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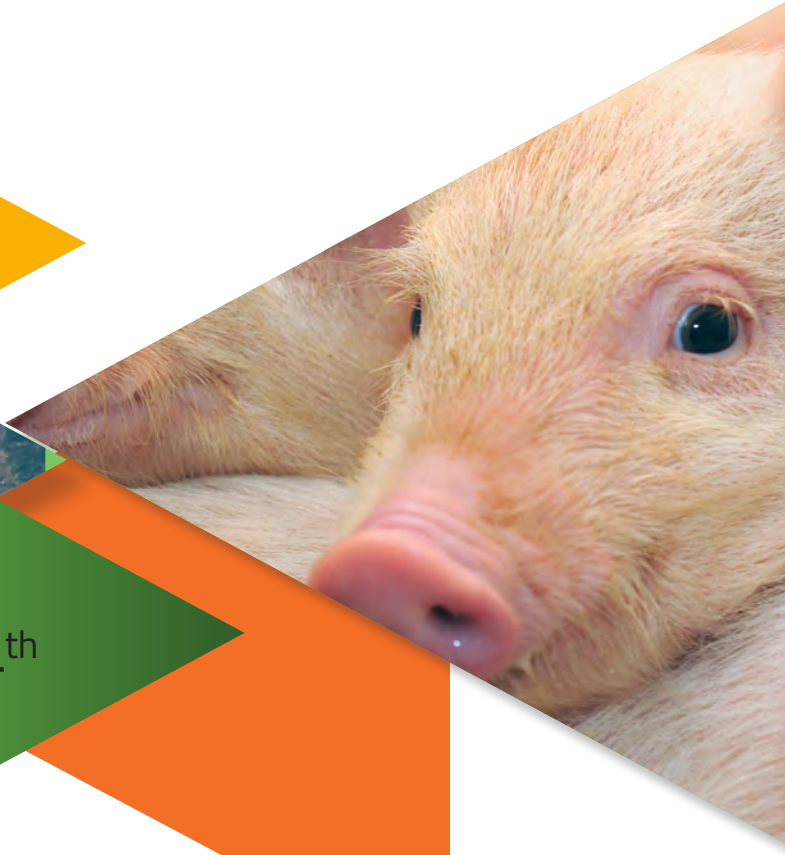

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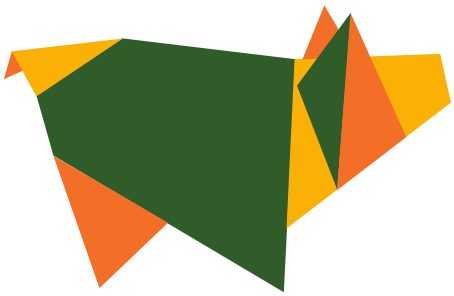

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Stimulating a protective adaptive immune response to *Lawsonia intracellularis* in pigs

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Lawsonia intracellularis-based diseases in pigs

Proliferative enteropathy (PE) is an enteric infectious disease caused by the obligate intracellular Gram-negative bacteria *Lawsonia intracellularis* (McOrist et al. 1993; Biester and Schwarte 1931). This pathogen is endemic throughout the world and it is a major cause of weight loss, mortality in pigs and significant economic losses for the swine industry (McOrist 2005). PE in pigs has two distinct clinical and pathologic forms, porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE). Porcine intestinal adenomatosis (PIA) affects young, post-weaned pigs, usually between 6 and 20 weeks of age and the major symptoms include diarrhea, anorexia and weight loss (Vannucci and Gebhart 2014). This disease is often mild, and it is subclinical in form with major characteristics including thickening of the wall of intestine due to proliferating immature crypt epithelial cells (McOrist et al. 1996). Evidence of infection is usually only observed at slaughter when pathologic lesions can be observed in the alimentary tract, especially in the terminal ileum. Despite these lesions, the pig immune system does not respond to infection with infiltration by inflammatory cells at this stage of the disease, which may indicate that the bacteria have an immunosuppressive effect on the host's immune system (Lawson and Gebhart 2000). Although clinical signs are mild, infected animals shed the bacteria in the feces and are a source of disease transmission. Despite the overt lack of clinical signs of disease, PIA impedes weight gain, thus negatively affects barn profitability, making it an important swine disease. PHE is the acute form of the disease that mainly affects young, adult pigs, 4 to 12 months of age such as finishing pigs, gilts, and boars (Kroll et al. 2005). The main symptoms of PHE are profuse hemorrhagic diarrhea leading to the sudden death of the animals. The pathological findings include thickened and rugose mucosa of the terminal ileum replete with blood in the intestinal lumen. Histological analysis indicates that the ileal crypts have undergone extensive proliferation and there may be evidence of bacteria in the apical cytoplasm of the epithelial cells, in macrophages in the *lamina propria* and submucosa, within epithelial capillaries, and within the lymphatics (Guedes et al. 2017; Rowland and Lawson 1974). An obvious inflammatory response is present in the PHE form of the disease, and this inflammatory response may be the reason for more severe symptoms and high mortality rate relative to the PIA form of the disease.

The adaptive immune response

The mammalian intestinal adaptive immune system has evolved to provide antigen-specific memory responses to intestinal pathogens, while at the same time maintaining tolerance to environmental, food or commensal antigens. Innate immune cells in the intestinal mucosa provide the necessary signals to respond to antigens which is communicated to and impacts the cells of the adaptive immune system. Adaptive immunity in the gut consists mainly of humoral immunity mediated by secreted IgA antibodies, induction of protective cell-mediated adaptive immunity mediated by CD4+ and CD8+ T cells, and induction of Treg cells that promote gut homeostasis and tolerance to food antigens (Gormley et al. 1998; Kiyono and Fukuyama 2004). The Peyer Patches and lymphoglandular complexes of the gut-associated lymphoid tissues (GALT) are the primary inductive sites in the gut, but the functions of the isolated lymphoid follicles and cryptopatches are unclear (Cesta 2006). The maturation of GALT depends on the presence of commensal bacteria and their products (Goto and Ivanov 2013). The relationship between microbiota and immune cells in GALT is bi-directional with both host and commensal bacteria cells contributing to immune homeostasis.

GALT is rich in effector T cells and it is the major site of secretion of antigen-specific IgA (Gormley et al. 1998; Kiyono and Fukuyama 2004). IgA is the main mediator of specific humoral immune protection, it is non-inflammatory and an important regulator of the commensal population (Pabst 2012). IgA antibodies are produced by plasma cells that stem from activated B lymphocytes which have undergone class-switch recombination in GALT (Pabst 2012). IgA antibodies are bound by polymeric immunoglobulin receptors (pIgRs) on the basolateral membrane of intestinal epithelial cells (IECs) and are then actively transcytosed to the apical surface and released into the lumen as secretory IgA (sIgA) (Pabst 2012; Johansen and Kaetzel 2011). Once in the lumen, sIgA can bind to pathogens or toxins and prevent their interaction with intestinal epithelial cells (Mantis and Forbes 2010). Alternatively, IgA can bind pathogens in the *lamina propria* then bind to pIgR on the basolateral surface of the IECs resulting in transport and the

release of the pathogen into the lumen. Intestinal IgA antibodies comprise around approximately 70% of all antibodies produced in mammals, making it the most abundant antibody class (Macpherson et al. 2008). Oral vaccination that target activation of antigen-specific long-lived IgA secreting plasma cells may be a feasible strategy to induce long-lasting protection against enteric pathogens while at the same time maintaining necessary immune balance in the intestinal mucosa.

The cell-mediated immunity in the intestine

Adaptive cell-mediated immunity is mediated by T cells present in GALT including in close proximity to the intestinal epithelium. T-cells are differentiated by the expressed surface receptors $CD4^+$ and $CD8^+$ cells. CD4+ T cells can be distinguished into distinct subsets by their distinct effector function and cytokine secretion in response to the antigen and co-stimulatory molecules presented on APCs or cytokines secreted in the intestinal epithelial environment. They play multiple roles in immune protection against intestinal pathogens and they play an important role in maintaining gut homeostasis in an environment rich in food and commensal antigens.

CD4 helper T cells (T_H) recognize antigens presented in major histocompatibility complexes class II (MHCII) after processing by APCs (Konjar et al. 2017). CD4+ T_H cells further differentiate into effector cells with specific functions in Th1, Th2, and Th17 immunity with cellular plasticity in intestinal tissue (Brucklacher-Waldert et al. 2014). Th1-type T-cells produce IFNγ cytokine, Th2-type T cells produce IL-4, IL-5, and IL-13 (Mosmann and Coffman 1989) and Th-17-type T cell cells produce IL-17A and IL-17F and IL-22 which induce neutrophil production and promote maintenance of epithelial barrier integrity (Weaver and Murphy 2007; Veldhoen et al. 2006). CD4+ regulatory Foxp3 expressing T cells (Tregs) are a subset of T cells that play a major factor in immune regulation and tolerance and a high proportion of these subsets reside in intestinal *lamina propria* (Agace and McCoy 2017). CD8 expressing cytotoxic T cells recognize antigens presented in MHCI on host cells, deriving from viral or intracellular bacterial pathogens that infected host cells. When $CD8^+$ T cells engage with MHCI presenting intracellular antigen, they are activated and they differentiate into effector cells which results in the production of IFNγ or they have cytotoxic activity directed towards infected cells. IFNγ is a strong activator of innate immune cells such as macrophages and an inducer of class-switch recombination of B cells to immunoglobulin IgG isotypes that opsonize microbes for enhanced uptake by phagocytes (Weaver et al. 2013).

The gut epithelium is home to an abundant population of $CD8^+$ T cells located in between epithelial cells or dispersed in underlying tissue (Kato et al. 2014). The $CD8^+$ T-cells are distinguished by expression of αβ or γδ TCRs, which determines their specific localization and role in the intestinal immune system (Gerner, Kaser, and Saalmuller 2009). The γδ T-cells are intraepithelial lymphocytes (IELs) while αβ T-cells are located in the *lamina propria*. Subsets of γδ T-cells are present in low numbers in the circulation but they are abundant in epithelial tissues and constitute between 10–100% of T-cells in the epidermis of the skin and the epithelial tissues in gastrointestinal tract (Nielsen, Witherden, and Havran 2017). They play an important role in maintaining epithelial barrier, regeneration of epithelium, homeostasis and providing a balance between commensal tolerance and pathogen clearance (Nielsen, Witherden, and Havran 2017).

L. intracellularis vaccine development

Currently, there are two registered vaccines against *L. intracellularis* available for prevention of PE in pig farms. The first registered vaccine against *L. intracellularis* (Enterisol, Boehringer Ingelheim) was an avirulent live vaccine developed by multiple consecutive passaging of bacterial isolate B3903 in McCoy cells (Kroll, Roof, and McOrist 2004). The attenuation of virulence is achieved between 20 and 40 passages *in vitro* which is represented by attenuation and down-regulation of important genes responsible for virulence and cell metabolism in bacteria (Vannucci et al. 2013). The vaccine is administered orally to animals and each dose is comprised of approximately 10^5 bacteria (Kroll, Roof, and McOrist 2004). After challenge with virulent bacteria, vaccinated pigs showed partial protection of pigs with a significant reduction in intestinal lesions, the absence of clinical symptoms, and higher average daily gains compared to the unvaccinated control group (Kroll, Roof, and McOrist 2004). Although fecal shedding was reduced in vaccinated group (47% and 40% reduction on day 35 and 42, respectively), vaccinated animals still shed bacteria in great numbers and therefore may be a source of transmission of *L. intracellularis* in naïve pigs (Kroll, Roof, and McOrist 2004). A study from Nogueira et al 2013 compared prescribed 1x oral dose ($10^{4.9}$ $TCID_{50}$) to 10x oral ($10^{5.9}$ $TCID_{50}$) and 1x intramuscular (i.m.) dose and found that pigs that received 10x higher oral dose showed increased serum and mucosal concentrations of IgM and IgG antibodies, increased TNF-α and TGF-β1 in the intestinal mucosa that trended towards higher levels of serum IFNγ and IL-6 on day 17 (Nogueira et al. 2013). Levels of serum IgG titers were increased between day 9 and day 17 for all vaccinated groups and those levels were similar for oral and 1x i.m. vaccinated animals. In contrast, serum and mucosal levels of IgA titers were below detection limits in all vaccinated groups (Nogueira et al. 2013).

Vaccinated and control animals were challenged orally with pathogenic bacteria (25 ml of a suspension containing around 10^9 bacteria) and after challenge, the control and all groups of vaccinated animals had an increase of serum cytokines levels IFNγ, IL-6, IL-10 and TNF-α from day 0 to day 21 post-challenge and significant increases of serum *L. intracellularis* specific IgG and IgA (Nogueira et al. 2013). Although all vaccinated pigs in this trial had significantly reduced fecal shedding of *L. intracellularis* after virulent challenge compared to non-vaccinated control group, animals vaccinated with 10x dose shed significantly fewer bacteria in their feces and had less percentage of the affected area in the ileum than other 2 vaccinated groups (Nogueira et al. 2013). The observed immune protection of oral 10x dose might be attributed to stronger priming of both cell-mediated and humoral immunity in intestinal mucosa than a 1x oral dose or 1x i.m. dose. Intramuscular administration of 1x dose of live avirulent vaccine was also able to provide a protection from virulent challenge by reducing fecal shedding and intestinal pathology suggesting that serum antibodies might play role in preventing entry of bacteria into the cells or by antibody-dependent cell-mediated cytotoxicity (ADCC) (Nogueira et al. 2013).

A second commercially available vaccine against *L. intracellularis* contains inactivated whole cell bacteria in an oil-in-water emulsion and adjuvant based on mineral oil and alpha-tocopherol (Vitamin E) (Roerink et al. 2018). (Porcilis Ileitis, Merck Animal Health, Madison, NJ, USA). Protection properties have been assessed under field conditions when the vaccine was administered intramuscularly to 22-25 days old pigs, just before weaning (Roerink et al. 2018). The vaccine induced significant serum antibody titres and upon subsequent challenge, these animals had 15-fold reduced bacterial fecal shedding and significantly reduced bacterial burden and microscopic lesions in ileum compared to control animals (Roerink et al. 2018). Vaccinated animals had preserved gut integrity and preserved goblet cells suggesting that the vaccinated animals had reduced incidence and severity of clinical symptoms of PE, and reduced colonization and duration of fecal bacterial shedding (Roerink et al. 2018). In a field study trial, 75 piglets were allotted to three groups vaccinated at 4 or 5 weeks of age with either Porcilis® Lawsonia in adjuvant or mixed with Porcilis® PCV M Hyo vaccine (group 1), with Enterisol (group 2), or unvaccinated (group 3) (Jacobs et al. 2019). They were challenged 3,4 or 17 weeks post-immunization. Pigs vaccinated with Porcilis® Lawsonia demonstrated by lower clinical scores, improved weight gain, reduction of Lawsonia intracellularis shedding and reduction of macroscopic as well as microscopic ileum lesion scores when compared to the controls relative to the live vaccine (Jacobs et al. 2019). Studies need to be undertaken to determine whether the immune correlates of protection include induction of humoral immunity response.

The above commercial vaccines represent a live, attenuated and a killed vaccine, respectively. However, subunit vaccines are an attractive option for vaccine development because they are very safe (they cannot revert to virulence), the type of immune response induced can be modified by co-formulation with adjuvants, and because only 1 or at most a few antigens are usually included, one can look at serum antibodies to discern whether the animals have been vaccinated (i.e. antibodies against only select antigens are present) or whether they have been infected (antibodies against numerous *L. intracellularis* antigens will be present).

Subunit B cell antigen selection and analysis

To develop a subunit vaccine, we need to identify immunogenic proteins to include. While the *L. intracellularis* antigenic properties have still not been fully elucidated, several proteomic studies have been performed. On an SDS-PAGE gel, *L. intracellularis* has 25 to 27 visible protein bands, from which 22 are conserved in six different isolates (McOrist et al. 1995). The protein masses of the major bands are 53 kDa, 42 kDa, 37 kda and 30 kDa (McOrist et al. 1995). The serum polyclonal 1999PAb antibody targets five outer membrane proteins (OMPs) with masses of 77, 69, 54, 42 and 36 kDa while the monoclonal 2001MAb targets OMP of 77 kDa, and monoclonal IG4 targets a protein of 18 kDa (Guedes and Gebhart 2003). Other select proteins that constitute the outer membrane have been detected and characterized such as LsaA (27 kDa), which is involved in cell attachment and invasion (Guedes and Gebhart 2003) and LatA autotransporter protein (72 kDa) (Watson et al. 2011). LsaA is predicted to have role in cell attachment and invasion and is expressed during *in-vitro* infection of IEC-18 cells and also in infected enterocytes from ileum tissue (Guedes and Gebhart 2003). This antigen is recognized by the monoclonal antibody VPM53 using IHC detection in both IEC-18 cells and tissue samples (Guedes and Gebhart 2003). Based on its similarity to proteins of other Gram-negative bacteria, LsaA is believed to play a role in pathogenicity. Further studies are needed to characterize its function and cellular networks. The outer membrane protein LatA, (LI0649) belonging to the family of autotransporter proteins was detected using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Watson et al. 2011). The predicted LatA molecular mass was 91.9 kDa but a 72 kDa form of LatA was observed on SDS-PAGE gels which implies that protein cleavage occurred (Watson et al. 2011). This gene was cloned and the purified protein was recognized by the immune serum from pigs infected with *L. intracellularis* indicating that is antigenic (Watson et al. 2011). This protein is highly specific to *L. intracellularis* and it can be exploited for development of diagnostic tests for PE.

The same researchers applied shotgun proteomic analysis on two *L. intracellularis* isolates and identified 19 proteins, including LI0841 and LI0902 that were predicted as outer membrane-associated proteins (Watson et al. 2014). The rLI0841 protein was identified as a putative invasin with sequence similarity to proteins that promote invasion. The rLI0902 protein was predicted to be involved in protein-protein interactions. Both recombinant proteins were recognized by serum antibodies from infected pigs (Watson et al. 2014). Recognition of these two recombinant proteins by pig immune sera indicate that they are expressed and available to host immune recognition thus making them potentially good diagnostic tools or antigens for a subunit vaccine. *L. intracellularis*, like all Gram-negative bacteria, have lipopolysaccharide (LPS) in the outer membrane which has been used as an antigen target for ELISA antibody detection in pig serum (27). LPS based ELISA are not ideal diagnostic tools due to the high cost of LPS antigen extraction and differences in background LPS reactivity among animals.

Analysis of 20 genes highly expressed by *L. intracellularis* when it resides in the enterocyte cytoplasm led to identification of 7 unknown genes whose corresponding proteins are part of the outer membrane surface (Won and Lee 2018). These hypothetical OMPs could play roles in attachment and invasion of host cells and thus might be recognized by the immune system of the host. Further investigation into their immunogenicity is needed before it can be determined if these proteins are critically required for *L. intracellularis* infection of enterocytes.

An antigenic and functional study was performed on the flagellin-like protein (LI0570) that was recently identified using *in silico* computational approaches (Won and Lee 2018). The recombinant LI0570 protein was detected in Western blots using mouse anti- *L. intracellularis* hyperimmune sera indicating that this protein is immunogenic (Won and Lee 2018). Further, HEK-Blue cells incubated with the rLI0570 protein led to stimulation of TLR-5 signaling and IL-8 production which implies its function in innate immune cell, *in-vivo* (Won and Lee 2018). Due to the potential immune-stimulatory effect of rLI0570, this recombinant protein has a dual role as antigen and adjuvant and may be an excellent candidate to develop recombinant subunit vaccine. Currently proteins which comprise the Type III secretion system (T3SS), autotransporter protein (LatA), LI0841, and LI0902 were characterized and shown to have immunogenic properties (Watson et al. 2014; Watson et al. 2011; Won and Lee 2018). Other uncharacterized bacterial proteins that are localized to the surface localization and that play a role in infection are potentially important immunogens

As an alternative approach to identify *L. intracellularis* antigens, we used functional analysis coupled with two-dimensional gel electrophoresis (2DE), Western blot analysis and mass spectrometry to help select the antigens that are targeted by the humoral immune system. Bacterial components and mechanism that facilitate attachment of bacteria to enterocytes has not yet been fully determined but they are possible facilitators for invasion of intestinal epithelial cells and are therefore potential immunogens (Vannucci and Gebhart 2014). We sought to identify these proteins. Briefly, we lysed the avirulent *L. intracellularis* from the Enterisol vaccine and labeled the proteins present with Cy5- dye (Obradovic et al. 2019). These labeled proteins were then incubated with plated but undifferentiated porcine intestinal epithelial cells (IPEC-1). The cells were rinsed then ruptured and the IPEC-1 proteins and adherent Cy5-labeled L. intracellularis proteins were subjected to 2DE. The proteins were then transferred to a nitrocellulose membrane and probed with serum from rabbits that were vaccinated with Enterisol plus Incomplete Freund's Adjuvant adjuvant multiple times. The *L. intracellularis* proteins that were bound by the rabbit antibodies are antigens of interest and they were sent for mass spectrometry for analysis. We identified 11 putative antigens (Obradovic et al. 2019) and cloned 6 of them: Flagellin (fliC, LI0710), Putative outer protein N (LI1153), ABC dipeptide transport system (LI0169) and autotransporter (LI0649), LatA. Among the 11 proteins detected in this study, proteins Chaperonin GroeL (LI0625) and 5'-nucleotidase/2', 3' cyclic phosphodiesterase (LI1171) were also reported previously using a shot-gun proteomic approach (Watson et al. 2014). The genes for these select proteins were cloned into plasmids, expressed in E. coli and purified. Western blot analysis was performed using both rabbit serum from Enterisol vaccinated rabbits as well as serum from pig barns that had been infected with pathogenic *L. intracellularis* to confirm that these recombinant proteins were immunogenic. Flow cytometric analysis on select antigens confirmed that neutralizing antibodies reduced the ability of L. intracellularis to invade IPEC-1 cells (Obradovic et al. 2019).

To establish whether these recombinant proteins were immunogenic, we performed several vaccine trials in pig using multiple vaccine adjuvants. We immunized pigs with a trivalent vaccine (FOG vaccine consisting of rFLiC, rOppA protein (a ABC Type dipeptide transport system) and rGroEL (a stress response protein)) and a divalent vaccine (CM vaccine consisting of rClpP (an ATP-dependent Clp protease proteolytic subunit) and rMetK (a S-adenosyl methionine synthase)) all formulated with the oil-in-water adjuvant Emulsigen® (Fourie et al. 2021). Relative to the control pigs, pigs immunized with the FOG vaccine produced robust and significantly higher serum IgG antibodies against rFliC and rGroEL, and significantly higher anti-FliC and anti-GroEL IgA antibodies in jejunal (GroEL only) and ileal intestinal mucosa (Fourie et al. 2021). Pigs immunized with CM vaccine produced significantly higher serum antibodies against rClpP and rMetK and significantly higher anti-rClpP IgA antibodies in the ileum relative to the control pigs. Quantitative polymerase chain reaction (qPCR) analysis showed that 18 days after challenge with infectious *L. intracellularis*, challenged/control pigs and pigs that received the CM vaccine, but not the pigs vaccinated

with the FOG vaccine, shed significantly more bacteria in feces than the unchallenged controls pigs. These data suggest that the FOG vaccinated pigs showed some, albeit limited, protection. These data suggest that antigens FliC, GroEL and ClpP should be investigated further using different adjuvants to promote robust humoral and cell-mediated immunity.

Subunit T cell antigen selection and analysis

Whether pigs are infected or vaccinated prior to re-exposure can impact the cell-mediated immune responses observed. Following primary infection of 5-6 week old piglets, some piglets were reinfected and some were vaccinated. The IFNγ responses to the initial infection were moderate with around 50% of the pigs responding with Ag-specific IFNγ above background level (100 pg/mL), and with several pigs showing a sustained high Ag-specific IFNγ response even at day 48 pi (Cordes et al. 2012). After rechallenge (RE), the RE pigs showed a memory recall cell-mediated immune response which was significantly stronger compared to the primary response in age-matched challenge control (CC group) pigs as assessed by whole blood IFNγ assay and by flow cytometry. The major IFNγ producing cells were identified as CD8+ and CD4+CD8+ double positive lymphocytes. However, when a similar trial was undertaken comparing the CMI response between re-infected group (RE group) and Enterisol-vaccinated group (VACC group), a difference in immune response and protection was observed (Riber et al. 2015). The VACC group showed antigen-specific proliferation at background levels after challenge, whereas the RE-pigs at 2nd challenge showed significantly increased responses, especially in the $CD4^{+}CD8^{+}$ T cell population, peaking at day 18 or 26 post challenge with a decline at day 33 post challenge, compared to VAC-group, which in turn showed responses comparable with the responses in the non-vaccinated CC-group. Only the RE-challenged pigs were fully protected against clinical disease suggesting that mediators of protective immunity against *L. intracellularis* were likely CD8+ effector cells and CD4+CD8+ double positive memory T cells (Riber et al. 2015).

Identification of T cell epitopes from the entire bacterial or viral proteome is a very challenging field. We have devised a functional assay wherein we use extensive gel electrophoresis of the entire L. intracellularis proteome to test which proteins are responsible for induction of the T cell-mediated IFNg secretion from PBMCs from LI-challenged and/or vaccinated pigs (manuscript in preparation). For our identification of T cell epitopes, we have ensured sufficient number of memory T cells in the PBMC population and an effective protein purification method that reverses denatured proteins back to biological active form in a low cytotoxic buffer. Further, we have performed extensive immunoinformatic analysis to identify T cell epitopes that will then be employing reverse vaccination. From a pool of ~ 1350 recorded *L. intracellularis* proteins, we focused on known expressed proteins in a pathogenic strain as well as proteins with unknown functions. Next, entries were selected based on antigenicity at ≥ 0.5 thresholds out of 1 which narrowed the number of proteins down to approximately 400. The solubility, high content of transmembrane helices, toxicities and physio-chemical properties of selected proteins further narrow down the selection. The analyses help us to exclude more proteins from the list due to having largely nonpolar contents and low solubility, in other cases, they compose 20% in minimum for transmembrane segments or toxicity observation danger enough to interfere with the piglets' metabolism. The remaining candidates were separately used as an input for Immune Epitope Database Analysis Resource (IEDB) and NetMHCpan 4.1 servers to be processed and for obtaining epitopes bound to piglets' swine leukocyte antigen (SLA) receptors. The higher ranked epitopes were extracted for evaluating their antigenicity, toxicities and to analyze their protease sensitivities. Among all epitopes, the selected candidates demonstrated predicted resistance to degradation from a diverse range of proteinases e.g., trypsin, chymotrypsin, and many others. All chosen epitopes were then used as inputs for homology modelling providing three-D conformations and docking tools producing SLA - epitopes binding poses to further be analyzed by means of Molecular Dynamics (MD) simulations. Twenty-eight epitopes were finalized for conducting MD simulations of SLA receptors with their docked epitopes. We performed 300 nanoseconds of MD production run and included the following criteria: stability, solvent-surface accessibility, radius of gyration and binding free energy between each epitope and its SLA. The epitopes with large negative free energy ≥ - 20 kJ/mol and the best conformational stability were the end-point selections for generating the multiepitope vaccine construct (manuscript in preparation). A synthetic gene coding for the selected epitopes are being developed and the corresponding proteins will be tested for antigenicity in a subunit vaccine in pigs.

Conclusions

Overall, we predict that a subunit vaccine would be a very attractive option for producers because comparative analysis of antibody response can be used to discern whether the animal has been vaccinated or infected. Once antigens combined with appropriate adjuvants are selected to stimulate neutralizing antibodies and/or robust CD8+ and CD4+CD8+ T cell activation, we predict reliable protection against disease. Taken together, these data indicate that ideal vaccine should be able to elicit balanced immune response where both cell-mediated and local humoral immunity work in concert to eliminate *L. intracellularis*-associated disease.

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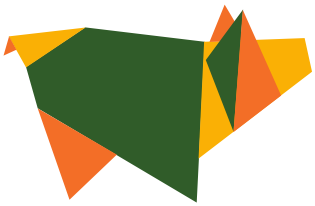

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