viral particles; blue dashed line) or supernatants (extracellular viral particles; black plain line) of (A) BMDMΦs or (B) MARC-145 pre-treated with TUL (1mg/mL) for 1h and then infected with PRRSV at an m.o.i of 0.1 for the indicated time points. (C-D) PRRSV-infected MARC-145 were stained at 24 hours p.i with a FITC-conjugated anti-PRRSV nucleocapsid SR30F antibody to allow the observation of PRRSV foci. MARC-145 nuclei were revealed using DAPI. (C) Fluorescence ratio (DAPI/SR30F) in double stained MARC-145 was calculated using the image J software. n = 4. (D) Representative microscopic images of a PRRSV foci (green) in MARC-145 infected by PRRSV at 24h post-infection. MARC-145 nuclei are stained in blue (DAPI). Pictures are representative of 4 independent experiments Bar = 100µm.


(A)
PFU/mL
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
2
8
12
24
48
Incubation time (h)
(B)
PFU/mL
$10^7$
$10^6$
$10^5$
$10^4$
2
8
12
24
48
Incubation time (h)
extracellular
Virus
Tulathromycin + virus
Intracellular
Virus
Tulathromycin + virus
(C)
Fluorescence ratio
(DAPI pixels to SR30F pixels)
3
2
1
0
Control
TUL
(D)
Virus
Tula + PRRSV
DAPI
PRRSV

## 3.2 Immunomodulatory effects of tulathromycin *in vitro*.

### 3.2.1 Tulathromycin and PRRSV act synergistically to induce BMDMΦs apoptosis.

Given that both PRRSV and tulathromycin have been shown to be pro-apoptotic *in vitro* (see sections 1.2.5.3 and 1.3.2), we wanted to determine whether these two components would synergize to induce apoptosis. Detection of mono-and oligonucleosomes using cell death ELISA revealed that tulathromycin (1 mg/mL) induced apoptosis after 24 h of incubation but not after 2 h or 12 h (figure 10). Consistently, PRRSV (m.o.i = 0.5) was found to induce apoptosis after 24 hours (figure 10). Staurosporine (STS), a positive pro-apoptotic control, significantly induced apoptosis compared to control at 2, 12 and 24 hours post-treatment (figure 10). Subsequently, we measured apoptotic levels of cells pre-treated with tulathromycin (1mg/mL; 1h) and inoculated with PRRSV (m.o.i = 0.5) for 24 hours. When combined, the drug and the virus synergized to induce BMDMΦs apoptosis compared to treatment and infection alone (figure 11A). To confirm cell death ELISA data, we stained our cells with annexin V, a phospholipid-binding protein with high affinity for phosphatidylserine (PS) is a known marker of early apoptosis. Annexin V staining showed similar results. At 24 hours, both tulathromycin and PRRSV significantly induced apoptosis compared to control values (4 times fold increase vs. control) (figure 11B and C). Fluorescence ratios of cells treated with tulathromycin and infected were almost 8 times higher than control and 2 times higher compared to tulathromycin treatment and PRRSV infection alone (figure 11B and C). Considering these results and to limit the impact of tulathromycin and PRRSV-induced apoptosis on macrophage numbers and functions, we treated our cells with tulathromycin at a concentration of 0.5 mg/mL and decreased PRRSV m.o.i from 0.5 to 0.1. If the cells were treated at a concentration of 1mg/mL, functional analysis were performed before 12 hours of incubation.

### 3.2.2 Tulathromycin prevents PRRSV-induced early necrosis.

In addition to being pro-apoptotic, PRRSV has been shown to induce necrosis (see section 1.2.5.3). To investigate if the virus was pro-necrotic in our experimental model, we measured lactate dehydrogenase (LDH) levels following tulathromycin treatment (1mg/mL) and/or PRRSV infection (m.o.i = 0.5) for 2 to 24 hours (figure 12). Tulathromycin treatment did not induce necrosis throughout the course of the experiment (figure 12). Consistently, PRRSV infection did not increase LDH levels at 2 hours post-infection. However, PRRSV-induced necrosis was observed after 12 and 24 hours of incubation (figure 12). Importantly, tulathromycin pre-treatment significantly reduced PRRSV cell necrosis at 12 hours. This protective effect was lost at 24 hours (figure 12). 0.1% Triton-X (Trit-X), used as a pro-necrotic positive control, induced necrosis at all time points compared to control values (figure 12).

### 3.2.3 Tulathromycin inhibits PRRSV-induced BMDMΦs pro-inflammatory signaling.

#### *3.2.3.1 Tulathromycin prevents PRRSV-induced morphological changes in BMDMΦs.*

Considering that L929 supernatant might contain cytokines other than M-CSF (such as IL-10 and IL-4) that may induce macrophage polarization, we decided to grow our cells in medium containing only pig serum when investigating on macrophage pro-inflammatory signaling. Consequently, our differentiated macrophages were not polarized or activated prior to tulathromycin and/or PRRSV infection.

Microscopic observations of infected macrophages showed that some cells exhibited a strong change in morphology compared to resting cells (figure 13A). While most resting cells were circular, some PRRSV infected cells exhibited a fibroblast-like morphology with a thin elongated cytoplasm and numerous dendritic projections (figure 13A). We quantified the number of cells exhibiting this morphology after PRRSV exposure for 12 hours (m.o.i. = 0.1) (figure 13B). In non-

infected cells (control and TUL), less than 20% of cells exhibited this change in morphology, this number was 3.5 times higher when cells were exposed to the virus (nearly 60%). Tulathromycin pre-treatment reduced this number by half – around 30% - (figure 13B). Since macrophage morphology and functions are closely correlated, we hypothesized that PRRSV-induced morphological changes were associated with a change in macrophage activation.

*3.2.3.2* Tulathromycin inhibits PRRSV-induced CXCL-8 secretion.

To test whether PRRSV infection and TUL treatment had an impact on macrophage activation and or functions, we measured the production of CXCL-8 (a potent pro-inflammatory cytokine) by macrophages. Briefly, BMDMΦs were treated with HBSS (control) or pre-treated with tulathromycin (0.5 mg/mL) for 1 hour (TUL) and then infected with PRRSV (m.o.i= 0.1; virus and TUL+virus) for 24 hours. A 6 times fold increase of CXCL-8 secretion by PRRSV-infected cells was observed at 24 hours p.i. compared to control and tulathromycin values (figure 14). PRRSV-induced CXCL-8 secretion was significantly inhibited when macrophages were pre-treated with tulathromycin, but cytokine levels did not return to control values (figure 14). LPS, a known CXCL-8 inducer was used as a positive control (3µM) (figure 14).

*3.2.3.3* Tulathromycin inhibits mitochondrial ROS production.

We then measured the production of mitochondrial ROS, a hallmark of pathological oxidative damage. BMDMΦs were treated and infected as described above (section 3.2.3.2), Similarly to what we observed with CXCL-8, PRRSV significantly increased intracellular ROS production in resting cells compared to control (figure 15). In contrast, tulathromycin treatment did not result in intracellular ROS production. Moreover, tulathromycin pre-treatment was found to significantly inhibit PRRSV-induced mitochondrial ROS (figure 15). The same trends were observed in LPS activated cells (figure 15).

*3.2.3.4* Tulathromycin prevents PRRSV inhibition of IL-10.

As our results indicated that tulathromycin inhibits macrophage pro-inflammatory signaling, another set of experiment assessed the effects of the drug on IL-10, a cytokine with potent anti-inflammatory properties. BMDMΦs were treated and infected as described above (see section 3.2.3.2), IL-10 levels were measured at 2, 12 and 24 hours. In our experimental model, resting macrophages produced significant levels of IL-10 (around 300 pg/mL at 2 and 12 hours post-infection) (figure 16). IL-10 levels did not significantly change when the cells were treated with tulathromycin alone. However, PRRSV-infected cells secreted significantly less IL-10 compared to control cells at 2 and 12 hours p.i. (figure 16). PRRSV-induced IL-10 inhibition was prevented when the cells were pre-treated with tulathromycin at 2 and 12 hours post infection. All of these effects were lost at 24 hours post infection (figure 16).

### 3.2.4 Tulathromycin restores non-opsnonized and opsonized phagocytosis of infected BMDMΦs.

At present, the mechanisms resulting in the increase of secondary infections during PRRSV infections remain largely unknown. However, decreased phagocytic capacities of macrophages could play a significant role in this process [187]–[191]. To determine if the NSVL-98-7895 PRRSV strain had an impact on phagocytosis by BMDMΦs, we pre-treated the cells with tulathromycin (1mg/mL) for 1 hour and then infected them with the virus at an m.o.i of 0.5 for 2 or 12 hours. Following infection, we exposed the cells to either fluorescently labeled Texas-red non-opsonized zymosan particles (figure 17A) or IgG-coated latex beads (figure 18A) at a 10:1 ratio (beads/zymosan:cell) to examine non-opsonized and opsonized phagocytosis respectively. BMDMΦs were incubated with zymosan particles for 1 hour or with latex beads for 45 minutes

(figure 17A and 18A). To ensure removal of non-phagocytosed particles, cells were washed 3 times with PBS.

In both experimental settings tulathromycin treatment alone had no impact on BMDMΦs phagocytosis. In contrast, PRRSV infection was sufficient to significantly decrease the number of phagocytosing cells (figure 17 and 18). The percentage of BMDMΦs capable of phagocytosing non-opsonized zymosan particles dropped by 20% compared to control values (figure 17B). Similarly, the percentage of IgG-phagocytosing cells decreased by 14% after infection (figure 18B). This inhibition was prevented when the cells were pre-treated with tulathromycin (figure 17B and 18B). To further assess the phagocytic potential of macrophages, we calculated the number of phagocytosed particles per phagocytosing BMDMΦs. During phagocytosis, macrophages can engulf multiple antigens at the same time [7], [10]. To differentiate between low basal phagocytosis and active phagocytosis, we arbitrarily determined that BMDMΦs that engulfed more than 5 particles had a “high phagocytic” potential, whereas BMDMΦs that engulfed less than 5 particles had a “low phagocytic” potential. Similarly, to what we observed before, tulathromycin treatment did not change the number of “highly” phagocytic BMDMΦs compared to control. However, PRRSV infection significantly reduced the number of “highly” phagocytic macrophages compared to control and tulathromycin (TUL) (figure 17C and 18C). More precisely, PRRSV decreased the number of highly phagocytic macrophages by 18% compared to control cells when non-opsonized phagocytosis was measured (figure 17C). This number was decreased by 19% during opsonized phagocytosis experiments (figure 18C). Tulathromycin pre-treatment was sufficient to prevent phagocytosis inhibition by the virus in both experimental conditions (figure 17 and 18). Finally, we observed that PRRSV inhibited BMDMΦs opsonized phagocytosis as early as 2 hours post-infection (figure 19).

**Figure 10. Tulathromycin and PRRSV induce BMDMΦs apoptosis in a time-dependent manner**. Levels of apoptotic BMDMΦs treated with HBSS (control) or tulathromycin (1mg/mL) for 1 hour and then infected with PRRSV (m.o.i = 0.5) for the indicated time points were measured using cell death ELISA. Staurosporine (STS) served as positive pro-apoptotic control. Control denotes untreated and uninfected cells; TUL denotes tulathromycin-treated cells; Virus denotes PRRSV-infected cells; TUL+Virus denotes tulathromycin-treated and PRRSV-infected cells; STS denotes staurosporine-treated cells. Values are ratios versus control. Mean values ± SEM n=4-5/group. # = $P<0.05$ vs. control; * = $P<0.05$ vs tulathromycin; Δ = $P<0.05$ vs virus.

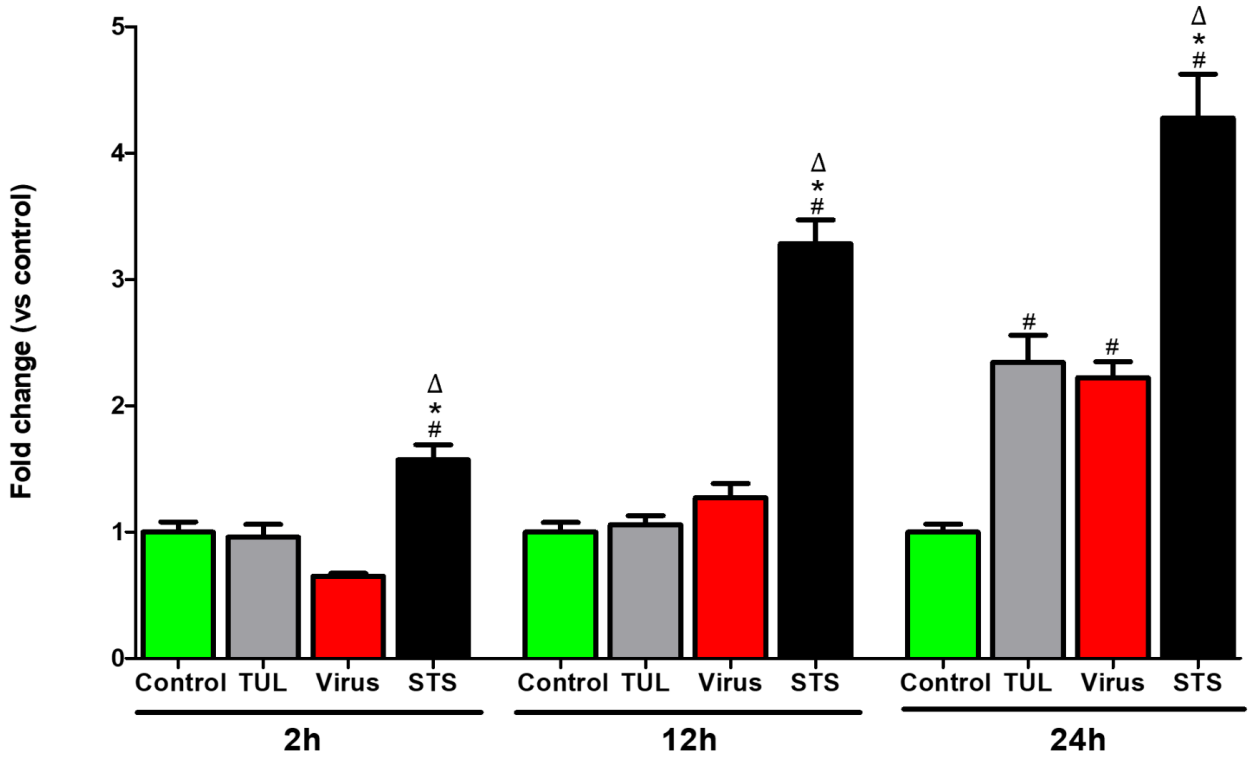

Fold change (vs control)
0
1
2
3
4
5
Δ
*
#
Control
TUL
Virus
STS
2h
12h
24h

**Figure 11. Tulathromycin and PRRSV act synergistically to induce BMDMΦs apoptosis**. Levels of apoptotic BMDMΦs following HBSS (control) or tulathromycin (1mg/mL; 1 hour; TUL) treatment and PRRSV infection (m.o.i = 0.5; 24 hours; virus and TUL+virus) were measured using (A) cell death ELISA and (B-C) Annexin V FLUOS staining. Staurosporine (STS) served as positive pro-apoptotic control. Values are expressed as (A) absorbance and (B) fluorescence ratio versus control. (A-B) Data represent mean ± SEM. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Δ = $P<0.05$ vs virus. (A) n = 4/group, (B) n = 5-7/group. (C) Pictures are representatives of 3 independent experiments.

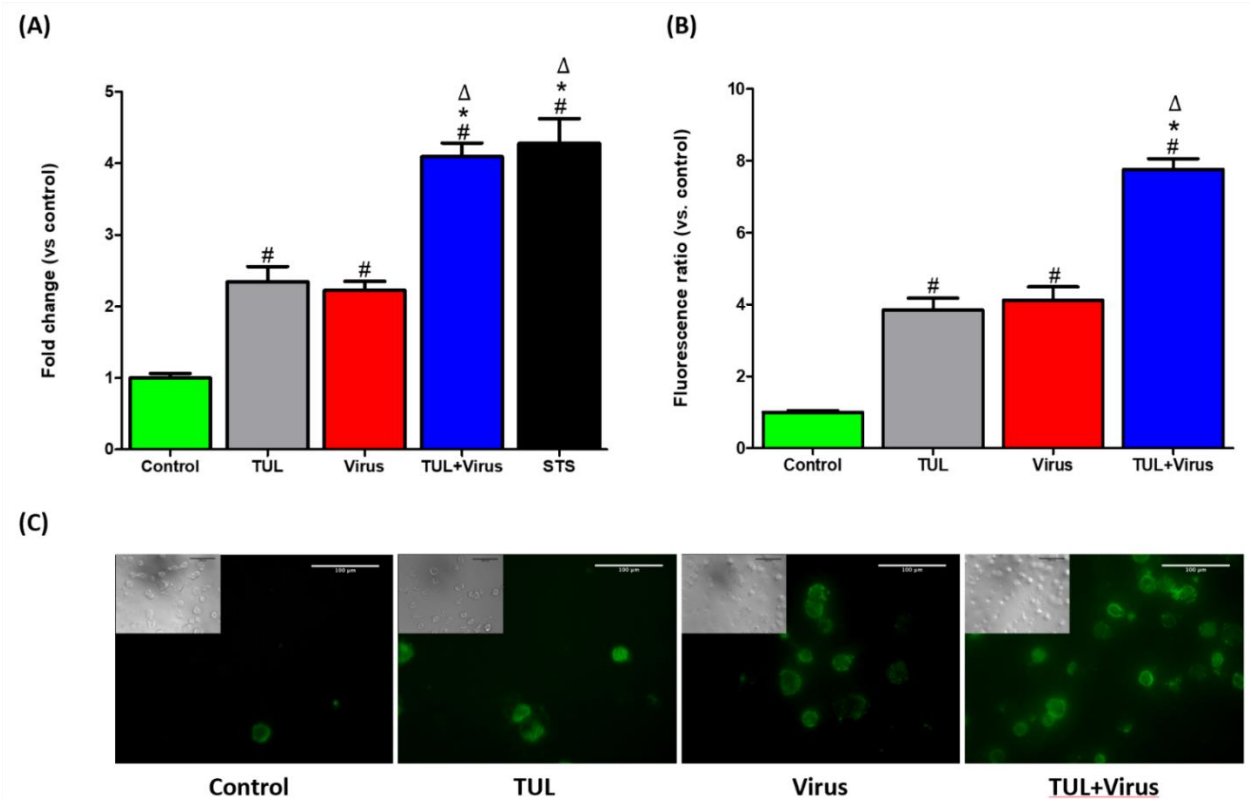

(A)
Fold change (vs control)
0
1
2
3
4
5
#
#
Δ
*
#
Δ
*
#
Control
TUL
Virus
TUL+Virus
STS
(B)
Fluorescence ratio (vs. control)
0
2
4
6
8
10
#
#
Δ
*
#
Control
TUL
Virus
TUL+Virus
(C)
Control
TUL
Virus
TUL+Virus

**Figure 12. Tulathromycin prevents early PRRSV-induced necrosis**. Levels of necrotic macrophages were measured using lactate dehydrogenase (LDH) assay. Macrophages were incubated with HBSS, (control) or treated with tulathromycin (1mg/mL; TUL) for 1 hour and then infected with PRRSV (m.o.i = 0.5; virus and TUL+virus) for the indicated time points. Cell supernatants were collected and LDH protein levels were quantified. Triton X served as positive pro-necrotic control. Values are expressed as fold change versus control. Data represent mean ± SEM, n=5/group. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Ω = $P<0.05$ vs tulathromycin + virus.

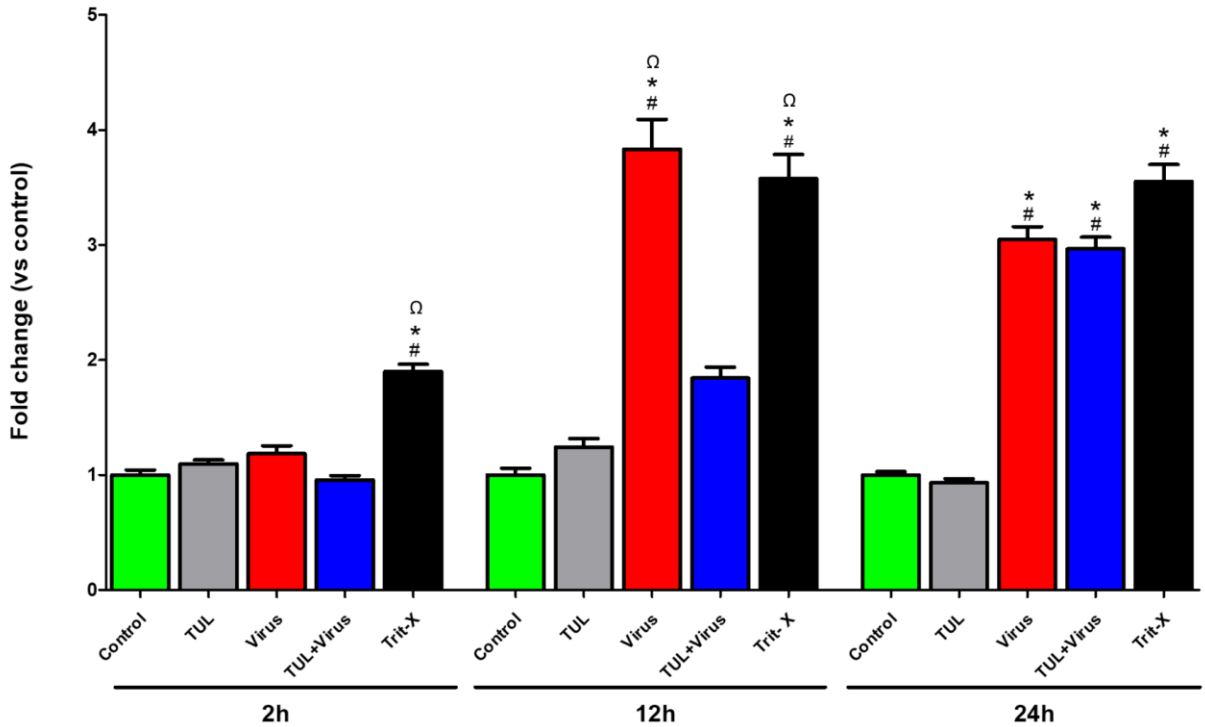

Fold change (vs control)
0
1
2
3
4
5
Control
TUL
Virus
TUL+Virus
Trit-X
2h
12h
24h
Ω
*
#

**Figure 13. Tulathromycin prevents PRRSV-induced morphological changes of BMDMΦs.** To assess any morphological changes following PRRSV infection, 7-days-old macrophages were treated with HBSS (control) or tulathromycin (0.5 mg/mL; TUL) for 1h and infected with PRRSV at an m.o.i. of 0.1 for 12 hours (Virus and TUL+ Virus). (A) 7-day-old MDMΦs cultures were fixed and stained using Diff-Quik stain to examine cytosolic and nuclear morphology. Representative microscopic images of 5 independent experiments Bar = 100µm. (B) Percentage of macrophage exhibiting morphological changes. At least 150 macrophages were counted for each experimental group. Data represent mean ± SEM, n=5. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Ω = $P<0.05$ vs tulathromycin + virus.

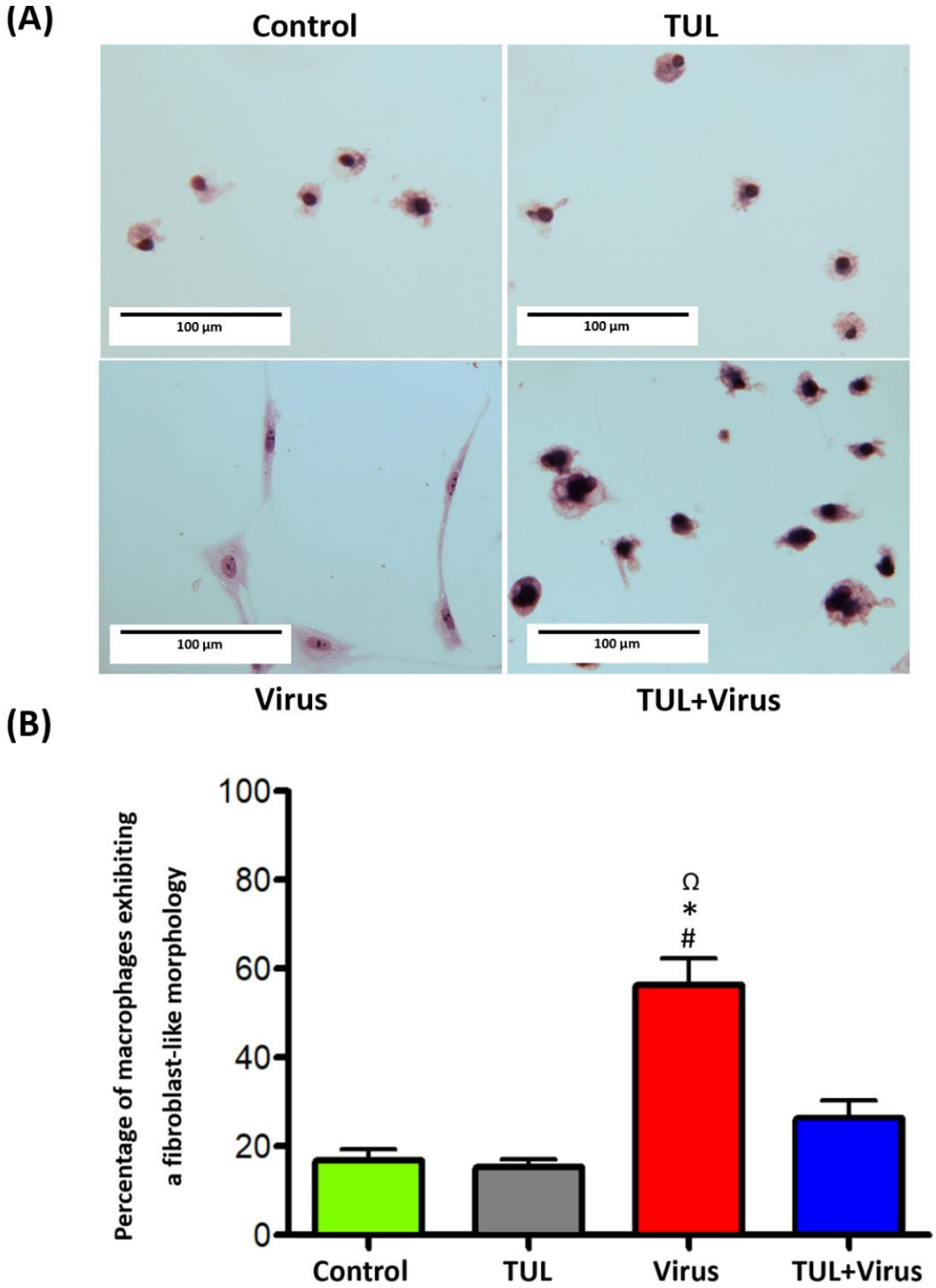

(A)
Control
TUL
100 µm
100 µm
100 µm
100 µm
Virus
TUL+Virus
(B)
Percentage of macrophages exhibiting a fibroblast-like morphology
100
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL+Virus

**Figure 14. Tulathromycin inhibits CXCL-8 secretion**. Levels of secreted CXCL-8 by 7-days old BMDMΦs following HBSS (control) or TUL treatment (0.5mg/mL; TUL) for 1 hour and PRRSV infection (m.o.i = 0.1; virus and TUL+virus) for 24 hours were measured using CXCL-8 ELISA. Lipopolysaccharides (LPS; 1µg/mL) served as a positive control. Mean ± SEM, n = 4 per group. # = P<0.05 versus control; * = P<0.05 vs tulathromycin; Ω = P<0.05 vs virus.

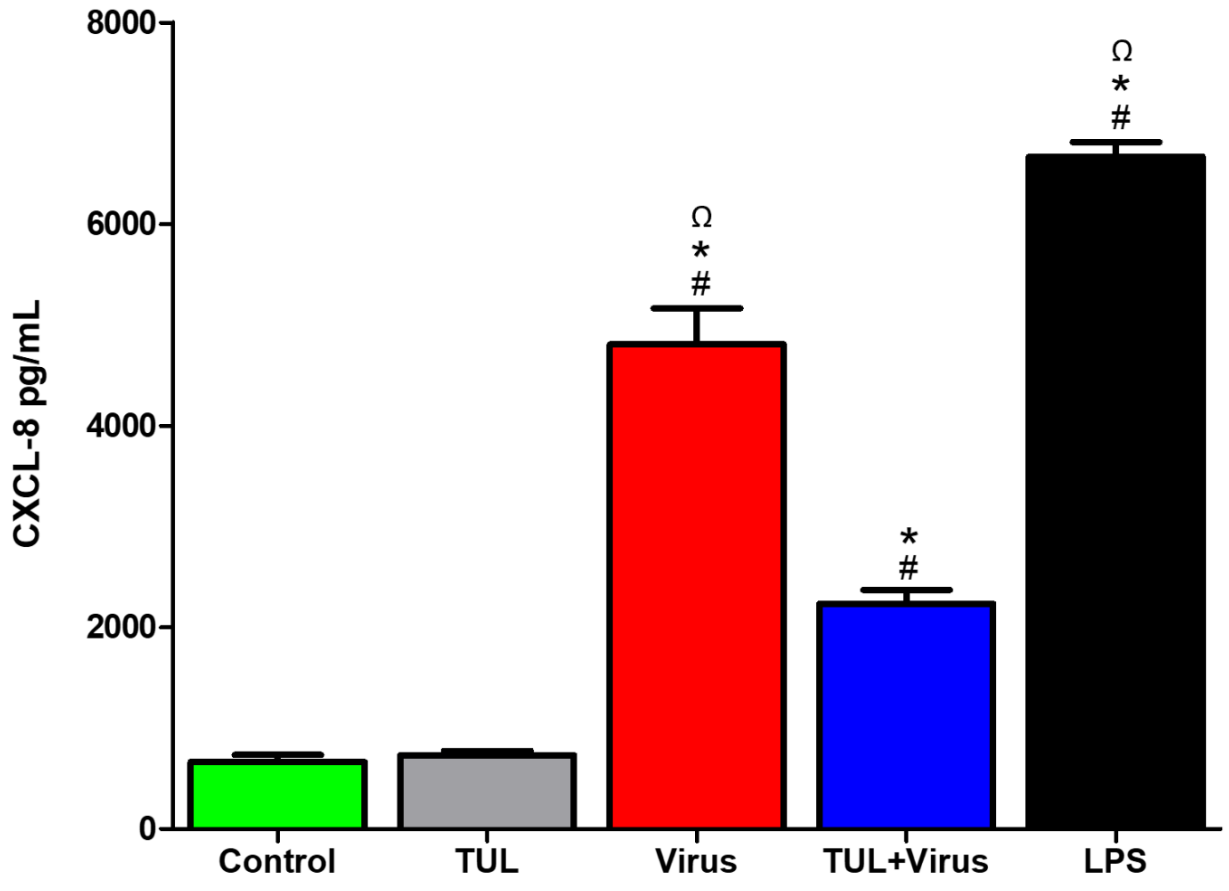

8000
6000
4000
2000
0
CXCL-8 pg/mL
Ω
*
#
Ω
*
#
*
#
Control
TUL
Virus
TUL+Virus
LPS

**Figure 15. Tulathromycin inhibits mitochondrial reactive oxygen species production**. Levels of intracellular ROS in 7-days old BMDMΦs were measured following HBSS treatment (control) or tulathromycin treatment (0.5mg/mL; TUL) for 1 hour and PRRSV infection (m.o.i. = 0.1; virus and TUL+virus) for 24 hours. Lipopolysaccharides (LPS; 1µg/mL) served as a positive control. Mean ± SEM, n=4/group. # = P<0.05 versus control; * = P<0.05 vs tulathromycin; Ω = P<0.05 vs tulathromycin + virus.

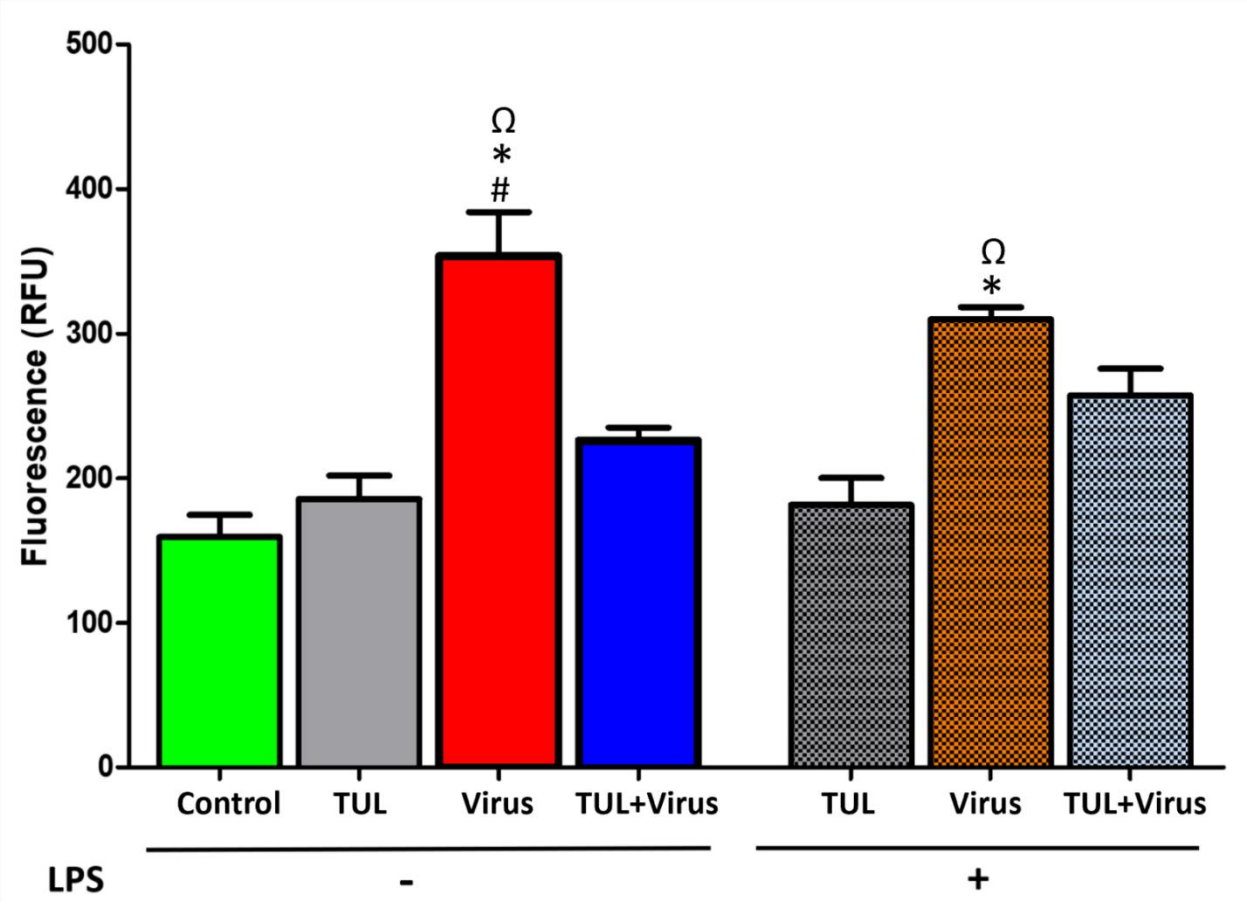

500
400
300
200
100
0
Fluorescence (RFU)
Ω
*
#
Ω
*
Control
TUL
Virus
TUL+Virus
TUL
Virus
TUL+Virus
LPS
-
+

**Figure 16. Tulathromycin prevents PRRSV-inhibition of IL-10 secretion in resting cells**. Levels of secreted interleukin-10 by 7-days old BMDMΦs were measured following HBSS (control) or TUL treatment (0.5mg/mL; TUL) and PRRSV infection (m.o.i. = 0.1, Virus and TUL+Virus) for the indicated time points. Mean ± SEM, n=3. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Δ = $P<0.05$ vs virus.

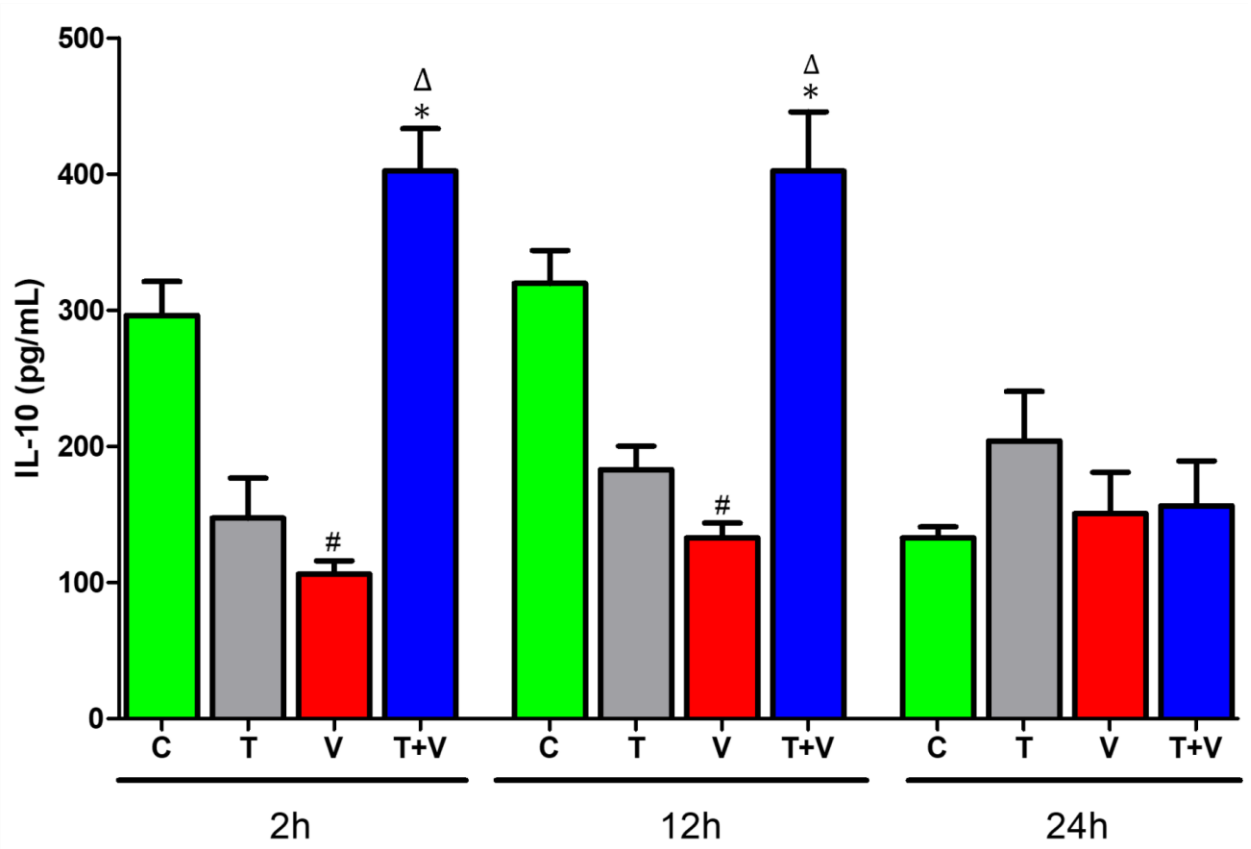

IL-10 (pg/mL)
500
400
300
200
100
0
Δ
*
#
C
T
V
T+V
2h
12h
24h

**Figure 17. Tulathromycin restores non-opsonized phagocytosis of PRRSV-infected BMDMΦs**. The effects of tulathromycin treatment and PRRSV infection on macrophage non-opsonized phagocytic capacities of BMDMΦs was measured using fluorescently labeled zymosan particles. BMDMΦs were treated with HBSS (control) or with tulathromycin (1mg/mL; TUL) for 1 hour and then infected with PRRSV (m.o.i. = 0.5; virus and TUL+virus) for 12 hours at 37°C. Following treatment and infection, BMDMΦs were incubated with fluorescently labeled zymosan particles (10:1 ratio; Zymosan: MDMΦs) for 1h. Cells were washed 3 times with PBS to remove free particles (A) Representative microscopic images of phagocytic macrophages. (B) Percentage of macrophages able to phagocytose at least 1 zymosan particle. (C) Percentage of macrophages that phagocytosed at least 5 zymosan particles. n=150-300 macrophages/group. Images and histograms are representative of 5 independent experiments. Means ± SEM. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Ω = $P<0.05$ vs tulathromycin + virus.

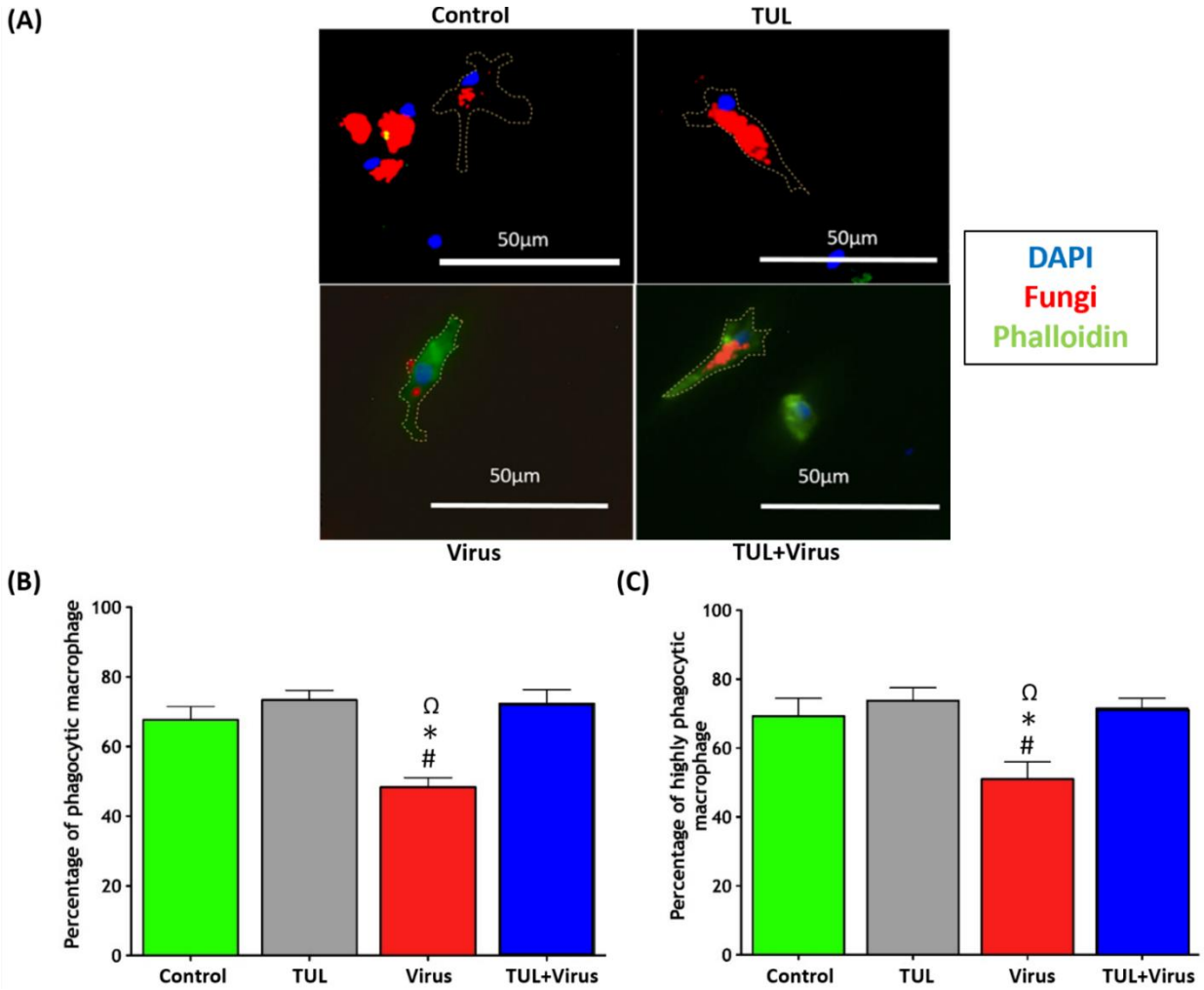

(A)
Control
TUL
Virus
TUL+Virus
50µm
DAPI
Fungi
Phalloidin
(B)
Percentage of phagocytic macrophage
100
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL+Virus
(C)
Percentage of highly phagocytic macrophage
100
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL+Virus

**Figure 18. Tulathromycin restores Fc-mediated phagocytosis of PRRSV-infected BMDMΦs**. To measure the effects of PRRSV infection and tulathromycin on Fc-mediated phagocytosis, BMDMΦs were treated with HBSS (control) or with tulathromycin (1mg/mL; TUL) for 1h at 37°C. and then infected with PRRSV (m.o.i. = 0.5; virus and TUL+virus) for 12 hours at 37°C. Then, BMDMΦs were incubated with IgG-coated latex beads (10:1 ratio; Zymosan: BMDMΦs) for 45 minutes at 37°C. Cells were washed 3 times with warm PBS to remove free beads (A) Representative images of macrophages that have phagocytosed latex beads (indicated by black arrows). Bar = 20µm. (B) Percentage of macrophages able to phagocytose at least 1 latex bead. (C) Percentage of macrophages that phagocytosed at least 5 IgG-coated latex beads. n=150-200 macrophages/group. Images and histograms are representative of 4 independent experiments. Means ± SEM. # = $P<0.05$ versus control; * = $P<0.05$ vs tulathromycin; Ω = $P<0.05$ vs tulathromycin +virus.

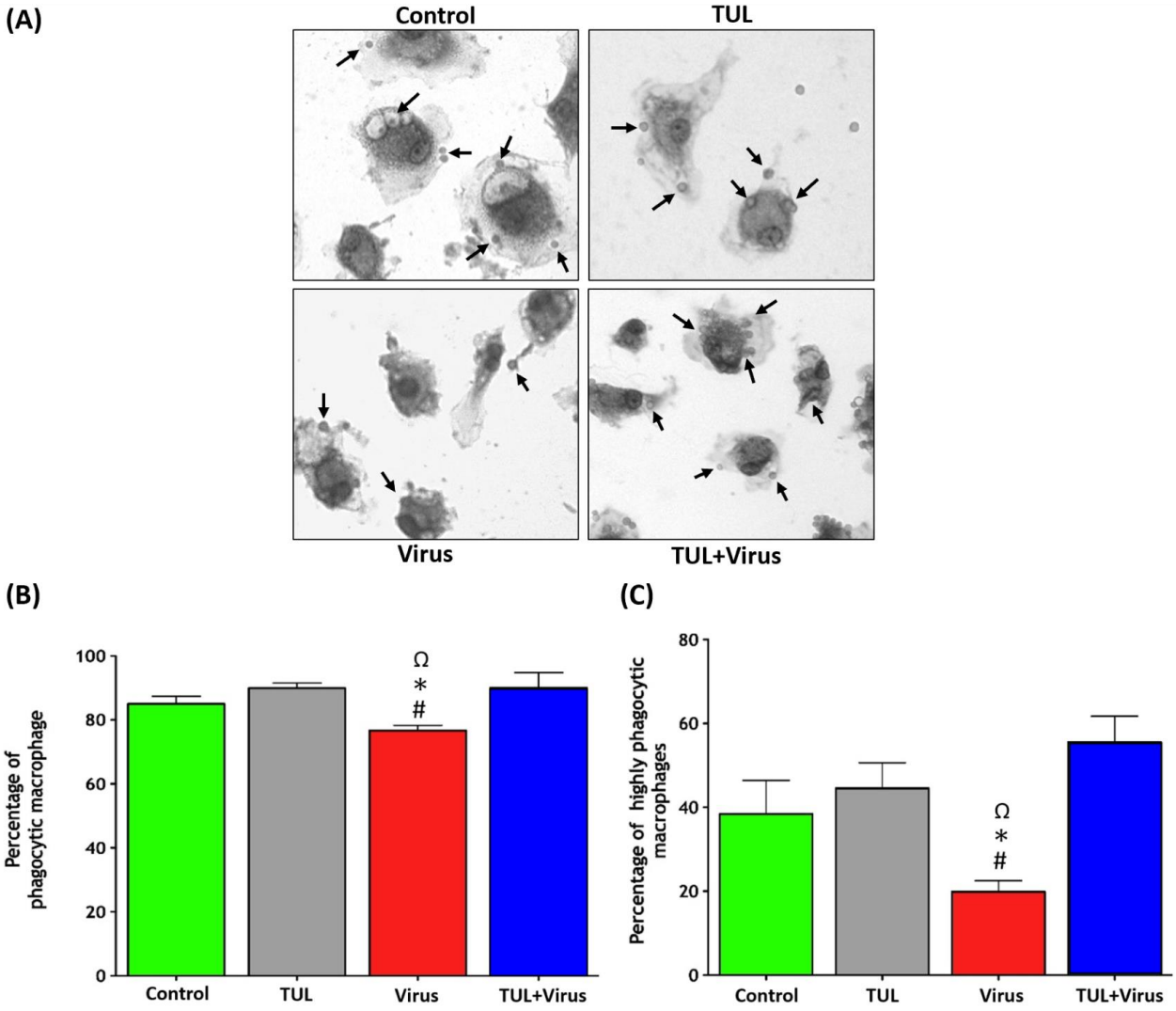
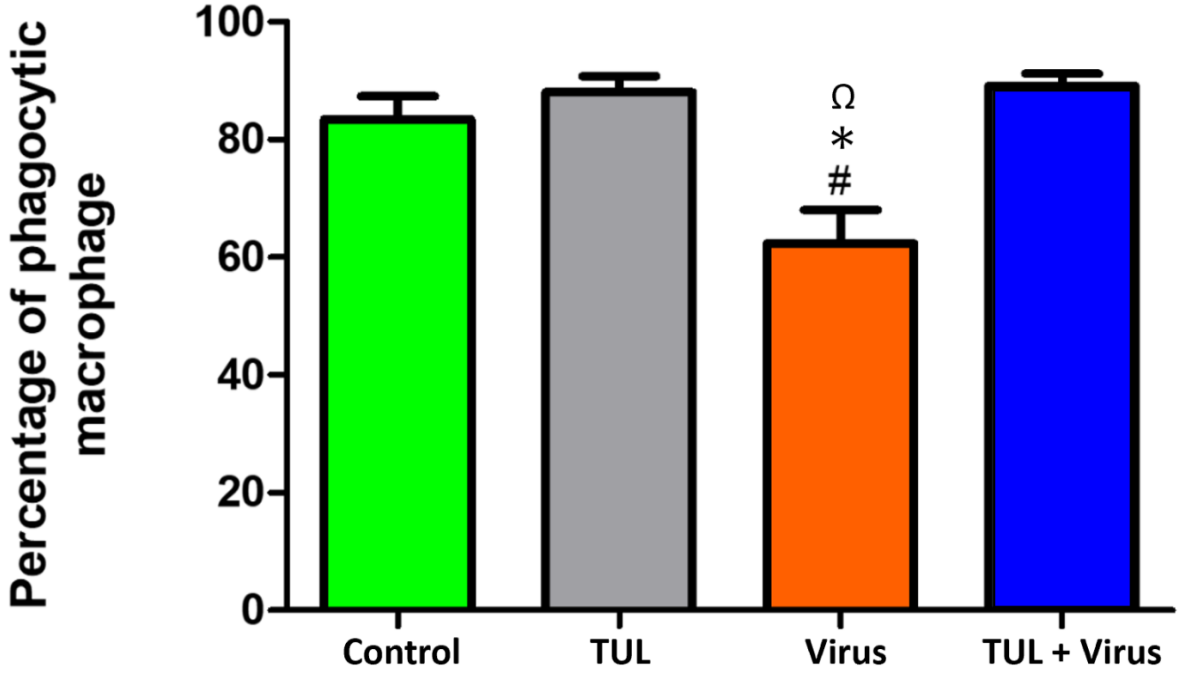

(A)
Control
TUL
Virus
TUL+Virus
(B)
Percentage of phagocytic macrophage
100
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL+Virus
(C)
Percentage of highly phagocytic macrophages
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL+Virus

**Figure 19. PRRSV replication is not necessary to inhibit BMDMΦs phagocytosis**. To measure the effects of PRRSV infection and tulathromycin on early Fc-mediated phagocytosis, 7-days old BMDMΦs were treated with HBSS (control) or with tulathromycin (1 mg/mL; TUL) for 1h at 37°C. and then infected with PRRSV (m.o.i. = 0.5; virus and TUL+virus) for 2 hours at 37°C. Then, MDMΦs were incubated with IgG-coated latex beads (10:1 ratio; Zymosan: MDMΦs) for 45 minutes at 37°C. Cells were washed 3 times with warm PBS to remove free beads Percentage of macrophages that phagocytosed at least 1 IgG-coated latex bead. n=150-200 macrophages/group. Histograms are representative of 3 independent experiments. Means ± SEM. # = P<0.05 versus control; * = P<0.05 vs tulathromycin; Ω = P<0.05 vs tulathromycin + virus.


Percentage of phagocytic macrophage
100
80
60
40
20
0
Ω
*
#
Control
TUL
Virus
TUL + Virus

# 4 DISCUSSION

Present in most swine producing countries, PRRS is one of the most economically important porcine diseases. Despite 50 years of research, there is currently no effective treatment to control PRRS outbreaks. Given the viral nature of PRRS etiological agent, most studies aiming to develop control strategies focused on vaccine development. However, due to the remarkable evolution rate and genetic diversity of PRRSV, commercially available vaccines are not effective to protect the pigs. In fact, current vaccines will not provide protection against heterologous strains and can also revert to virulence [93], [94]. These limitations advocate for the development of different tools to control PRRSV infection. Interestingly, during PRRSV infection, the severity of the disease and the development of fatal clinical symptoms is closely related with the ability of the virus to dysregulate macrophages functions and induce inflammation (see section 1.2). Therefore, targeting either of these components could be of critical importance to treat infected animals.

Anti-inflammatory and immunomodulatory properties of macrolides are well established (see section 1.3.1). In addition, these antibiotics have been shown to accumulate with high affinity in macrophage, the main cellular target of PRRSV, where they may interact with receptors and second messenger to modulate cell behavior [219]–[221]. Together, these data suggest the potential clinical benefits of macrolides to treat inflammatory-driven respiratory viral diseases such as PRRS. With its anti-inflammatory and immunomodulatory properties as well as its superior clinical efficacy compared to other macrolides (see section 1.3.2), tulathromycin seems to be the perfect candidate to investigate whether such antimicrobials may help protect swine against PRRS. In the present study, we evaluated the anti-viral and immunomodulating properties of tulathromycin in PRRSV–infected porcine macrophages.

## 4.1 Anti-viral effects of tulathromycin *in vitro*

Due to PRRSV strong tropism for the cells of the monocyte/macrophage lineage, there is very few cellular models to study PRRSV infection. To date, the two most common cellular models are PAMs and MARC-145 cells. However, these two models have significant limitations. While PAMs isolation requires bronchoalveolar lavages which are time-consuming and stressful for the animals, MARC-145 are epithelial cells which do not originate from pigs. Moreover, shortly after the initiation of a respiratory infection, alveolar macrophages are replaced by monocyte-derived macrophages, which therefore represent a key cell population to interact with PRRSV. Therefore, we sought to develop and use an easy to isolate, culture and manipulate model system that would be biologically relevant in the context of the infection. Previous *in vitro* studies showed that the virus could infect blood monocyte-derived macrophages [156]. Since our laboratory routinely works with blood monocyte-derived macrophages (BMDMΦs) to study the effects of various macrolides including tylvalosin and tulathromycin on macrophages functions and survival, we isolated and cultivated monocytes for 7 days. Consistent with previous findings, monocytes were less susceptible to PRRSV than differentiated monocyte-derived macrophages [156]. The addition of L929 supernatant during macrophage differentiation significantly increased their susceptibility to the virus compared to macrophages cultivated in medium supplemented with pig serum alone. L929 supernatant is a source of M-CSF and is used to induce macrophage differentiation. It is likely that other cytokines are contained in the supernatants increase macrophages susceptibility to PRRSV. Since viral receptors expression is dependent on cytokines expression, we wanted to assess whether this increase in susceptibility was associated with an upregulation of viral receptor expression. We performed immunostaining for the two main PRRSV receptors (CD163 and CD169). Interestingly, in our experimental model, macrophages cultured in pig serum alone

expressed both receptors. The addition of L929 strongly upregulated CD163 (29% positive cells to 89% positive cells) but abolished CD169 expression. These results indicate that L929 supernatant modulates PRRSV receptor expressions and that CD163 alone is sufficient for PRRSV infection. This is consistent with recent observations showing that CD163, but not CD169, was sufficient to enable non-permissive cells to become permissive to PRRSV, and that increased CD163 correlates with increased susceptibility to PRRSV [58], [114]–[116], [156]. In addition, in our experimental setting, we were not able to detect positive 1-day old monocytes for CD163 and CD169. Therefore, the lack of expression of these two viral receptors might explain why differentiated BMDMΦs are more susceptible to PRRSV than blood monocytes. L929 supernatant is known to contain M-CSF, but very little is known about other cytokines and chemokines present in this supernatant [248]. Considering that CD163 and CD169 expression can be induced by IL-10 and IFN-y respectively and that IL-10 treatment increases PRRSV infectivity while IFN-y decreases it, we could hypothesize that L929 supernatant contains IL-10, which hence would potentiate PRRSV infection through the upregulation of CD163 expression [124], [156], [157], [249]. Future characterization of this L929-supernatant is warranted. More importantly, our data show that BMDMΦs can readily be infected by PRRSV, and hence represent a useful cellular model to study PRRSV pathogenesis.

Some macrolides such as tilmicosin and tylvalosin have been recently demonstrated to possess direct anti-viral effects against PRRSV [244], [245]. Interestingly, we did not measure any change in infectious viral particles counts when BMDMΦs and MARC-145 cells were pre-treated with tulathromycin. These data suggesting that tulathromycin does not seem to possess direct anti-viral activity, are consistent with other reports demonstrating that erythromycin does not affect PRRSV replication [239]. The studies on tilmicosin and tylvalosin referenced above used RT-PCR

and/or $TCID_{50}$ to quantify PRRSV titers. In contrast, our experiments used plaque assays. Despite being long, labor-intensive and more inconsistent than RT-PCR, plaque assay allows the enumeration of the number of live infectious viral particles in a sample with high precision. On the other hand, RT-PCR is rapid and highly precise but measure variation in viral gene expression and thus does not necessarily account for change in infectious virion titers [250]–[252]. PRRSV foci staining with an anti-PRRSV nucleocapsid antibody in MARC-145 showed similar results. In fact, consistently with plaque assays data, we did not observe any change in PRRSV foci number with or without tulathromycin. In addition, PRRSV foci size was not altered upon pre-treatment with the drug suggesting that the drug has no effect on intercellular spread. Future experiments using RT-PCR, should be performed to determine whether tulathromycin can impact viral gene expression without changing viral infectivity. Consistently, TUL pre-treatment did not change viral receptor expression compared to untreated cells. All together, these data strongly indicate that tulathromycin does not possess any direct anti-viral properties and that any change in macrophage functions and behavior following tulathromycin treatment and PRRSV exposure does not merely reflect altered infectious viral titers.

## 4.2 Immunomodulatory effects of tulathromycin during PRRSV infection *in vitro*.

A hallmark of PRRSV pathogenesis resides in its ability to alter macrophages survival and function, hence predisposing the host to secondary infections (see section 1.2.5 and figure 4). Recent research in our laboratory demonstrated that TUL was also able to modulate macrophages function and survival, both in pigs and cattle (see section 1.3.2). Therefore, we sought to investigate macrophage survival and function following TUL treatment in the context of PRRSV infection.

Macrophages are essential components of the pulmonary immune defense against various pathogens. Uncontrolled cell death of these cells can result in the immunosuppression of the host associated with an increased incidence of secondary infections. In addition, Apoptotic removal of macrophages is crucial to limit self-sustained inflammation and to promote the resolution of inflammation [3], [30]. Consistent with previous studies, we found that PRRSV and TUL induced macrophage apoptosis (at 24 hours post-exposure in the present experiments). Moreover, TUL and PRRSV synergized to induce apoptosis at 24 hours. During acute lung injury, Fas-induced apoptosis of recruited macrophages as of neutrophils is essential for the resolution of inflammation [259]. Therefore, by increasing BMDMΦs apoptosis, tulathromycin may help promote the resolution phase of inflammation. Future *in vivo* experiments are necessary to determine if tulathromycin-induced apoptosis of macrophages might dampen pulmonary inflammation of PRRSV-infected pigs. Interestingly, while TUL induces apoptosis through caspase 3 and caspase 8 activation, PRRSV was found to induce apoptosis in caspase 3, caspase 8 and caspase 9 dependent fashion [177]–[179], [229]. Future western blotting and caspase inhibition experiments will help determine whether these synergistic effects may result from the hyperactivation of the intrinsic pathway and/or from the combined activation of the intrinsic and extrinsic pathways. In addition, future experiments will help to determine whether increased apoptosis causes immunosuppression in pigs and/or attenuates lung injury during the acute phase of inflammation in live PRRSV-infected pigs. Aside from apoptosis, we observed that PRRSV significantly induced cell death through necrosis after 12 hours. Importantly, TUL could prevent early PRRSV-induced necrosis (at 12 hours p.i. but not 24 hours p.i). Necrosis is known to exacerbate local inflammation through the release of cytotoxic molecules that lead to extensive tissue damage [49], [50]. Thus, by inhibiting early PRRSV-induced necrosis, TUL may attenuate virus-induced

inflammation and lung tissue damage. In our experimental model, apoptosis was observed after 24 hours of exposure whereas necrosis was measured as early as 12 hours p.i., therefore it is not likely that PRRSV-induced necrosis simply reflects secondary necrosis following apoptosis. Nonetheless, it remains unclear as to why at 24 hours p.i. TUL was not able to prevent necrosis. Future experiments may determine whether in this specific case, necrosis might be due to secondary necrosis. Future experiments using caspase inhibitors will help to answer this question. To limit the impact of apoptosis and necrosis during functional experiments, we used a lower TUL concentration (0.5 mg/mL) and a lower multiplicity of infection (0.1).

In the past few years, the correlation between macrophage morphology and function has been established providing an easy way to monitor change in macrophage polarization [253]. In this study, we found that PRRSV infection dramatically altered macrophage morphology compared to control cells. Indeed, control macrophages were characterized by a small round shaped cytoplasm whereas infected macrophages exhibited an elongated fibroblast-like morphology with numerous cytoplasmic extensions (or pseudopods). Tulathromycin pre-treatment was sufficient to prevent the PRRSV-induced morphological alterations as the cells were morphologically similar to their untreated and uninfected counterparts. Interestingly, a recent paper suggested that these pseudopods promote intercellular junction as a mean to escape the immune system through direct intercellular spread [254]. In this article, the authors claim that these intercellular junctions allow the virus to spread to neighboring cells, bypassing neutralizing antibodies and the humoral response, and hence that they may contribute to viral pathogenesis [254]. Therefore, it is possible that by preventing these intercellular junctions, TUL might hinder PRRSV ability to spread from cell to cell and escape the immune system, a hypothesis that requires

more research. In the light of these observations, we hypothesized that the change in morphology was associated with a change in macrophage functions.

To test this hypothesis, we measured the production of prototypical pro- and anti-inflammatory cytokines (respectively CXCL-8 and IL-10) as well as the production of mitochondrial ROS. CXCL-8, a potent neutrophil chemoattractant secreted by macrophages and other cell types, is crucial for neutrophil infiltration to the site of an infection and participates in the onset of the inflammation [20]. Our data demonstrate that PRRSV significantly induces CXCL-8 at 24 hours p.i. CXCL-8 secretion was inhibited when the cells were pre-treated with TUL. These results suggest that TUL might attenuate PRRSV-induced inflammation through CXCL-8 inhibition, which will need to be further characterized in live infection models. Whether CXCL-8 production correlates with the apoptotic death of the cells remains to be fully assessed. However, considering that the cells pre-treated with TUL secreted less CXCL-8 than infected cells alone, and that previous research on TUL in our laboratory demonstrated that CXCL-8 inhibition was independent on caspase activity or cell death, it is not likely that CXCL-8 levels are dependent on cell death in this system [228]. To confirm the anti-inflammatory potential of TUL during PRRSV infection, the level of other pro-inflammatory cytokines should be assessed in the future.

Mitochondrial ROS production is a hallmark of cell stress and inflammation and contributes to PRRSV induced tissue damage [166], [178]. Here we demonstrate that PRRSV indeed induces ROS production in porcine macrophages and that this production is inhibited when the cells are pre-treated with the drug. Interestingly, in a paper published in 2007, Lee et Kleiboeker demonstrated that mitochondrial ROS production is involved in PRRSV-induced apoptosis in MARC-145 [178]. In our model system, TUL inhibited PRRSV-induced ROS but synergized to induce apoptosis. Given, that ROS activates the intrinsic pathway of apoptosis via cytochrome c

release and caspase 9 activation, we can hypothesize that TUL may induce apoptosis exclusively through the extrinsic pathway and inhibits the intrinsic pathway. The mechanisms by which TUL inhibits mitochondrial ROS production remain to be elucidated but $cPLA_2$ seems to be a good potential target. Indeed, TUL has been shown to inhibit $cPLA_2$ a known inducer of mitochondrial ROS [255].

A final set of studies sought to determine whether TUL inhibition of the viral-induced pro-inflammatory CXCL-8 coincided with an increase in anti-inflammatory signaling. To that extent, we measured IL-10 levels over time. Surprisingly, PRRSV and TUL did not induce IL-10 secretion by macrophages. On the contrary, we found that the virus inhibited IL-10 secretion at 2 and 12 hours post infection. These data are in contrast with those found in the scientific literature. Indeed, it is generally accepted that PRRSV induces IL-10 production to increase its infectivity [156]. In fact, IL-10 activated cells are more permissive to PRRSV than unstimulated and M1 polarized cells [156]. Furthermore, in homeostatic conditions (see section 1.1.2.1), PAMs are in a suppressive state mediated partly by IL-10 [6], [56]. In view of our results indicating that TUL pre-treatment may prevent early PRRSV-induced IL-10 inhibition, one may wonder whether TUL pre-treatment could increase early PRRSV infectivity *in vivo*. Tulathromycin did not induce IL-10 secretion at 24 hours suggesting that CXCL-8 and mitochondrial ROS inhibition by TUL was not dependent on IL-10 production.

Taken together the present findings strongly support the hypothesis that tulathromycin might attenuate PRRSV-induced inflammation by preventing early anti-inflammatory signaling inhibition and by inhibiting of pro-inflammatory signaling at later stage of the infection. It is unclear whether the pro-inflammatory signaling increase and the anti-inflammatory signaling

decrease by PRRSV is a direct effect of the virus or results from the activation of the cell following viral detection.

Pathogen engulfment and degradation through phagocytosis is a crucial function of macrophages and is integral to immune surveillance in the lungs [6], [56]. Phagocytosis is triggered when phagocytic receptors including opsonic receptors (FcR) or pattern recognition receptors such as the mannose receptor are activated [7], [8]. Field analyses have reported that PRRSV-infected pigs are often infected by secondary pathogens [137], [210]. One theory suggests that the increase in secondary infections results from PRRSV ability to inhibit macrophage phagocytosis [187]–[192]. Considering previous results demonstrating TUL's ability to increase macrophage efferocytosis of neutrophils (i.e the ingestion and elimination of apoptotic cells by macrophages), we sought to investigate on the phagocytic potential of infected BMDMΦs following TUL treatment. To that extent, we exposed TUL treated and/or PRRSV-infected macrophages to either non-opsonized zymosan particles or IgG-coated latex beads. In both cases, we found that our viral strain significantly inhibited phagocytosis. Indeed, the NSVL-98-7895 PRRSV strain reduced the number of phagocytosed particles inside macrophages, and the ability of macrophages to phagocytose multiple particles. Hence, it appears that PRRSV inhibition of phagocytosis is not FcR or PRR selective. These results are consistent with previous reports showing decreased phagocytosis of dextran, latex beads, or live bacteria (*Streptococcus suis*) upon PRRSV infection [133], [190], [192]. Interestingly, a paper published in 2012 demonstrated that a European genotype strain of PRRSV inhibited phagocytosis through its interaction with sialoadhesin (also referred to as CD169). However, in our model system, L929 cultivated BMDMΦs were negative for CD169 suggesting either that the mechanisms for phagocytosis inhibition are strain- and/or genotype-dependent, or that PRRSV inhibits phagocytosis through

multiple pathways [192]. A reduction of macrophage phagocytic function has the potential to dramatically increase the incidence of secondary infections. Indeed, another report recently demonstrated that the same NSVL-98-7895 strain impaired phagosomal maturation and reduced NADPH oxidase-mediated respiratory burst [193]. PRRSV inhibition of phagocytosis and antimicrobial functions is likely to hinder the ability of infected macrophages to eliminate secondary pathogens. Importantly, TUL pre-treatment was able to prevent the inhibition of phagocytosis, suggesting that this drug might help control secondary infections during PRRSV infections, not only through its antimicrobial properties, but also by averting the inhibition of phagocytosis in macrophages. The mechanisms by which tulathromycin prevents PRRSV-induced inhibition of phagocytosis require further elucidation. Based on the data presented herein and the literature, we speculate that phagocytosis modulation by PRRSV and tulathromycin involves the cytoskeleton. Firstly, tulathromycin was not found to change the expression of viral receptors (CD163 and CD169). Second, the morphology of infected macrophages is significantly different than the morphology of control and TUL-treated cells. This suggests that both the virus and the drug are able to induce cytoskeleton remodeling. Third, phagocytosis of particles targeting different phagocytic receptors is impaired, indicating that PRRSV inhibition of phagocytosis is not receptor specific. Similarly, TUL treatment restored non-opsonized and opsonized phagocytosis and increased neutrophil efferocytosis indicating a receptor independent mechanism. Phagocytosis inhibition by viruses such as HIV-1 is well documented and is known to be dependent on the actin cytoskeleton [256]. Moreover, in a recent paper, PRRSV was found to induce cytoskeleton remodeling for intercellular spread through nanotubes [254]. Drugs targeting the cytoskeleton strongly inhibit PRRSV infection, indicating a role for cytoskeletal effects in viral pathogenesis [257]. The ability of macrolides to induce cytoskeletal changes has not been extensively

documented, but a few studies report that some macrolides are able to inhibit actin remodeling by sequestering actin monomers [258]. All together, these data support the hypothesis that both TUL and PRRSV might modulate phagocytosis through actin cytoskeleton remodeling. To test this hypothesis, cell migration and actin polymerization assays could be performed in presence of tulathromycin and/or PRRSV. Interestingly, PRRSV inhibition of phagocytosis was observed as soon as 2 hours post-infection in the present study. This is consistent with the findings by De Baere et al. showing that the mere entry of PRRSV into macrophages was sufficient to inhibit phagocytosis [194]. By preventing PRRSV-induced phagocytosis, tulathromycin might significantly decrease the incidence of secondary bacterial infections and thus increase pig survival in the field. *In vitro* phagocytosis experiments using live bacteria with or without *tulathromycin* would be useful to test this hypothesis. However, due to tulathromycin's bacteriostatic properties, such experiments will need to address the difficulty of assessing the drug's anti-inflammatory benefits versus its antimicrobial properties. Due to a lack of a reliable anti-PRRSV antibody, we were not able to clearly measure the percentage of PRRSV-infected macrophage in our experimental system. Therefore, we cannot fully assert whether PRRSV modulated macrophages functions directly (in infected cells) or indirectly (bystander cells). According to the literature, only about 2 to 10% of AMs are infected by PRRSV throughout the course of the infection suggesting that PRRSV modulates macrophages functions indirectly [98], [124]. Flow cytometry and immunostaining approach will be helpful to answer this question. Finally, *in vivo* testing of tulathromycin will be required to fully assert whether this drug can significantly reduce the prevalence of secondary pathogens during PRRSV infection.

## 4.3 Summary

In this study, we investigated the anti-viral and immunomodulatory effects of tulathromycin in a non-microbial *in vitro* system using porcine blood monocyte-derived macrophages. Our data demonstrate that porcine blood monocyte-derived macrophages can be readily infected by PRRSV and represent a useful alternative to MARC-145 and PAMs to study PRRSV pathogenesis *in vitro*. Importantly, PRRSV titers and viral receptor expression analyses strongly suggest that tulathromycin does not possess any direct anti-viral properties against PRRSV. Nonetheless, we found that the drug exhibited potent immunomodulatory properties in PRRSV-infected macrophages. Tulathromycin acts synergistically with PRRSV to induce apoptosis in a time-dependent fashion but reduced early PRRSV-induced necrosis, in further support of the pro-resolution properties of tulathromycin. In addition, we demonstrated that the drug was able to prevent PRRSV-induced CXCL-8 secretion, mitochondrial ROS production and early IL-10 inhibition in porcine monocyte-derived macrophages. Tulathromycin pre-treatment was also found to prevent non-opsonized and opsonized phagocytosis inhibition by PRRSV. A schematic illustrating the immunomodulatory actions of tulathromycin in PRRSV infected porcine macrophages is presented below (figure 20).

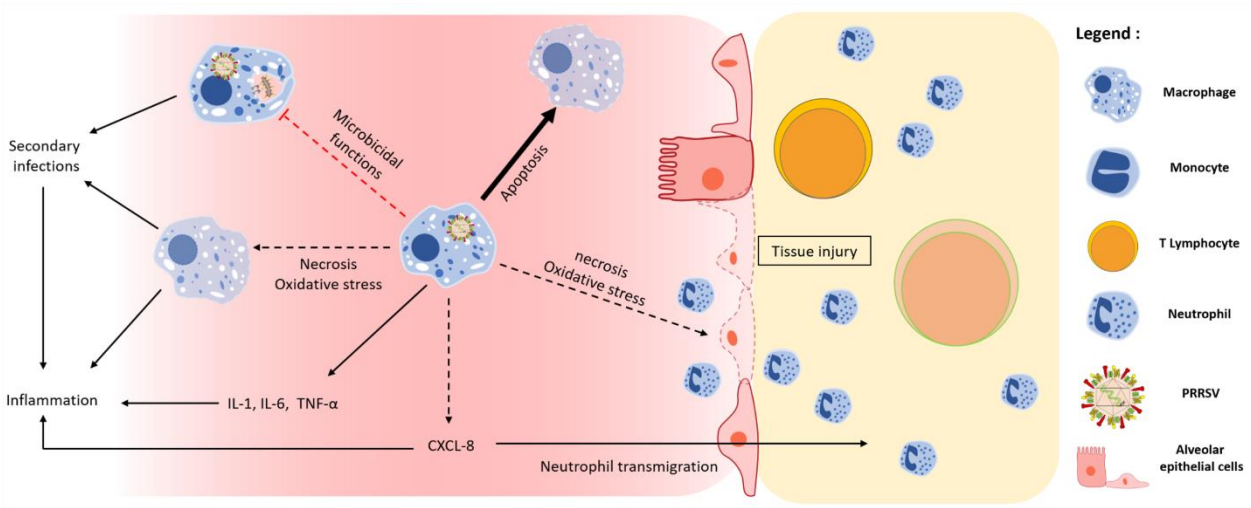



**Figure 20. Speculative immunomodulatory properties of tulathromycin in PRRSV-infected BMDMΦs**. Hatched arrows indicate inhibitory effects of tulathromycin on PRRSV modulation of macrophage functions and survival. Thick arrows indicate synergistic effects of tulathromycin on PRRSV modulation of macrophage functions and survival. Thin solid arrows indicate hypothetical immunomodulatory effects of tulathromycin in PRRSV-induced inflammation which have yet to be determined.

# 5 CONCLUSION

Continuously evolving and causing numerous outbreaks during the last 30 years, porcine reproductive and respiratory syndrome virus (PRRSV) remains one of the major challenges for the swine industry. Current control strategies hinged around vaccines have yielded inconsistent results, advocating for the development of different approaches to control PRRSV infection. During the past decade, macrolides received increasing interest for their potent anti-inflammatory and immunomodulatory properties. Given the inflammatory nature of PRRSV pathogenesis, we sought to investigate on the potential therapeutic effects of tulathromycin, a macrolide used for the treatment and prevention of bacterial respiratory diseases in pigs, during PRRSV infection. Findings from our study demonstrate that tulathromycin, attenuates PRRSV-induced macrophage pro-inflammatory signaling and prevents phagocytosis inhibition. These effects occur in the absence of a direct anti-viral activity. Together, these data suggest the potential clinical benefits of macrolides in the context of a virus-induced inflammation. Perhaps more importantly, this research shed the light on novel directions for the development of new anti-viral anti-inflammatory drugs.

# 6 FUTURE DIRECTIONS

The results presented herein suggest the potential clinical benefits of tulathromycin in the context of an *in vitro* model of PRRSV-induced inflammation. However further studies are required to fully understand how tulathromycin modulates macrophage functions during PRRSV infection. These results might help understand PRRSV pathogenesis and shed light on how a macrolide like tulathromycin may modulate immune cell function during a virus-induced inflammatory disease. Further *in vitro* and *in vivo* studies are warranted to investigate whether and how tulathromycin may yield clinical benefits in the context of PRRSV infections in pigs.

*In vitro* studies:

- To characterize the composition of L929-supernatant and further assess how it increases macrophage susceptibility to PRRSV.
- To determine the percentage of PRRSV-infected macrophages over time in infection *in vitro*.
- To clarify the mechanisms by which PRRSV and tulathromycin act synergistically to induce apoptosis.
- To elucidate the mechanisms by which tulathromycin prevents early PRRSV-induced necrosis and assess whether late necrosis observed in the present studies was due to secondary necrosis.
- To further characterize the anti-inflammatory and pro-resolving potential of tulathromycin during PRRSV infection through lipidomic analyses.
- To characterize the mechanisms by which tulathromycin inhibits mitochondrial ROS production.

- To further identify the direct/indirect mechanisms through which PRRSV and tulathromycin modulate macrophage phagocytosis.
- To determine whether the anti-inflammatory and immunomodulatory properties of tulathromycin against PRRSV are strain- and or genotype-dependent
- To characterize the anti-inflammatory and immunomodulatory actions of tulathromycin in co-infected macrophage models (*Actinobacillus pleuropneumoniae*/PRRSV or PCV2/PRRSV for instance).

*In vivo* studies:

- To assess the effects of tulathromycin on growth, survival and disease progression including lung injury in piglets infected with live PRRSV.
- To determine whether tulathromycin alters the occurrence and/or incidence of secondary infections in PRRSV-infected animals.
- To measure cytokines and lipid mediators levels (pro- and anti-inflammatory as well as pro-resolving) from bronchoalveolar lavage fluid following tulathromycin treatment in PRRSV infected pigs
- PRRSV is known to modulate lipid metabolism, but the production of lipid mediators in the lungs during PRRSV infection remains obscure. Moreover, tulathromycin inhibits pro-inflammatory lipid mediators ($LTB_4$) and promotes pro-resolving mediators (LXA4) production in inflamed porcine lungs *in vivo*. Therefore, studying the effects of an antimicrobial drug on virus-induced lipid mediators production would be extremely novel.

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