ABSTRACT

Title of Document: ALTERED PORCINE ALVEOLAR MACROPHAGE PHENOTYPE BY ALL-TRANS RETINOIC ACID AND *ASCARIS SUUM* INFECTION

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Ascaris suum is known to cause ascariasis of both pigs and humans. Ascariasis is associated with a reduced control of intracellular pathogens and decreased vaccine efficacy. All-trans retinoic acid (ATRA), an active metabolite of Vitamin A (VA), is known to mediate a diverse array of physiological and biological processes including immune and inflammatory responses. The phenotype of alveolar macrophages (AM), the most abundant cell in the lung, has not been critically examined during chronic infection with *A. suum* nor has the effect of administration of dietary ATRA alone or during infection. This combination of treatments is likely common in underdeveloped regions where infection with *Ascaris* is prevalent and public health initiatives to control nutrient deficiencies often include VA supplementation. In this study we showed that a low dose and repeated “trickle” infection with *A. suum* alone and with ATRA administration altered the AM cell surface antigen phenotype.

ALTERED PORCINE ALVEOLAR MACROPHAGE PHENOTYPE BY ALL-TRANS RETINOIC ACID AND *ASCARIS SUUM* INFECTION

By

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List of Abbreviations

AAMs – Alternatively Activated Macrophages
ANOVA – Analysis of variance
Arg I – Arginase I
ATRA – All-trans Retinoic Acid
BAL – Bronchial alveolar lavage
CAMs – Classically Activated Macrophages
CO – Corn oil
DAI – Days after infection
DC – Dendritic cells
ECM – Extracellular Matrix
FSC – Forward scatter
GM-CSF – Granulocyte macrophage colony-stimulating factor
HAI – Hours after infection
IgE – Immunoglobulin E
IgG – Immunoglobulin G
IL – Interleukin
IL-4R – Interleukin 4 receptor
INF-γ – Interferon gamma
MFI – Mean fluorescence intensity
NK – Natural killer
NKT – Natural killer T
PBS – Phosphate-buffered saline
PDL-1 – Programmed death ligand 1
PFA – Para formaldehyde
PI – Post inoculation
RMs – Regulatory Macrophages
SSC – Side scatter
SD – Standard deviation
SED – Super-enhanced D-max
Th1 – CD4+ T helper cell type 1
Th2 – CD4+ T helper cell type 2
TLR – Toll-like receptor
TNF-α – Tumor necrosis factor alpha
VA – Vitamin A

CHAPTER 1: INTRODUCTION

1.1 Ascaris suum

1.1.1 The parasite

Parasitic worms (helminths) infect an estimated 2.5 billion people living in the developing world with over 500 million people being infected with at least two species [1]. The most common intestinal parasite in humans is an extracellular gastrointestinal nematode, *Ascaris lumbriocoides*, that has a high prevalence among children <12 years of age and is associated with malnutrition, disease, and poor growth and cognition. [1-2]. Infection with *A. lumbriocoides* is most common in tropical and sub-tropical countries, but it also affects an estimated 4 million people per year in the United States [3]. Because infection depends largely on poor sanitation, it has the potential to be controlled by health education and better hygienic practices; although domestic animals such as pigs and even chickens can also act as transport/paratenic hosts for *Ascaris* eggs [2].

Ascaris suum, a closely related large roundworm species, is known to cause ascariasis of both pigs and humans. Infective *A. suum* eggs from pigs have the potential to be transmitted to humans through fecal contamination of soil thus posing a human health problem given that >50% of all pigs raised for human consumption are infected [4]. *Ascaris suum* is the most common helminth parasite in modern swine operations including confinement facilities that fail to control parasite exposure [5]. Infections can cause the producer major economic losses by decreased feed efficiency, reduced growth rates and increased time to market, decreased carcass

value, and condemnation of livers [6]. Further economic losses are incurred by producers due to the nature of the immune response to *A. suum*. Pigs infected with *A. suum* exhibit an altered immune response skewed to protect against extracellular parasites which leads to diminished vaccine efficacy and a reduced control of intracellular pathogens [7-9]. Vaccination is one of the most cost effective practices swine producers can implore to benefit the health, growth, and welfare of their livestock. Likewise, vaccination is the most cost effective and efficient practice to prevent human disease. Several animal models to test vaccination efficacy have shown the ability of chronic helminth infections to affect successful vaccination outcomes [9]. For example, *A. suum* infection resulted in higher mean percentage of lung pathology, a skewed Th2 response in the lung and draining lymph nodes, and a reduction in the percentage of pigs that sero-converted by 25% in pigs vaccinated against *Mycoplasma hyopneumoniae* [10].

1.1.2 Life cycle of *Ascaris suum*

Ascaris suum has an intricate life cycle that involves the migration through multiple host organ systems (**Fig. 1**). Infection is initiated by ingestion of eggs containing infective larvae that have embryonated in the environment for a minimum of 10-14 days at 30°C [1]. As the ingested egg comes into contact with bile acids, the second (L2) or third stage larva (L3) is released from the egg casing [1]. Between 6-8 hours after infection (HAI) newly hatched larvae burrow through the ceacum and proximal colon and into the liver where between 10 and 36 HAI they develop into parasitic L3 [11]. By 7 days after infection (DAI) developing late L3 are found

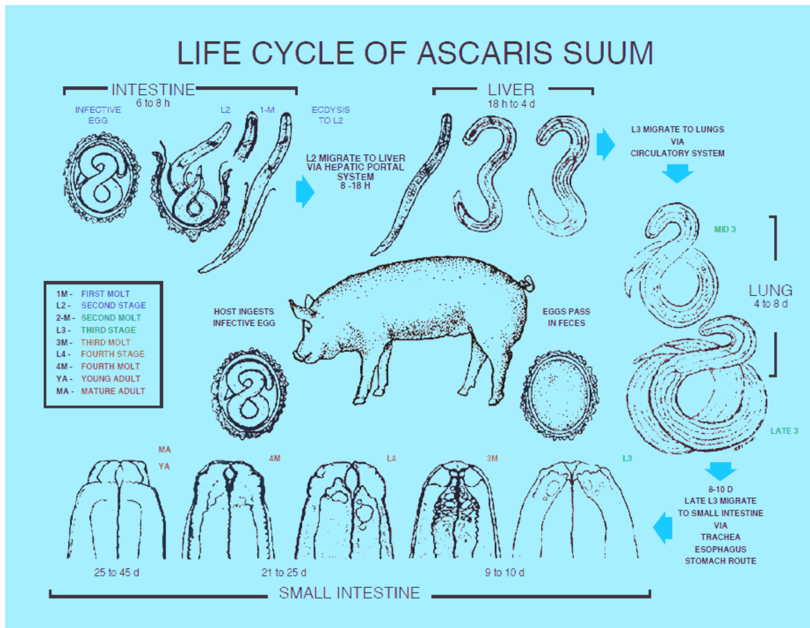


FIGURE 1. Life cycle of Ascaris suum. Migration through host organ systems and larval stages [72].

accumulating in the lungs [11]. Late L3 penetrate into the alveoli where they are coughed up into the trachea, swallowed, and migrate into the small intestine. [1,11]. At 10 DAI, the larvae arrive in the jejunum where they molt to fourth stage (L4), and 23-25 DAI larvae mature into fifth stage (L5) or young adult [11]. By 6 to 8 weeks after infection adult female ascarids begin to produce thousands of eggs daily, which pass out of the host in the feces [12]. Adult ascarids inhabit the lumen of the jejunum and feed on the intestinal contents robbing the host of nutrients [1]. In both humans and pigs, migrating larvae produce liver lesions (milk spot) and eosinophilic pneumonitis in the lungs; they may also migrate into unusual sites such as the bile duct or peritoneum where they have the potential to cause acute and life threatening disease [1,13].

1.1.3 Immune response induced by *Ascaris suum*

Infection Characteristics

In both humans and pigs the immune response to *A. suum* is characterized by a classical helminth induced Th2 response associated with elevated levels of IL-4, IL-5, and IL-13 cytokine production and gene expression, elevated levels of circulating and cell bound immunoglobulin E (IgE), eosinophilia, and intestinal mucosal mast cell hyperplasia [4,14]. The L4 induce a partial self-clearing response from the jejunum between 14 and 21 DAI that is associated with a localized mast cell-dependent immediate type hypersensitivity response to *A. suum* antigens [4,15-16]. In both humans and pigs eosinophilic pneumonitis and an immediate type I hypersensitivity is induced by L3 that migrate through the alveolar spaces of the lung [13,17].

Protective Immune Response to Ascaris suum

Ascaris suum infection is more common among young developing piglets than among adult pigs, indicating a protective immune response to subsequent parasite infections. Further evidence of protective immunity in swine is evident by a reduced number of liver milk spots after subsequent re-infection and a reduced recovery of larvae from the liver and lungs [18-19]. In pigs immunized with adult worms and then treated with anthelmintics prior to reinoculation exhibit a strong protective response characterized by peripheral blood eosinophilia and *A. suum* specific antibody secreting cells draining the sites of infection, as well as a reduced recovery of larvae from the lungs and small intestine [20]. A similar phenomenon is seen in children where a strong correlation between elevated levels of parasite specific IgE antibodies and reduced intestinal worm burden supports a similar mechanism of protective immunity [21].

1.2 All-trans retinoic acid

1.2.1 All-trans retinoic acid and Vitamin A

Sources of Vitamin A

All-trans retinoic acid (ATRA), an active metabolite of Vitamin A (VA), is an essential vitamin that mediates a diverse array of physiological and biological processes including, vision, growth, reproduction, the control of cell growth and differentiation as well as immune and inflammatory responses [22]. VA can be found in two main forms in food. One form comes from animal sources such as liver, eggs, and dairy products in the form of retinyl ester (preformed vitamin A) or commercially

produced forms such as retinyl palimate, that are converted to retinoic acid in the small intestine. [22-23]. Another form is from vegetables such as carrots, and leafy greens in the form of carotenoids (pro-vitamin A), which converts to retinol then retinoic acid [22-23].

Vitamin A deficiency and immunity

VA deficiency is a serious health problem in the developing world associated with weakened innate and adaptive immune responses, enhanced susceptibility to infection, diseases and developmental defects [24]. Deficiency is estimated to affect approximately 127 million preschool children and 7.2 million pregnant women worldwide [25]. A close link between VA nutrient status and immune function has been known to exist for more than 70 years with early observations of improved outcomes during infections with supplemental VA extracts or VA rich food sources in malnourished and deficient animals [22]. Further understanding of the mechanism in which VA regulates immunity has been possible by the discovery of two families of VA nuclear receptors that regulate gene transcription, RA receptor (RAR) and the retinoid X receptor (RXR) [22, 26-27]. Homodimers (RXR/RXR) or heterodimers (RAR/RXR) are formed by these receptors and are known to regulate over 500 different genes [24].

1.2.2 Vitamin A, immune function, and helminth infections

Studies in both humans and mice indicate that VA status alters Th1, Th2 and T regulatory immune responses [22]. It is proposed that VA deficiency shifts the immune response towards a Th1 cell mediated response whereas VA supplementation boosts a Th2 response [28]. In several animal studies, VA deficiency significantly

biased the immune response to Th1 while high levels of VA supplementation biased a Th2 [29]. For example, in a mouse model of asthma, high VA supplementation was associated with an increase in disease severity and pulmonary Th2 responses to ovalbumin, while deficiency was associated with decreased disease severity and Th2 responses [29-30]. Coexistence of malnutrition and parasitism with ascarids have been well documented and the health effects associated with VA status and *Ascaris* infections have been studied extensively [31]. Despite frequent coexistence, little is known about their biological interactions, the affect on tissue regeneration, and the host pulmonary immune response [31]. *Ascaris suum* infection severely damages host tissue during migration causing extensive hemorrhaging and inflammation. One theory is that the Th2-type immune response elicited by these parasites may also function to promote a wound healing response and regenerate damaged tissue, however there is little evidence in its support.

1.3 Macrophages

1.3.1 Macrophage phenotypes and classifications

Macrophages exist in nearly all tissue and have a diverse functional repertoire including host defense, homeostasis, immune regulation, and wound repair [32]. They are highly susceptible to changes in their microenvironment and display highly plastic activation states able to rapidly change and adapt [33]. The ability of macrophages to change according to their environment gives rise to different cell populations with distinct functions. Several phenotypes or classifications of macrophages have been described [32]. However, the majority of our understanding

of macrophage activation lies within two main categories, Classically Activated Macrophages (CAMs) and Alternatively Activated Macrophages (AAMs). A third category of macrophage activation worth mentioning, although our understanding is limit, is Regulatory Macrophages (RMs) [34].

Classically Activated Macrophages(CAM)

Priming with IFN-γ induces the differentiation of CAMs. IFN-γ is a cytokine that is produced by both innate (NK cells) and adaptive (Th1 cells) immune cells. These cells exhibit enhanced microbicidal activity, increased superoxide anion and nitric oxide production, and secrete pro-inflammatory cytokines such as TNFα, IL-6, IL-1, and IL-12. Classical activation is important for host defense, especially against intracellular pathogens. However, activation of these cells can cause extensive damage to the host tissue and their activation must be tightly controlled [32].

Alternatively Activated Macrophages (AAM)

The cytokines IL-4 and/or Il-13, secreted by innate or adaptive immune cells, are sufficient to induce differentiation of AAMs. Cells of innate immunity responsible for IL-4 and IL-13 induced AAMs include eosinophils, basophils, mast cells, NKT cells, and even macrophages themselves [32,35] However, the main source of IL-4 and IL-13 cytokine production comes from Th2 cells of an adaptive immune response. AAMs are characterized by expression of the mannose receptor (CD206), reduced pro-inflammatory cytokine production, lower production of superoxide anion and nitric oxide production, and inefficient killing of intracellular pathogens [32]. AAMs are widely known for their ability to promote wound healing and extracellular matrix (ECM) reconstruction by secreting components of the ECM, such as

fibronectin, and by inducing ECM production of nearby fibroblasts [36-37]. In addition, alternative activation induces the upregulation of macrophage Arginase I (Arg I). Arg 1 converts arginine to ornithine, a precursor of proline and polyamine biosynthesis [32]. Like CAMs the dysregulation and uncontrolled activation of AAMs can be detrimental to the host. AAMs have been associated with several pathologies including tissue fibrosis, severe allergy, and asthma. [32].

Regulatory Macrophages(RM)

RMs are the least studied and were first identified by David Mosser and colleagues. They found that in-vitro stimulation of macrophages with a TLR agonist in the presence of high density IgG immune complexes lead to the development of a population of cells that produced high levels of IL-10. In addition to high IL-10 production these cells exhibit a repressed production of IL-12. IL-10 is a potent inhibitor of inflammation and can inhibit the activity and production of several pro-inflammatory cytokines, such as IFN-γ, IL-2, and TNF-α. Like AAMs, RMs have a reduced ability to kill intracellular parasites, however, unlike AAMs, RMs do not appear to contribute to the wound healing processes. Other factors besides immune complexes can differentiate RMs, such as prostaglandins, apoptotic cells, IL-10, and ligands for G-protein coupled receptors. However, a second stimulus, such as a TLR ligand, must be present in order to induce RM activation [32].

1.3.2 Alveolar Macrophages (AM)

As the most abundant cell in the lung and alveolar spaces, AM play a crucial role in regulating pulmonary immune responses and inflammation. They serve as the first line of defense against inhaled antigens, including pathogens, allergens, and

particulate debri. Alveolar macrophages have been described as having a unique phenotype; one that is comparable to Dendritic cells (DCs) [38]. When cultured in-vitro they also appear to be significantly better at presenting antigen than macrophages from other tissues, however, many studies indicated that they have a reduced antigen presentation ability that serves to limit the lung inflammatory response [38-41]. Like DCs, AM express CD11c at remarkably high levels. Peritoneal macrophages and bone marrow derived macrophages that have been adoptively transferred into the lung assume a DC-like phenotype, suggesting the lung environment, which is rich in GM-CSF and surfactant proteins, is the reason for the unique phenotype of AM [38]. It is believed that AM act as immunosuppressant cells that serve to limit T cell-mediated tissue damage of the lung airways [42]. Many studies refer or compare them to macrophages that have been alternatively activated [40,43]. In fact, AM have high CD206 expression. During infection with the gastrointestinal nematode parasite *Nippostrongylus brasiliensis,* AM from mice up-regulate several genes associated with alternative activation, such as ARG1, FIZZ1, and YM1 [40].

1.3.3 Macrophages in helminth infections

Helminth infections, including *Ascaris*, induce an immune response that is predominately characterized by elevations in Th2 cytokines, IL-4, IL-5, IL-13, IL-9, Il-21, and IL-25 [8]. As previously discussed, macrophages activated during Th2 responses exhibit an alternatively activated phenotype, therefore, macrophages involved and observed in a variety of helminth infections are considered to be alternatively activated [8]. During helminth infections, AAMs function to control

Th1-type inflammation, promote wound healing, and aid in worm expulsion and to promote resistance [8]. The mechanisms in which AAMs carry out these host protective functions are not well understood.

1.3.4 Vitamin A and macrophage function

Data on VAs role in regulating macrophages function is scarce. However, there is some evidence that VA status can influence macrophage immune function. One study showed the ability of VA to inhibit mouse macrophage IL-12 production in vitro, while another study in VA deficient mice demonstrated increased nitric oxide production by mouse peritoneal macrophages after LPS stimulation. These studies suggest that VA deficiency causes increased inflammation that is mediated by macrophage pro-inflammatory cytokine production. [28,44-45].

1.4 Pig as a model for Ascaris infection

A rodent model for *Ascaris* infection is not practical; they are not natural hosts and will not support the development of the late larval and adult stages in the intestine [15,46]. Human studies are limited and in most aspects unfeasible and unethical. However, using the pig as a model for *Ascaris* infection has several advantages. Pigs are naturally susceptible to helminth infections such as *Ascaris* and generally have more anatomical and physiological similarities to humans than many other animal models [15,46]. Detailed analysis of the pig, human, and mouse genomes associated with the immune response demonstrated a greater similarity between human and pig than human and mouse [15,46]. However, there are limitations to using the pig

model, such as limited immunological reagent. Hopefully with the completion of the pig genome more effort will be put into this area [15,46].

1.5 Summary and significance

Ascaris infection is a worldwide problem in both human health and in agricultural production of swine. Infection by this parasite can lead to malnutrition, disease, and poor growth [1]. It is well known that these parasites induce a Th2-like immune response in which AAMs play a crucial role in the hosts' protective immune response [14]. Our understanding of how these parasites affect host pulmonary immune responses and alveolar macrophage phenotype and function is lacking. More studies in this area should be pursued given the evidence for a reduced vaccine efficacy in pigs, as has also been reported in humans [8,10]. Alveolar macrophages are the most abundant cell type found in the alveolus of the lung. They serve as the first line of defense against invading pathogens and foreign matter making them a key player in pulmonary immunity. Alveolar macrophages have multiple functions that are critical to host defense, inflammation, tissue repair, and homeostasis. One objective of this study is to gain insight into how chronic *A. suum* infection affects and alters AM phenotype, which could shed light on the function of these macrophages.

A second aim of this study was to observe the effects of VA supplementation, in the form of All-trans Retinoic Acid (ATRA), on macrophage phenotype in both *A. suum* infected and uninfected pigs. VA is an essential nutrient and its deficiency is associated with the severity and progression of numerous infections and metabolic

disease states in humans. Notably, in the developing world where malnutrition is widespread, high dose VA supplementation of children is commonly practiced. Given the prevalence of *A. suum* in these same populations, it could be assumed that a majority of children receiving supplemental VA are also co-infected. Currently, little is known about the biological and immune interactions between *A. suum* and VA supplementation and how it affects host health. Likewise there is little data on how VA may regulate macrophage function. Therefore, it is important to study how VA in the context of *A. suum* infection affects macrophage phenotype. This could also be relevant to animal and human studies that suggest a role of VA supplementation in asthma [29,47].

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Twelve pigs (derived from boars from a four-way crossbred composite BX line [Duroc X maternal Landrace X terminal Landrace X Yorkshire] designed by scientists at the USDA ARS US Meat Animal Research Center, Clay Center, NE to be genetically similar to genetics in the commercial swine industry at the time they were born) between the ages of 16 and 22 weeks were selected for this study. All were male pigs, born, weaned, and maintained at the swine facility located at the Beltsville Agricultural Research Center. Pigs were kept two per pen in a parasite-free sealed concrete flooring facility and were provided unrestricted access to fresh water and appropriate feed rations. Diet consisted of a corn-soybean meal formulation containing 16% crude protein, vitamins and minerals that exceeded National Research Council guidelines [48].

2.2 Experimental Design

All procedures were supported by protocol#10-012 and approved by the Beltsville Area Animal Care and Use Committee. Pigs used in this study were divided into four treatment groups with three pigs per group. The treatments consisted of 1) ATRA in corn oil, 2) corn oil only (control), 3) *A. suum* infected and corn oil, and 4) *A. suum* infected and ATRA in corn oil. The inoculation dose for *A. suum* “trickle” infection was predetermined by a trial infection of 50,000 eggs that yielded approximately 1,000 L3 collected from the liver four days post inoculation (PI). The

stock preparation of 50,000 eggs/ml was diluted 1:20 and 1ml was given to each pig thrice per week from week 0 to 5. The 2,500 infective egg inoculation dose represents approximately 50 L3 migrating through the liver per day. Pigs were orally administered ATRA in corn oil at 0.1 mg/kg of body weight while control pigs were given an equal amount of corn oil. ATRA dose used in this study was selected because it is comparable to doses administered to humans and is physiologically relevant [4,49]. ATRA and corn oil were administered six times at days -1, +1, +3, +24, +26, and +28 from the start of the trickle infection on day 0. Pigs were euthanized 35 days into the study via an overdose of Ethasol (Virbac Animal Health, Fort Worth, TX, USA).

2.3 Bronchial alveolar lavage

Bronchial alveolar lavage (BAL) was obtained from the lungs of all pigs. The lobes of the excised lung was gravity-filled with 200-250 ml of phosphate-buffered saline (PBS), followed by a 30 second massage and draining of the cell suspension into 50 ml polypropylene tubes.

2.4 Flow Cytometry

Cells were isolated from the BAL fluid by washing twice with PBS supplemented with 0.5% bovine serum albumin and were resuspended at 10×10^6 cells/ml in pig staining buffer (PBS, 2% porcine serum, 1µg/100µl porcine IgG, and 0.9 mg/ml sodium azide) and incubated for 30 minutes at 4°C to block non-specific binding of antibodies. A volume of 100µl of the cell suspension (1×10^6) was

transferred into wells of a 96-well V-bottom plate, and the appropriate antibody was added and incubated at 4°C in the dark for 30 minutes. Cells were washed with 200µl PBS twice, and centrifuged at 200 rpm for three minutes. Cells were then fixed with 200µl 1% Para formaldehyde (PFA). Data was acquired by running samples on a Attune™ Acoustic Flow Cytometer using Attune Cytometric software version 1.2 (Applied Biosystems, Carlsbad, CA, USA) Data obtained were analyzed via Flowjo software version 9.3.2 (Tree Star, Inc., Ashland, OR, USA). Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software (SED).

2.5 Antibodies

The following antibody was purchased from BD Pharmingen (San Jose, CA, USA); anti-porcine ITGB1 CD29-PE (552369). Anti-human ENTPD1 CD39-PE (MCA1268PET), anti-pig CD163-FITC (MCA2311F), and anti-porcine CD203a SWC9-FITC (MCA1973F) was obtained from AbD Serotec (Raleigh, NC, USA). Anti-human PD-L1 CD274-PE (329706), and isotype control rat IgG2b-PE (400636) were obtained from Biolegend (San Diego, CA, USA). Anti-human/mouse TREM-2-PE was obtained from R&D Systems (Minneapolis, MN, USA). Anti-porcine CD-172a SWC3-PE (4525-09), isotype control mouse IgG1-PE (0102-02), isotype control mouse IgG2a-PE (0103-09), and isotype control mouse IgG2b-PE (0104-09) were obtained from Southern Biotech (Birmingham, AL, USA). Anti-human MRC1 CD206-PE (IM2741) were obtained from Beckman Coulter (Miami, FL, USA) and

anti-human/mouse IL-4Receptor-PE (I8426-70B) was obtained from US Biological (Marblehead, MA, USA).

CHAPTER 3: RESULTS

3.1 Bronchial alveolar lavage cell populations

The BAL specimens from each pig were stained with monoclonal antibodies against porcine macrophages (CD203a/SWC9) and porcine granulocytes (CD172/SWC3). SWC9 is a surface antigen expressed specifically by mature macrophages [50]. The antigen SWC3 is expressed by granulocytes including, eosinophils, basophils, and neutrophils, and macrophages [51]. Gating of live cells from pigs from the four treatment groups and assigning quadrants based on isotype control staining revealed the presence of three main cellular profiles, SWC3/SWC9 double negatives, SWC3 single positives, presumed eosinophils, and SWC3/SWC9 double positives, macrophages (**Fig. 2A**). Since the SWC3 antigen is expressed on several other cell types, a cyto-spin preparation of the BAL cells stained by diff-quick confirmed the predominate granulocyte found in the BAL from all pigs were eosinophils (data not shown). Lymphocytes were identified according to side scatter and forward scatter profiles, the lack of SWC3 and/or SWC9 expression (**Fig 2A,B**), and by cyto-spin preparations and diff-quick staining (data not shown).

3.1.1 Percentage of $SWC3^+/SWC9^+$ alveolar macrophages in BAL fluid

To ensure the phenotypic analysis presented in this study includes a relatively pure population, AM were gated according to side and forward scatter and the percentage of AM was calculated after determining the percentage of double stained $SWC3^+/SWC9^+$ macrophages (**Figs. 2B and 3**). All treatment groups were greater

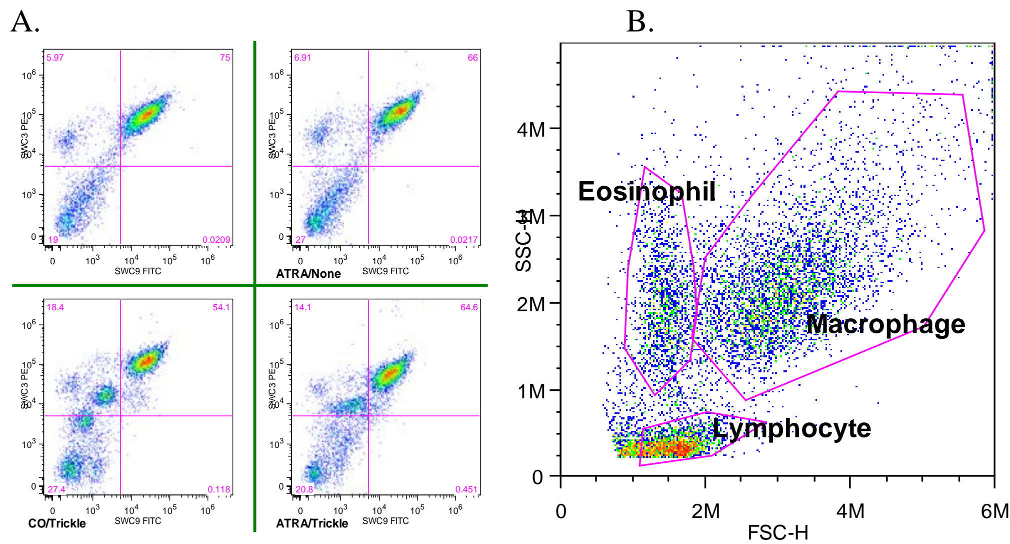


FIGURE 2. Bronchial alveolar lavage cell populations during Ascaris suum trickle infection and ATRA treatment. Cells were stained with porcine monoclonal antibodies against porcine macrophages (CD203a/SWC9) and porcine granulocytes (CD172a/SWC3) and analyzed by flow cytometry. (A) Quadrants were set based on isotype control staining. Lower left quadrants: lymphocytes. Upper left quadrants: eosinophils. Upper right quadrants: alveolar macrophages. (B) Alveolar macrophages, lymphocytes, and eosinophils were gated according to SSC/FSC from infected animals.

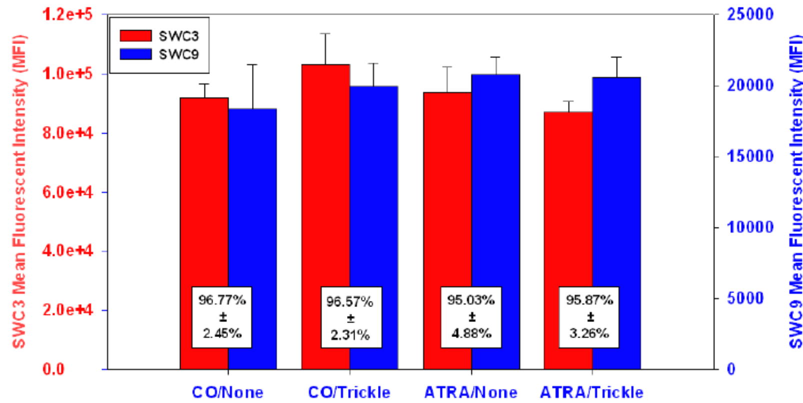


FIGURE 3. Mean Fluorescent Intensity (MFI) and percentage of alveolar macrophages co-expressing porcine macrophage marker SWC9 and porcine granulocyte marker SWC3. Cells were stained with porcine monoclonal antibodies against macrophages (CD203a/SWC9) and porcine granulocytes (CD172a/SWC3) and analyzed by flow cytometry. MFI and percentage that co-express SWC3 and SWC9 was calculated. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. Inlay within graph represents the mean ± SD percentage of alveolar macrophages co-expressing SWC3 and SWC9. Bars on graph represent the mean of MFI ± SD from three pigs per group and evaluated by ANOVA.

than 95% ± SD pure AM. This gating was used throughout this study to discriminate macrophages from other cell populations recovered from the BAL fluid.

3.1.2 Population dynamics during *A. suum* trickle infection and ATRA treatment.

To investigate the cellular changes induced by recurrent *A. suum* larvae passing through the lungs and the influence of in-vivo ATRA administration, the cellular components of the BAL was characterized in all 4 treatment groups 35 days after the beginning of the trickle infection and 7 days after the last ATRA administration. Flow cytometric analysis showed that $SWC3^{+}/SWC9^{+}$ AM were the dominant cell type found in the BAL fluid of all four treatment groups, however, pigs infected with *A. suum* had significantly ($P < 0.05$) reduced percentages of AM compared to uninfected pigs (**Fig. 4**). Uninfected pigs fed ATRA had a lower percentage of AM compared to corn oil fed pigs but slightly higher percentages than infected pigs; however these differences were not statistically significant as determined by ANOVA. As previously described [52] there was a significant ($P < 0.05$) induction of lung $SWC3^{+}/SWC9^{-}$ eosinophils in the BAL of ATRA fed and *A. suum* trickle infected pigs. Infection alone also resulted in an increase in the percentage of lung eosinophil; however these results were found to be statistically insignificant since one out of the three pigs analyzed had eosinophil percentages similar to that of uninfected pigs. The percentage of lymphocytes recovered from the BAL was not significantly affected by *A. suum* infection or infection plus ATRA compared to corn oil fed uninfected pigs. However, ATRA alone when compared to corn oil uninfected pigs had significantly ($P < 0.05$) higher percentages of

A.

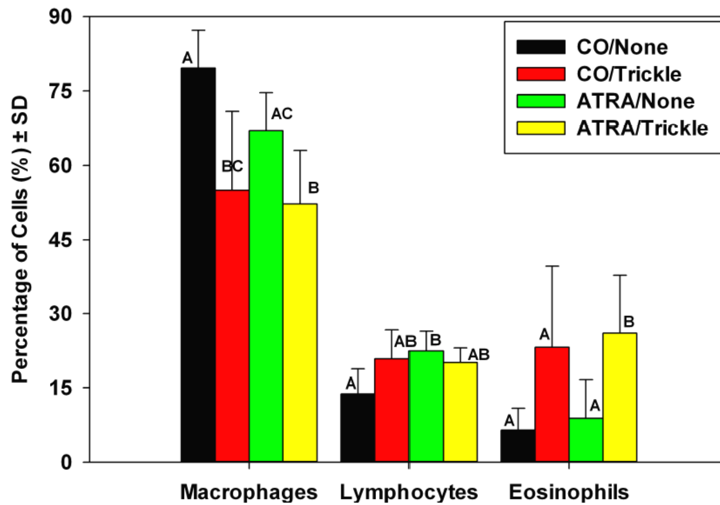


FIGURE 4. Bronchial alveolar lavage cell population dynamics during A. suum trickle infection and ATRA treatment: percentages of cells recovered from the BAL of 3 pigs per group. Cells were stained with porcine monoclonal antibodies against macrophages (CD203a/SWC9) and porcine granulocytes (CD172a/SWC3) and analyzed by flow cytometry. Percentage of cell populations was determined by quadrant. Results are expressed as the mean ± SD from three pigs per group and evaluated by one-way ANOVA. Superscripted letter annotation of treatment groups represents the statistical difference between the groups scoring P values of <0.05.

lymphocytes but not when compared to infected or infected and ATRA fed pigs (**Fig. 4**).

3.2 Altered alveolar macrophage phenotype induced by all-trans retinoic acid and Ascaris suum trickle infection.

Many helminth infections, including *Ascaris suum*, are known inducers of Th2-type immune responses in which AAM play a pivotal role in expulsion of the parasite, controlling inflammation, and promoting tissue repair. [53-54].

ATRA is known to mediate a diverse array of biological processes including the control of cell growth and differentiation as well as many immune and inflammatory responses [22]. In previous observations, (Dawson, unpublished) primary explanted AM stimulated in-vitro with ATRA demonstrate a highly polarized AAM phenotype characterized by an up-regulation of several cell surface markers associated with alternative activation as well as mRNA induction of alternatively activated chemokines. One objective of this study was to determine if in-vivo ATRA administration and/or long-term exposure to *A. suum* alters AM expression of CD206.

3.2.1 Alveolar macrophages express alternative macrophage activation marker CD206.

The mannose receptor (CD206) is a well-known marker used to identify the alternative activation of macrophages [35] and can be easily measured via flow cytometric techniques. To determine if in-vivo ATRA administration alone or in the presence of *A. suum* trickle infection or infection alone had an influence on AM CD206 expression, cells recovered from the BAL fluid were stained with anti-human CD206 and AM populations were gated according to FSC/SSC. Alveolar

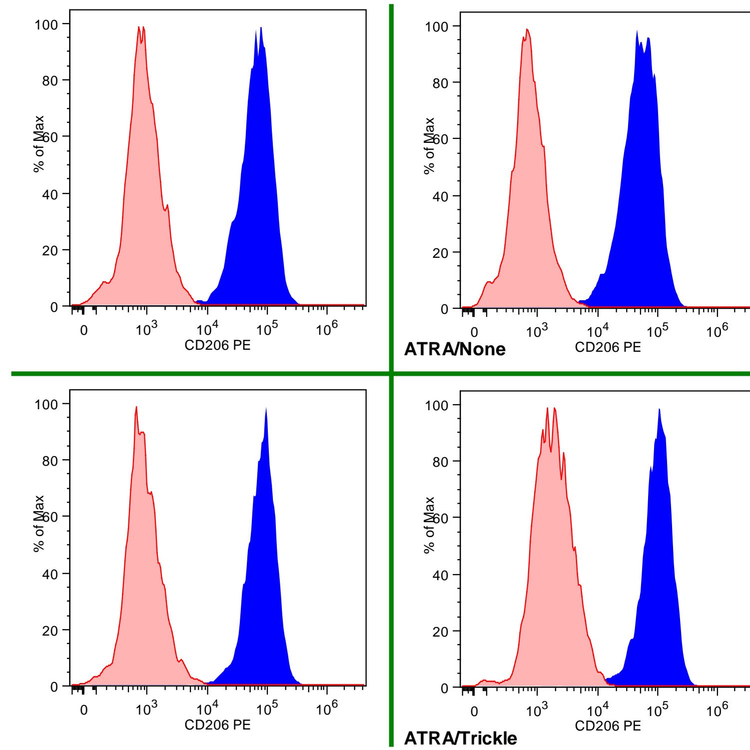
macrophages isolated from control corn oil uninfected pigs revealed a constitutive expression of CD206 (**Fig 5A**). CD206 expression was also observed on BAL cells from pigs in the three other treatment groups with no significant increase in mean fluorescent intensity. The percentage of macrophages expressing CD206 was greater than 95% ± SD for all treatment groups (**Fig. 5B**).

3.2.2 Ascaris suum infection induces a higher expression of CD163 on alveolar macrophages.

CD163 is a cystine rich, hemoglobin scavenger receptor expressed exclusively on monocytes and macrophages [55]. It scavenges hemoglobin by binding and internalizing haptoglobin-hemoglobin complexes with high specificity and affinity [56]. Macrophages expressing high levels of CD163 have been identified in the resolution phase of inflammation [57], indicating a possible role in regulating inflammation and tissue repair.

The surface expression of CD163 on AM from the BAL cells of pigs from all treatment groups was analyzed to determine if CD163 may have a role in regulating inflammation during *A. suum* infection and could be modulated by administration of ATRA in-vivo. CD163 is constitutively expressed on AM of corn oil uninfected pigs and is not significantly affected by ATRA administration; however both groups of infected pigs, regardless of ATRA treatment status, exhibited significantly ($P < 0.01$) elevated CD163 expression (**Fig. 6A and B**) ATRA did not significantly affect CD163 expression during infection. The percentages of AM expressing CD163 are greater than 95% ± SD all four treatment groups (**Fig. 6B**).

A.



B.

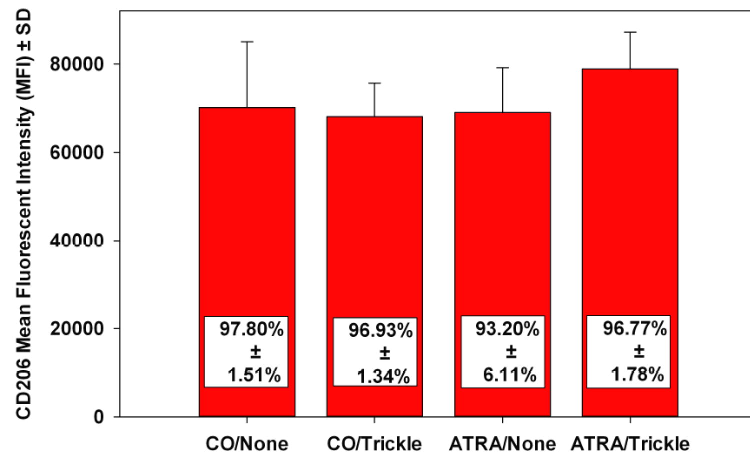
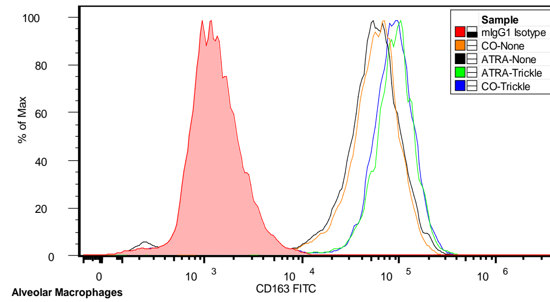


FIGURE 5. Alveolar macrophages express alternative activation marker CD206. Cells isolated from the BAL were stained with anti-human CD206, alveolar macrophage populations were determined by FSC/SSC gating of live cells and analyzed by flow cytometry. (A) Flow cytometry histograms of alveolar macrophage CD206 expression in all groups. Isotype control is illustrated in red, CD206 surface levels are represented in blue. Profiles are representative of three biological replicates. (B) MFI and percentage of gated alveolar macrophage expressing CD206. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. Results are expressed as the mean MFI ± SD and mean percentage cells expressing CD206 ± SD evaluated by ANOVA.

A.



B.

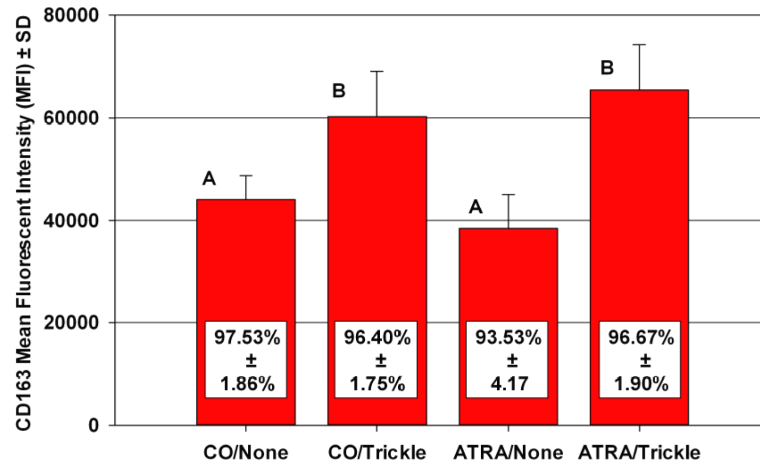


FIGURE 6. Alveolar macrophages have a higher expression of CD163 during A. suum trickle infection. Cells isolated from the BAL of three pigs per treatment group were stained with anti-pig CD163, alveolar macrophage populations were determined by FSC/SSC gating of live cells and analyzed by flow cytometry. (A) Representative flow cytometry histograms of alveolar macrophage CD163 expression. Representative isotype control staining is illustrated in red, CD163 expression for Co/Trickle is in blue, ATRA/Trickle in Green, ATRA/None in black, and CO/None in orange. Profiles are representative of three biological replicates. (B) MFI and percentage of alveolar macrophage expressing CD163 Results are expressed as the mean ± SD evaluated by ANOVA. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. Superscripted letter annotation of treatment groups represents the statistical difference between the groups scoring P values of <0.01.

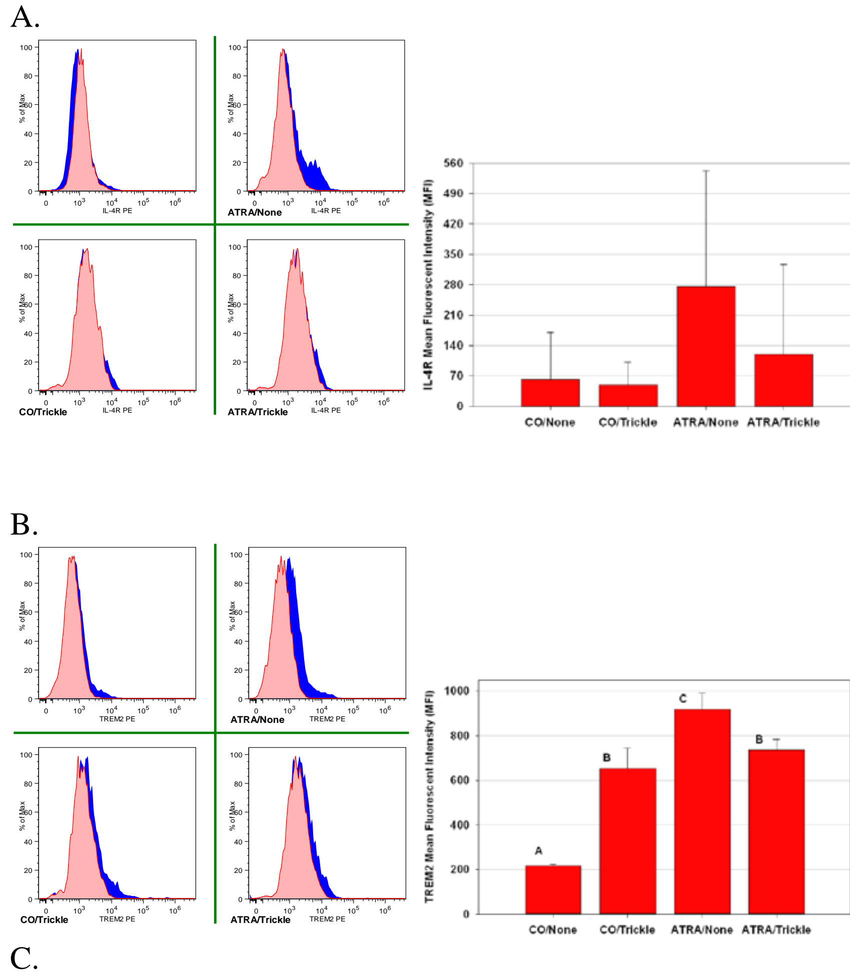
3.2.3 The expression of IL-4R and TREM2 associated with macrophage alternative activation are altered during all-trans retinoic acid administration and *A. suum* trickle infection.

The signaling pathway induced by the Th2 cytokines, IL-4 and IL-13, are dependent on the expression of IL-4 receptors. The functional IL-4 receptor is a complex consisting of IL-4Rα chain and the IL-2 gamma common (γc) chain [54]. Studies using mice report an increase in macrophages expressing both CD206 and the IL-4Rα and also the requirement of the IL-4Rα to induce macrophage alternative activation essential for survival during helminth infection [58-59].

ATRA treatment in vitro of primary explanted porcine AM and porcine cell lines have shown an increase in the IL-4Rα expression indicating a possible modulation of it expression by this VA metabolite (Dawson, data unpublished). In the current study the surface expression of the IL-4R was measured to determine if *A. suum* trickle infection or in-vivo administration of ATRA had an effect on its expression. IL-4R expression in control corn oil fed pigs was very low and remained low in infected pigs regardless of ATRA status, however uninfected pigs fed ATRA had higher levels of its expression (**Fig. 7A**). Uninfected ATRA fed pigs also displayed a significantly higher percentage of cells expressing IL-4R, 33.82% ± 10.89%, compared to the other treatment groups. Although the expression of IL-4R on ATRA fed and infected pigs was not significantly elevated above controls, it was noted that the percentage of cells expressing IL-4R in infected pigs fed ATRA was significantly (P>0.05) higher, 14.76% ± 3.12% compared to approximately 3-5%, than corn oil only and corn oil infected pigs (**Fig. 7C**) .

A.

B.



C.

Marker	CO/None	CO/Trickle	ATRA/None	ATRA/Trickle
	Percent (mean ± SD)			
IL-4R	3.20% ± 2.54%^{A}	5.53% ± 2.14%^{A}	33.82% ± 10.89%^{B}	14.76% ± 3.12%^{C}
TREM2	30.97% ± 7.60%^{A}	51.80% ± 9.07%^{B}	60.20% ± 14.31%^{C}	31.17% ± 5.63%^{A}

FIGURE 7. Surface expression and percentage of cells expressing IL-4R and TREM2 on alveolar macrophages increase in ATRA fed pigs. Cells isolated from the BAL of three pigs per treatment group were stained with anti-human/mouse IL-4R or anti-human TREM2 and analyzed by flow cytometry. Representative Flow cytometry histograms and MFI graph of alveolar macrophages (A) IL-4R and (B) TREM2 expression. Representative isotype control is illustrated in red, measured marker expression is represented in blue. Alveolar macrophage expressing IL-4R and TREM 2 was determined by FSC/SSC gating of live cells. (C) Percentage of cells expressing IL-4R/TREM2. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. All results are expressed as the mean ± SD evaluated by ANOVA. Superscripted letter annotation of treatment groups represents the statistical difference between the groups scoring P values of <0.05.

Another receptor expressed by macrophages reported to increase in its expression during alternative activation is TREM2 [54]. In mice TREM2 expression has been reported on infiltrating thioglycollate elicited peritoneal macrophages and tissue resident macrophages in the presence of Th2 cytokines and serves to inhibit macrophage cytokine production in response to TLR ligands [60]. TREM2 expression was significantly (P>0.05) increased in pigs fed ATRA only and in infected pigs both with and without ATRA treatment compared to control corn oil fed pigs; ATRA treatment alone had the highest expression of TREM2 (**Fig. 7B**). ATRA only fed pigs also had the highest percentage of cells expressing TREM2, 60.20% ± 14.31%. Corn oil fed and infected pigs had the next highest percentage of cells expressing TREM2, 51.80% ± 9.07%. The percentage of cells expressing TREM2 in both CO/ATRA and CO/Trickle pigs was found to be significantly (P<0.05) different from control, 30.97±7.60%, and ATRA/Trickle pigs, 31.17%±5.63%, (**Fig. 7C**).

3.2.4 PDL-1 (CD274) alveolar macrophage expression is altered by *Ascaris suum* infection and in-vivo ATRA administration.

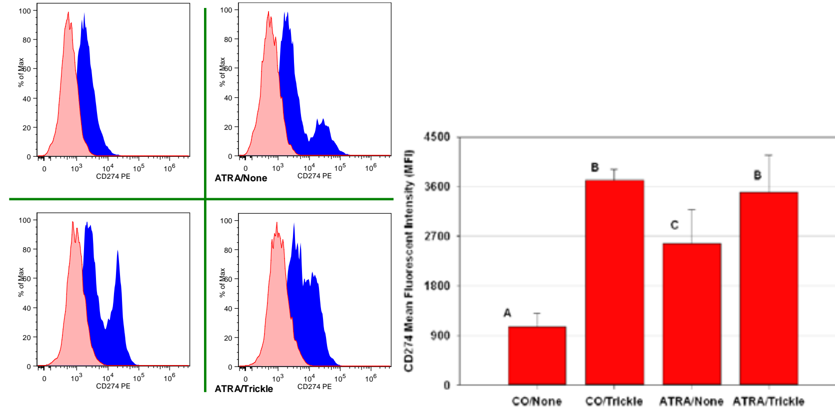
A previous experiment by Siracusa et al. involving the infection of mice with the parasitic helminth *Nippostrongylus brasiliensis* showed constitutive expression of CD274 by AM that significantly increased in its expression 13 days post-infection. [40]. In the current study, the surface expression of CD274 was measured to determine if it expression profile was affected by *A. suum* trickle infection and/or administration of ATRA. Cells from the BAL fluid of pigs from all four treatment groups were stained with anti-human CD274 (PDL-1) monoclonal antibody. Analysis of AM from control corn oil fed pigs revealed a constitutive expression of CD274, which significantly increased during infection on both CO/Trickle and ATRA/Trickle

treated pigs (**Fig. 8A**). ATRA alone also significantly increased CD274 expression compared to control corn oil fed pigs but had slightly lower expression compared to infected pigs. The percentage of cells expressing CD274 did not significantly change during infection and/or ATRA administration (**Fig 8B**).

3.2.5 Surface phenotype of alveolar macrophages during *A. suum* infection and in-vivo administration of ATRA.

The surface expression of CD29 and CD39 were also analyzed in this study. Alveolar macrophage CD29 expression significantly ($P<0.05$) decreased during *A. suum* infection in both CO/Trickle and ATRA/Trickle pigs but was found to be significantly ($P<0.05$) increased on AM of pigs only administered ATRA (**Fig. 9A**) Trickle infection greatly reduced the percentages of cells expressing CD29 compared to control corn oil and ATRA fed pigs ($P<0.01$). Approximately 80% of AM express CD29 in control and ATRA pigs compared to 0-5% in infected pigs (**Fig. 9C**). The molecule involved in adenosine metabolism CD39 was significantly ($P<0.01$) affected by ATRA because its expression was elevated only in pigs fed ATRA in the absence of infection (**Fig. 9B**). Likewise the percentage of AM expressing CD39 was significantly elevated only in pigs fed ATRA (**Fig. 9C**).

A.

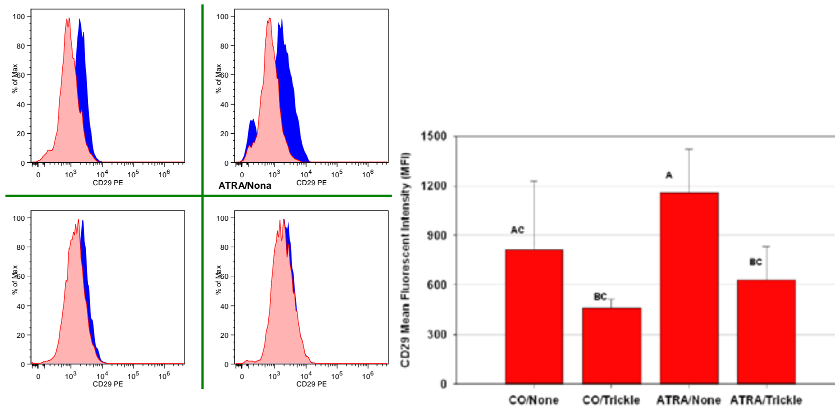


B.

Marker	CO/None	CO/Trickle	ATRA/None	ATRA/Trickle
CD274	Percent cells (mean ± SD)			
	80.23% ± 7.16%	85.80% ± 4.96%	83.40% ± 6.70%	88.43% ± 2.20%

FIGURE 8. Ascaris suum Infection and/or ATRA increase alveolar macrophages PDL-1 expression. Cells were isolated from the BAL of three pigs per treatment group and stained with anti-human CD274 (PDL-1) and analyzed by flow cytometry. Representative flow cytometry histograms and MFI graph of alveolar macrophages (A) CD472 expression. Representative isotype control staining is illustrated in red, measured marker expression is represented in blue. Alveolar macrophages were determined by FSC/SSC gating of live cells. (C) Percentage of cells expressing CD274. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. All results are expressed as the mean ± SD evaluated by ANOVA. Superscripted uppercase letter annotation of treatment groups represents the statistical difference between the groups scoring P values <0.05.

A.



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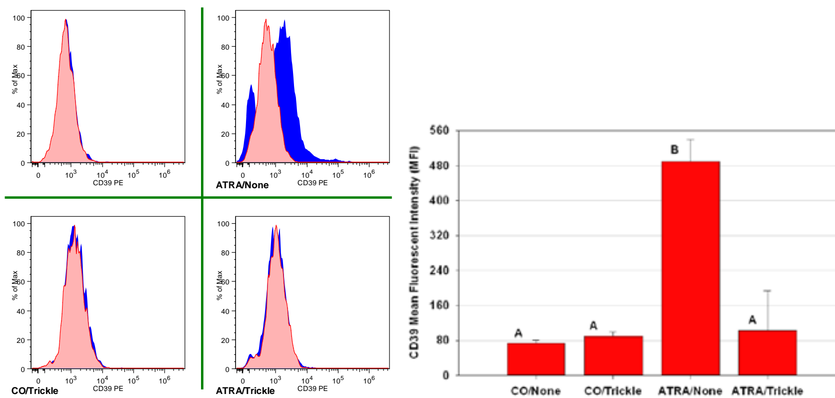

C.

Marker	CO/None	CO/Trickle	ATRA/None	ATRA/Trickle
	Percent cells (mean ± SD)			
CD29	76.70% ± 9.36^{A}	0% ± 0 %^{B}	82.57% ± 14.02%^{A}	5.0% ± 8.66%^{C}
CD39	0.57% ± 0.80%^{A}	0.18% ± 0.16%^{A}	35.73% ± 5.7%^{B}	0.6% ± 0.52%^{A}

FIGURE 9. Surface phenotype of alveolar macrophages. Cells were isolated from the BAL of three pigs per treatment group and stained with anti-human CD29 or anti-human CD39 and analyzed by flow cytometry. Representative Flow cytometry histograms and MFI graph of alveolar macrophages (A) CD29, (B) CD39 expression. Representative isotype staining is illustrated in red, measured surface molecule expression is represented in blue. Alveolar macrophages determined by FSC/SSC gating of live cells. (C) Percentage of cells that express the measured surface molecule. Percentages were determined using Flowjo Super-enhanced D-max Subtraction statistical software. All results are expressed as the mean ± SD evaluated by ANOVA. Superscripted uppercase letter annotation of treatment groups represents the statistical difference between the groups scoring P values <0.05.

CHAPTER 4: DISCUSSION

The phenotype of alveolar macrophages (AM) during chronic trickle infection with *A. suum* has not been critically examined nor has the effect of administration of ATRA in the diet alone or during infection. This combination of treatments is likely common in humans from underdeveloped regions where infection with *Ascaris* is prevalent and where public health initiatives to control nutrient deficiencies often include supplementation with VA. In this study we showed that a low dose and repeated "trickle" infection with *A. suum* alone and with ATRA administration altered the AM cell surface antigen phenotype. An infective dose of 2,500 eggs was given three times per week for five weeks to pigs. Oral administration of ATRA was given at six different time points throughout a five-week period to both infected and uninfected pigs. Bronchial alveolar lavage (BAL) fluid was collected to obtain an enriched and reliable source of AM. The collection of BAL is a widely used technique that has been shown to accurately reflect cellular exudates from the lung parenchyma during lung injury [61]. Flow cytometry was used to measure the cellular and fluorescent characteristics of these cells after labeling with fluorochrome conjugated monoclonal antibodies and fixation. Cell populations were determined by their FSC/SSC profiles and the cell surface antigen expression of CD172a and CD203a was indicated by staining with two monoclonal antibodies anti-porcine SWC3-PE and anti-porcine SWC9-FITC, respectively. Alveolar macrophage were gated and analyzed for purity, which exceeded >95%, and for the expression 14 different cell surface markers.

Our data indicated that chronic *A. suum* trickle infection induced changes in the cellular composition of the BAL. Infection increased the percentage of eosinophils present in the BAL 25-28% compared to 6-8% in uninfected pigs. This increase in the percentage of eosinophils was accompanied by a reciprocal decrease in the percentage of macrophages. In pigs given a primary single dose *A. suum* the level of eosinophilia is significantly higher than those observed in the current study with up to 70% eosinophils in the BAL at approximately two – three weeks after inoculation [52]. Since eosinophils are an indicator of inflammation and important for acquired immunity to parasitic infections, the relatively lower percentage of eosinophilia observed in this study could indicate an acquired immunity to chronic infection or a result of fewer migrating larvae in the lung following multiple exposures or a down regulatory effect induced by chronic infection. Several studies indicated an acquired immunity to *A. suum* after repeated exposure; however, the mechanism remains elusive [19, 62-63]. It is not clear which organ system is most prominent in the resistance to migrating *A. suum* larvae. Urban et al. (1988) reported a reduced "milk spot" formation in the liver following trickle infection that suggested a pre-hepatic immunity. However, Serrano et al (2000) observed a low number of larvae in the lung compared to the number of "milk spots" and suggesting this reflects the trapping of larvae in the liver and indicates a more pronounced immunity in the lung. A recent study using green fluorescent IL-4 reported mice and truncated larval infection identified the lung as an important site for priming CD4 T-cell-mediated protective immunity against *N. brasiliensis*, a gastrointestinal nematode parasite of rodents that exhibits similar characteristics to *A. suum* infection in the lung [64]. It would be of

future interest to identify if the acquired protection of pigs to *A. suum* infection in the lungs expresses a similar mechanism. A greater understanding of the key immunological processes that lead to the development of acquired immunity could lead to new vaccine strategies that contribute to improved human and animal health.

One objective of this study was to determine if ATRA and/or long-term exposure to *A. suum* altered AM expression of CD206. The mannose receptor (CD206) is a type 1 trans membrane glycoprotein expressed by macrophages and certain populations of endothelial and dendritic cells. CD206 expression on macrophages has become a well know marker of macrophage alternative activation [34]. Tissue resident AM are known to constitutively expression CD206 indicating these cells are already polarized to an alternatively activated macrophage (AAM) phenotype. A study in humans with lung fibrosis showed an up-regulation CD206 in these patients when compared to normal control patients, indicating a highly polarized AAM phenotype [65]. Since, *A. suum* larval migration induces extensive lung damage and tissue fibrosis and is an inducer of Th2-type immune responses in which it is believed AAM play a pivotal role, we investigated the effect of infection on AM CD206 expression [53-54]. In previous studies, (Dawson et al, unpublished) primary explanted AM stimulated in-vitro with ATRA demonstrated a highly polarized AAM phenotype characterized by an up-regulation of several cell surface markers associated with alternative activation as well as mRNA induction of several genes associated with alternative activation. In the current studies using freshly explanted cells isolated from BAL, no changes were seen in CD206 expression to an already highly polarized AAM phenotype in response to in-vivo administration of

ATRA or to *A. suum* trickle infection. Interestingly, CD163 expression in AM of *A. suum* infected pigs was significantly up-regulated compared to corn oil treated uninfected controls. The percentage of cells expressing CD163 (~95%) remained the same for all groups; however the MFI greatly increased on cells from *A. suum* infected pigs. This may be of significance given that the up regulation of CD163 expression is induced by IL-10, IL-6, and glucocorticoids, but down-regulated by IL-4, IL-13, IFNγ, and TNFα [66]. CD163 expression appears to be tightly controlled by pro- and anti- inflammatory mediators, indicating that it may play an important role in the control of inflammation [67]. Macrophages with high CD163 expression have been identified during the resolution phase of inflammation, indicating a possible role in regulating tissue repair [57]. CD163 functions as a scavenger receptor that binds and internalizes circulating haptoglobin-hemoglobin (Hp–Hb) complexes. Internalization of these complexes then fuels an anti-inflammatory response via the secretion of IL-10 that is mediated by heme metabolites [67-68]. Hemorrhage and increasing red blood cell content in the alveolar spaces is a feature of the early response to migrating *A. suum* L3 in the lung that is rapidly resolved with time after infection. In our data, the high expression of CD163 on AM of *A. suum* trickle infected pigs may indicate a macrophage phenotype that differs from the AAMs reported during primary parasite exposure. The macrophages we observed may fit more into the category of regulatory macrophages (RM) or into a spectrum between AAM and RMs proposed by Mosser and Edwards (2008). Macrophages expressing high levels of CD163 have been compared to and even suggested to be

AAM, however, data indicating that IL-4 and IL-13 down-regulate this molecule on macrophages goes against this theory. CD163 expressing macrophages have also been implicated as having an important role in wound repair processes; however RMs are thought to not be involved in this process. Given this contradiction, further investigation into the phenotype, function, and role of these cells during chronic *A. suum* infection seems warranted.

We also examined the expression of two other surface molecules associated with macrophage alternative activation, the IL-4 receptor and the triggering receptor expressed on myeloid cells 2 (TREM2). Only ATRA fed pigs had a higher MFI and higher percentage of macrophages expressing IL-4R. It is important to note that two out of the three pigs examined had higher IL-4R expression whereas one pigs had expression levels comparable to controls pigs, therefore these data were not statistically significant. It is possible that ATRA was inefficiently administered to this pig or that it became oxidized, although the multiple exposures to ATRA make this unlikely. The out-bred genetic background of these animals could indicate a pig unresponsive to the dose of ATRA that was given. TREM2 MFI expression was very low on control corn oil treated pigs but was found to be significantly up-regulated on ATRA fed pigs as well as infected pigs with the highest expression seen on ATRA treated pigs. Likewise the percentage of cells positive for TREM2 expression were highest on ATRA fed pigs (33%) and *A. suum*-infected pigs fed ATRA (14.76%) compared to controls (3%) and

infection only treated pigs (5%). It has been proposed that VA deficiency shifts the immune response towards a Th1 cell mediated response whereas VA supplementation boosts a Th2 response [28]. In our study the pigs are not VA deficient, however given the observed up-regulation of these two surface molecules on pigs fed ATRA in the absence of infection it is possible that ATRA is acting on resident AM to induce a skewed Th2 immune response in pigs. Functional studies of these cells are lacking and further studies would have to be performed to verify this theory. However, archived samples of isolated RNA could be used associate changes in gene expression with phenotype. TREM2 has been found to be up-regulated on macrophages exposed to IL-4 and IL-13 and on infiltrating macrophages independent of presence of alternative activation [60]. Since infiltrating macrophages express TREM2, this could provide one explanation for the observed TREM2 expression and lack of IL-4R expression on AM of infected pigs. Interestingly, infected pigs fed ATRA have a higher percentage of cells expressing TREM2 than pigs infected only. It is possible that ATRA is having some effect on resident macrophages while infection is inducing the infiltration of TREM2 expressing macrophages. Again, further studies need to be performed to verify this theory.

The expression of the programmed death ligand 1 (CD274) was found to be constitutively expressed on AM of control pigs, however its expression was significantly increased in pigs from all the other treatment groups, with the highest expression on AM from pigs infected and treated with ATRA. The percentage of cells expressing CD274 was similar for all groups. CD274 is a member of the B7 family of immune-regulatory ligands involved in T-cell co-stimulation. It functions to bind to

its receptor, programmed death 1 (PD-1), on activated T-cells to down-regulate T cell responses [69]. Similar to our results, Siracusa et al [40] infected mice with *N. brasiliensis* and showed constitutive expression of CD274 by AM that significantly increased at 13 days post-infection. It was suggested that the expression of CD274 on AM indicated a more anti-inflammatory response during the later stages of infection. In our study, it is possible that CD274 expression on AM functions to suppress T-cell activity in order to avoid further inflammatory tissue damage during long-term exposure to *A. suum* that could be a target of immune regulation by ATRA. Further research is necessary to determine if these cells are immunosuppressive.

We also investigated the expression of nine other surface molecules, CD25, CD29, CD39, CD62L, CD86, CD95, CD273, CD275, and CD279. Only two out of these nine, CD29 and CD39 were expressed by AM. CD29 was only expressed on cells from control corn oil treated uninfected pigs and ATRA fed pigs. Interestingly, CD29 was completely absent from AM from infected pigs regardless of ATRA treatment status. Our data did not indicate a role for ATRA in regulating CD29 expression on AM; however infection may negatively regulate CD29 expression. CD29 is a part of the integrin family involved in immune cell trafficking from blood to tissues [70]. CD39 is an integral membrane protein with triphosphate diphosphohdrolase activity that converts ATP to AMP [70]. Only pigs fed ATRA in the absence of infection expressed CD39, whereas all other treatment groups did not.

In conclusion, we observed marked changes in several surface molecules expressed by AM in response to *A. suum* trickle infection and/or feeding ATRA at a physiological concentration to pig. The changes observed in AM phenotype during

infection indicated a population of cells that express characteristics of both AAMs and RMs that suggested functional inhibition of T-cell responses and inflammation. Administration of ATRA in the absences of infection induced a population of AM that could potentially boost type-2 like immunity to pathogens. Given these results, further studies to define the dynamics and functional role of AM during *A. suum* infection and in response to feeding ATRA could model the health consequences in humans faced with similar interactions.

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