IDENTIFICATION OF VACCINE RESPONSIVENESS BIOMARKERS THROUGH KINOME ANALYSIS IN PIGLETS

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ABSTRACT

Individual variability in immune responses to vaccination can result in vaccinated individuals who fail to develop protective immunity. These "vaccine low-responders" remain at risk for infection and compromise the protection achieved through herd immunity. Biomarkers of vaccine unresponsiveness could enable rapid identification of susceptible low-responders while discerning mechanisms of vaccine-induced immune responses. To investigate biomarkers of vaccine unresponsiveness, piglets (n=117) were vaccinated with a commercial *Mycoplasma hyopneumoniae* bacterin, and vaccine-induced serum IgG titers were quantified 35 days following vaccination. High (HR) and low (LR) vaccine responders within the 80^{th} and 20^{th} percentile of serum IgG titers were stratified, respectively, and split into discovery (n=12) and validation (n=8) cohorts. Within the discovery cohort, kinome analysis conducted on peripheral blood mononuclear cells collected from HR and LR revealed multiple differential phosphorylation events before and 6-days following vaccination. Differential phosphorylation events before vaccination were enriched in cytokine signaling pathways, a result supported by the quantification of higher plasma interferon-gamma (IFNγ) and interleukin-1beta (IL-1β) in LR compared to HR before vaccination. Additionally, LR had lower birth weight than HR, thus establishing significant associations between vaccine responsiveness and kinase signaling, plasma cytokines, and birth weight. Analysis of the validation cohort verified the differential phosphorylation events identified within the discovery cohort, but there were no differences in birth weight or plasma cytokines between LR and HR. In a second trial, piglets (n=67) from a different facility were vaccinated with the same *Mycoplasma hyopneumoniae* bacterin to further evaluate plasma cytokines and birth weight as biomarkers of vaccine unresponsiveness. Piglets in the second trial all seroconverted, and serum IgG titers varied less than the first trial. While the second trial found no associations between vaccine unresponsiveness and either birth weight or plasma cytokines, it revealed piglets had age- and litter-dependent differences in plasma IFNγ and IL-1β concentrations within the first 2-months of life. Collectively, these data suggest that though plasma cytokines or birth weight can be associated with vaccine unresponsiveness, their temporal and individual variability can make them inconsistent biomarkers. Phosphorylation biomarkers offered consistent discrimination of HR and LR and provided insight into potential mechanisms regulating vaccine-induced immunity.

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A_{650}	Absorbance at 650 nm
ELISA	Enzyme-linked immunosorbent assay
FC	Fold-change
FDR	False-discovery rate
FMDV	Foot and mouth disease virus
HBV	Hepatitis B virus
HR	High responder
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
LLOQ	Lower limit of quantification
Log2	Logarithm to the base 2
LR	Low responder
MHC	Major histocompatibility complex
PBMC	Peripheral mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PIIKA	Platform for Intelligent, Integrated Analysis
PRR	Pattern recognition receptor
SNP	Single nucleotide polymorphisms
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha

1. INTRODUCTION

1.1 Vaccination and Disease Prevention

Vaccination is one of the most effective and economical means of protecting humans and animals against infectious diseases. Vaccination aims to induce an immune response within the individual and establish immunological memory against a molecular fragment that mimics the pathogen (Zepp, 2016). This preemptive development of vaccine-specific immunity typically allows the individual to mount an immune response to the live-infectious agent (Pollard and Bijker, 2021). However, biological variability among individuals can result in inconsistent vaccine immunogenicity, particularly within populations that are heterogeneous with respect to genetics, age, and health status. Individuals may develop weak vaccine-induced immune responses resulting in the immune system being incapable of preventing or minimizing infection by the vaccine-associated pathogen. Therefore, individuals who are incapable of responding (termed "non-responders") or respond insufficiently (termed "low-responders") to the vaccine are a potential detriment to the health of the population and require appropriate management.

1.1.1 Individual Variability in Vaccine Responses

Individual variation in vaccine responses is a natural occurrence that can result in low vaccine responders within a population. This phenomenon has been observed for multiple vaccine regimens in a range of species. For example, 5-10% of individuals vaccinated against hepatitis B virus (HBV) failed to develop protective levels (anti-HBs>10 mIU) of anti-HBV antibody (Averhoff *et al*., 1998; Poland and Jacobson, 2004; Walayat *et al*., 2015). Vaccines against the influenza virus can be an effective means of disease control, yet antibody responses following influenza virus vaccination consistently vary among populations, independent of influenza strain/season (Keitel *et al*., 2006; Levine *et al*., 2016; Nakaya *et al*., 2015). Within animal health, piglets vaccinated against tetanus toxoid can exhibit a range of seropositive and seronegative antibody responses 28-days following vaccination, suggesting inconsistent levels of protection (Adler *et al*., 2015). Recently, pigs vaccinated with a commercial *Mycoplasma hyopneumoniae* (*M. hyopneumoniae)* bacterin had highly variable *M. hyopneumoniae*-specific antibody responses, with only 80% of pigs having persistent antibody responses 118-days post-vaccination (Blanc *et al*., 2021). Others have observed variable antibody responses in populations vaccinated with both

commercial and experimental foot-and-mouth-disease virus (FMDV) vaccines (Braun *et al*., 2018; Jouneau *et al*., 2020; Knight-Jones *et al*., 2015). While factors influencing the magnitude of vaccine responses are explored later (**Section 1.3**), these previous studies demonstrate that a variety of vaccines used in both humans and animals can result in individuals classified as vaccine low-responders.

1.1.2 Protection through Herd Immunity

The protection afforded by vaccination is achieved at the level of the individual and the population. For individuals, vaccination programs usually prioritize either reducing infection susceptibility or promoting pathogen clearance, while from the population perspective, the priority is to reduce the incidence of exposure and limit infections (Rose and Andraud, 2017; Smith, 2010). A vaccine's effectiveness depends on multiple factors, including the pathogen transmission potential, vaccine coverage, and vaccine efficacy. If the transmission potential of the pathogen is high, the vaccine coverage must compensate to establish protection in the population. The basic reproduction number of a pathogen (R_0) is the average number of secondary infections a single infectious individual infects within a susceptible population. R_0 is directly proportional to the number of protected individuals needed to establish population-wide protection, known as herd immunity (Fine *et al*., 2011; Smith, 2010). Pathogens with a high R_0, such as measles virus (R_0 ~ 12 to 18), require a higher vaccine coverage of the population than other pathogens with a lower R_0, such as influenza virus (R_0 ~ 1 to 2) (Biggerstaff *et al*., 2014; Guerra *et al*., 2017). The R_0 is highly dependent on biological and sociodemographical factors but can be estimated using "$P = 1-(1/R_0)$", where P = the proportion of the population to vaccinate to stop transmission (Guerra *et al*., 2017; Smith, 2010). So, when a sufficient proportion of the population has been vaccinated against the pathogen, pathogen transmission is hindered and reduces the probability of encountering unvaccinated and susceptible individuals (Rose and Andraud, 2017).

Vaccine efficacy, the ability to reduce disease incidence, relies on vaccine immunogenicity, the vaccine's ability to trigger an immune response (Mahanty *et al*., 2015; Smith, 2010). Therefore, factors that compromise vaccine immunogenicity inevitably reduce vaccines efficacy by promoting the development of vaccine non-responders (Weinberg and Szilagyi, 2010). In addition, vaccine non-responders challenge the achievement of herd immunity, requiring a higher vaccine coverage threshold (Fine *et al*., 2011; Heininger *et al*., 2012). Thus, not only do

non-responders impair protection of individuals, but they also compromise efforts to achieve herd immunity and should be identified and managed accordingly.

1.1.3 Biomarkers of Vaccine Responses

Identifying vaccine non-responders/low-responders usually requires quantifying surrogate metrics of vaccine outcomes, such as antigen-specific antibody titers or T-cell responses, in the weeks following immunization (Plotkin, 2001). This approach to quantifying vaccine responses has several practical limitations. First, there is a window of opportunity between vaccination and quantification of vaccine outcomes when pathogens may infect and be transmitted by non-responders within the population. Second, quantifying these surrogate metrics requires additional time and economic cost to the health care system or livestock producer. This problem creates a need for more rapid and inexpensive identification of non-responders. Early identification of vaccine non-responders through biological markers (termed "biomarkers") that reduce the time of identification and/or the cost of testing could provide a valuable tool for improving individual and population health. The discovery of biomarkers capable of predicting future vaccine responses could allow immediate identification of vaccine non-responders and facilitate strategic management decisions, such as revaccination, physical isolation, or, in the livestock industry, culling or genetic selection of individual animals (te Beest *et al*., 2011; Knight-Jones *et al*., 2015; Mallard *et al*., 2015). As well, biomarkers providing a molecular basis of vaccine unresponsiveness could be utilized to improve vaccine delivery strategies to minimize the frequency of vaccine non-responders (Pulendran *et al*., 2010). Research into understanding the molecular events within the host immune system before and after vaccination may prove fruitful in discovering possible biomarkers of vaccine responsiveness.

1.2 Immune Responses to Vaccination

The primary objective of vaccination is to induce an immune response against a specific pathogen that can prevent clinical symptoms of infection (Zepp, 2016). A variety of pathogen components can act as vaccine antigens, including biomolecular components of the pathogen (e.g. protein subunits, peptides, toxins, polysaccharides, nucleic acids) or an inactivated/killed version of the pathogen itself. Alternatively, attenuated pathogens can be generated by removing the virulent effects that harm the host, but pathogens retain the ability to cause infection so the immune

system can generate immunity (Pollard and Bijker, 2021). In either case, vaccination prepares the immune system to detect, mediate, and respond against a future attack by the pathogen.

1.2.1 The Innate Immune Response

The innate immune system represents the initial line of defense against invading pathogens and consists of phagocytic cells, protein complement, and physical barriers. Innate responses occur within minutes to hours of infections and are hallmarked by being relatively nonspecific and lacking immunological memory to recognize recurring infectious agents (Coffman *et al*., 2010). The breadth of detection by the innate immune system is derived from its ability to recognize a spectrum of bacteria, viruses, parasites, or tissue damage. Detecting infectious agents is facilitated by pathogen recognition receptors (PRRs) on host cells, including phagocytic dendritic cells. PRRs like Toll-like receptors (TLRs) recognize and bind pathogens through evolutionary distinct but highly conserved molecules called pathogen-associated molecular patterns (Pulendran and Ahmed, 2011; Rosadini and Kagan, 2017). PRRs bind a range of pathogen-associated molecular patterns (e.g. lipopolysaccharide, viral nucleic acids) to activate signal transduction events. PRR activation results in the expression of genes encoding proteins involved in defensive host activities including pro-inflammatory cytokine release, complement cascades, pathogen opsonization, and the recruitment of phagocytes (Mogensen, 2009; Takeda and Akira, 2004). These processes promote pathogen clearance, minimize damages to the host, and, importantly for vaccination, initiate antigen-specific responses.

A critical cell type for transitioning from an innate immune response into an adaptive immune response are the dendritic cells. The dendritic cells sense, engulf, and lyse pathogens to present antigenic fragments on their extracellular surface (Pulendran and Ahmed, 2006). This stimulation of dendritic cells differentiates the cell into an antigen-presenting cell, where cytokine signals coordinate the migration of activated dendritic cell to the draining lymph nodes (Iwasaki and Medzhitov, 2004). Here, antigen-presentation cells prime and direct T-cell differentiation by presenting antigenic fragments on major histocompatibility complex (MHC), along with other costimulatory molecules, to naive T-lymphocytes (Iwasaki and Medzhitov, 2004; Reis e Sousa, 2004). Vaccines must stimulate specific T- and B-lymphocyte responses to establish immunological memory and elicit a rapid, precise response against subsequent infection.

1.2.2 The Adaptive Immune Response

Compared to the innate immune response, the adaptive immune response develops more slowly, is pathogen-specific, and leads to immunological memory. Pathogens have evolved mechanisms to evade innate immune responses, so establishing an adaptive immune response enables the immune system to recognize pathogen-specific antigens (Janeway *et al*., 2001). T-lymphocytes deliberate effector and regulatory functions, while B-lymphocytes primarily mediate antigen-specific antibody responses (Zepp, 2016). T- and B-lymphocytes express clonal antigen recognition receptors, called T-cell receptors and B-cell receptors, respectively, that bind specifically to antigen peptides and lead to T- and B-lymphocyte activation and differentiation (Janeway *et al*., 2001; Pulendran and Ahmed, 2011). Recognizing the roles of lymphocytes and their activities within the vaccine-induced immune response may be critical for distinguishing responders from non-responders.

1.2.2.1 T-lymphocytes and Cellular Responses

T-cells have many functions that largely depend on their distinct functional subset (Kumar *et al*., 2018). Nucleated cells intracellularly infected with a pathogen communicate their infection by processing, loading, and presenting antigenic fragments of the pathogen onto the cell surface through MHC class 1 proteins. MHC-I resides on the surface of all nucleated cells and bear both endogenous degradation of self and foreign antigens (Hewitt, 2003). T-cells bearing the surface protein CD8 (CD8+ T-cells) recognize foreign antigens through surface T-cell receptors and bind the antigen-MHC-I complex, leading to a cytotoxic response and killing infected cells with cytotoxic factors. Conversely, exogenously-derived foreign antigens are presented by antigen-presenting cells on MHC Class II receptors which are recognized by T-cell receptors on T-cells bearing the surface protein CD4 (CD4+ T-cells) (Hewitt, 2003). Analyses of vaccine responders to HBV vaccination found recessive haplotypes of MHC-II human histocompatibility leukocyte antigen in non-responders, suggesting that there are associations between mutations in the MHC-II presentation system and impaired vaccine responses (Alper *et al*., 1998; Kruskall *et al*., 1992). T-cell receptor stimulation, combined with co-stimulatory and cytokine signals, commits the CD4+ T-cell into one of many helper T-cell subsets. Helper T-cells have many supportive functions in activating innate immune cells, promoting T-cell differentiation, promoting antibody formation, adhesion and regulating inflammatory responses (Crotty, 2015; Hennecke and Wiley,

2001). Current vaccines must optimize the coordination among the vaccine antigen, helper T-cells, and B-cells to induce protective vaccine-specific immune responses.

1.2.2.2 B-lymphocytes and Antibody Responses

B-lymphocytes, are the primary cell of the humoral response due to their ability to generate and secrete antibodies specific to an antigenic fragment (Alberts *et al*., 2002). Antigen-specific antibody generation is initiated when whole proteins or antigenic fragments bind the B-cell receptor. Antigens can bind B-cell receptors either with (T-cell-dependent) or without (T-cell-independent) co-stimulation from antigen-specific T-cells recognizing the same antigen, yet T-cell-independent binding typically generates a weaker response and lacks memory (Pollard and Bijker, 2021; Pulendran and Ahmed, 2006). T-cell-dependent activation leads to downstream signaling effectors that result in cytoskeletal rearrangement and the maturation and differentiation of the antigen-specific B-lymphocyte (Crotty, 2015; Janeway *et al*., 2001; Li *et al*., 2019). Activated B-lymphocytes produce antigen-specific antibody isotypes like immunoglobulin G (IgG) or IgA with high affinity and differentiate into antibody-producing plasma cells or memory B-cells. Memory B-cells differentiate with the help of CD4+ T-cells to provide rapid antibody responses following antigen re-exposure, while plasma cells continuously secrete antibodies into the circulation (Alberts *et al*., 2002; Pollard and Bijker, 2021; Zepp, 2016). However, the generation of antigen-specific antibodies may not protect against infections, as observed with intracellular pathogens like *Mycobacterium tuberculosis* or HIV (Siegrist, 2018). Therefore, vaccine optimization requires recognizing the antigen-specific effectors needed for protection against a given pathogen (Pulendran and Ahmed, 2011).

Establishing innate and adaptive immune responses requires highly integrated cellular communication through various signaling cascades to achieve an effective, antigen-specific response. As a result, modulating the expression and activity of receptors, transcription factors, signaling molecules, or changes in cell frequencies can profoundly influence the immune response to vaccines. These modulations are known to be driven by vaccine, host, environmental, and developmental factors.

1.3 Factors affecting Vaccine Responses

Numerous vaccine and host factors modulate the immune response within both the innate and adaptive immune systems. Inconsistent vaccine responses can reflect inherent problems

related to the vaccine, such as antigen optimization, adjuvant selection, vaccine type, and administration method. In addition, the individual variation in vaccine-induced immune responses is primarily attributed to heterogeneity within a population determined by genetic differences, prior environmental exposures, perinatal factors, nutrition, and behavior, to name a few (Zimmermann and Curtis, 2019). Vaccination must overcome these factors to elicit an antigen-specific immune response.

1.3.1 Vaccine-Dependent Factors

1.3.1.1 Vaccine Adjuvants

Adjuvants, such as mineral salts, oil and water emulsions, PRR ligands, and cytokines, modulate vaccine responses to increase vaccine antigen immunogenicity. Adjuvants enhance immune responses by multiple mechanisms. This includes stimulating the innate immune system, forming antigen depots and sustaining the release of the antigen, upregulating cytokines, activating inflammasomes, recruiting leukocytes to the injection site, or activating antigen-presenting cells (Awate *et al*., 2013). For example, AS03, an oil-in-water emulsion, enhances antigen uptake and antigen presentation by stimulating nuclear factor-κB transcriptional activity and cytokine and chemokine responses to activate monocytes into antigen-presenting cells at the site of injection (Coffman *et al*., 2010; Garçon *et al*., 2014). AS03 facilitates the activation of CD4+ T-cells to promote stronger adaptive immune responses in current influenza-virus vaccines (Garçon *et al*., 2014). Furthermore, adjuvants in a vaccine formulation can strengthen vaccine-induced immune responses thereby reducing the incidence of vaccine non-responders. Within livestock, sheep vaccinated against FMDV revealed higher virus neutralization titers when vaccinated with a vaccine containing a water-in-oil emulsion adjuvant compared to an unadjuvanted vaccine antigen (Jouneau *et al*., 2020). In developing novel *M. hyopneumoniae* vaccines, researchers found that experimental vaccine formulations differing only in adjuvant selection (various PRR ligands or squalene-in-water emulsions) led to highly variable vaccine-specific IgG titers, CD4+, and CD8+ T-cell responses within vaccinated pigs (Matthijs *et al*., 2019). The selection of different adjuvants is just one method that allows vaccine manufacturers to modify the immune response required to combat a pathogen (Coffman *et al*., 2010).

1.3.1.2 Vaccine Type and Antigen Selection

Vaccine type, antigen selection, and pathogen strain can also influence vaccine-specific immune responses. For example, a small cohort of humans vaccinated with trivalent influenza vaccine had statistically higher hemagglutination inhibition titers than a cohort vaccinated with live-attenuated influenza virus (Nakaya *et al*., 2011). Similarly, in a study by Jouneau *et al*. (2020), sheep vaccinated with an inactivated FMDV responded with higher post-vaccination virus-neutralization titers over one year than sheep vaccinated with a replication-defective adenovirus 5 vector expressing FMDV capsid proteins. This study suggested there are substantial differences in vaccine-induced immune responses depending on the vaccine type (Jouneau *et al*., 2020). As vaccine immunogenicity relies on the antigen's ability to trigger the T- and B-cell receptors, optimal antigen selection is critical for developing vaccines that stimulate immune responses (Mahanty *et al*., 2015). Also, diversity in the strain of pathogen used to formulate the vaccine can impact the vaccine-induced immune response, as evidenced in the development of influenza virus vaccines that use different strains than the pathogen circulating in the environment (Keitel *et al*., 2006; Levine *et al*., 2016; Maes *et al*., 2021). Indeed, all aspects of the vaccine, up to and including administration technique, should be optimized to generate a strong vaccine-induced immune response (Zhang *et al*., 2015). Nevertheless, the formulation of the vaccine does not explain why individuals given the same vaccine can generate highly variable vaccine responses. To further investigate this phenotype, host factors that influence the immune response need to be considered.

1.3.2 Host-dependent Factors

1.3.2.1 Genetics and Heritability

Genetic factors can partially explain the heterogeneity of vaccine-induced immune responses in a population. In a meta-analysis by Posteraro *et al.* (2014), genes involved in antigen recognition and cytokine responses had single nucleotide polymorphisms (SNPs) that were associated with vaccine-induced antibody responses following measles virus, HBV, or meningococcus vaccination. However, there was not a consistent set of genes common across all vaccine responses, suggesting individual genetic differences could have vaccine-specific effects (Posteraro *et al*., 2014). A high number of genetic associations were found between human histocompatibility leukocyte antigen gene polymorphisms in humans and their post-vaccination influenza-specific antibody titers, highlighting the importance of antigen-presentation proteins in developing the vaccine response (Poland *et al*., 2008). Further evidence that the magnitude of

vaccine-specific responses is associated with genetic polymorphisms was observed in investigations that follow measles virus vaccination. SNPs in the genes coding cell surface receptors, CD46 and signaling lymphocyte-activation molecule, have been associated with allele-dependent variations in measles-specific antibody responses, while SNPs in TLR coding genes have been inconsistently associated with measles immunity (Dhiman *et al*., 2007; Ovsyannikova *et al*., 2011, 2014). Dairy cattle classified as either antibody-mediated immune responders or cell-mediated immune responders were genotyped and revealed a high representation of SNPs in bovine MHC genes, complement protein genes, and cytokines associated with immune responsiveness (Thompson-Crispi *et al*., 2014). Altogether, there is strong evidence that gene polymorphisms in antigen presentation proteins, T-cell recognition proteins, and cytokine signaling genes are associated with variations in the vaccine response.

Additional evidence suggests that genomic factors contribute to, but cannot fully explain, the total heterogeneity of vaccine responses. A study by Newport *et al.* (2004) evaluated the contributions of genetic and environmental factors on vaccine-induced immune responses using a cohort of dizygotic and monozygotic twins. Here, the heritability of vaccine-induced antibody responses was 44-78%, depending on the vaccine antigen (tetanus toxin, diphtheria toxin, oral poliovirus, and HBV) (Newport *et al*., 2004). Another large (n=210 pairs) human twin study that analyzed immune cell subsets and serum protein composition revealed that heritable factors contributed to <20% of the total variation in 61% of the measured cell populations and 69% of the measured serum proteins (Brodin *et al*., 2015). Genome-wide association studies of piglets vaccinated against *M. hyopneumoniae* identified multiple SNPs positively correlated to *M. hyopneumoniae*-antibody responses. The heritability of *M. hyopneumoniae*-antibody response in this population was determined to be between 0.46 and 0.57, further demonstrating that environmental and non-genetic factors must be determinants of variability in vaccine responses (Blanc *et al*., 2021). Finally, not all non-/low-responders are permanently incapable of responding to vaccines, as demonstrated by a study on human neonates with low (anti-HB titer<100 mIU/mL) antibody response following hepatitis B vaccination. This study revealed that revaccination of non-responders and low-responders increased antibody titers after additional doses, suggesting vaccine unresponsiveness can be a temporal phenotype (Han *et al*., 2012). Thus, vaccine responsiveness is dynamic, and challenges to the immune system may critically influence the vaccine-induced immune responses.

1.3.2.2 Inflammation and Immune Activation

Inflammation is a natural host response to pathogens, toxins, and cell damage (Chen *et al*., 2018). While adjuvants may stimulate an acute inflammatory response to activate innate immunes cells and recruit cells to the site of danger, a chronic inflammatory condition can be detrimental to vaccine-induced immune responses by exhausting cytokines and lymphocytes and desensitizing antigen-presenting cells to PRR stimulation (Alter and Sekaly, 2015; Muyanja *et al*., 2014; Panda *et al*., 2010). This chronic inflammatory phenotype has been partially characterized by greater circulating inflammatory cytokines and increased immune activation, which contributes to a negative association with vaccine-induced immune responses (Frasca *et al*., 2014; Muyanja *et al*., 2014).

Much of the research relating to inflammation and vaccine unresponsiveness has been done in the context of aging. Vaccine-specific antibody responses to influenza vaccination consistently declines with age (Fourati *et al*., 2016; Lambert *et al*., 2012; Panda *et al*., 2010; Poland *et al*., 2014). Investigations into the cause of this observation have identified a low-grade, chronic inflammation within older persons (termed "inflammaging"), as well as a reduced T cell repertoire (Lambert *et al*., 2012). Frasca *et al.* (2012) found that while B-cells collected from older (>65 years) persons had baseline higher levels of TNFα expression compared to younger (≤64 years) persons, the B-cells from older person produced lower TNFα levels in response to lipopolysaccharide stimulation than the B-cells from younger persons. As well, incubating B-cells with TNFα before lipopolysaccharide stimulation decreases the expression and secretion of TNFα, demonstrating there are differences in immune responsiveness between older cells with greater exposure to pro-inflammatory cytokines and younger cells (Frasca *et al*., 2012a). Increased circulating pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin (IL)-6 are observed in older individuals with poor B-cell responses to influenza virus vaccination compared to younger individuals with more robust B-cell responses (Frasca *et al*., 2014; Trzonkowski *et al*., 2003). Poland *et al.* (2014) reviewed the possible effects of aging on the vaccine response, citing immunosenescence, diminished innate immunity responses, decreases in T-cell and B-cell receptor diversity, and dysregulated release of cytokines as significant factors (Poland *et al*., 2014). The age-related impairment of vaccine responses provides evidence that the vaccine responsiveness phenotype can differ over time.

Similar to aging, body weight, namely, excess adipose (obesity) and low birth weight, are other phenotypic markers associated with chronic inflammation and vaccine responsiveness. Obesity has been a risk factor for reduced vaccine responsiveness in humans following multiple vaccination programs (Eliakim *et al*., 2006; Painter *et al*., 2015; Weber, 1985). A common factor between the obesity and aging phenotype is the increased chronic inflammatory condition, as demonstrated by greater expression and circulating levels of TNFα, IL-1β, and IL-6 in obese humans and mice fed a high-fat diet (Frasca *et al*., 2016; Trayhurn and Wood, 2004). Following influenza vaccination, mice on a high-fat diet had lower neutralizing antibody titers and fewer influenza-specific CD8+ T-cells than normal-weight mice (Park *et al*., 2014). While the mechanisms by which the inflammatory condition affects vaccine responses are not exact, researchers have proposed that chronic inflammation can lead to immune cell tolerance, suboptimal macrophage functions, and impaired cytokine responses (Honce and Schultz-Cherry, 2019; Park *et al*., 2014).

Body weight at other time points in life, such as birth, may also have negative effects on vaccine responses. For example, a study of adolescent humans revealed that being small for gestational age (based on birth weight) resulted in the lowest probability of generating vaccine-specific serum IgG titers to typhoid vaccination (McDade *et al*., 2001). These individuals with lower serum IgG titers had greater plasma concentrations of the pro-inflammatory marker, C-reactive protein, than individuals with higher serum IgG (McDade *et al*., 2011). Thus, dysregulation of immune function due to variables like age, body weight, and polymorphisms in cytokine and inflammatory signaling genes appear to contribute to increased circulating inflammatory signals that might hinder the development of the vaccine-specific immune response.

1.3.2.3 Microbiome and Antibiotic Use

Gut microbiomes contain trillions of bacteria (that outnumber the eukaryotic cells of the host) and have the propensity to shape host processes, such as metabolism (Turnbaugh *et al*., 2006), pharmacokinetics (Sousa *et al*., 2008), and immunity (Belkaid and Hand, 2014). For this reason, multiple research groups have begun to surmise the microbiome's role in vaccine-induced immune responses. Evidence collected by Oh *et al.* (2014) revealed antibiotic-treated mice had reduced influenza-specific IgM and IgG following influenza virus vaccination. However, when vaccination of microbiota-deficient mice was combined with exogenous administration of the

TLR5 ligand, flagellin, IgG titers were restored to levels similar to that in mice untreated with antibiotics. These results support the hypothesis that microbial products can act as natural adjuvants to enhance host responsiveness against unadjuvanted vaccines (Oh *et al*., 2014). Further studies demonstrated that antibiotic treatment of mice at a young age diminished commensal bacteria and impaired vaccine-specific antibody responses to multiple vaccines. The reduction in vaccine-specific antibody responses could be reversed by fecal microbiota transfer of commensal bacteria to antibiotic-treated mice, providing further evidence for an essential role of gut microflora in modulating host vaccine responses (Lynn *et al*., 2018). The effect of antibiotic usage on vaccine responses in pigs vaccinated against *M. hyopneumoniae* was investigated as well, but there were no differences in vaccine-specific antibody responses when comparing antibiotic-treated subjects and controls (Munyaka *et al*., 2019). Remarkably, microbiota analysis in an independent cohort of pigs vaccinated against *M. hyopneumoniae* revealed that pigs with the highest and lowest vaccine-induced antibody responses had differences in operational taxonomical units belonging to the genus *Prevotella* on the day of vaccination (Munyaka *et al*., 2020). Together, these studies illustrate that specific bacterial species may influence certain immune responses to vaccination.

Only a few variables that influence vaccine responsiveness have been described here, yet many others have been studied, including environmental, behavioral, maternal, and nutritional factors (Zimmermann & Curtis, 2019). Each factor can contribute to variation in an individual's cellular function (i.e. gene expression, cell population frequency, immune tolerance) (Brodin *et al*., 2015). Given the interactions among these factors, it can be challenging to identify a single cause for impaired vaccine responses using reductionist approaches. A systems biology approach may better identify the mechanisms driving vaccine-induced immune responses (Pulendran *et al*., 2010).

1.4 Systems Biology Analysis of the Vaccine Response

Researchers employed systems biology approaches to elucidate the global molecular events and cellular responses of vaccine-induced immune responses. Some of the earliest work done in the field of "systems vaccinology" used global analysis techniques to characterize immune responses to vaccination with human vaccines (Pulendran *et al*., 2010). This emerging field aims to delineate the mechanisms and define predictive models of vaccine unresponsiveness. Both aims

generate knowledge behind the phenotype and influence the development of future vaccines (Wimmers and Pulendran, 2020).

1.4.1 Correlates of Vaccine Outcomes Following Vaccination

Early systems biology applications of vaccine responsiveness identified gene expression and cellular responses following vaccination. In a cohort of adults vaccinated against Yellow Fever 17D, Gaucher *et al.* (2008) observed consistent patterns of gene expression, such as the expression of interferon pathway genes, complement system components, TLR-associated genes, and B-cell activation genes, at 3- and 7-days post-vaccination. Polychromatic flow cytometry of peripheral blood mononuclear cells (PBMCs) from vaccinated individuals identified an overall increase in T-cells within 14 days of vaccination, while a cytometric bead assay identified unique PBMC cytokine responses to Yellow Fever-derived peptides at 60-days post-vaccination. Through combining transcriptomics, cytometry, and cytokine profiles, researchers obtained a comprehensive view of the Yellow Fever vaccine response (Gaucher *et al*., 2008). Querec *et al.* (2009) also conducted early research to identify gene networks associated with vaccine responsiveness following Yellow Fever vaccination. This study observed the expression of genes involved in complement cascades, T-cell activity, and virus-sensing responses early after vaccination against the Yellow Fever 17D virus (Querec *et al*., 2009). A study by Li *et al.* (2013) augmented this research by conducting a multi-cohort analysis exploring the gene expression events in blood leukocytes following vaccination against Yellow Fever 17D virus, influenza virus, and meningococcus to identify a potential "universal" vaccine response. This study found distinctive patterns of gene expression corresponding to the particulars of the vaccine, reinforcing that vaccine factors, such as strain or vaccine type, can influence the associated vaccine-specific immune responses (Li *et al*., 2014). Together, these studies demonstrate that systems biology approaches can identify correlates of various vaccine outcomes early after vaccination. However, these studies did not answer the question regarding the mechanism(s) underlying the vaccine non-responder phenotype.

1.4.2 Post-vaccination Events in Vaccine Non-Responders

Given the ability of systems biology to describe the transcriptional and cellular changes in response to successful vaccinations, researchers sought to utilize these approaches to characterize what happens in vaccine non-responders. For example, in a study by Nakaya *et al.* (2011),

transcriptomic responses were evaluated before vaccination, and at 3- and 7-days following influenza virus vaccination. Microarray analysis was conducted on subjects classified as "high" and "low" responders based on hemagglutination-inhibition antibody titers 28-days following vaccination using PBMCs collected 0-, 3-, and 7-days post-vaccination. This analysis revealed differentially expressed genes capable of predicting independent vaccine responders with high (>85%) accuracy (Nakaya *et al*., 2011). These results indicated that systems biology approaches could identify molecular events associated with non-response and be instrumental in predicting unresponsiveness. Subsequent studies used similar strategies to investigate the differences between vaccine responders and non-responders following vaccination with influenza virus (Nakaya *et al*., 2015; Panda *et al*., 2010; Zimmermann *et al*., 2017), HBV (Bartholomeus *et al*., 2018; Shannon *et al*., 2020), measles virus (Haralambieva *et al*., 2018), and *M. hyopneumoniae* (Munyaka *et al*., 2019). As a result, there is a growing body of work determining the gene expression signatures associated with non-responders following various vaccination programs.

Research into the molecular mechanisms of vaccine unresponsiveness in livestock species using systems biology technology has been sparse. This is possibly due to curated databases containing the transcriptomes and proteomes of livestock species being not as well-annotated as model organisms such as humans and mice (Bick *et al*., 2019). Translating these technologies to livestock species such as cattle, pigs, or sheep, can substantiate research programs with large animal models that are highly comparative to human physiology and health (Facciuolo *et al*., 2020; Hein and Griebel, 2003). Transcriptional studies on PBMCs collected from pigs following tetanus toxoid (Adler *et al*., 2015) and *M. hyopneumoniae* vaccination (Munyaka *et al*., 2019) did not reveal significant differences in gene expression between high and low vaccine responders either before or after vaccination. Others have adapted computational analyses designed for humans to work on other species such as sheep and swine (Braun *et al*., 2018; Jouneau *et al*., 2020; Matthijs *et al*., 2019). These new computational frameworks were used in sheep vaccinated against FMDV to identify downregulated T-cell activities and platelet activation in high vaccine responders compared to low vaccine responders (Jouneau *et al*. 2020). The computational adaptation for different species, specifically livestock, has enabled a broader scope of systems vaccinology analyses for other model organisms.

1.4.3 Pre-vaccination Events of Vaccine Non-Responders

In contrast to defining gene expression events and frequencies of cell populations that follow vaccination, few researchers have characterized how the immune environment of the host at the time of vaccination impacts vaccine-induced immune responses. Pre-vaccination factors such as genetic predispositions, inflammation, and microbiomes have been examined in isolation, but systems biology analyses could provide insight into the global host characteristics (i.e. cell populations and cellular processes) that influence the vaccine-induced immune response. Tsang *et al.* (2014) demonstrated that inter-individual differences in gene expression and cell population frequencies prior to influenza virus vaccination correlated with influenza-specific responses in humans. Metrics such as pre-vaccination serum IgG titers and subsets of lymphocytes had power for predicting post-vaccination influenza-specific responses and provided a model for future predictions (Tsang *et al*., 2014). A similar analysis found baseline predictors of vaccine responsiveness to influenza vaccination in elderly adults (60 to 89 years); transcription of apoptosis-related genes prior to vaccination was positively correlated with strong vaccine-specific antibody responses. (Furman *et al*., 2013). In a study of humans vaccinated against HBV, older individuals with lower HBV-specific antibody responses had multiple differentially expressed genes involved in inflammation prior to vaccination than younger individuals with high antibody responses (Fourati *et al*., 2016). However, transcriptional analysis of pre-vaccination blood leukocytes has not consistently identified correlates of post-vaccination vaccine responses (Munyaka *et al*., 2019). Thus, while an individual's pre-vaccination immune state might not always predict their post-vaccination response, it remains a valuable control for describing individual variation prior to vaccination.

1.4.4 Integrating Omic Analyses

Transcriptional analyses have been a significant component of systems vaccinology research, yet these studies might not reflect the cell's phenotype. mRNA levels do not always directly correlate with protein abundance due to mRNA degradation, miRNA silencing, or protein half-lives, and provide little detail on a protein's activity (Anderson and Seilhamer, 1997; Greenbaum *et al*., 2003; Liu *et al*., 2016). Proteomic approaches that quantify a protein's abundance or characterize a protein's activity can supplement the current understanding of vaccine-induced immune responses (Galassie and Link, 2015). For example, mass spectrometry of proteins extracted from whole blood of cattle vaccinated against *Mycoplasma bovis* identified

upregulation of proteins involved in TLR-signaling and JAK-STAT signaling compared to unvaccinated controls (Lopez *et al*., 2018). Others have conducted cytokine profiling of human subjects before and after vaccination to identify correlations between circulating cytokine concentrations with vaccine-induced immune responses (Fourati *et al*., 2016; Qiu *et al*., 2018; Querec *et al*., 2009). To discover vaccine responsiveness biomarkers, Furman *et al.* (2013) used peptide fragments of the hemagglutinin protein of the influenza virus to detect pre-existing antibodies against the viral protein. Individuals with lower vaccine-specific antibody responses had higher pre-existing antibodies against peptide fragments, suggesting pre-vaccination reactivity to these peptides could be a biomarker for rapidly identifying vaccine responders. (Furman *et al*., 2013).

Similarly, metabolomic analyses have been notable for describing immune processes by quantifying the changes in metabolic intermediates perturbed by vaccination or other stimuli (Diray-Arce *et al*., 2020). One investigation into the human immune response to shingles virus vaccination integrated transcriptomic with metabolomics data to elucidate vaccine response biomarkers. This investigation found early time point metabolites involved in inositol phosphate metabolism, glycerophospholipid metabolism, and sterol signaling correlated with vaccine-specific T-cell and antibody responses (Li *et al*., 2017). Furthermore, patients given antibiotics following human influenza vaccination had decreased influenza virus-specific IgG1, secondary bile acid metabolites, and increased inflammasome activity compared to vaccinated controls. These metabolic changes between impaired and unimpaired vaccine responders demonstrated the combined use of metabolomic, transcriptomic, and microbiomic analyses for identifying a potential effect of gut dysbiosis on vaccine responsiveness (Hagan *et al*., 2019).

While proteomic and metabolomic approaches have proven valuable in providing insights into the dynamic state of vaccine responsiveness, these approaches require expertise and sophisticated equipment, such as mass spectrometers, that are not available in many labs (Clish, 2015). In addition, depending on the number of samples and analytes, metabolomic profiling can be highly expensive and time-consuming, potentially restricting their usage to well-funded laboratories or clinics (Diray-Arce *et al*., 2020). Therefore, there is need for techniques that can capture and describe the immediate phenotype of the cell, complement the use of other systems

biology approaches, yet remain cost-effective and do not require a high degree of technical and analytical specialization.

1.5 Kinomics

Post-translational modifications are covalent changes (i.e. additions, cleavages) to proteins that modulate aspects of their function, such as changes to activity, cellular localization, or interaction affinity. Phosphorylation is one of the most fundamental post-translational modifications for regulating protein function (Rauch *et al*., 2011). Protein phosphorylation is mediated by protein kinases that catalyze the transfer of a phosphate group from ATP to the hydroxyl group of serine, threonine, or tyrosine residues of cellular proteins (Ardito *et al*., 2017). Extracellular signals (e.g. pathogen-associated molecular patterns, cytokines) activate kinase-mediated phosphorylation events that initiate intracellular phosphorylation-mediated signaling cascades and trigger cellular responses, such as gene transcription, cell division, cell motility, and differentiation (Mogensen, 2009; Rauch *et al*., 2011; Schroder *et al*., 2004). The kinetics of protein phosphorylation permit a rapid cellular response following stimulation. Given that phosphorylation is an essential mechanism for modulating protein function, there has been a focus on analyzing the global cellular kinase activity within the cell, or "kinome analysis", to build upon the understanding of cellular phenotypes.

One high-throughput approach for analyzing the kinome uses peptide microarrays to characterize the active kinases catalyzing phosphorylation reactions of kinase-substrates. Proteins are one such kinase substrate. The kinase's specific phosphorylation site can be mimicked using short (15-amino acid) peptides containing the consensus target sequence (Kreegipuu *et al*., 1998). These peptides can be printed onto a solid-state array (termed "peptide arrays") (Jalal *et al*., 2009). Biological samples containing active kinases phosphorylate the known peptide substrates on the peptide array, and the reactions can be quantified using either radiolabeled ATP, phosphorylation-specific antibodies, or staining for phosphorylated residues with fluorescent dyes (Arsenault *et al*., 2011). Peptide arrays containing a large number of unique peptide substrates have established a need for software to computationally quantify, transform, and visualize the kinome data (Li *et al*., 2012; Trost *et al*., 2013a). Early experiments identifying phosphorylation events following lipopolysaccharide stimulation of human PBMCs provided proof-of-concept for the use of kinome analysis to delineate biological processes (Diks *et al*., 2004). Kinome analysis has since been

integrated for species-specific use, including applications in plants (e.g. barley, *Arabidopsis*), insects (e.g. honeybees), and livestock (e.g. cattle, swine, poultry) (Arsenault *et al*., 2012; Napper *et al*., 2015; Régnier *et al*., 2017; Ritsema *et al*., 2009; Robertson *et al*., 2014). Together, the development and employment of kinome analysis has facilitated the identification of immune-related signaling events for understanding immune mechanisms and discovering drug targets or biomarkers (Facciuolo *et al*., 2020).

1.5.1 Identifying Immune Mechanisms using Kinome Analysis

One application of kinome analysis has been to decipher complex, polygenic immune mechanisms related to pathogenesis and host responses within livestock. For example, bovine monocytes were infected with *Mycobacterium avium* subsp. *paraturberculosis* and kinome analysis was conducted on lysates of infected and uninfected monocytes to better understand bacterial pathogenesis. *M. avium* subsp. *paraturberculosis*-infected monocytes revealed differential phosphorylation of proteins involved in interferon-gamma signaling (Arsenault *et al*., 2012) and TLR9 mediated signaling (Arsenault *et al*., 2013) compared to uninfected monocytes, providing insight into the specific molecular mechanisms mediating this host-pathogen interaction. In a separate investigation, kinome analysis of Ebola virus-infected human liver cells revealed modulation of TGF-β signaling within host cells. This study led to the identification and application of kinase inhibitors that increased the survival of Ebola virus-infected mice compared to untreated, infected controls (Kindrachuk *et al*., 2014). Other *in vitro* and *ex vivo* kinome analyses have explored the pathogenesis of *Mycoplasma bovis* and bovine viral diarrhea virus infections in cattle and *Salmonella* Enteritidis and *Salmonella* Heidelberg infections in chicken (He *et al*., 2018; Mulongo *et al*., 2014; Van Wyk *et al*., 2016). Together, the details of various immune mechanisms have been elucidated through the analysis of kinase signaling.

1.5.2 Identifying Biomarkers using Kinome Analysis

Kinome analysis has also been pertinent in discovering biomarkers and predicting phenotypes in the context of stress and disease resilience. An investigation of the responses within PBMCs of cattle to restraint stress revealed signaling events implicating carbohydrate metabolism and apoptosis (Chen *et al*., 2016). This work supported the use of plasma glucose as a simple biomarker of stress in cattle. In another investigation, Robertson *et al.* (2014) used kinome analysis to discover a panel of phosphorylation events that correlated with the susceptibility of honeybees

to Varroa mite infestation. These phosphorylation events were subsequently used to predict the Varroa mite susceptibility of independent honeybee colonies prior to infestation, presenting proof-of-concept that kinome analysis has value in predicting immunity-associated phenotypes (Robertson *et al*., 2014, 2020).

1.5.3 Kinases Implicated in the Vaccine Response

Previous transcriptomic investigations on vaccine responses have identified protein kinases as being differentially expressed when comparing vaccine responders and non-responders. For example, calmodulin-dependent kinase IV, a kinase involved in dendritic cell survival, was negatively correlated with humoral responses specific to influenza vaccination in humans (Nakaya *et al*., 2011). Similarly, following Yellow Fever-17D vaccination, the kinase eukaryotic initiation factor 2α-kinase 4 was included in gene expression signatures to predict CD8+ cell responses. (Querec *et al*., 2009). Kinases are heavily involved in immune response processes, such as cytokine and chemokine signaling, cell motility, and leukocyte proliferation and differentiation. However, aside from scarcely discovering gene expression events of kinases, there has been no exploration of global kinase activity within blood leukocytes before and after vaccination. Thus, there is a potential opportunity for kinome analysis to offer new perspectives into vaccine responses as was provided by other multi-omics studies (Régnier *et al*., 2017).

1.6. *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is an intracellular bacterial pathogen that is a primary cause of enzootic pneumonia in pigs and also contributes to the porcine respiratory disease complex (Thacker, 2004). Infection with *M. hyopneumoniae* is characterized clinically by a chronic, unproductive dry cough. *M. hyopneumoniae* is also known to adhere to the ciliated epithelium of the respiratory tract, leading to a reduction in both cilia and ciliary activity (DeBey and Ross, 1994). *M. hyopneumoniae* infection has been found to predispose pigs to concurrent infections with other pathogens such as swine influenza virus, porcine reproductive and respiratory syndrome virus, or porcine circovirus type 2 (Thacker, 2004). These viral infections result in significant economic losses to the pig industry due to decreased performance, reduced growth, higher treatment costs, and increased mortality of pigs (Maes *et al*., 2018). Treatment with antimicrobials has been used to combat *M. hyopneumoniae* infections, reducing mycoplasmal pneumonia under experimental conditions (Thacker *et al*., 2006). However, due to the prevalence

of antimicrobial resistance, alternative options such as vaccination are being encouraged (Laxminarayan *et al*., 2013).

1.6.1 *Mycoplasma hyopneumoniae* Vaccines

Vaccination against *M. hyopneumoniae* has proven effective in decreasing clinical symptoms (e.g. lung lesions) of *M. hyopneumoniae*-infection (Maes *et al*., 2021; Thacker *et al*., 1998). Many commercial and experimental vaccines for *M. hyopneumoniae* have used whole-cell inactivated bacteria (bacterins) or subunit antigens (Djordjevic *et al*., 1997; Matthijs *et al*., 2019; Thacker *et al*., 1998). For example, RespiSure-One (Zoetis, USA) is an inactivated *M. hyopneumoniae* bacterin vaccine containing the adjuvant, Amphigen. RespiSure-One can be given in one dose as early as 1-day of age to reduce the severity of colonization and shedding of *M. hyopneumoniae*. Vaccination using whole-cell bacterins stimulates both humoral and cell-mediated responses (Bandrick *et al*., 2008). Vaccine-induced serum IgG titers were not correlated with a reduction in the severity of lung lesions in *M. hyopneumoniae*-infected piglets, suggesting that antibody responses are not fully protective against infection and T-cell responses are critical (Djordjevic *et al*., 1997).

Piglets vaccinated with different *M. hyopneumoniae* vaccines have resulted in highly variable serum antibody responses (Blanc *et al*., 2021; Matthijs *et al*., 2019; Munyaka *et al*., 2020). As well, *M. hyopneumoniae*-specific antibodies have been detected in unvaccinated piglets farrowed from sows which were vaccinated against *M. hyopneumoniae* vaccines prior to farrowing, demonstrating piglets can passively acquire vaccine-specific antibody (Sibila *et al*., 2008). Given that these *M. hyopneumoniae* vaccines induce variable magnitudes of antibody responses, they provide a valuable tool for evaluating vaccine immunogenicity. The ability of these vaccines to stimulate a range of readily quantifiable antibody responses provides an opportunity to discover biomarkers associated with vaccine responsiveness and interpret the mechanisms the vaccine-induced immune responses.

RATIONALE

Variability in vaccine immunogenicity exists in both humans and livestock. This can result in vaccine non-responders who remain at risk for infection and threaten individual and population health. One solution to this problem is to identify biomarkers that can predict vaccine antibody responders and non-responders. Given the numerous factors that can influence vaccine-induced immune responses, combined with the necessity of cell signaling to coordinate the vaccine response, this thesis used kinome analysis to characterize the cell signaling events within peripheral blood leukocytes collected from pigs prior to vaccination and early after vaccination. This analysis may provide insight into the immune environment of the host and the resulting signaling events that follow perturbation, respectively. To establish high vaccine responders and low vaccine responders, the *M. hyopneumoniae* vaccine, RespiSure-One, is used to induce variable vaccine-specific antibody responses. While these antibody responses may not be considered protective against infection, they were utilized as a metric of vaccine immunogenicity for identifying early markers of low vaccine responsiveness.

HYPOTHESIS

There are differences in kinase-mediated signaling within porcine PBMCs both before and after vaccination that are significantly associated with vaccine-induced IgG responses. These differences in kinase activity can be used to infer molecular mechanisms involved in vaccine responsiveness and identify biomarkers for predicting an individual's antibody response to vaccination.

OBJECTIVES

1. Using a phenotype-first approach, identify high and low vaccine responders to investigate biochemical and physiological differences that exist prior to and following vaccination as potential biomarkers for vaccine responsiveness in piglets.
2. Evaluate the predictive capability of physiological and phosphorylation biomarkers within a second set of high and low vaccine responders.
3. Validate and expand knowledge of the physiological associations identified between vaccine responsiveness and differences in birth weight and pro-inflammatory cytokines using a population of vaccinated pigs from an independent facility.

2. MATERIALS AND METHODS

2.1 Reagent List

All reagents and chemicals, their suppliers, and their supplier's addresses are provided **(Table 2.1)**.

Table 1: Reagent, supplier, and supplier address list.

Reagent/Chemical	**Supplier**
Acetonitrile	EMD Biosciences
Aprotinin	Sigma-Aldrich
Adenosine triphosphate	New England Biolabs
Beta-glycerophosphate	Sigma Aldrich
Bio-Plex Pro magnetic COOH beads	Bio-Rad
Brij-35	Sigma Aldrich
Bovine serum albumin	Sigma-Aldrich
Ethylene glycol tetraacetic acid (EDTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EGTA)	Sigma-Aldrich
Glycerol	GE Healthcare
Ficoll	Sigma-Aldrich
Fluortrac 200 96F microplate	Greiner Bio-One
Leupeptin	Sigma-Aldrich
Magnesium chloride hexahydrate	EMD Biosciences
Microseal B Adhesive Seals	BioRad
New Zealand pig serum	RMBIO
Phosphate-buffered Saline (PBS) pH 7.4	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
ProQ Diamond phosphoprotein stain	Invitrogen
RespiSure-One	Zoetis Canada Inc.
Sodium acetate	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium fluoride	Sigma-Aldrich
Sodium pyrophosphate	Sigma-Aldrich
Sodium vanadate	Sigma-Aldrich
Streptavidin R-phycoerythrin	Agilent Technologies
Triton X-100	Sigma-Aldrich
Tris	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Cytokine	**Supplier**
Interferon alpha (200 pg/mL)	Genentech
Interferon gamma (2000 pg/mL)	VIDO-InterVac
Interleukin 1 beta (IL-1β) 681PI010 (5000 pg/mL)	R&D Systems

Interleukin 6 (IL-6) 686PI025 (5000 pg/mL)	R&D Systems
Interleukin 8 (IL-8) RP0109S-005 (200 pg/mL)	Kingfisher Biotech Inc.
Interleukin 10 (IL-10) PSC0104 (5000 pg/mL)	Fisher Scientific
Interleukin 12 (IL-12) 912PL025 (5000 pg/mL)	R&D Systems
Interleukin 13 (IL-13) RP0007S-005 (5000 pg/mL)	Kingfisher Biotech Inc.
Interleukin 17 alpha (IL-17α) RP0128S-005 (500 pg/mL)	Kingfisher Biotech Inc.
Tumor Necrosis Factor alpha (TNFα) 690PT025 (5000 pg/mL)	R&D Systems
Capture Antibody	**Supplier**
Mouse anti-pig IFN-alpha antibody, GTX11408	GeneTex
Mouse anti-pig IFN gamma antibody, Clone: P2F6, ENMP700	Fisher Scientific
Mouse anti-Porcine IL-1 beta/IL-1F2 Monoclonal Antibody IgG1 Clone # 77724, MAB6811	R&D Systems
Goat anti-Porcine IL-6, Polyclonal IgG, AF686	R&D Systems
Rabbit anti-pig Interleukin-8 Antibody, AHP2392	Bio-Rad Laboratories
Mouse anti-pig IL-10 Monoclonal Antibody, 945A4C437B1	Fisher Scientific
Mouse anti-swine IL-12 p70 Monoclonal Antibody (clone G9), MA0413S	Kingfisher Biotech Inc.
Goat anti-swine IL-13 Polyclonal Antibody, PB0094S-100	Kingfisher Biotech Inc.
Rabbit anti-swine IL-17A Polyclonal Antibody, KP0498S-100	Kingfisher Biotech Inc.
Porcine TNF-alpha Antibody, Monoclonal Mouse IgG1 Clone # 103304, MAB6902	R&D Systems
Detection Antibody	**Supplier**
Mouse anti-Porcine IFN-alpha Antibody, 27105-1 (200 ng/mL)	R&D Systems
Rabbit anti-Porcine IFN gamma, Polyclonal Antibody, PIPP700 (400 ng/mL)	Fisher Scientific
Goat anti-Porcine IL-1 beta /IL-1F2 Biotinylated Antibody, BAF681 (500 ng/mL)	R&D Systems
Goat anti-Porcine IL-6 Biotinylated Antibody, BAF686 (500 ng/mL)	R&D Systems
Goat anti- Porcine IL-8/CXCL8 Biotinylated Antibody, BAF535 (400 ng/mL)	R&D Systems
Mouse anti-IL-10 Monoclonal Antibody Biotin, 945A1A926C2 (500 ng/mL)	Fisher Scientific
Mouse anti-Porcine IL-12/IL-23 p40 Biotinylated Antibody, BAM9122 (500 ng/mL)	R&D Systems
Goat anti-Swine IL-13 Polyclonal Antibody – Biotinylated, PBB0096S-050 (500 ng/mL)	Kingfisher Biotech Inc.
Rabbit anti-Swine IL-17A Polyclonal Antibody – Biotinylated, KPB0499S-050 (500 ng/mL)	Kingfisher Biotech Inc.

Mouse anti-Porcine TNF-alpha Biotinylated Antibody, BAM6903 (500 ng/mL)	R&D Systems
Supplier	**Supplier Address**
Agilent Technologies	Mississauga, ON, CAN
Bio-Rad Laboratories	Mississauga, ON, CAN
EMD Biosciences	Oakville, ON, CAN
Fisher Scientific	Ottawa, ON, CAN
GE Healthcare	Mississauga, ON, CAN
Genentech	Mississauga, ON, CAN
GeneTex	Irvine, CA, USA
Invitrogen	Oakville, ON, CAN
Kingfisher Biotech Inc.	Burlington, ON, CAN
New England Biolabs	Whitby, ON, CAN
R&D Systems	Toronto, ON, CAN
Rocky Mountain Biologicals, Inc. (RMBIO)	Missoula, MT, USA
Sigma-Aldrich	Oakville, ON, CAN
VIDO-InterVac	Saskatoon, SK, CAN
VWR	Mississauga, ON, CAN
Zoetis Canada Inc.	Kirkland, Quebec, CAN

2.2 Animal Housing and Animal Care

This work was approved by the University of Saskatchewan Animal Care Committee (AUP: AUP20190084) and the University of Alberta Animal Care and Use Committee (AUP: AUP00001125) following Canadian Council of Animal Care guidelines. All piglets were under the attention and care of licensed veterinarians. Piglets were monitored for changes in weight gain, behaviour, and physical injury throughout each trial.

Piglets were weaned at 21 ± 2 days of age and remained grouped with littermates. Piglets were vaccinated intramuscularly with one dose (1 mL) of RespiSure-One at 28 ± 2 days of age (Day 0) and received a booster vaccination (1 mL) at 52 ± 2 days of age (Day 24). The trial was terminated when piglets were 63 ± 2 days of age (Day 35).

In both vaccine trials, *M. hyopneumoniae*-specific antibodies were not measured for sows. However, the facilities housing the sows and piglets had not used *M. hyopneumoniae* vaccines or infected animals with live *M. hyopneumoniae* for at least 5 years prior to this study to ensure vaccine-induced maternal antibodies were not transferred to the piglets used in this study.

The methods from the First Trial (2015) have been previously described in detail (Lipsit *et al*., 2020) and any modifications made to these methods have been described below.

2.2.1 First Trial (2015)

All sows and piglets were housed and managed at the Swine Research & Technology Centre (Edmonton, Alberta). Twenty sows (parity = 3-8) bore litters from which 6 piglets (3 males and 3 females) of average-litter birth weight were selected. Piglets that were not of average-litter birth weight were not monitored for this study. The selected piglets provided a population of 117 healthy *M. hyopneumoniae*-free piglets ((Large White × Landrace) × Duroc; 59 male, 58 female). Three piglets died before the end of the study and were excluded from analyses. Whole blood for PBMC isolation was collected at 28-(Day 0), 30- (Day 2) and 34- (Day 6) days of age. Plasma was collected at 28-days (Day 0) of age prior to vaccination, and serum was collected at 63-days of age (Day 35). Body weight was measured at birth, 24-days of age (weaning), and 63-days of age (Day 35). A nasal swab taken from each piglet on Day 0 confirmed that all animals tested negative for *M. hyopneumoniae*.

2.2.2 Second Trial (2020)

All sows and piglets were housed and managed at Prairie Swine Centre (Saskatoon, Saskatchewan). Six Camborough Plus sows (parity = 0-3) bore litters (n=8-14 piglets/litter) for a total of 67 piglets (37 male; 30 female). Eleven piglets died prior to the end of this study and were excluded from analyses. Plasma was collected from piglets at 0-, 7-, 14-, 21-, 28- (Day 0), and 63-days (Day 35) of age. Serum was collected on Day 0 (prior to primary vaccination) and Day 35 (11 days after booster vaccination). Plasma collections consistently took place within the same two-hour period of the day each week to minimize possible effects of circadian rhythms on plasma cytokine concentration. Body weight was measured at 0- (birth), 7-, 14-, 21-, 24- (weaning), and 63-days of age.

2.3 Peripheral Blood Mononuclear Cell, Serum, and Plasma Isolation

2.3.1 Peripheral Blood Mononuclear Cell Isolation

Whole blood was collected from the jugular vein using 0.4% EDTA in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) as an anticoagulant. Whole blood was centrifuged at 1400 x g for 20 min without brake, and the buffy coat was collected and diluted 1:2 in PBS + 0.1% EDTA. Buffy coats were layered onto 15 mL Ficoll and centrifuged at 2000 x g for 20 min without break.

PBMCs were collected from the interphase of the Ficoll and the plasma, washed with cold PBS + 0.1% EDTA and centrifuged at 300 x g at 4 °C for 8 min. PBMC pellets were washed with cold PBS and centrifuged at 300 x g, 4 °C for 8 min twice. PBMCs were counted using trypan blue exclusion using a hemocytometer. PBMC pellets of 10 x 10^6 cells were flash-frozen in liquid nitrogen and stored at -80 °C for kinome analysis.

2.3.2 Serum and Plasma Isolation

Whole blood from the jugular vein was collected into serum separation tubes and K_2EDTA-coated Vacutainer tubes (Becton Dickinson) to isolate serum and plasma, respectively. Serum tubes were incubated at room temperature for 30 min. Serum and plasma tubes from the 2015 trial were centrifuged at 15,000 x g for 10 mins, 4 °C. Serum and plasma tubes from the 2020 trial were centrifuged at 2000 x g for 20 mins, 4 °C without break. Aliquots of serum and plasma were stored at −80 °C.

2.4 Serum *Mycoplasma hyopneumoniae*-IgG Quantification

2.4.1 First Trial (2015)

One mL serum from each piglet collected on Day 35 was shipped to Biovet (Saint—Hyacinthe, Quebec, Canada), where serum *M. hyopneumoniae*-specific IgG titers were quantified using an IDEXX *Mycoplasma hyopneumoniae* Antibody Test Kit ELISA (IDEXX Laboratories, Inc.). *M. hyopneumoniae*-specific IgG titers were transformed using a z-score Log_2 scale.

Plasma collected on Day 0 from high (n=10) and low (n=10) responders was centrifuged (20,000 x g, 30 min) and shipped to Prairie Diagnostic Services (Saskatoon, SK, Canada), and *M. hyopneumoniae*-specific antibody titers were quantified using an IDEXX *Mycoplasma hyopneumoniae* Antibody Test Kit ELISA (IDEXX Laboratories, Inc.). Samples were considered seronegative for *M. hyopneumoniae*-specific IgG if the S/P ratio ($\frac{Sample A_{650} - negative\ A_{650}}{positive\ A_{650} - negative\ A_{650}}$) at a 1:40 dilution was S/P<0.3 and seropositive if S/P>0.4, as per the manufacturer's instructions. Positive and negative controls were commercially (IDEXX Laboratories, Inc.) provided porcine anti-*M. hyopneumoniae* serum and porcine serum non-reactive to *M. hyopneumoniae*, respectively.

2.4.2 Second Trial (2020)

One mL serum collected on Day 0 and Day 35 from each piglet was shipped to Prairie Diagnostic Services (Saskatoon, SK, Canada), and *M. hyopneumoniae*-specific antibody titers were quantified

using an IDEXX *Mycoplasma hyopneumoniae* Antibody Test Kit ELISA (IDEXX Laboratories, Inc.). Serum was considered seronegative for *M. hyopneumoniae*-specific IgG if the S/P ratio at a 1:40 dilution was S/P<0.3 and seropositive if S/P>0.4, as per the manufacturer's instructions. Serum *M. hyopneumoniae*-specific IgG titers were determined for each piglet on Day 35 and quantified using a modified endpoint titration ELISA (IDEXX *M. hyo* Ab Test Kit ELISA). Briefly, Day 35 sera were serially diluted 1 in 4 starting with an initial 1:40 dilution. ELISA reactions were quantified using A_{650} absorbance. Endpoint titers were calculated by subtracting the negative control A_{650} and taking the reciprocal of the highest dilution with an A_{650} absorbance greater than the mean negative control. Endpoint titration ELISAs were completed by Biovet (Saint-Hyacinthe, QC, Canada).

2.5 Stratification of High and Low Responders

2.5.1 First Trial (2015)

Twelve piglets were stratified into a discovery cohort of low (LR; n=6) and high (HR; n=6) responders using the 10th percentile and 90th percentile of serum IgG titers, respectively. Eight additional piglets were selected from the 20^{th} percentile and 80^{th} percentile of serum IgG titers for a validation cohort of low (n=4) and high (n=4) responders, respectively. Multiple animals were excluded from LR and HR cohorts based on the availability of archived samples. These piglets were, however, included in the *M. hyopneumoniae*-specific IgG titer population analysis. HR5 (ID: "338B") had usable Day 0 and Day 2 samples but not on Day 6. Therefore, HR5 was included in the validation cohort for Day 0 and Day 2 analyses but was substituted with another HR (ID: "893R", HR11) for Day 6 analyses.

2.5.2 Second Trial (2020)

Twelve piglets within the 10^{th} and 90^{th} percentile of serum IgG titers, respectively, were stratified as LR (n=6) and HR (n=6) to match the discovery cohort of the first trial (2015).

2.6 Kinome Array Experiment

Frozen pellets of 10 x 10^6 PBMCs were lysed with ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 mM PMSF) and incubated for 10 min on ice. Lysates were centrifuged for 10 min at 14,000 x g, 4 °C. Supernatant was combined with 8:1 activation mix (50% glycerol, 50 µM ATP,

60 mM $MgCl_2$, 0.05% Brij-35, and 0.25 mg/mL bovine serum albumin) for 10 min on ice. Samples were incubated on the peptide array for 2 h at 37 °C. Arrays were washed with PBS + 1% Triton X-100, submerged in ProQ Diamond phosphoprotein stain, and incubated for 1 h with agitation. Arrays were destained with 20% acetonitrile + 50 mM sodium acetate, pH 4.0 for 10 min. Arrays were washed with distilled deionized water and centrifuged for 5 min at 800 x g to remove excess moisture. Phosphorylation intensity was collected using a GenePix Professional 4200A Microarray Scanner at 532 nm to 560 nm with a 580 nm filter. Images were captured using the GenePix Pro 6.0 software (MDS) to collect spot intensity. Kinome array experiments for each time point (Day 0, Day 2, and Day 6) were conducted independently.

2.7 Kinome Array Data Transformation and Analysis

2.7.1 Data Transformation

Peptide microarrays (JPT Peptide Technologies, Berlin, Germany) with 282 unique peptides representing sequences surrounding selected phosphorylation sites within specific porcine proteins were utilized for this study (Jalal *et al*., 2009; Li *et al*., 2012). Each peptide was 15 amino acids in length and represented by 9 technical replicate spots on the peptide microarray. Individual phosphorylation intensities were determined by subtracting the background F632 intensity from the foreground F632 intensity using Platform for Integrated, Intelligent Kinome Analysis (PIIKA2) software (Trost *et al*., 2013a). PIIKA2 subtracted background intensities from foreground intensities and transformed differences using variance-stabilizing normalization (VSN). Arrays from the discovery cohort at all time points were transformed together, independent of the validation cohort. Arrays from the validation cohort at all time points were subsequently transformed with the discovery cohort datasets to allow for comparable scales. The technical replicates were averaged together and fold-change (FC) for each peptide phosphorylation intensities was calculated using Log_2 values. FC=2^d, where "d = (average intensity of group y – average intensity of group x)". The negative reciprocal of FC was calculated when d<1 for interpretation purposes.

2.7.2 Principal Component Analysis

Principal component analysis (PCA) was conducted and visualized using ClustVis version 1.0 with the parameters: transformation = "no transformation", Row scaling = "unit variance scaling", PCA method = "SVD with imputation" (Metsalu and Vilo, 2015).

2.7.3 Pathway Analysis

Functional enrichment analysis of differential phosphorylation events was performed using gProfiler on default parameters (Raudvere *et al*., 2019). Results were considered statistically significant at a threshold of $p \leq 0.05$.

2.8 Fluorescent Microsphere Immunoassay

All incubations were done at room temperature with agitation at 750 rpm. Plates were covered in foil to reduce light exposure. Following each incubation, plates were washed with PBS pH 7.4 + 0.5% Tween 20 using a Bio-Plex PRO II wash station (30-sec soak, 3 cycles). Antibodies for IFNα, IFNγ, IL-1β, IL-6, IL-8, IL-12, IL-13, IL-17α and TNFα were conjugated to individual BioPlex Max-Plex C magnetic beads (BioRad) using a method previously described (Christopher-Hennings *et al*., 2013). TNFα multiplex analysis was conducted on a separate plate to avoid cross-reactivity **(Table 2.2)**.

Cytokine standards were diluted 1:3 in New Zealand pig serum to account for serum inhibitory effects. Plasma samples from Trial 2015 were diluted 1:2 and 1:4 in porcine diluent (PBS, 1% New Zealand pig serum, 0.05% sodium-azide). Plasma samples from Trial 2020 were diluted 1:3 in the porcine diluent. All plasma samples were run in triplicate. Plasma samples were incubated for 1 h with 1200 beads/well in a Fluortrac 200 96F microplate. After a wash, samples were incubated for 30 min with specific biotinylated antibodies specific to the corresponding cytokine. After another wash, samples were incubated for 30 min with 5 μg/mL streptavidin R-phycoerythrin conjugate. After a final wash, samples were incubated for 5 min with TE buffer (50 mM Tris, 25mM EDTA, pH 8.0). Plates were read on a BioPlex 200 reader (Bio-Rad Laboratories Inc.) with the settings "50 beads per region, 45-second time-out, and 60 μL volume". All replicates and dilution factors for each animal were averaged for a final sample concentration. Technical replicates below the lower limit of quantification were not calculated in the average result. Samples below the limit of detection were recorded as ½ the lower limit of quantification value. Lower limit of quantification values for all cytokines were: IFNα (1.57 pg/mL), IFNγ (14.4 pg/mL), IL-1β (37.5 pg/mL), IL-6 (40.3 pg/mL), IL-8 (2.72 pg/mL), IL-12 (39.1 pg/mL), IL-13 (40.0 pg/mL), IL-17α (15.5 pg/mL), and TNFα (39.8 pg/mL).

Table 2: Porcine cytokine concentration, bead region, biotinylated antibody concentration.

rPorc Cytokine	Standard (starting concentration)	Bead region	Capture Antibody	Biotinylated Antibody (concentration)
Interferon alpha	Genentech (200 pg/mL)	BioRad MC10045-01	GeneTex GTX11408	R&D 27105-1 (200 ng/mL)
Interferon gamma	Fisher (2000 pg/mL)	BioRad MC10043-01	Fisher ENMP700	Fisher PIPP700 (400 ng/mL)
Interleukin 1 beta	R&D 681PI010 (5000 pg/mL)	BioRad MC10026-01	R&D MAB6811	R&D BAF681 (500 ng/mL)
Interleukin 6	R&D 686PI025 (5000 pg/mL)	BioRad MC10065-01	R&D AF686	R&D BAF686 (500 ng/mL)
Interleukin 8	Kingfisher RP0109S-005 (200 pg/mL)	BioRad MC10027-01	AbD Serotec MCA1660	R&D BAF535 (400 ng/mL)
Interleukin 10	Fisher PSC0104 (5000 pg/mL)	BioRad MC10028-01	Fisher ASC0104	Fisher ASC9109 (500 ng/mL)
Interleukin 12	R&D 912PL025 (5000 pg/mL)	BioRad MC10036-01	Kingfisher MA0413S	R&D BAM9122 (500 ng/mL)
Interleukin 13	Kingfisher RP0007S-005 (5000 pg/mL)	BioRad MC10052-01	Kingfisher PB0094S-100	Kingfisher PBB0096S-050 (500 ng/mL)
Interleukin 17 alpha	Kingfisher RP0128S-005 (500 pg/mL)	BioRad MC10062-01	Kingfisher KP0498S-100	Kingfisher KPB0499S-050 (500 ng/mL)
Tumor Necrosis Factor alpha	R&D 690PT025 (5000 pg/mL)	BioRad MC10034-01	R&D MAB6902	R&D BAM6903 (500 ng/mL)

2.9 Statistical and Data Analysis

All data analysis and visualization were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, California USA). The correlation matrix **(Fig. 12A)** was created in R using the package corrplot (Wei and Simko, 2021). P-values were considered statistically significant at $p \leq 0.05$, and were considered to be a statistical trend at $0.05 < p \leq 0.1$.

2.9.1 First Trial (2015)

The Log_2-transformed serum *M. hyopneumoniae*-specific IgG titer data and the variance-stabilizing normalization-transformed kinome data were determined to be normally distributed (Kolmogorov-Smirnov test, $p > 0.1$). The birth weight of the piglets was assumed to follow a normal

distribution as the sample size (n=117) was large, the samples were measured independently, and the mean (1.48 kg) was approximately the median (1.5 kg). Plasma cytokine concentrations from the discovery cohort and validation cohort were determined to not be normally distributed (one-sample Kolmogorov-Smirnov test, $p<0.1$). A two-tailed unpaired Student's t-test was conducted to analyze differences in Log_2-transformed serum *M. hyopneumoniae*-specific IgG titer, and weight at birth, weaning, or the end of the trial, between HR and LR within the discovery cohort. A Mann-Whitney U-test was conducted to determine differences in cytokine concentrations between HR and LR in the discovery cohort. A Mann-Whitney U-test was conducted to analyze differences in Log_2-transformed serum *M. hyopneumoniae*-specific IgG titer, plasma cytokine concentrations, birth weight, and weaning weight between HR and LR within the validation cohort. A Pearson linear regression was conducted for the correlation analysis of Log_2-transformed serum *M. hyopneumoniae*-specific IgG titer and birth weight. A Spearman Rank Correlation was conducted to correlate Log_2-transformed serum *M. hyopneumoniae*-specific IgG titer and plasma IFNγ concentrations.

A repeated-measures two-way ANOVA with Geisser-Greenhouse correction was conducted using the average transformed intensity for each peptide with the factors "Day" and "Response" to determine differential phosphorylation events between HR and LR within the discovery cohort. Sidak's multiple comparisons were conducted between HR and LR within the discovery cohort for each Day. A false-discovery rate (FDR) of 5% was applied using a Benjamini-Hochberg Correction to P-values for each Day. Phosphorylation events were considered differentially phosphorylated under two criteria: there was an effect ($p<0.05$) of either the "Response" variable or the "Response x Day" variable, and there was a difference ($FDR<0.05$) between HR and LR after FDR-correction. A Mann-Whitney U-test was conducted to analyze differences in mean phosphorylation intensity between HR and LR in the validation cohort.

2.9.3 Second Trial (2020)

The Log_2-transformed *M. hyopneumoniae*-specific serum IgG titer from the second trial was treated as normally distributed as the sample size (n=67) was large, samples were measured independently, and the mean (11.6) approximated the median (11.8). A two-sided Kolmogorov-Smirnov test was used to test if the distributions of Log_2-transformed *M. hyopneumoniae*-specific IgG titers were different between the first and second trials. A two-tailed, unpaired Student's t-test

with Welch's correction was used to determine differences in titers of HR between trials and differences in titers of LR between trials (F-test of equality of variances, $p<0.05$). Plasma cytokine concentrations were not normally distributed on individual days (Kolmogorov–Smirnov test, $p<0.1$). Therefore, a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons was used to detect differences in IFNγ and IL-1β concentrations when comparing among days. Plasma cytokine concentrations were normally distributed when grouped by litter (Kolmogorov–Smirnov test, $p>0.1$). Therefore, a two-way ANOVA with a Geisser-Greenhouse correction was conducted using the factors "Litter" and "Day". Tukey's multiple comparisons were conducted between litters on each Day due to the effect of "Litter x Day" ($p<0.001$). A one-way ANOVA was conducted to detect a mean difference in Log_2-transformed *M. hyopneumoniae*-specific IgG titers between litters. A Spearman Rank Correlation was conducted to correlate Log_2-transformed *M. hyopneumoniae*-specific IgG titer and plasma cytokine concentrations. The body weight of all piglets at birth, weaning, and at the end of the trial were determined to be normally distributed (Kolmogorov–Smirnov test, $p>0.1$). A two-tailed, unpaired Student's t-test with Welch's correction was conducted to analyze mean differences in birth, weaning, and end of trial weight between HR and LR and mean differences in Log_2-transformed *M. hyopneumoniae*-specific IgG titer between the highest and lowest birth weight piglets.

3. RESULTS

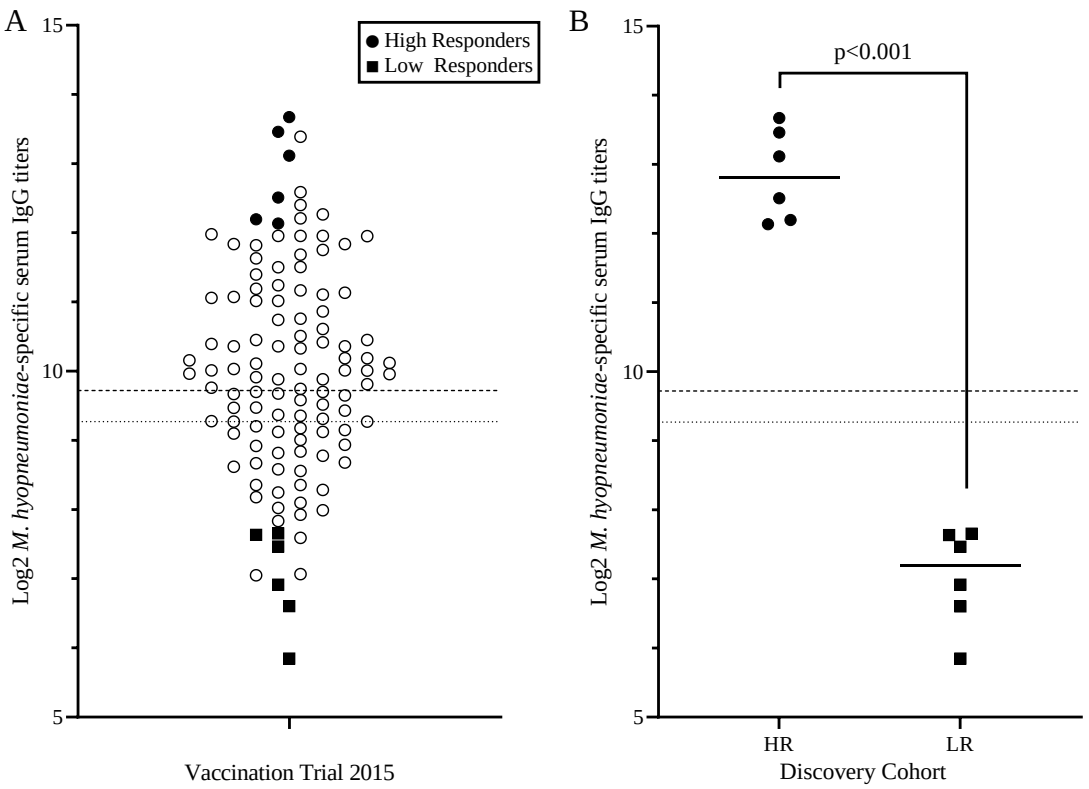
3.1 Identifying Vaccine Response Biomarkers in a Discovery Cohort

Results from **Section 3.1** have been previously described (Lipsit *et al*., 2020). Modifications to statistical tests, sample cohorts, and visualizations have been made and are described here.

3.1.1 Variation of *M. hyopneumoniae*-specific IgG Titers following Vaccination

A population of piglets (n=117; 58 male, 59 female) selected from 20 litters (6 piglets/litter; 3 male, 3 female) were vaccinated with a commercial *M. hyopneumoniae* vaccine (RespiSure-One; Zotetis) at 28- and 52-days of age (Day 24). Serum *M. hyopneumoniae*-specific IgG titers were quantified at 63-days of age (Day 35) using an IDEXX *M. hyo* Ab ELISA. Piglets exhibited a broad variation in serum *M. hyopneumoniae*-specific IgG titers 11-days following the booster vaccination (range, 5.85-13.67; median, 9.96) **(Fig. 1A)**. No sex-dependent effect was observed when comparing serum IgG titers ($p=0.34$; Student's T-test with Welch's correction). Samples were collected from piglets in 4 batches and no batch-dependent effect was observed ($p>0.99$; ordinary one-way ANOVA). There was a mean difference in serum IgG titers among the 20 litters selected for this study ($p=0.047$; ordinary one-way ANOVA). However, multiple comparisons among all litters failed to identify significant differences between any two litters ($p>0.11$; Tukey's multiple comparisons) and all litters were included in subsequent analyses. To classify piglets as seronegative ($S/P<0.3$), suspected ($0.3<S/P<0.4$), and seropositive ($S/P>0.4$), thresholds of serum IgG titers were calculated at Log_2 values of 9.27 ($S/P=0.3$) and 9.72 ($S/P=0.4$) based on instructions from the ELISA manufacturer (IDEXX). Thus, this trial population consisted of 36 seronegative, 65 seropositive, and 16 suspected animals for *M. hyopneumoniae* on Day 63.

Within this trial, high (HR) responders (n=6; titer range = 12.13-13.67; median = 12.81) and low (LR) responders (n=6; titer range = 5.85-7.65; median = 7.19) were stratified based on serum IgG titers at Day 35 to establish a "discovery cohort" **(Fig. 1B)**. HR from this discovery cohort had a 48-fold greater ($p<0.001$) median serum *M. hyopneumoniae*-specific IgG titers than LR. While other piglets (empty shapes) shown in **(Fig. 1A)** may have had higher titers than HR and lower titers than LR subjects, they were excluded from HR and LR cohorts due to a lack of PBMCs required for kinome analysis. HR and LR were seronegative ($S/P<0.1$) for *M. hyopneumoniae*-specific IgG titers prior to vaccination.



882

883 **Figure 1: Defining high and low responders among vaccinated piglets (Trial 2015) using**
884 ***Mycoplasma hyopneumoniae*-specific IgG titers 11-days following booster RespiSure-One**
885 **vaccination. A.** Variability of *M. hyopneumoniae*-specific IgG titers among piglets in Trial 2015
886 (n=117; open and solid symbols). Solid symbols indicate high (HR; n=6; circles) and low (LR;
887 n=6; squares) responders selected for further study. **B.** Comparison of median *M. hyopneumoniae*-
888 specific IgG titers of HR and LR selected for the discovery cohort. Dashed and dotted lines
889 represent the threshold for seropositive and seronegative cutoffs, respectively. P-values were
890 determined using a two-tailed, unpaired Student's t-test. Figures 1A and 1B are modified from
891 Lipsit *et al.* (2020).

892

3.1.2 Kinome Analysis of Pre-vaccination PBMCs

Kinome analysis was performed on PBMCs collected immediately prior to vaccination (Day 0) and 2- and 6- days post-vaccination (Days 2 and 6) from HR and LR within the discovery cohort that were to determine the phosphorylation events within blood leukocytes associated with vaccine responses. PBMC lysates were incubated over peptide microarrays containing 282 unique peptide targets representing known phosphorylation sites of porcine proteins. The magnitude of phosphorylation of each peptide target was quantified to generate a kinome profile for each piglet.

3.1.2.1 Principal Component Analysis of Global Kinome Profiles

Principal component analysis (PCA) was conducted on HR and LR for each Day using the 282-phosphorylation event kinome profile. PCA is a method of unbiased cluster analysis that separates samples with a large number of variables into fewer dimensions (called "principal components") that retain the variability of the original data (Jolliffe and Cadima, 2016). There was a high intra-group similarity within LR subjects on Day 0, suggesting similar kinome activities prior to vaccination. In contrast, HR displayed greater variability when considering the phosphorylation events of all 282 peptide targets **(Fig. 2A)**. Kinome profiles on Day 2 did not cluster separately based on vaccine responsiveness phenotypes and showed overlapping 95% confidence intervals between LR and HR kinome profiles **(Fig. 2B)**. Finally, PCA of Day 6 kinome profiles revealed that PC1 (23.3%) could separate HR and LR kinome profiles when considering the 282-kinome profile, suggesting multiple phosphorylation events were different between HR and LR **(Fig. 2C)**. Comparative analysis of individual HR and LR phosphorylation events was conducted to reveal specific phosphorylation biomarkers that strongly associated with vaccine responsiveness.

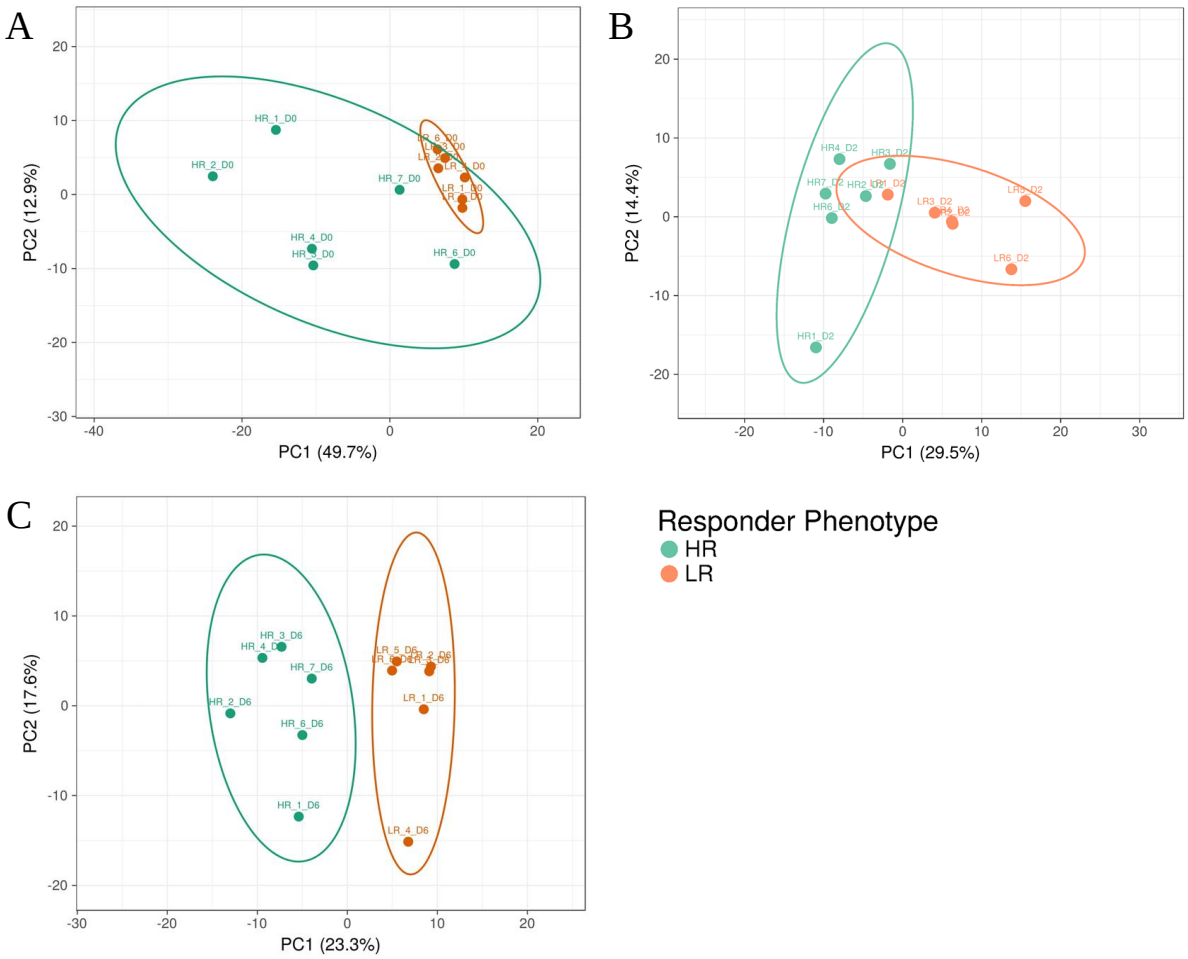


Figure 2: Principal component analysis of the high and low responders from the discovery cohort. Principal component analysis of high (HR; n=6; green) and low (LR; n=6, orange) responders using phosphorylation events of 282 peptides on **A.** Day 0, **B.** Day 2, and **C.** Day 6. The two principal components (PC) with the highest variance (%) are shown. Ellipses represent 95% confidence intervals for each phenotypic group.

3.1.2.2 Comparative analysis of high and low responders

A repeated-measures two-way ANOVA using the factors "Response" (High vs Low) and "Time" (Day 0 vs Day 2 vs Day 6) was conducted using a post-hoc Sidak's multiple comparisons test to identify significantly different phosphorylation events between HR and LR. A false-discovery rate (FDR) of 0.05 (5%) was applied to the 282 tests on each time point using the Benjamini-Hochberg method. FDR corrections are helpful in big data analyses to reduce the number of false-positive results from conducting multiple hypothesis testing.

3.1.2.3 Differences prior to vaccination (Day 0)

On Day 0, 10 differential (FDR-corrected P-value<0.05) phosphorylation events were identified between HR and LR on Day 0 **(Table 3)**. Eight of the 10 phosphorylation events had higher levels of phosphorylation (FC>1) in LR compared to HR. Within the Day 0 list, there was differential phosphorylation of peptide targets representing mediators of immune-function such as B-cell linker protein (BLNK), IL-6 receptor (IL6ST), TNF receptor-associated factor 6 (TRAF6), and cell signaling mediators such as AKT1, protein phosphatase 2 catalytic subunit alpha (PPP2CA), and calmodulin (CALM1).

3.1.2.4 Differences following vaccination (Day 2 and 6)

No individual phosphorylation events were significantly different between HR and LR at an FDR of 5% on Day 2 (**Table 3**). However, on Day 6, 11 differential (FDR-corrected P-value<0.05) phosphorylation events were identified between HR and LR **(Table 3)**. Within the Day 6 list, differential phosphorylation events were observed on peptide targets representing proteins involved in mediating cell signaling, such as phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) and receptor of activated C kinase 1 (RACK1), cytoskeletal proteins like stathmin 1 (STMN1) and PPP2CA, and proteins with known immunological signaling functions like TRAF6, SYK, and nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1). Seven of the 11 differential phosphorylation events had higher levels of phosphorylation (FC>1) in the LR than the HR.

In comparing the biomarkers determined on Day 0 and 6, three identical phosphorylation events (TRAF6_Y353, STMN1_S15, and PPP2CA_T304) were consistent over time with similar fold-changes at each time point. Together, these results demonstrate that molecular differences between HR and LR can be detected both before and after vaccination.

Table 3: Differential phosphorylation events within PBMCs between low and high responders on Day 0 and Day 6 in the discovery cohort.

	Target Name	Target Site	UniProt ID	FDR[a] q-value	FC[b]
Day 0	TRAF6	Y353	Q9Y4K3	0.014	-2.33
	AKT1	T308	P31749	0.021	-2.10
	PPP2CA	T304	P67775	0.016	-2.04
	STMN1	S15	P16949	0.014	-1.89
	STAT4	S722	Q14765	0.025	-1.88
	FGFR1	Y653	P11362	0.016	-1.70
	CALM1	Y99	P0DP23	0.016	-1.66
	RPS6KB1	S447	P23443	0.040	-1.62
	BLNK	Y178	Q8WV28	0.016	1.55
	IL6ST	S782	P40189	0.016	1.75
Day 6	STMN1	S37	P16949	0.014	-2.66
	TRAF6	Y353	Q9Y4K3	0.014	-2.16
	PPP2CA	T304	P67775	0.014	-1.96
	RAB5A	T202	P20339	0.014	-1.84
	RACK1	Y194	P63244	0.025	-1.80
	PIK3R1	Y556	P27986	0.014	-1.65
	STMN1	S15	P16949	0.014	-1.62
	KEAP1	Y141	Q14145	0.036	1.27
	SMAD1	S214	Q15797	0.014	1.40
	SYK	Y348	P43405	0.036	1.48
	NFATC1	S245	O95644	0.014	2.41

[a] False-discovery rate (FDR) was applied to Sidak's multiple comparison tests between high and low responders on each time point. FDR was set at 0.05.

[b] Fold-change (FC) is calculated as a change from low responders (x) to high responders (y).

3.1.3. Pathway analysis with Gprofiler

To determine potential functions associated with differentially phosphorylated peptides between HR and LR, a functional enrichment analysis using Gprofiler (version e104_eg51_p15_3922dba), an online software for conducting over-representation analysis, was performed for each time point. Functional enrichment analysis finds statistically significant cell signaling pathways and processes involving the genes/proteins within the query set. Thus, it may provide insight into the function of the differential phosphorylation events. Fold-changes and target sites of peptides were not included in the analysis because the function of all phosphorylation sites are not curated in the databases being queried.

3.1.3.1 Pathway analysis of Day 0

Functional enrichment of the protein IDs corresponding to the differential phosphorylation events contributed to numerous immune-associated signaling pathways, including "*Signaling by Interleukins*" (Reactome; $p=3.1x10^{-4}$), "*Cytokine Signaling in the Immune System*" (Reactome; $p=3.5x10^{-3}$), *IL-6 signaling pathway* (WikiPathways; $p=1.8x10^{-3}$), and *Interleukin-11 Signaling Pathway* (WikiPathways; $p=1.9x10^{-3}$). The protein IDs contributing to the enrichment pathways included TRAF6, BLNK, IL6ST, PPP2CA, AKT1, and STAT4. Overall, there was a high representation of pathways implicating differential phosphorylation of proteins involved in cytokine signaling within PBMCs prior to vaccination.

3.1.3.2 Pathway analysis of Day 6

Repeating the functional enrichment analysis with the differential phosphorylation events on Day 6 revealed pathways such as "*Fc epsilon receptor (FCERI) signaling*" (Reactome; $p=4.4x10^{-3}$), "*RANKL/RANK (Receptor activator of NFKB (ligand)) Signaling*" (WikPathways; $p=4.5x10^{-5}$) and "*B cell receptor signaling pathway*" (KEGG; $p=6.1x10^{-3}$). The protein IDs that contributed to these enrichments included TRAF6, NFATC1, PPP2CA, and PIK3R1. However, the enrichment analysis of the Day 6 differential phosphorylation events did not elude biological processes that could be verified with readily available plasma samples.

The Day 0 functional enrichment analysis suggested differential cytokine signaling between HR and LR prior to vaccination. Multiplex assays have been developed and validated in swine to quantify an array of analytes in parallel, including panels of cytokines (Lawson *et al*.,

2010) Together, this led to the investigation of plasma cytokines concentrations prior to vaccination within HR and LR.

3.1.4 Multiplex Plasma Cytokine Analysis

The numerous cytokine signaling pathways identified in the functional enrichment analysis of HR and LR PBMCs prior to vaccination suggested there could be differences in cytokine signaling. To determine if cytokine signaling pathways were differentially active in HR and LR prior to vaccination, nine cytokines (IFNα, IFNγ, IL-1β, IL-6, IL-8, IL-12, IL-13, IL-17α, and TNFα) were quantified in plasma collected on Day 0. LR had a higher ($p=0.016$) plasma concentration of IFNγ compared to HR **(Fig. 3A)**. Furthermore, within the HR and LR, there was a significant ($p=0.028$) negative ($r=-0.64$) correlation between plasma IFNγ concentrations on Day 0 with vaccine-induced serum IgG titers on Day 35 **(Fig. 3B).** There was also a trend of higher IL-1β ($p=0.06$) plasma concentrations within LR compared to HR **(Fig. 3A)**. Additional samples are needed to determine if there were a difference in plasma TNFα ($p=0.12$) between HR and LR **(Fig. 3A)**. HR and LR showed no significant difference ($p>0.05$) in IFNα, IL-6, IL-8, IL-12, IL-13, or IL-17α plasma concentrations on Day 0 **(Fig. 3C)**. Correlation analyses were not conducted for IL-1β and TNFα due to the low number of data points available for these cytokines in the HR and LR cohorts. These data suggest that elevated pro-inflammatory cytokines circulating in the blood prior to vaccination negatively associate with vaccine responses.

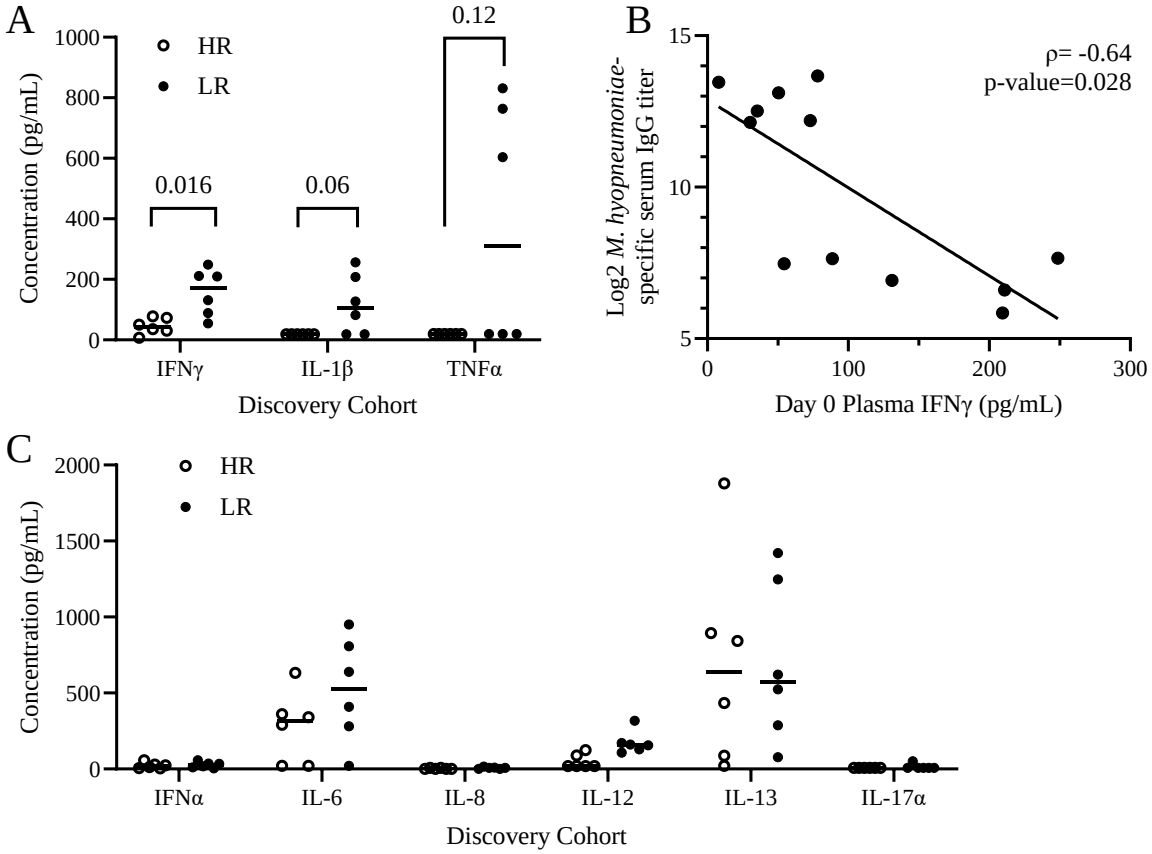


Figure 3: High and low responders differ in plasma concentration for multiple pro-inflammatory cytokines prior to vaccination. A. Comparison of median plasma concentrations of IFNγ, IL-1β, and TNFα between high (HR; n=6; open circles) and low (LR; n=6; closed circles) responders on Day 0 (prior to vaccination). **B.** Correlation analysis between *M. hyopneumoniae*-specific IgG titers and Day 0 IFNγ plasma concentrations within HR and LR. Best fit lines determined using Spearman Correlation. **C.** Comparison of median plasma concentrations of IFNα, IL-6, IL-8, IL-12, IL-13, and IL-17α between HR and LR on Day 0. P-values were determined using a Mann-Whitney U-test. Figures 3A and 3B are modified from Lipsit *et al.* (2020).

3.1.5 Body Weight Analysis of Vaccine Responders

Body weight is a common metric for evaluating livestock health and their likelihood of animal survival (Milligan *et al*., 2002a). Therefore, piglet body weight at birth, weaning (24-days of age), and at the end of the trial (63-days of age) were investigated for potential relationships with vaccine responsiveness.

There was considerable variation in birth weights (range, 1.0-1.9 kg; mean, 1.5 kg), weaning weights (range, 4.6 – 10 kg; mean, 6.9 kg), and trial end weights (range, 19 – 38 kg; mean, 25 kg) among piglets. LR had lower birth weight (p=0.002) and lower weaning (p=0.035) weight compared to HR **(Fig. 4A)**. At the end of the experiment (63-days of age), there was no difference (p=0.28) in weight between HR and LR, which suggests that LR did not have inherent long-term growth impairments compared to the HR **(Fig. 4A)**. Within the HRs and LRs, there was a significant (p=0.002) positive (r=0.63) correlation between birth weight and serum IgG titer **(Fig. 4B).** However, a strong (p=0.024; r=0.04) correlation between birth weight and serum IgG titer was not evident within the entire (n=117) trial population **(Fig. 4C)**. Therefore, associations between vaccine responsiveness, plasma IFNγ concentrations, and birth weight observed within the highest and lowest vaccine antibody responders are not apparent across the entire population.

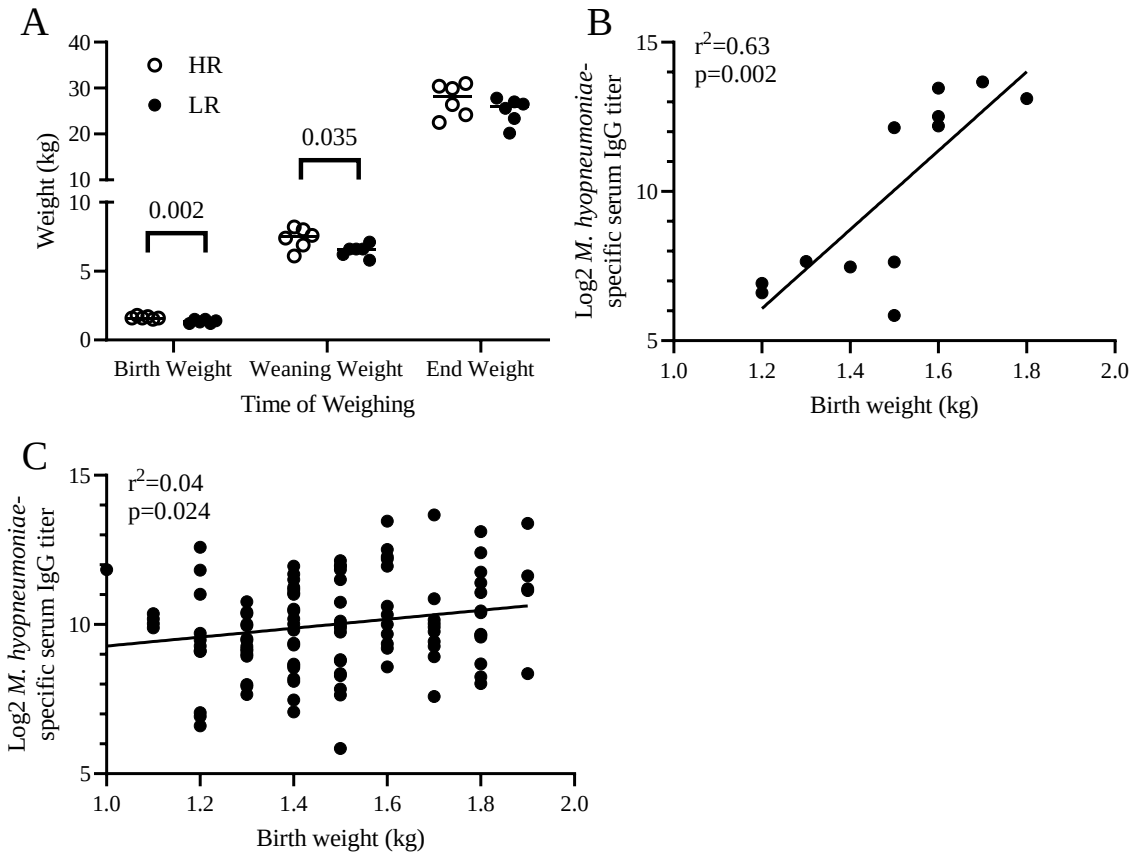


Figure 4: Body weight is associated with vaccine responsiveness in high and low responders prior to vaccination. A. Comparison of median body weight between high (HR; n=6; open circles) and low (LR; n=6; closed circles) responders at birth, weaning (24-days of age), and at the end of the trial (63-days of age). **B.** Correlation analysis between *M. hyopneumoniae*-specific IgG titers and piglet birth weight of HR and LR. **C.** Correlation analysis between *M. hyopneumoniae*-specific IgG titers and piglet birth weight for the trial population. P-values were determined using a two-tailed, unpaired Student's t-test. Best fit lines determined using Pearson Correlation. Figures 4A, 4B, and 4C are modified from Lipsit *et al.* (2020).

3.1.6 Conclusions to Section 3.1

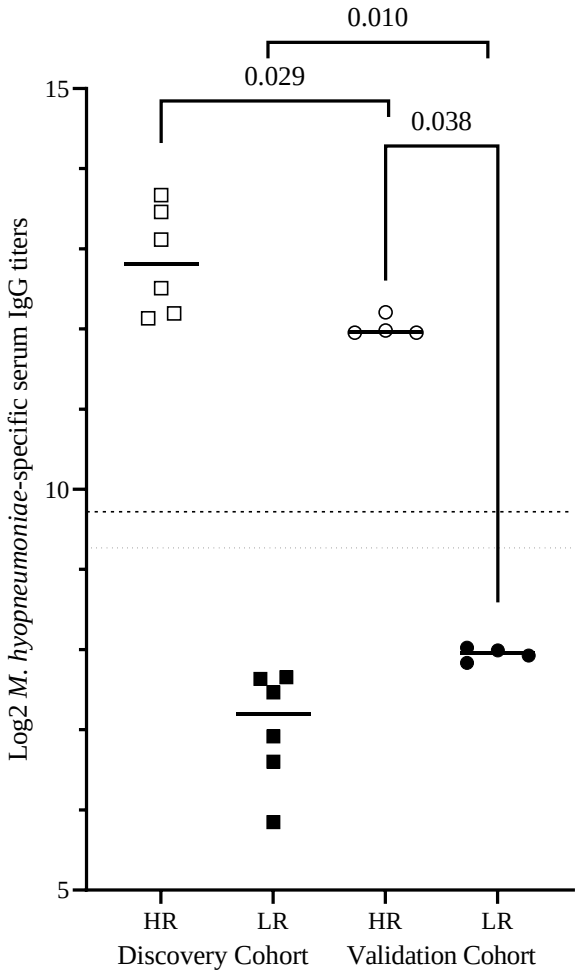
Vaccinating a trial population of piglets with a two-dose regimen of RespiSure-One resulted in broad variation in vaccine-induced antibody responses. Approximately 31% of piglets were considered seronegative for *M. hyopneumoniae*-specific antibodies at 11-days following booster vaccination. High and low vaccine responders were apparent and revealed multiple variables associated with vaccine responsiveness. Kinome analysis of PBMCs collected from HR and LR highlighted molecular differences among vaccine responders immediately before and 6-days after vaccination. The pre-vaccination differential phosphorylation events were associated with cytokine signaling pathways and elevated plasma IFNγ and IL-1β within LR compared to HR were consistent with kinome data. These data align with the observations of others who identified higher concentrations of serum cytokines in low vaccine responders at the time of vaccination (Fourati *et al*., 2016; Frasca *et al*., 2014; Trzonkowski *et al*., 2003). Also, LR and HR had differences in body weight prior to vaccination, specifically at birth and weaning. Similar observations between birth weight and vaccine responsiveness were reported for human infants and adolescents following typhoid vaccination (McDade *et al*., 2001; Moore *et al*., 2004). These data support using birth weight and plasma cytokine levels as biomarkers for detecting likely low responders in a herd of piglets. Further exploration of whether these variables are host characteristics that affect vaccine responsiveness or are indicators of immunological mechanisms that affect vaccine responsiveness is required. Since validation that these biomarkers are consistent in other vaccine responders was needed, the relationships between vaccine responsiveness, phosphorylation events, plasma cytokines, and body weight were investigated in a second cohort of high and low vaccine responders.

3.2 Validating Vaccine Response Biomarkers in a Validation Cohort

3.2.1 Establishing a Validation Cohort of High and Low Responders

A second cohort of HR (n=4) and LR (n=4) corresponding to the 80^{th} and 20^{th} percentile of serum IgG titers were stratified as a validation cohort to test the predictive capability of the biomarkers identified in the discovery cohort. HR and LR within the validation cohort were seronegative (S/P<0.1) prior to vaccination. HR within the validation cohort had higher (p=0.038) serum IgG titers than the LRs in the validation cohort. When comparing HR between the discovery and validation cohorts, validation HR had a lower rank-sum difference (p=0.029) than discovery HR **(Fig. 5)**. Similarly, the validation LR had a higher rank-sum than the discovery LR (p=0.010).

Thus, the validation cohort consists of high and low responders with a more moderate phenotype than the discovery cohort.



1080

1081 **Figure 5: High and low responders from the validation cohort have less divergent serum IgG**
1082 **titers 11-days following booster RespiSure-One vaccination than the discovery cohort.**
1083 Median *M. hyopneumoniae*-specific IgG titers of high (HR; empty shape) and low (LR; filled
1084 shape) responders within the discovery cohort (squares; n=6/cohort) and the validation cohort
1085 (circles; n=6/cohort). Dashed and dotted lines represent the threshold for seropositive and
1086 seronegative cutoffs, respectively. P-values were determined using a Mann-Whitney U-Test.

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3.2.2 Biochemical and Physiological Markers within the Validation Cohort

In the discovery cohort, LR had higher levels of plasma IFNγ, IL-1β, and TNFα on Day 0 than HR **(Fig. 3A)**. The discovery cohort also revealed LR had lower body weight at birth and weaning than HR **(Fig. 4A)**. Therefore, these plasma cytokines and body weights were quantified in HR and LR within the validation cohort prior to vaccination to test their capability of discriminating vaccine responders.

There were no differences in IFNγ ($p=0.68$), IL-1β ($p<0.99$), or TNFα ($p<0.99$) between HR and LR within the validation cohort **(Fig. 6A)**. When comparing HR and LR within the validation cohort, there was also no difference in either birth weight ($p=0.58$) or weaning weight ($p=0.74$) **(Fig. 6B)**. Similar to the cytokine observations, this may indicate that as the vaccine responsiveness phenotype becomes less extreme, physiological differences in body weight between vaccine responders are less apparent.

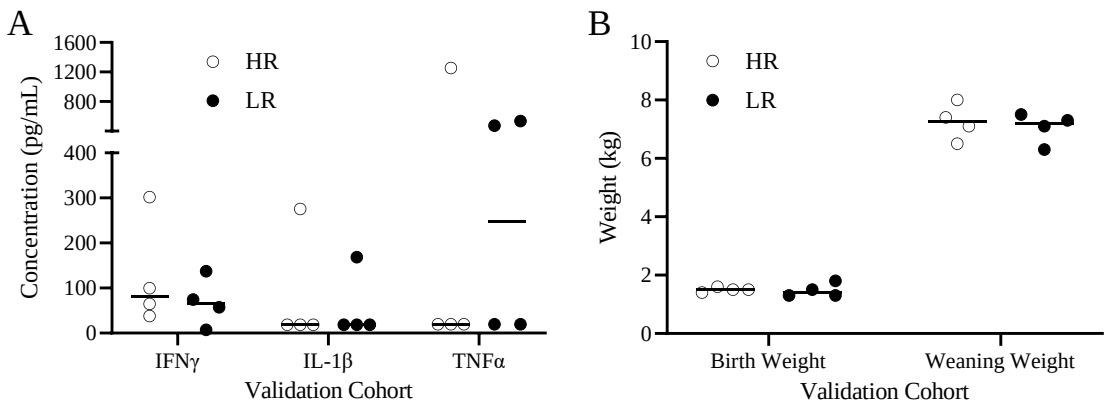


Figure 6: Plasma cytokines at Day 0 and body weight of the validation cohort do not differ between high and low responders within the validation cohort. A. Median plasma cytokine concentrations of IFNγ, IL-1β, and TNFα of high (HR; n=4; empty circles) and low (LR; n=4; filled circles) within the validation cohort prior to vaccination. **B.** Median body weight of HR (empty circles) and LR (filled circles) at birth and weaning (24-days of age). P-values were determined using a Mann-Whitney U-Test.

3.2.3 Validation of Phosphorylation Biomarkers

To validate the robustness of the vaccine responsiveness phosphorylation biomarkers identified in the discovery cohort, the capability of the biomarker phosphorylation events to cluster HR and LR kinome profiles was tested using PCA. Subsequently, a comparative analysis was conducted to determine if individual biomarker phosphorylation events showed consistent phosphorylation patterns between HR and LR of the validation cohort as was observed in the discovery cohort.

3.2.3.1 Validation of Phosphorylation Biomarkers on Day 0

PCA of HR and LR within the validation cohort on Day 0 using the untargeted, 282-peptide kinome profile did not clearly separate kinome profiles based on vaccine responsiveness phenotype **(Fig. 7A)**. Then, piglets within the validation cohort were given new IDs to blind vaccine response phenotypes, and only the phosphorylation intensities of the 10 phosphorylation events determined from the discovery cohort were used for PCA. The cluster analysis using the 10 phosphorylation biomarkers alone suggests that PC1 (52%) reduces the inter-group overlap between HR and LR within the validation cohort compared to the untargeted kinome profile **(Fig. 7B)**. Together, these cluster analyses suggest that a signature of 10 biomarker phosphorylation events better separates HR and LR phenotypes than the complete array of phosphorylation events.

Next, phosphorylation intensities of the 10 biomarker phosphorylation events between the discovery HR and LR were quantified within the validation cohort. This analysis revealed that multiple phosphorylation biomarkers observed in the discovery cohort were consistently (similar direction of change) differentially phosphorylated between HR and LR within the validation cohort. **(Fig. 7C).** Differential phosphorylation events of peptide targets such as STMN1_S15, TRAF6_Y353, and PPP2CA_T304 were consistently differentially phosphorylated ($p=0.029$) in the discovery cohort. CALM1_Y99 showed a trend ($p=0.057$) of being differentially phosphorylated, while the remaining phosphorylation events did not show a trend ($p>0.1$) between HR and LR in the validation cohort. Altogether, multiple phosphorylation events observed in the discovery cohort on Day 0 persist between HR and LR within the validation group, even when plasma cytokines or body weight did not significantly associate with vaccine responsiveness.

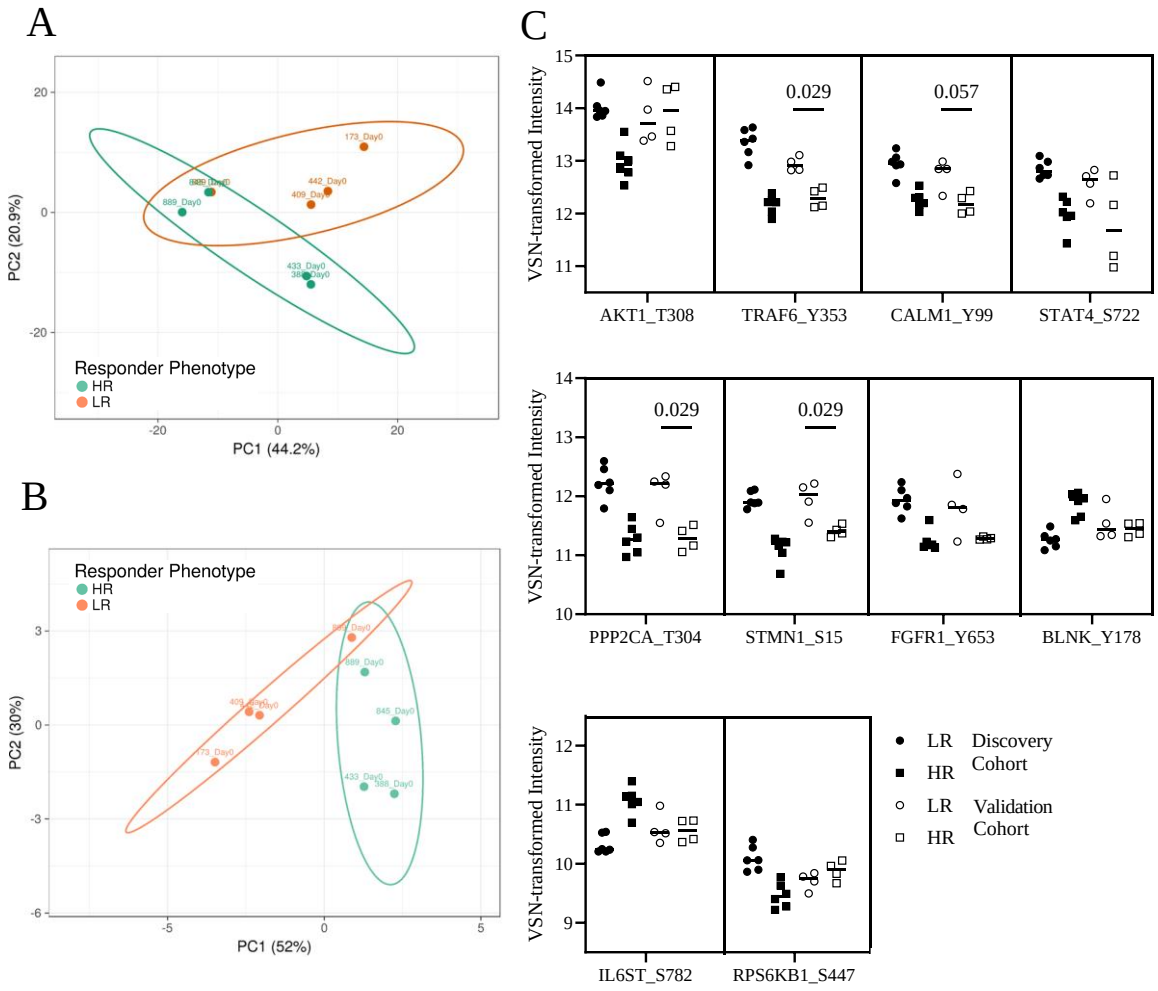


Figure 7: Biomarker phosphorylation events between high and low responders within the discovery and validation cohorts on Day 0. Principal component analysis of the high (HR; n=4; green) and low (LR; n=4, orange) responders within the validation cohort using **A.** 282 peptide phosphorylation events represented on the kinome array and **B.** 10 peptide phosphorylation events determined to be differentially phosphorylated between HR and LR within the discovery cohort on Day 0. Principle components (PCs) with the highest variance (%) are shown. Ellipses represent 95% confidence intervals. **C.** Phosphorylation intensities of the 10 differentially phosphorylated peptides on Day 0 of LR (circles) and HR (squares) within the discovery (filled shape) and validation (empty shape) cohorts. The horizontal line represents the group median. P-values in C are only shown for comparisons between LR and HR within the validation cohort. P-values were determined using the Mann-Whitney U-test.

3.2.3.2 Validation of Phosphorylation Biomarkers on Day 6

Similar to the Day 0 analyses, the discriminatory capability of the vaccine response biomarkers identified within the discovery cohort on Day 6 was evaluated using PCA and comparative analysis. One piglet classified as HR in the validation cohort ("388B") on Day 0 did not have PBMCs collected on Day 6 and was substituted with another HR ("893R") for subsequent Day 6 validation cohort analyses.

On Day 6, PCA of the validation HR and LR using the 282-peptide kinome profile revealed indistinct clustering of HR and LR, suggesting the complete array of peptide phosphorylation events cannot differentiate the vaccine responsiveness phenotype **(Fig. 8A)**. As with the Day 0 analysis, the PCA was repeated using only the 11 biomarker phosphorylation events identified in the discovery cohort. Reducing the consideration to the 11 biomarker phosphorylation events on Day 6 shows a combination of PC1 (45%) and PC2 (21.7%) can separate HR and LR within the validation cohort **(Fig. 8B)**. This improvement from overlapping 95% CIs using the untargeted kinome profile to completely distinct 95% CIs using the 11 biomarker phosphorylation events supports the conclusion that these biomarkers are associated with the magnitude of vaccine responsiveness.

Comparative analysis of the 11 phosphorylation events identified in the discovery cohort revealed consistent differences between HR and LR in the validation cohort **(Fig. 8C)**. Specifically, the peptide targets STMN1_S15 and Nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1)_S245 were differentially phosphorylated ($p=0.029$), while Kelch-Like ECH-Associated Protein 1 (KEAP1)_Y293/5, and SYK_Y348 were close to the threshold of a trend ($p=0.11$) for having consistent differential phosphorylation. These 4 phosphorylation events demonstrate highly similar patterns of direction and magnitude of change in each cohort. For the other biomarker phosphorylation events, there are no significant differences in intensity between the HR and LR of the validation cohort, indicating not all phosphorylation events are consistently different in less extreme phenotypes.

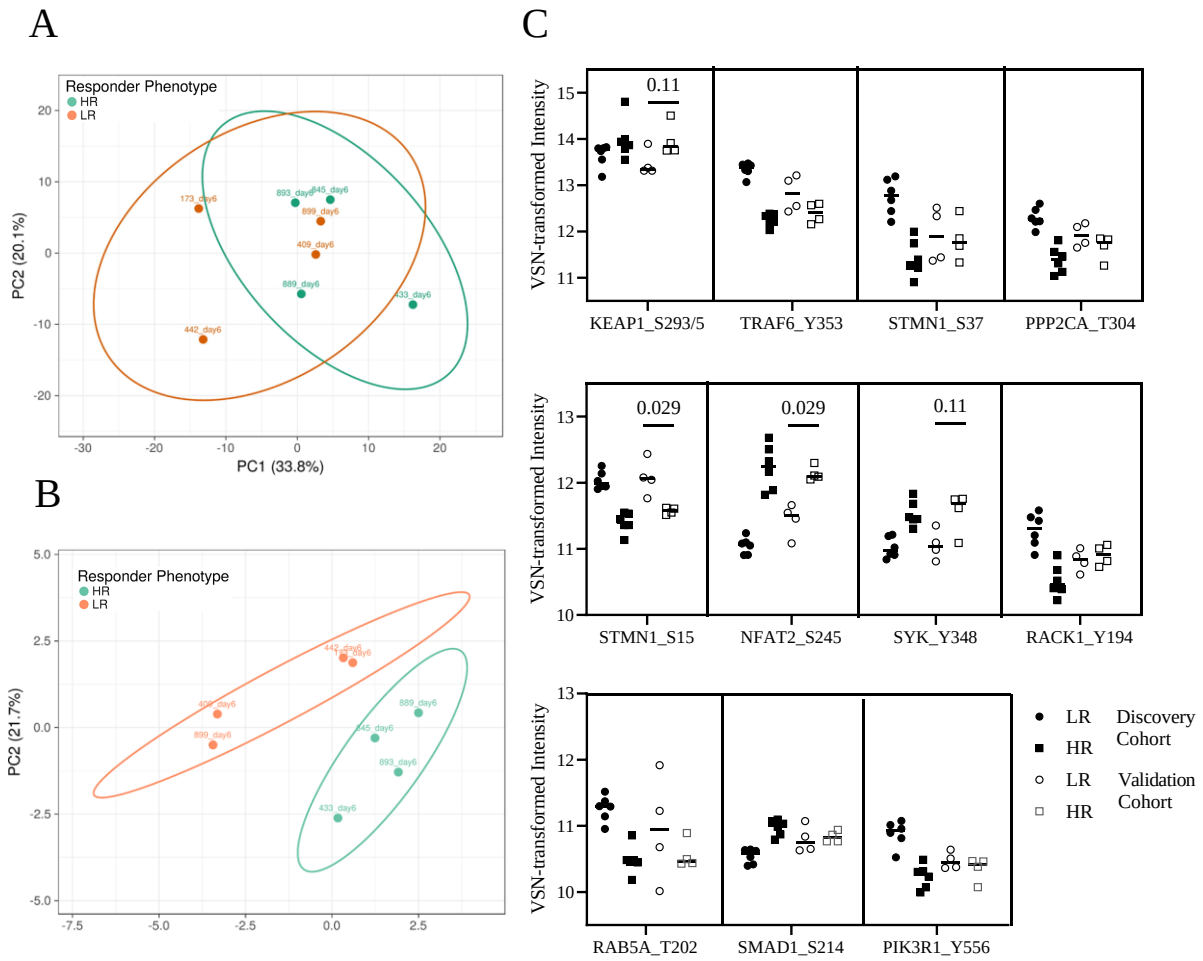


Figure 8: Biomarker phosphorylation events between high and low responders within the discovery and validation cohorts on Day 6. Principal component analysis of the high (HR; n=4; green) and low (LR; n=4, orange) responders within the validation cohort using **A.** 282 peptides phosphorylation events represented on the kinome array and **B.** 11 peptide phosphorylation events determined to be differentially phosphorylated between HR and LR within the discovery cohort on Day 6. Principle components (PCs) with the highest variance (%) are shown. Ellipses represent 95% confidence intervals. **C.** Phosphorylation intensities of the 11 differentially phosphorylated peptides on Day 6 of LR (circles) and HR (squares) within the discovery (filled shape) and validation (empty shape) cohorts. The horizontal line represents the group median. P-values in C are only shown for comparisons between LR and HR within the validation cohort. P-values were determined using the Mann-Whitney U-test.

3.2.4 Conclusions to Section 3.2

Using an independent subset of high and low vaccine responders, phosphorylation events had greater discriminatory capabilities than physiological factors such as circulating plasma cytokine concentrations or birthweight, at least within this trial population of piglets. Multiple differential phosphorylation events found in the discovery cohort were also differentially phosphorylated in the validation cohort; these phosphorylation events improved cluster separation of HR and LR compared to an untargeted kinome profile. HR and LR within the validation cohort did not reveal differences in plasma cytokines or body weight, suggesting that phosphorylation biomarkers better discern vaccine responsiveness than these physiological markers. Following these studies, a second vaccine trial was initiated to again evaluate the use of plasma cytokines and body weight as biomarkers of vaccine responsiveness and explore the dynamics of circulating cytokines and piglet growth prior to vaccination.

3.3 Validating Plasma Cytokine and Body weight Biomarkers in a Second Trial

A second vaccination trial ("Trial 2020") was modelled after the first vaccine trial ("Trial 2015") to test the hypothesis that plasma cytokines and body weight are associated with vaccine responsiveness. Piglets (n=67; 30 female, 37 male) born from multiple litters (n=6 litters; 8-14 piglets/litter) were vaccinated (RespiSure-One) at 28-days of age (Day 0) and given a booster at 52-days of age. *M. hyopneumoniae*-specific serum IgG titers were quantified prior to vaccination and at 63-days of age (Day 35) using the commercial IDEXX *M. hyo* Ab ELISA (IDEXX Laboratories, Inc.). All piglets within this trial (n=67) were seronegative (S/P<0.3) for *M. hyopneumoniae*-specific serum IgG prior to vaccination and all piglets were seroconverted (S/P>0.4) on Day 35.

3.3.1 Variability of Vaccine Responses between Trials

To quantify vaccine-induced antibody responses, post-vaccination serum was serially diluted 5-fold, and endpoint serum IgG titers were calculated. There was an 8-fold difference between the highest and lowest Log_2 serum IgG titers (titer range = 10.0 –13.2; median (95% CI) = 11.8 (11.0 – 12.1)). In contrast with piglets within the first trial (titer range = 5.85 – 13.67; median (95% CI) = 9.96 (9.65 – 10.2)) described in **Section 3.1**, piglets within the second trial had a greater distribution ($p<0.001$) of serum IgG titers **(Fig. 9A)**. Within the second trial, there was

no sex-dependent (p=0.13; Mann-Whitney U-test) effect on post-vaccination serum IgG titers. Serum samples from piglets within the second trial were collected in two batches (3 litters/batch) on different experimental days, and no batch-dependent (p=0.35; Mann-Whitney U-test) effect was observed. Altogether, piglets from the second trial had higher serum IgG titers with less variation than piglets from the first trial.

Similar to the first trial, piglets from the second trial representing the 90^{th} percentile and 10^{th} percentile of serum IgG titers on Day 35 were stratified into HR (n=6) and LR (n=6), respectively, to establish cohorts of piglets with the most extreme differences in serum IgG titers. When comparing the post-vaccination serum IgG titers of HR and LR between trials, there was no difference (p=0.89) between HR of the second (mean ± SD = 12.9 ± 0.18) and first (mean ± SD = 12.9 ± 0.64) trials **(Fig. 9B)**. In contrast, all LR within the second trial were seropositive (S/P>0.4) and had significantly higher (p<0.001) post-vaccination serum IgG titers (mean ± SD = 10.3 ± 0.11) than the seronegative LRs within the first trial (mean ± SD = 5.85 ± 0.71) **(Fig. 9B)**. Thus, the LR within the second trial did not phenotypically represent seronegative non-responders. LR from the second trial more appropriately represent seropositive vaccine responders with lower serum IgG titers than the HR. However, since LR from the second trial had statistically lower serum IgG titers than the HR from the second trial, they were used to test the previously identified associations between vaccine-induced antibody responses and either plasma cytokines or birth weight.

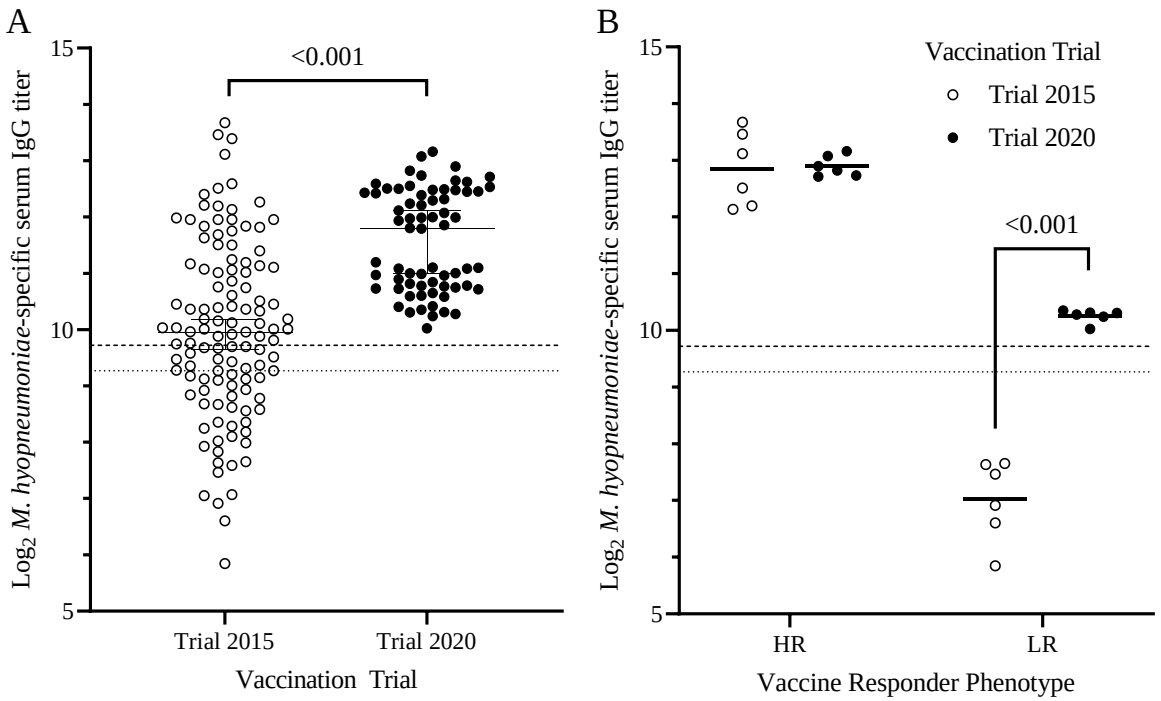


Figure 9: *Mycoplasma hyopneumoniae*-specific serum IgG titers of piglets from the first (Trial 2015) and second (Trial 2020) trials 11-days following booster RespiSure-One vaccination. A. Median (±95% confidence interval) *M. hyopneumoniae*–specific serum IgG titers of piglets from the first (n=117; open circles) and second (n=67; solid circles) trials. Differences were determined using a 2-sample Kolmogorov–Smirnov test. **B.** Mean *M. hyopneumoniae*–specific serum IgG titers of the high (HR) and low (LR) responders within the first (n=6/cohort; open circles) and second (n=6/cohort; closed circles) trials. The horizontal bar represents the group mean. Dashed and dotted lines represent the threshold for seropositive and seronegative cutoffs, respectively. Differences were determined using a two-tailed, unpaired Student's t-test with Welch's correction.

3.3.2 Plasma Cytokines and Vaccine Responsiveness in the Second Trial

Associations between plasma cytokines and vaccine-induced antibody responses were identified in the first trial. To test the relationship between Day 0 plasma cytokine concentrations and vaccine-induced antibody responses further, correlation and comparative analyses were conducted on piglets from the second trial. Here, Day 0 plasma IFNγ levels prior to vaccination did not reveal a significant (ρ=0.10, p-value=0.41) correlation with Day 35 serum IgG titers among all piglets (n=67) **(Fig. 10A)**. Similarly, Day 0 plasma IL-1β levels prior to vaccination and Day 35 serum IgG titers were not significantly correlated (ρ=0.13, p-value=0.28) **(Fig. 10B)**. Limiting the analysis to only the HR (n=6) and LR (n=6) within the second trial revealed no rank-sum differences in Day 0 plasma IFNγ (p=0.34) between HR and LR and a trend towards higher plasma IL-1β (p=0.12) in HR than LR **(Fig. 10C)**. Overall, no associations between pre-vaccination plasma cytokine concentrations and post-vaccination vaccine-induced serum IgG titers were identified in the second trial. There were no significant differences (p=0.81) in post-vaccination serum IgG titers among litters **(Fig. 10D)**. While plasma IFNγ and IL-1β concentrations at the time of vaccination were associated with vaccine responsiveness within HR and LR from the first trial, these data suggest the elevated plasma cytokine concentrations do not always correlate with the magnitude of vaccine-induced antibody responses.

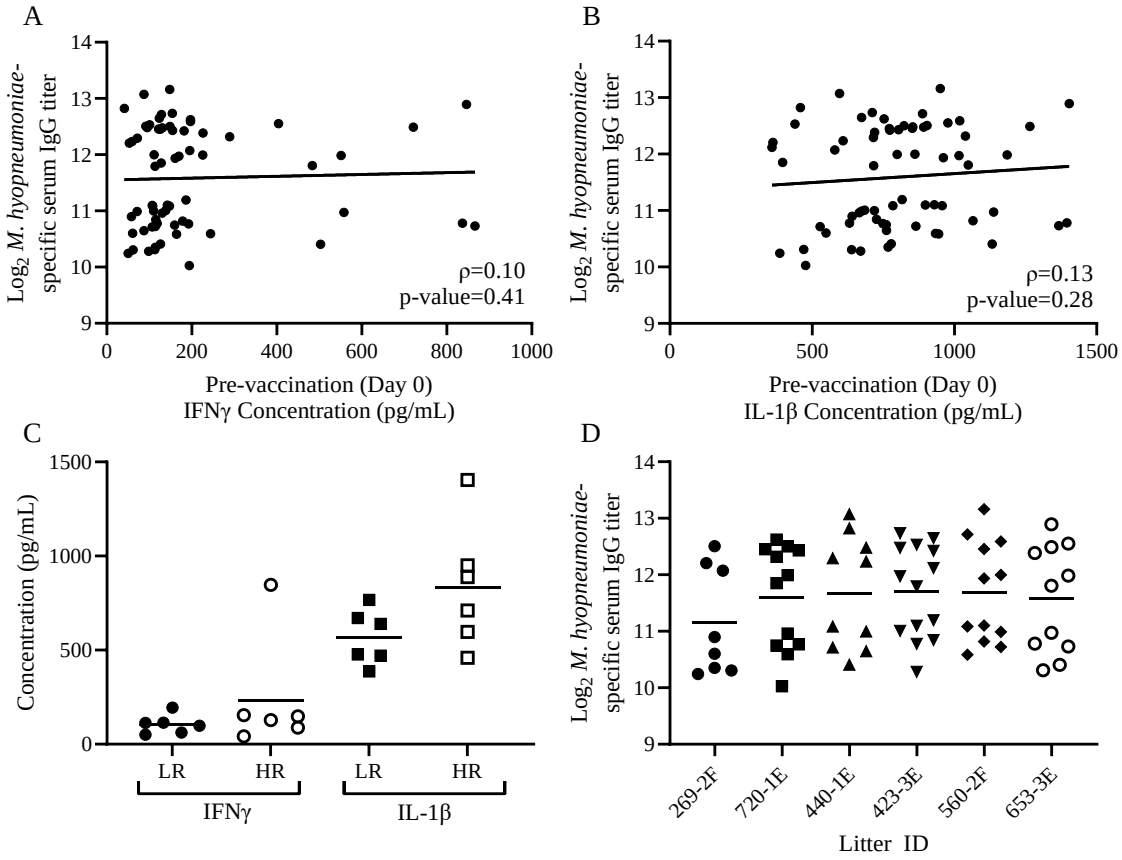


Figure 10: Plasma cytokine concentrations do not correlate with *Mycoplasma hyopneumoniae*-specific IgG titers in the second trial. A. Correlation analysis of post-vaccination *M. hyopneumoniae*-specific serum IgG titers with Day 0 plasma IFNγ and **B.** Day 0 plasma IL-1β concentrations within piglets of the second trial (n=67). Best-fit lines were determined using Spearman Rank Correlation. **C.** Mean Day 0 plasma concentrations of IFNγ (circles) and IL-1β (squares) in low (LR; n=6; solid symbol) and (HR; n=6; open symbol) within the second trial. Differences were determined using a Student's t-test with Welch's correction. **D.** Mean (±SEM) Log_2 *M. hyopneumoniae*-specific serum IgG titer of piglets from the second trial grouped by the factor "Litter". Differences were determined using a one-way ANOVA.

3.3.3 Dynamic Concentrations of Plasma Cytokines

The first trial revealed that piglets with lower vaccine-induced antibody responses had elevated plasma cytokines, specifically IFNγ and IL-1β, prior to vaccination compared to age-matched piglets with higher vaccine-induced antibody responses. However, these plasma cytokines were measured only at the time of vaccination (28-days of age; Day 0), and it is unknown if differences in cytokine concentrations persisted at different time points. Though plasma cytokines were not associated with vaccine responsiveness in piglets from the second trial, the opportunity remained to investigate additional time points to evaluate the stability of plasma cytokines in young piglets for future reference.

3.3.3.1 Temporal Variability of Plasma Cytokine Concentrations in Piglets

Plasma IFNγ and IL-1β were measured at 7-day intervals beginning at birth to 28-days of age (primary vaccination) as well as at 63-days of age to determine the temporal stability of cytokine biomarkers prior to and following vaccination. Within the second trial, there was a significant time-dependent effect ($p<0.001$) on plasma IFNγ and IL-1β concentrations within all piglets. Plasma IFNγ concentration was highest at birth and decreased significantly ($p<0.001$) by 14-days of age. Piglets had the lowest concentrations of plasma IFNγ at 63-days of age ($p<0.001$) **(Fig. 11A)**. In contrast, IL-1β did not show age-dependent changes between birth and 28-days of age. However, IL-1β concentrations were significantly lower ($p<0.001$) at 63-days of age than at birth **(Fig. 11B)**. Temporal variation in plasma cytokines following birth could limit the use of plasma IFNγ or IL-1β as vaccine response biomarkers if they do not consistently associate with vaccine-induced immunity.

3.3.3.2 Litter-specific differences in Plasma Cytokines

Piglets were grouped within litters and a two-way mixed-effects ANOVA with Tukey's multiple comparisons was conducted using the factors "Litter" and "Time" to account for litter-dependent variability in plasma IFNγ and IL-1β concentrations among litters at each time point. There was a significant ($p<0.001$) interaction between "Litter" and "Time" for mean plasma IFNγ concentrations. One litter, "653-3E" ($n=11$), had consistently higher levels of plasma IFNγ than the other 5 litters at 0-, 7-, 14-, 21- ($p<0.001$), and 28-days of age ($p<0.01$) **(Fig. 11C)**. This difference in plasma IFNγ levels between Litter 653-3E and the other 5 litters waned and was not significant at 63-days of age. Few litter-dependent differences were revealed among other litters

as Litter 720-1E (n=12) had higher ($p<0.05$) plasma IFNγ than 3 other litters at birth, but these differences were not consistent at subsequent time points. Differences in plasma IFNγ concentration among litters were not observed at 63-days of age except for few individual differences that were not consistent with other time points. All litters had lower ($p<0.05$) plasma IFNγ at 63-days of age than birth, except for Litter 269-2F (n=8), which only showed a trend towards ($p=0.07$) lower concentrations **(Fig. 11C)**.

A two-way mixed-effects ANOVA using the factors "Litter" and "Time" was conducted to identify differences in mean plasma IL-1β concentrations among litters within the second trial. There was a significant ($p<0.001$) interaction between "Litter" and "Time" on IL-1β concentrations. A comparison among litters revealed Litter 653-3E had significantly higher plasma IL-1β concentrations than the other 5 litters at birth ($p<0.005$), four litters at 7- and 14-days of age ($p<0.005$), and four litters at 21- and 28-days of age ($p<0.05$) **(Fig. 11D)**. At 63-days of age, all litters had lower ($p<0.01$) plasma IL-1β concentrations than at birth. Four litters had lower ($p<0.05$) plasma IL-1β concentrations at 63-days of age than all other time points. Litter 423-3E (n=12) had significantly lower ($p<0.005$) mean plasma IL-1β concentration than three other litters at 63-days of age, but this difference was not observed at other time points.

Routine monitoring and clinical assessment of piglets throughout the current study did not identify clinical signs of infection, physical injury, or trauma, supporting the conclusion that illness did not cause the elevated plasma IFNγ and IL-1β, specifically within Litter 653-3E. To determine if this difference in litter-specific plasma cytokines was related to the number of times a sow had given birth, we investigated the parity of sows and the level of plasma cytokines in piglets at birth. Sows used in this study had a parity of 0-3, and no relationship between parity and cytokine concentrations was found. Observing a litter (Litter 653-3E) with elevated plasma cytokines compared to other litters with unimpaired vaccine-induced serum IgG titers showed that high cytokine concentrations at the time of vaccination did not always classify piglets as low vaccine responders. However, the elevated IFNγ and IL-1β within this biologically-related litter from the second trial may have different roles than the individual LR piglets from the first trial. Data from the two trials suggest that the plasma cytokines biomarkers may be situationally dependent for predicting vaccine responders.

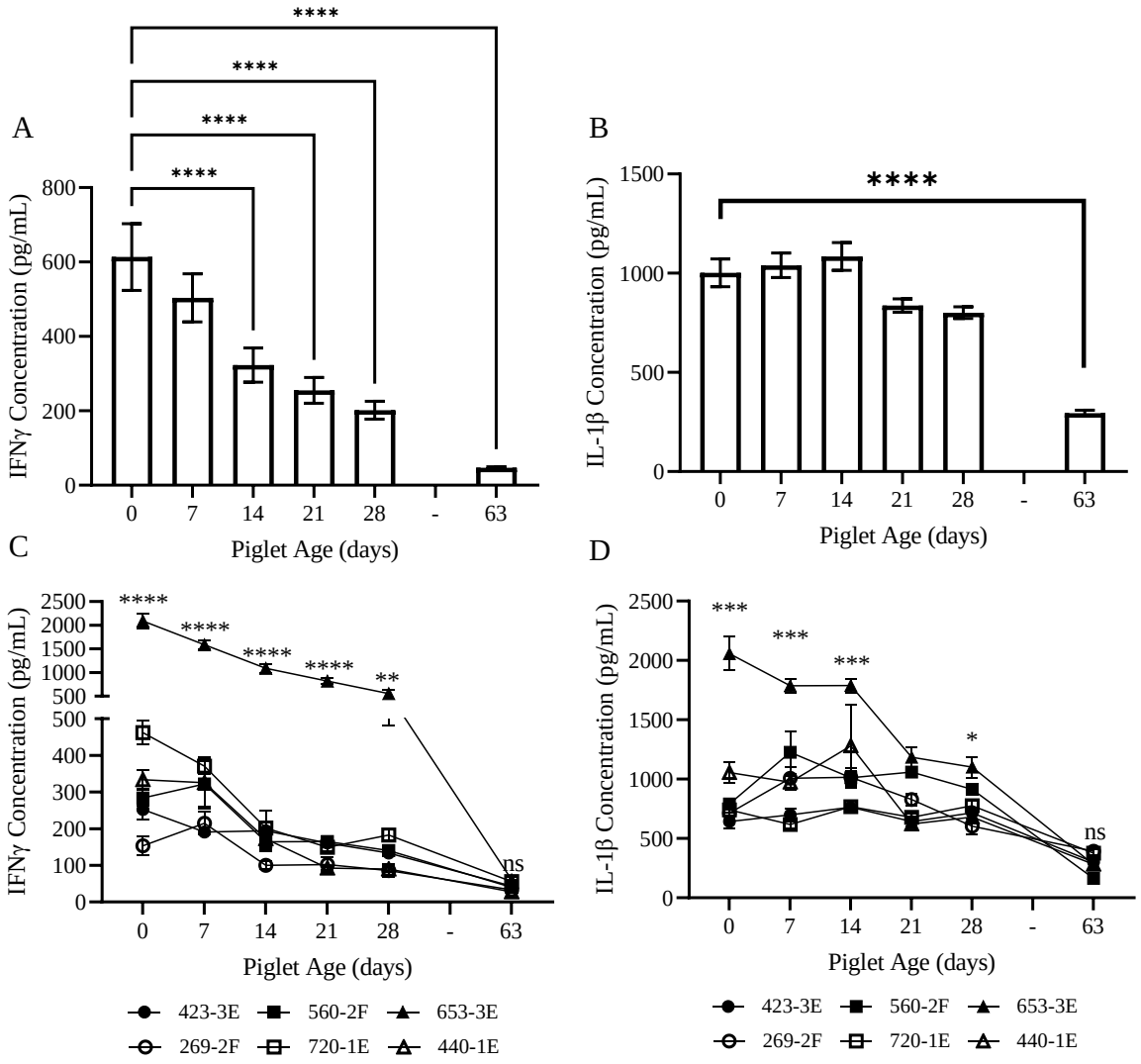
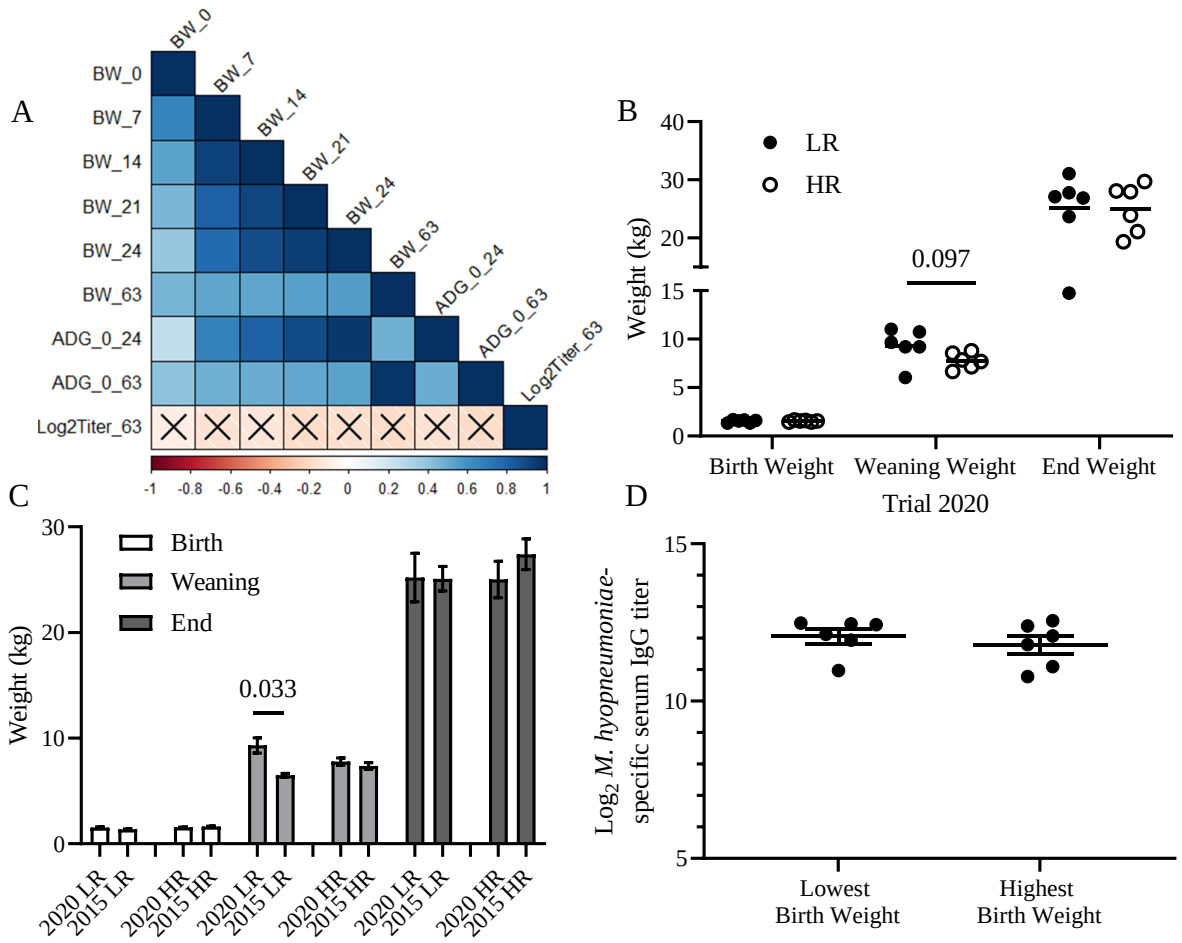


Figure 11: Plasma cytokine concentrations of piglets within the second trial from birth to 63-days of age at weekly intervals. A. Mean (±SEM) plasma interferon-gamma (IFNγ) and **B.** interleukin 1-beta (IL-1β) concentrations measured weekly from piglets within the second trial (n=67) from 0-days of age until 28-days of age and at 63-days of age. Differences were determined using a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons. **C.** Mean (±SEM) plasma IFNγ and **D.** IL-1β concentrations of piglets from the second trial were quantified weekly from 0-days to 28-days of age and at 63-days of age grouped by the factor "Litter" (n=8-14/litter). Differences were determined using a two-way mixed-effects ANOVA with Tukey's multiple comparisons. Asterisks designate differences in mean cytokine concentration among all litters. Only P-values representing the differences across all multiple comparisons among litters are shown. $^{n.s.}p>0.05$, *$p<0.05$; **$p<0.01$; ***$p<0.005$; ****$p<0.001$.

3.3.4 Birth weight and Vaccine Responsiveness in the Second Trial

As the first trial revealed a positive correlation between piglet body weight and vaccine-induced antibody responses, the hypothesis that there were associations between body weight and serum IgG titers was tested further in the second trial. A Pearson correlation analysis was conducted to determine if body weight and average daily gain (ADG) were associated with vaccine-induced antibody responses. Piglets from the second trial had positive ($r>0.4$) Pearson correlations ($p<0.05$) among body weights at 0- (birth), 7-, 14-, 21-, 24- (weaning), and 63-days of age **(Fig. 12A)**. Additionally, piglets had positive correlations between all body weights and their ADG from 0- to 24-days of age (birth to weaning) and from 0- to 63-days of age (birth to end of the trial) **(Fig. 12A)**. There were no significant correlations ($p>0.10$) between Day 35 serum IgG titers and body weights or ADGs of piglets within the second trial **(Fig. 12A)**. In the first trial, LR had lower birth weight and lower weaning weight than HR, but there was no difference in body weight at 63-days of age **(Fig. 12C)**. In the second trial, there was no difference in body weight between HR and LR at birth ($p=0.91$) or the end of the trial ($p=0.95$) **(Fig. 12B)**. While there was a trend of LR having a higher weaning weight than HR ($p=0.097$), this trend was not consistent with other time points **(Fig. 12B)**.

When comparing HR and LR between trials, there are no differences in body weights between HR of the first and second trials. Though LRs from the second trial have higher ($p=0.033$) body weight than LRs from the first trial at weaning, this difference is not observed at other time points **(Fig. 12C)**. To further test if birth weight was associated with vaccine responsiveness, post-vaccination serum IgG titers between the highest ($n=6$) and lowest ($n=6$) birth weight piglets were analyzed. This analysis revealed no difference ($p=0.48$) in serum IgG titers between the largest and smallest piglets at birth **(Fig. 12D)**. Altogether, there were no significant associations between body weight at birth or weaning with vaccine-induced antibody responses in the second trial.



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1376 **Figure 12: High and low vaccine responders in the second vaccine trial do not differ**
1377 **significantly in body weight. A.** Pearson correlation matrix of body weights at 0- (BW_0), 7-, 14-
1378 , 21-, 24-, and 63-days of age, pre-weaning average daily gain (ADG_0_24), total average daily
1379 gain (ADG_0_63) and Log_2 *M. hyopneumoniae*-specific serum IgG titer (Log2Titer_63) of piglets
1380 from the second trial (n=67). Scale represents Pearson correlation coefficients between
1381 comparisons. Crosses designate insignificant ($p>0.05$) Pearson correlations. **B.** Mean (±SEM)
1382 body weight (kg) of low (LR; n=6; filled circles) and high (HR; n=6; empty circles) at birth,
1383 weaning, and end of trial time points experiment. **C.** Mean (±SEM) body weight (kg) of LR (n=6)
1384 and HR (n=6) within the second and first trials at birth, weaning, and end of trial time points. **D.**
1385 Mean (±SEM) Log_2 *M. hyopneumoniae*-specific serum IgG titer of animals with the lowest (n=6)
1386 and highest (n=6) weight at birth. P-values were determined using a two-tailed, unpaired Student's
1387 t-test with Welch's correction.

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3.3.5 Conclusions to Section 3.3

The second vaccine trial provided multiple insights on using physiological markers as biomarkers of vaccine responsiveness. First, plasma cytokines such as IFNγ and IL-1β show time-dependent and litter-dependent effects in young piglets. Thus, a cytokine biomarker may need to account for these temporal changes. Second, piglets with the highest plasma cytokines concentrations at the time of vaccination did not have impaired vaccine responses. This suggests that while elevated plasma cytokines can be associated with impaired vaccine responses, they may not always be indicative of low vaccine responders. This is consistent with literature demonstrating that cytokine profiling of low vaccine-induced antibody responders does not always reveal correlations with IFNγ and IL-1β (Fourati *et al*., 2016; Qiu *et al*., 2018). As well, piglets with the lowest birth weights did not have lower vaccine responses, which was hypothesized as a risk factor for impaired immune responsiveness in humans and piglets (Bæk *et al*., 2020; McDade *et al*., 2001; Milligan *et al*., 2002a). Since LR piglets selected in the first trial were seronegative and LR piglets selected in second trial were seropositive, the inconsistencies between vaccine trials could result from the definition of "low responder". Further analysis is needed to verify whether seronegative low responders have elevated plasma cytokines and lower birth weight compared to seropositive responders. Together, data from the second trial provides further understanding of the immune-environment of piglets prior to vaccination and reveals situational-dependencies for utilizing physiological vaccine responsiveness biomarkers.

4. DISCUSSION

Vaccination remains one of the most effective methods for minimizing disease outbreaks and reducing infectious disease incidence in human and animal populations. Antibiotics are another tool for managing infectious diseases caused by bacterial infection, although antibiotic use (and misuse) can contribute to the rise of antibiotic resistance in pathogens (Potter *et al.*, 2008). Other strategies such as genetic selection could facilitate the development of a healthier population by selectively breeding for livestock with more protective immune responses and increased disease resistance (Mallard *et al.*, 2015). However, identifying highly heritable traits that correlate with disease resistance and other immune traits requires further optimization (Samorè and Fontanesi, 2016). One potential limitation to the effectiveness of vaccination is that variations in vaccine immunogenicity among individuals can result in unresponsive, susceptible individuals within the population. Low vaccine responders have the potential for contracting and transmitting disease agents, which compromises the protection afforded to the population by herd immunity. Identifying effective biomarkers that predict vaccine-induced immune responses is one potential solution to this problem. This thesis demonstrates complementary explorations for discovering and validating potential biomarkers of vaccine responsiveness.

4.1 Pre-vaccination Phosphorylation Biomarkers of Vaccine Responsiveness

Pre-vaccination biomarkers of vaccine responsiveness are beneficial in two ways: their detection could help identify low responders in a population, and they may provide insight into the molecular mechanisms of the vaccine responsiveness phenotype. Therefore, a phenotype-first approach identified high (HR) and low (LR) vaccine responders, and kinome analysis was conducted on PBMCs collected from piglets prior to, and following, vaccination to identify the signaling events associated with vaccine-induced antibody responses. Kinome analysis revealed numerous differences in phosphorylation prior to vaccination when comparing HR and LR within a discovery cohort. Differential phosphorylation of peptide targets representing proteins like BLNK, TRAF6, IL6ST, and the others **(Table 1)** suggest differential signaling events within PBMCs might influence processes involved in vaccine-induced antibody responses.

In an independent analysis that used the same vaccine responders from the first trial described in **Section 3.1**, RNA-seq was conducted on PBMCs collected from high and low responders at identical time points (Day 0, 2, and 6) (Munyaka *et al.*, 2019). Munyaka *et al.* (2019)

found no differences in gene expression between HR (n=15) and LR (n=15) on Day 0. While Munyaka *et al.* (2019) used a larger sample size (n=15/phenotype) than the kinome analysis (n=6/phenotype), their results suggested that HR and LR had identical transcriptional processes at the time of vaccination, which contrasts with the kinome analysis of the same subjects. These discrepancies could reflect different levels of sensitivity with each technology or the ability of the kinome analysis to identify phenotypic differences that do not require changes in gene expression. However, other transcriptional analyses have identified pre-vaccination differences in gene expression that correlate with vaccine-induced responses in piglets. Functional enrichment analysis of the gene expression events within PBMCs collected from 28-day old piglets prior to *M. hyopneumoniae* vaccination found processes and functions such as cell junction and adhesion, the extracellular matrix, signal transduction, and inflammation, were associated with vaccine-specific responses at 118-days post-vaccination (Blanc *et al*., 2021). Future vaccine responsiveness investigations utilizing genomic or transcriptomic approaches might gain complementing perspectives on the immune environment by incorporating systems analyses of protein post-translation modifications.

A previous kinome investigation conducted on PBMCs collected from humans and pigs demonstrated that individuals have temporally stable phosphorylation patterns that were unique to each individual, suggesting that individuals possess consistent signaling events independent of immune stimulation (Trost *et al*., 2013b). The hypothesis that the baseline state of the immune system affects immune responsiveness has been an area of recent exploration. Pre-vaccination gene expression of apoptotic genes were positively correlated with vaccine responses in a previous transcriptional analysis of humans vaccinated with an influenza virus vaccine, illuminating potential baseline gene expression markers of vaccine responses (Furman *et al*., 2013). Tsang *et al.* (2020) proposed that further understanding of an individual's baseline state can direct new methods of vaccine administration that modulate the immune system prior to vaccine delivery for improved responsiveness (Tsang *et al*., 2020). However, this study uses young (28-day old) piglets undergoing developmental changes similar to neonates in other species, such as changes in blood leukocyte populations, cytokine production, and the transition from passive to active immunity (Chase *et al*., 2008; Nguyen *et al*., 2007; Talker *et al*., 2013). Therefore, the "baseline" immune state at Day 0 might not model the baseline states described in studies investigating mature, human adults (Furman *et al*., 2013; HIPC-CHI Signatures Project Team and Consortium, 2017; Tsang *et*

al., 2014). More accurately, this work revealed novel phosphorylation events when comparing high and low vaccine responders, suggesting that differences in cellular activity at the time of vaccination can influence vaccine-induced antibody responses.

4.2 Post-vaccination Phosphorylation Markers of Vaccine Responsiveness

The molecular events of vaccine responsiveness have been primarily characterized through genomic, transcriptomic, cytometric, and microbiomic analyses to characterize the biological mechanisms associated with vaccine responses. In this study, kinome analysis of PBMCs collected from HR and LR at 2- and 6-days post-vaccination revealed a panel of 11 differential phosphorylation events at 6-days post-vaccination, yet there were no differences at 2-days post-vaccination. The inability to detect differences between HR and LR at a particular time point may result from strict statistical criteria for detecting differential phosphorylation events or could accurately reflect kinase signaling changes within PBMCs following vaccination. Also, the kinome array used in these analyses consisted of 282 phosphorylation events representing 148 proteins, a small fraction of the complete porcine proteome. The kinome array did not represent all possible protein targets involved in the innate immune signaling events 2-days post-vaccination. The most significant number of temporal phosphorylation changes within LR occurred between 2- and 6-days post-vaccination, suggesting that active processes occurring within the PBMCs may not have been captured on Day 2, but occurred between these two time points.

Compared to the transcriptional analysis by Munyaka *et al.* (2019), there were no differential gene expression events between high and low vaccine responders at either 2- or 6-days post-vaccination. However, a discrimination analysis was capable of partially discriminating high and low responders using gene expression events at 2-days post-vaccination only. Proteins encoded by the genes contributing to the distinction of high and low responders on Day 2 were not represented on the kinome array used in the analyses reported in this thesis (Munyaka *et al*., 2019). Therefore, it was not possible to directly compare the RNAseq data with the biomarkers identified in the kinome study, emphasizing differences in the scope of these two analyses. Ultimately, HR and LR selected in the first trial only had detectable phosphorylation differences, but no transcriptional differences, at 6-days post-vaccination. The kinetics of kinase signaling and gene expression likely influence their detection; signaling cascades can activate and decay within minutes of stimulation, while mRNAs may take tens of minutes or hours to accumulate (Ben-Ari

et al., 2010; Lemmon *et al*., 2016). Paired with the regulation of signal cascades and mRNA abundance, finding kinomic or transcriptomic changes within HR and LR could be highly dependent on the time of sample collection (Rauch *et al*., 2011).

Independent studies have observed transcriptomic changes that associate with antibody responses in other vaccination trials and at other post-vaccination time points within piglets. For example, Matthijis *et al.* (2019) demonstrated that gene expression of inflammatory and antigen presentation networks occur within PBMCs of piglets vaccinated with experimental *M. hyopneumoniae* vaccine formulations as early as 24-hours post-vaccination (Matthijs *et al*., 2019). Furthermore, although there were no differences in gene expression prior to vaccination, high and low antibody responders vaccinated against tetanus toxoid showed differences in gene expression between 2-4 weeks post-vaccination (Adler *et al*., 2015). Together, these studies show that high and low vaccine responder piglets can exhibit unique molecular changes at the transcriptomic level that correlate with vaccine outcomes. The post-vaccination kinome data provides additional evidence of molecular changes within PBMCs following vaccination.

4.3 Validation of Phosphorylation Biomarkers

Kinome analysis was performed on HR and LR representing the extremes of vaccine responses, namely, subjects within the 80^{th} and 20^{th} percentile of vaccine-induced antibody responses, respectively. One potential study design would utilize all 10 HR and 10 LR in a single discovery cohort to discover differential phosphorylation events. While such a study design would increase the statistical power to identify positive results with high confidence, it would lack the ability to validate any discovered phosphorylation events. Therefore, the data was split into a discovery cohort with 60% of vaccine responders (6 HR and 6 LR) and a validation cohort with the remaining 40% of vaccine responders (4 HR and 4 LR). Piglets from the validation cohort represented vaccine responders of less extreme phenotype compared to the discovery cohort. Among the differential phosphorylation events identified in the discovery cohort, multiple phosphorylation events were similarly differentially phosphorylated in the validation cohort. Indeed, the small sample size of the validation cohort could result in lower statistical power for detecting differences between HR and LR. As well, the difference in magnitude of vaccine responses between discovery and validation cohorts may explain the incomplete agreement in biomarker phosphorylation levels. Similar results were observed in an investigation using

phosphorylation biomarkers to predict the susceptibility of honeybees to Varroa mite infestation (Robertson *et al*., 2014, 2020). When applying phosphorylation biomarkers discovered in subjects of the most extreme phenotype, the phosphorylation biomarkers had markedly less intensity within the independent subjects of lesser phenotype.

A variety of other models used in systems vaccinology studies utilize sophisticated feature selection, novel algorithms, independent populations for testing the model, and methods of evaluating the model (Gonzalez-Dias *et al*., 2020). For example, a 10-fold cross-validation method was used to develop predictive models for predicting CD8+ T-cell responses to Yellow Fever vaccination and humoral responses to influenza vaccination in humans using gene expression data (Gaucher *et al*., 2008; Nakaya *et al*., 2011). In both studies, multiple independent trials were used to discover predictive biomarkers of the vaccine response and validate the sets of biomarkers. In comparison to observations reported in this thesis, the validation methods here included a comparative analysis of phosphorylation events, the clustering of the independent vaccine responders, and replication of the vaccine trial in an independent population to evaluate biomarkers. These validation methods might be improved in future trials with larger sample sizes.

4.4 Comparing Vaccine Responses between Trials

The two vaccine trials utilizing age-matched piglets exhibited different levels of seroconversion and range of antibody responses following vaccination with the same commercial vaccine. In the first trial (n=117), there was a 64-fold range in serum IgG titers resulting in both seropositive and seronegative piglets. Comparatively, piglets from the second trial (n=67) had less variance in serum IgG titers 11-days following booster vaccination than piglets from the first trial; only an 8-fold range in serum IgG titers was observed between HR and LR in the second trial. Interestingly, all the piglets from the second trial were considered seropositive (S/P<0.4) post-vaccination, demonstrating highly different distributions in vaccine responsiveness when comparing the two vaccine trials. There are several factors to consider when comparing the antibody responses of these two trials.

The first trial consisted of 117 piglets selected from 20 different litters, whereas the second trial had almost half the number of piglets (n=67) selected from only 6 different litters. The larger sample size in the first trial may have provided a higher probability of encountering vaccine non-responders that failed to seroconvert. Recently, a large population (n=182) of piglets from 47 sows

was vaccinated with an inactivated *M. hyopneumoniae* vaccine (Stellamune; Elanco) at 28-days of age. Broad variation of *M. hyopneumoniae*-specific IgG titers were observed at 21-, 35-, and 118-days post-vaccination. In agreement with the second vaccine trial described here, Blanc *et al.* (2021) reported almost all pigs to be seropositive (S/P>0.4) 11-days following booster vaccination. Only 19 piglets were seronegative (S/P<0.4) at 118-days post-vaccination (Blanc *et al*., 2021). This study provides further evidence that piglets often exhibit variable *M. hyopneumoniae*-specific responses following vaccination, albeit at a later post-vaccination time point than analyzed in the first vaccine trial described here.

Another potential issue is that the two vaccine trials reported in this thesis were conducted at different pig production facilities and there was a 5-year interval between studies. Both trials used RespiSure-One with Amphigen adjuvant (lot numbers not recorded), and there was no evidence that vaccine formulation changed within this timeframe (based on personal communication with the Associate Director of Zoetis, USA). The two facilities may have environmental differences, different animal handling practices that triggered a stress response, or have differences in diet and microbiome composition that could modulate the magnitude of antibody responses (de Groot *et al*., 2001; Tuchscherer *et al*., 2000). For example, the gut microbiota composition of weaned piglets has been found to vary among facilities (Luise *et al*., 2021) and may impact humoral responses similar to other studies conducted in mice, humans, and pigs (Hagan *et al*., 2019; Munyaka *et al*., 2020; Oh *et al*., 2014). Vaccine failure, a result of spoiled vaccine or improper injection, may result in poor vaccine-induced responses. However, this is unlikely to have occurred in the first trial because piglets were vaccinated in 4 batches (28-30 piglets/batch), and each batch contained both seropositive and seronegative vaccine responders.

Taken together, the two vaccine trials provide novel findings. First, vaccination with RespiSure-One can result in variable vaccine-induced antibody responses. Second, the level of seroconversion can either be complete, as in the second trial, or insufficient, as in the first trial. The implications of this are that the LR subpopulations defined in the first and second trials may not be biologically similar. For example, the LR selected for the first vaccine trial were seronegative, but the LR selected for the second vaccine trial were seropositive. Thus, the biomarkers discovered with piglets from the first trial might not be capable of identifying the LR selected in the second trial. However, LR from the second trial had significantly lower antibody

responses than the high responders and were utilized to evaluate the use of the pre-vaccination biomarkers in the second trial.

4.5 Body weight as Biomarkers of Vaccine Responsiveness

Birth weight is considered a predictor of overall health and survival within piglets; low birth weight piglets have a lower growth rate and a greater likelihood of pre-weaning mortality (Milligan *et al*., 2002a; Quiniou *et al*., 2002). In the first trial, only piglets of average litter weight were included to reduce inter-animal variability and ensure survival. Nevertheless, even with this selection criteria, LR piglets still had lower body weight at birth and weaning than HR piglets. Low birth weight had previously been associated with reduced vaccine outcomes in humans. For example, multiple cohorts of persons vaccinated against hepatitis B virus or typhoid vaccines demonstrated that reduced serum antibody titer was negatively associated with subject birth weight (Han *et al*., 2012; Moore *et al*., 2004). In a study of adolescents vaccinated with typhoid vaccine, low birth weight was significantly associated with reduced serum typhoid-IgG titers (McDade *et al*., 2001). Incidentally, individuals with reduced serum typhoid-IgG titers also had higher pro-inflammatory C-reactive protein concentrations in plasma later in life than adolescents with higher serum IgG titers (McDade *et al*., 2011). However, McDade *et al*. (2001, 2011) studied intra-uterine growth restricted individuals. Since intrauterine growth restriction is not strongly correlated with birth weight, McDade *et al*.'s conclusions may not implicate all individuals with low birth weight alone (McDade *et al*., 2001, 2011). This relationship between birth weight and vaccine responses is not fully understood, yet it is possible that pre- and postnatal factors have the potential to imprint an offspring's immune system. For example, piglets of lower birth weight receive less colostrum after birth than higher birth weight littermates, which has been observed to have consequences on a piglet's developing immune functions (Bæk *et al*., 2020; Milligan *et al*., 2002b). Additional experiments demonstrating an effect of piglet birth weight on vaccine-induced responses are needed to support the biological relevance of these empirical associations. Overall, findings from the first vaccination trial led to the hypothesis that low birth weight in piglets could provide a simple biomarker of impaired vaccine-induced antibody responses.

In contrast, the second vaccine trial did not reveal significant associations between either growth or weight with antibody responses in HR and LR piglets. To this effect, piglets with the lowest birth weight in the second trial had similar antibody responses than to the highest birth

weight piglets. Other studies have found that preterm infants and infants with low birth weight do not have lower vaccine-induced antibody responses than healthy weight controls (D'angio *et al*., 2011; D'Angio *et al*., 1995; Saari, 2003). These studies suggest that while being a metric of development and survival, birth weight does not always predispose individuals to fail to respond to vaccination. A recent study found that high antibody responders to *M. hyopneumoniae* vaccination had lower birth weights than low antibody responders, proposing a trade-off between immunity and growth in piglets (Blanc *et al*., 2021). While trial-specific differences (e.g. facility, animal handling) could have led to events that impacted vaccine responsiveness in low birth weight piglets, the identification of piglets with low birth weight who developed seropositive antibody responses in the second vaccine trial highlight the limitations of this biomarker.

4.6 Cytokines as Biomarkers of Vaccine Responsiveness

While prediction models have been valuable for identifying low vaccine responders, there is an equal necessity to understand the root mechanisms leading to reduced vaccine responsiveness. To gain insight into the immune mechanisms differentiating HR and LR, pathway analysis was conducted on the differentially phosphorylated peptide targets prior to vaccination, which implicated various cytokine signaling pathways. The differential phosphorylation of peptide targets that contributed to cytokine pathway enrichment included TRAF6, part of the NF-κB signaling pathway, IL6ST, a receptor for the pro-inflammatory IL-6 signaling pathway, and STAT4, a transcription factor activated through IL-12 and type-1 interferon-mediated signaling. As validation, cytokine analysis of plasma collected from HR and LR within the first vaccine trial revealed that LR had greater plasma concentrations of IFNγ and IL-1β than HR. This observation supported the hypothesis that pro-inflammatory environments at the time of vaccination can negatively impact vaccine-induced antibody responses.

Systemic, baseline inflammatory processes have been associated with poor vaccine responsiveness, which has been best defined in vaccine trials with older humans. For example, higher levels of serum TNFα were negatively associated with influenza-specific hemagglutination inhibition titers in older humans following influenza virus vaccination (Frasca *et al*., 2012b). Older humans vaccinated with an HBV vaccine had reduced anti-hepatitis B antibody responses, higher gene expression of genes involved in type II interferon signaling and complement pathways, and increased activation of innate immune cell populations compared to adult controls (Fourati *et al*.,

2016). Munanja *et al*. (2014) found that greater frequencies of pro-inflammatory monocytes, natural killer cells, and natural killer T-cells were present in circulation prior to vaccination against Yellow Fever virus 17D within an African cohort showing impaired humoral and cellular vaccine responses in comparison to a Swiss cohort showing unimpaired vaccine responses (Muyanja *et al*., 2014). The negative impact of inflammation on vaccine responses has also been observed in investigations comparing older and obese mice with younger and healthy-weight mice, respectively (Frasca *et al*., 2014; Park *et al*., 2014). Altogether, there is strong evidence that pre-vaccination inflammatory processes can negatively affect the magnitude of vaccine-induced antibody responses.

The second vaccine trial further explored the dynamics of plasma cytokines as robust, reliable biomarkers of vaccine responsiveness and revealed that plasma concentrations of IFNγ or IL-1β at the time of vaccination were not significantly correlated with vaccine-induced antibody responses. There was no difference in plasma IFNγ or IL-1β concentrations between the HR and LR within the second trial. While the LR of the second trial did not represent seronegative vaccine responders, the presence of seropositive piglets with high IFNγ and IL-1β concentrations at the time of vaccination indicates these cytokines have limitations as biomarkers of impaired vaccine responses. Indeed, cytokine profiling of vaccine responders has not always revealed correlations between pre-vaccination pro-inflammatory cytokines and vaccine-induced antibody responses (Fourati *et al*., 2016; Qiu *et al*., 2018). Other studies identifying an association between inflammatory conditions and vaccine-induced responses were conducted in adult mice and humans and may not translate to young, developing piglets.

However, it is possible that the cytokines detected within LR of the first trial and within Litter 653-3E of the second trial had different biological functions. Inflammation can be viewed on a spectrum, with basal, homeostatic levels at the lower end and disease-inducing levels at the upper end (Chovatiya and Medzhitov, 2014). Elevated pro-inflammatory cytokines can reflect both harmful (tissue damage, autoimmunities, necrosis) and beneficial (increased microbial killing, response to pathogenic stimuli) inflammatory mechanisms (Bent *et al*., 2018; Schroder *et al*., 2004). Perhaps, high IFNγ, IL-1β, and TNFα within genetically different LR of the first trial reflected innate immune-activation associated with poor vaccine-specific humoral responses, while high IFNγ and IL-1β within Litter 653-3E of the second trial implicated homeostatic

processes unrelated to vaccine responsiveness. In the future, seronegative low responders are necessary to thoroughly test the hypothesis that low responders have greater pro-inflammatory cytokines than high responders. If birth weight and plasma cytokines are associated with vaccine-induced antibody responses, it would suggest that low birth weight or elevated plasma cytokines do not immediately predispose animals to impaired vaccine responses.

4.7 Plasma Cytokines at an Early Age

Within the second trial, there were litter-specific differences in cytokine concentrations at birth. Specifically, there were consistent differences in IFNγ and IL-1β at various time points among litters. The most prominent difference was found in a litter of 11 piglets with 2-3x higher plasma IFNγ concentrations than the other 5 litters over multiple weeks. This litter of piglets was presumably not impacted by an external stimulus, such as infection or injury. Plasma cytokine concentrations may have been regulated by a maternal factor, such as genetics or epigenetics, or an immunomodulatory environmental factor, such as stress, that specifically affected Litter 653-3E (Tuchscherer *et al*., 2000). These differences were not apparent at 63-days of age, suggesting that elevated plasma cytokine concentrations reach a baseline level regardless of the concentration at birth. Such litter-dependent effects have been observed on other immune parameters in piglets, such as PBMC stimulation responses (de Groot *et al*., 2005). The presence of an entire litter of piglets with elevated plasma cytokines, yet unimpaired vaccine responses, was further evidence that plasma cytokines are not always associated with vaccine-induced antibody responses.

The second trial also revealed that plasma IFNγ and IL-1β exhibit temporal changes within the first 9 weeks of a piglet's life; specifically, plasma IFNγ concentrations declined after birth and this decline was evident by 14 days of age. Both IFNγ and IL-1β concentrations were lower at 9 weeks of age compared to concentrations at birth. These results augment previous work by Nguyen *et al.* (2007) who found piglets had decreasing serum concentrations of cytokines such as IFNγ, IL-4, and IL-6, within the first 2 weeks of life. Reasons for these decreases are not entirely understood but could reflect exogenous absorption of maternal cytokines and colostral cells via colostrum from the sow, followed by endogenous production by the piglet as it develops (Nguyen *et al*., 2007; Williams, 1993). Serum IL-1β and IL-6, but not TNFα, concentrations were elevated in human infants at 1-day of age and then significantly declined between 1- and 40-days of age (Sarandakou *et al*., 1998). The time-dependent and litter-dependent plasma cytokine

concentrations in piglets from the second trial further restrict the feasibility of using plasma cytokines as a possible biomarker of vaccine responsiveness.

4.8 Limitations and Future Directions

This research introduced new avenues for advancing the understanding of vaccine responsiveness and for recognizing the limited circumstances of biomarkers of vaccine responsiveness. However, these projects also possess limitations in their analysis that must be addressed when pursuing future investigations.

This research uses vaccine-specific antibody responses as the metric of vaccine responsiveness. Antibody-mediated immune responses to *M. hyopneumoniae* are not considered protective against infection (Djordjevic *et al*., 1997). Thus, a vaccine response does not equate to protection in this study. Possibly, LR developed strong cell-mediated responses that were not measured here yet would be considered a vaccine response in other studies. In the second vaccination trial, piglets had varying concentrations of cytokines but this had no significant effect on vaccine-specific antibody responses. It remains unknown whether elevated plasma IFNγ or IL-1β impedes vaccine-specific cell-mediated responses. Therefore, future investigations should incorporate cytometric techniques for investigating the heterogeneity of cell populations (ex. monocytes, natural killer cells, or naive T- and B-cells) and quantify cellular responses, as in other vaccine responsiveness studies (Gaucher *et al*., 2008; Li *et al*., 2017). Insight into cell populations may also provide context for the results of the kinome analysis. Since the kinome analysis in the first vaccination trial was conducted on an assorted population of PBMCs, different proportions of immune cell subpopulations may contribute to unique phosphorylation patterns.

When validating the phosphorylation biomarkers detected in the first vaccination trial, cluster analysis and statistical methods were used to evaluate if differential phosphorylated events consistently appeared in multiple cohorts. The opportunity remains to utilize more advanced techniques to classify ‘vaccine responders’ vs ‘vaccine non-responders’. Others have developed pipelines and models for predicting vaccine responsiveness using transcriptomic data, and a similar approach can be taken using phosphorylation intensity data (Furman *et al*., 2013; Lee *et al*., 2016; Nakaya *et al*., 2011; Querec *et al*., 2009; Tsang *et al*., 2014). Predictive capabilities become more robust as they are tested on independent populations, which is a vital component of this project; using the panels of phosphorylation biomarkers to predict vaccine responses in an independent

population of pigs would be an ideal test of reproducibility. As this kinome analysis focused on using vaccine responders with the highest and lowest serum IgG titers as verification, the inclusion of average/median vaccine responders may provide additional perspectives on the accuracy of these phosphorylation biomarkers (HIPC-CHI Signatures Project Team and Consortium, 2017; Thompson-Crispi *et al*., 2013). HR and LR from the first trial could be utilized to identify differential phosphorylation changes between vaccine responders following vaccination if the trial included age-matched, unvaccinated controls to account for the developmental immune changes independent of vaccination. Lastly, biomarkers must be tested using a different vaccine type, given that Li *et al*. (2014) found that different human vaccines can have unique vaccine type-specific transcriptional responses (Li *et al*., 2014).

The second vaccination trial evaluated the results of the first trial by testing plasma cytokines and birthweight as biomarkers for vaccine responsiveness in an independent trial population. The second trial provided results that provoked additional questions for understanding the immune physiology of neonate piglets. Are there physiological consequences of having greater circulating IFNγ at a young age? If so, what role in immunity and development do these cytokines play? Do these cytokines represent endogenous production from the piglet? Were cytokines transferred from the sow at or before birth? Or was it a combination of endogenous production directed by maternal factors? Future directions that investigate these questions should consider collecting a comprehensive set of samples from sow and offspring at time points immediately at and following birth. With archived cells such as PBMCs collected at a young age, there is an opportunity to measure IFNγ-stimulated genes if IFNγ concentrations are high in plasma. In addition, blood and colostrum samples collected from the sow may provide insight into the source of plasma cytokines in piglets. Finally, a longitudinal analysis of litters from the same sow may answer whether litter-specific differences in plasma cytokines represent a maternally-derived effect or a consequence of environmental factors.

5. CONCLUSIONS

Low and non-responders to vaccination create a challenge to achieving protective immunity, both in individuals and the population. The identification and subsequent management of low responders could have tremendous benefits for achieving a healthier livestock population and improving vaccination programs. This thesis identifies birth weight and elevated plasma cytokines at the time of vaccination as biomarkers that were not consistently associated with impaired vaccine responses in piglets. Plasma cytokines demonstrate highly variable concentrations between subjects and over time, suggesting their use as a stand-alone biomarker should be heeded. However, phosphorylation events had increased potential for prediction of vaccine responsiveness and may complement other systems approaches by offering a novel perspective of the host immune response to vaccination. Together, these data provide insight into molecular events that may be associated with vaccine responses within a critical livestock species and evaluate the feasibility of using specific physiological factors as biomarkers of vaccine responsiveness.

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