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KL07 - MEASURING AND INTERPRETING THE IMMUNE RESPONSE

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The immune system is a complex and interactive network of different organs, tissues and cells aimed at neutralizing and eliminating all "non self" agents, cells, and conditions. Immunity is continuously engaged in limiting the effects of microorganisms, chemical substances, "cells proliferation out of control" (pre-cancerous or cancerous), etc. by activating an immune response that is able to recognize, destroy, eliminate them and to create protection and memory for future exposure. The protection against these different pathological conditions depends on a complex system of overlapping and interlinked defence mechanisms that collectively destroy and control almost all invaders.

The interplay of the different components of the immune system (cells, chemical substances, proteins, receptors, etc.) is very complex and is based on interaction and signals that connect inflammatory, non specific and specific immune cells which determine the activation, proliferation and differentiation of these cells which are responsible for mounting an efficient defence mechanism.

MEASURING THE IMMUNE RESPONSE

According to the Oxford Dictionary the verb "to measure" has two main definitions: 1) Ascertain the size, amount, or degree of (something) by using an instrument or device marked in standard units; 2) Assess the importance, effect, or value of (something). So, "to measure" expresses a concept of graduation and enumeration of the quality (importance, effect, value) and quantity of an item, of a phenomenon by an instrument (tools). The question is: "Are we able to efficiently measure immunity as a whole? Based on the complexity of the immune system we cannot directly measure the overall efficiency.

Therefore, the complexity of the mechanisms playing a role in this system makes it impossible to measure the efficiency of immunity as a whole. Consequently, there is no tests available that can estimate the overall function of the immune system. We know, for instance, that ageing compromises the efficiency of the immune system, as do several diseases that directly affect it. Toxic substances can impair the integrity and efficacy of the immune system. Some mycotoxins can play a detrimental role on the immune system based on experimental studies. Sometimes the reduced efficiency of the immune system is supposed to be the causative condition sustaining misdiagnosed diseases.

As mentioned above, each contact between the immune system and a "non self agent" generates a **signal**, a trace of the interaction (i.e. serological response) that can persist for a certain period of time, sometimes for the entire life. The immune response is commonly used for **diagnostic purposes (indirect diagnosis)**. Serology is not aimed at demonstrating the presence of the etiological agent, but at showing that a specific agent was in contact with the defences of the host. That is the most frequently used "measurement" of the immune response.

In human and veterinary medicine in general and in porcine health management in particular, the immune system is required to generate **protection following vaccination.** In fact, vaccination is an "artificial infection" by attenuated or inactivated infectious agents or otherwise assembled parts of immune agents. Vaccines are given primarily to protect against disease, though protection against infection and even infectiousness may also be an important and required effects. In porcine health the switch from **individual immunity** (single pig) to **herd immunity** (population immunity) is necessary.

The most efficient way to measure the immune response to vaccination is the evaluation of clinical and immunological protection. Protection is evident, and measured, in terms of the reduction in the risk of the clinical endpoint of interest. Thus, the (protective) **vaccine efficacy (VE)** is defined as the percent reduction in risk of (for example) disease amongst vaccinated individuals compared to equally exposed unvaccinated individuals, or VE = (Rnv - Rv)/(Rnv), where Rnv and Rv represent incidence risk or rate of disease in non-vaccines and in vaccines respectively.

In the context of vaccines, **protection** implies an immunological mechanism (both humoral and cellular) to prevent or to reduce severity of infection or disease. Many aspects of these mechanisms are not yet understood. Protection is complex, not only in its intrinsic mechanism, but also in its manifestation. It may be **complete**, such that a protected individual suffers no ill consequences whatsoever if exposed to infection. It may be **incomplete**, implying that the severity of the consequences of the disease is reduced. (The term **partial protection** is sometimes used for this state, but is used in a different way for vaccines). Incomplete protection can be interpreted in two ways: a) the majority of the vaccinated population is protected but a few individuals are not protected at all (fully susceptible); b) the whole vaccinated population has an incomplete protection.

Sometimes, protection may be **situation-dependent**, related to the **infectious agent** (e.g. genetic plasticity, pathogenicity, exposure dose), to the **environment** or to **animal** status (e.g. age) and **concurrent infection**. These nuances complicate the evaluation of vaccines either directly, with disease reduction outcomes, or through **substitute immunological endpoints**, markers of the immune reaction correlated with protection. In figure 1, the induction of protective immunity by a vaccine and the **immune markers (IM)** also known as **correlates of protection** (parameters useful for **measuring the immune response**) are shown.

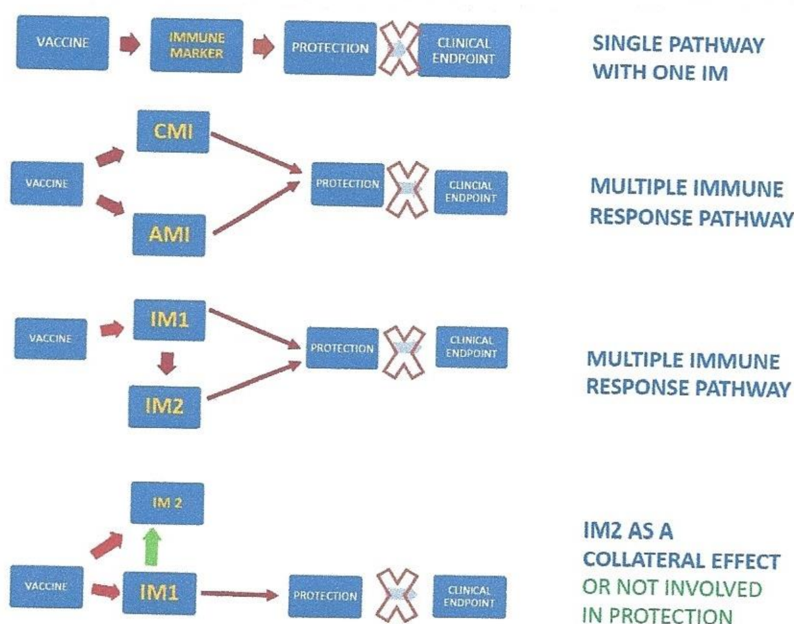


Figure 1. Different pathways of correlation between Immune Markers (IM or correlates of protection) and clinical protection.

THE IMMUNE SYSTEM AND IMMUNITY: GENERAL ASPECTS

The immune response to infectious agents is a very complex network of interactions among physical and humoral signals that interconnect inflammatory cells with those of innate and specific immunity, determining their activation, proliferation and differentiation with the ultimate goal of implementing an efficacious defence against pathogens.

Typically, immunity is divided into **innate or natural immunity** (non-adaptive immunity) and **acquired or specific immunity**. The latter is divided into the cell-mediated immunity and the humoral immunity. These two types of immune response are closely related through the close cooperation between their cellular components. Immunity is also coordinated with the neuroendocrine system.

The innate immunity: structural and functional features

The components of innate immunity are always ready to act as a "first, prompt line of defence against the pathogens" controlling and containing them at their entry site. Innate immunity is focused on the initial elimination of the pathogen and also on the differentiation and activation of the dendritic cell for efficient antigen presentation to specific immune cells such as lymphocytes.

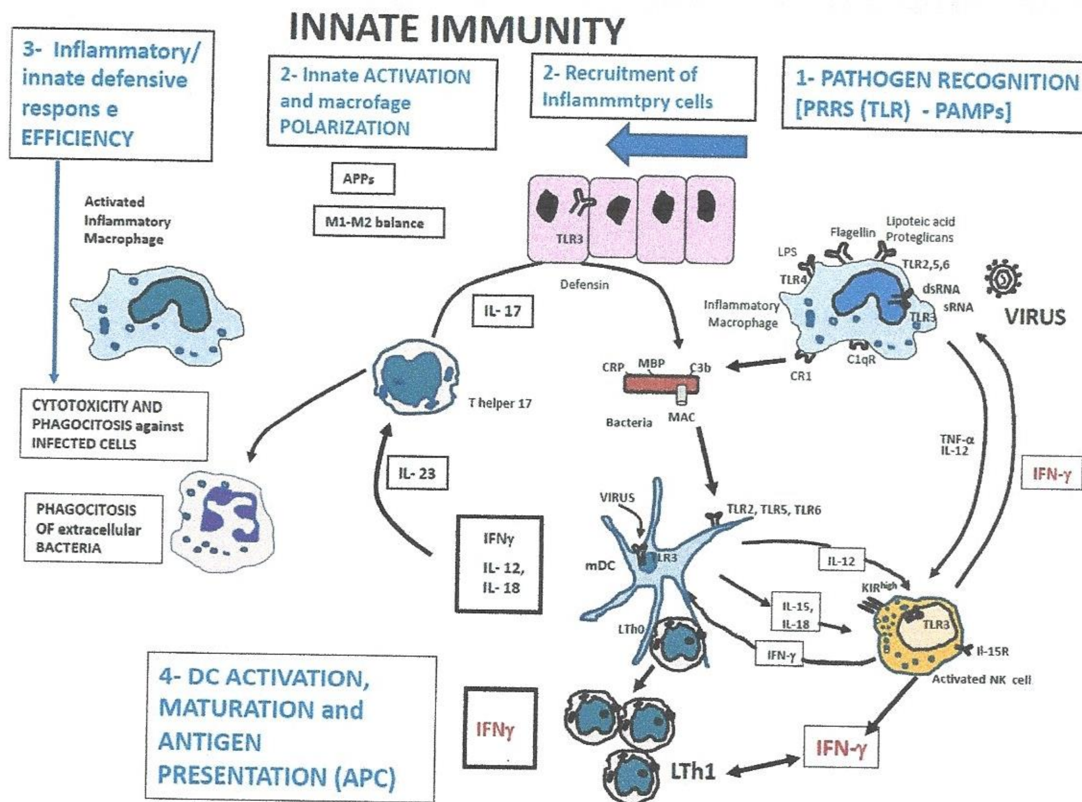


Figure 2. Cellular and molecular mechanism involved in the innate immune response.

An efficient innate response is essential for an efficient adaptive immune response. The natural recognition of the pathogen, mediated by natural receptors, with efficient production of inflammatory cytokines (IL-1, TNFalfa, IFN alfa/beta, chemokines) plays a major role in the connection between innate and acquired immunity; this allows the recruitment and activation of inflammatory and innate cells at the sites of infection and of lymphocytes and DC in lymphoid tissues and tributary lymph nodes. Moreover, cytokines from the innate cells induce the maturation of DCs for antigen presentation and for driving the differentiation of T helper Lymphocytes and establishing specific immunity.

Measurement of the innate immune efficiency upon viral infection.

The major markers for the measurement of the efficacy of the innate immune response are:

1) the expression of **TLR** by RT-qPCR from PBMC or in biological fluids (e.g. BAL fluid) or by IHC in the tissues;

2) the measurement of the main **proinflammatory** (type 1 INF, IL-1, TNF-alfa, IL-8, IL-6) and **proimmune cytokines** (IFN-gamma) by gene expression (RT- qPCR) and/or as protein secretion by ELISA or by IHC in tissues;

3) the measurement of the **blood level of innate immune cells** (i.e. NK and gamma/delta T-lymphocytes by Flow cytometry);

4) the evaluation of the secretion of the above-mentioned cytokines associated with **cell phenotype** (e.g. macrophages, Gamma/delta lymphocytes, NK) by means of Intracellular Staining (IS) and Flow cytometry.

The best method could be the qualitative and quantitative evaluation of cytokines by RT-qPCR or ELISA comparing different conditions (e.g. vaccinated vs. non-vaccinated; infected vs. non-infected etc.).

In pigs, there are two major Acute Phase Proteins (APPs), pig-MAP (Major Acute Protein) and Aptoglobin (HP), that show a strong increase during acute inflammation, stress and acute bacterial or viral infections. Other cytokines have been studied for their anti-inflammatory role and field application such as SAA and adiponectin.

2.2 Acquired (specific) immunity: structural and functional features

The acquired or specific immunity is more efficient both at systemic and mucosal level than innate immunity, and is able to definitively eliminate the pathogen from the tissues (clearance).

B and T immune cells have receptors for specific antigen determinants (epitopes) of the pathogen. The great capacity of B cells for antigen recognition is derived from genetic recombination processes that generate an enormous repertoire of immunoglobulins; the variability of antigenic recognition of receptors (TCR) of T-lymphocytes originates from similar mechanisms.

Specific immunity is triggered when **naïve B and T lymphocytes encounter their specific antigen in the secondary lymphoid tissues where the antigen arrives as it is or is presented by mature dendritic cells.**

The specific antigen recognition provides a survival stimulus for these cells, determining their activation and proliferation as specific B or T lymphocyte clones (**clonal proliferation**). The result is the onset of the effector immune response (**activation of the primary response**), with production of antibodies, cytotoxicity and phagocytosis of infected cells.

In parallel, a circulating pool of long-living immune cells (**T and B memory cells**) is established, which have memorized the first encounter (immunology memory) and that are prompt and more efficient to act upon a subsequent exposure to the same antigen.

The specific immunity against pathogens is regulated by **T helper lymphocytes (Th).** Naïve **helper T cells** (CD4+) recognize antigen-derived peptides of the pathogen that have been processed and associated with class II (MHC II) SLA molecules on the cell surface of **Antigen Presenting Cells** (APC).

Once the pathogen is captured, the APC, derived from an immature DC, migrates into the lymph node and matures, thus increasing the expression of MCH II and costimulation adhesion molecules (CD80/86) and the production of cytokines and chemokines that act on T helper cells.

T helper cells modulate the response through cytokine secretion so different subsets have been defined by the different cytokine pattern of secretion.

Th1 lymphocytes carry out their defensive role by reaching the sites of infection and secreting cytokines (IL-2, IFN-gamma), which mainly **regulate cell-mediated response** by activating specific **cytotoxic T cells** and innate immune cells, **NK cells and macrophages**, as effectors of cytotoxicity and phagocytosis. Thus, Th1 response plays a pivotal protective role in the defence against **intracellular microorganisms (viruses, intracellular bacteria and protozoa).**

Th2 lymphocytes act within the lymphoid tissue and promote the immune response by stimulating the proliferation and differentiation (isotype switching) **of B lymphocytes** involved in the **production of IgE and IgA (defensive immune response at mucosal level)**, via the main cytokines (IL-4, IL-5, IL-13, IL-10).

Th17 lymphocytes are numerous at mucosal level and have a key role in the **innate defensive response against bacteria and fungi, by recruiting an early, acute neutrophilic inflammation.**

The effectors of acquired immunity are **cytotoxic T lymphocytes (CTL)**, in cooperation with NK cells and macrophages (**Cell Mediated Immunity**), and **antibodies** [Immunoglobulins (Ig)] produced by the activated B lymphocytes, that proliferate and differentiate in Antibody Secreting Cells (Plasma Cell) (Antibody Mediated Immunity). (***Figure 3***)

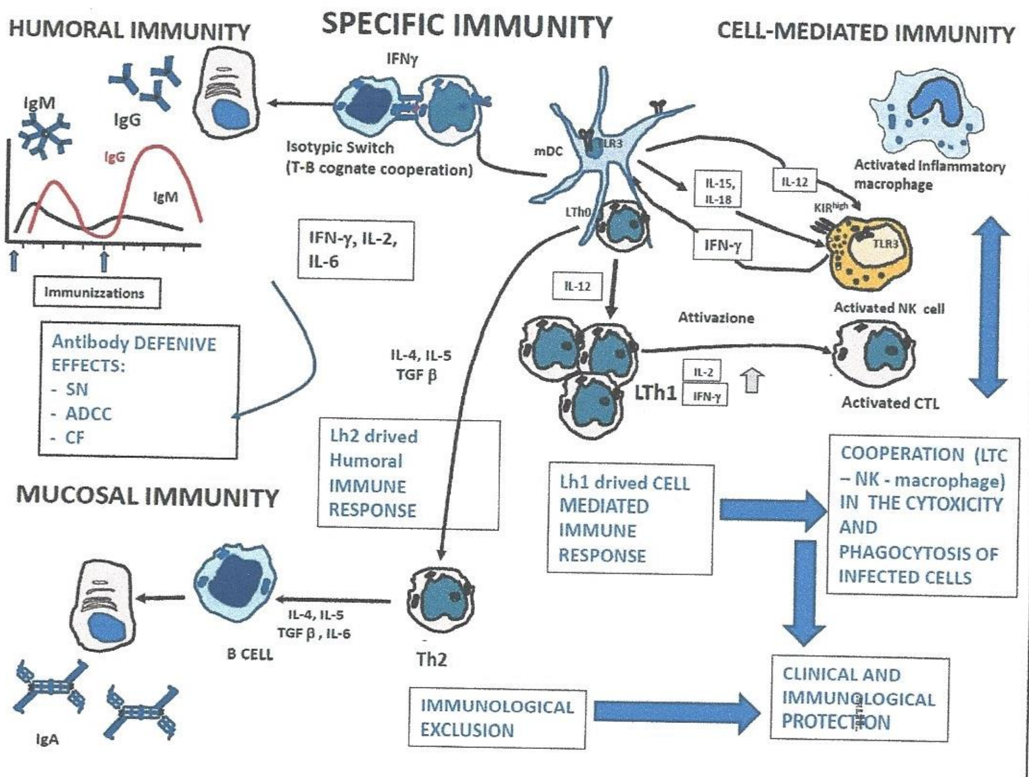


Figure 3. Cellular and molecular mechanism involved in the specific immune response to viruses at systemic and mucosal levels.

2.3 Control, "silencing" and immunological memory

Regulatory T (Treg) cells, which secrete IL-10 and/or TGF-Beta, play a fundamental role in limiting the clonal expansion and activation of effector T cells, which would otherwise lead to tissue damage. Regulatory T lymphocytes (Treg) arecharacterized by the **co-expression of CD4 and CD25 and transcription factor FoxP3** [the forkhead family factor (box P3)]. The protein encoded by the FoxP3 gene promotes the transcription of genes encoding for immuno-inhibitory cytokines (eg. IL-10, TGF-β).

A phase of "**silencing**" follows the clearance of the pathogen and it is characterized by the loss of effector T lymphocytes that undergo **anergy and apoptosis.**

The passage from an acute effector phase to a state of immunological memory occurs. In fact, after the resolution of infection, the **immunological memory** provides the ability to respond more efficiently (secondary response) to a subsequent infection with a homologous antigen mediated by **B** (memory B cells and plasma cells) and **T memory lymphocytes**.

It is believed that in pigs, the **double positive T helper population (CD4 + CD8 + low)**, characterized by a reduced requirement of costimulation, high production of IFN-gamma and prompt ability to respond to antigenic recall, can play an important role in the secondary response to viral antigens, accelerating viral clearance. Memory CTLs also show a more efficient capacity of activating and performing effector response compared with the primary response.

Together with neutralizing antibodies and amplification of the CD8+ response, the trend of cellular memory correlates with the degree of protection and is the immunological basis for vaccinology.

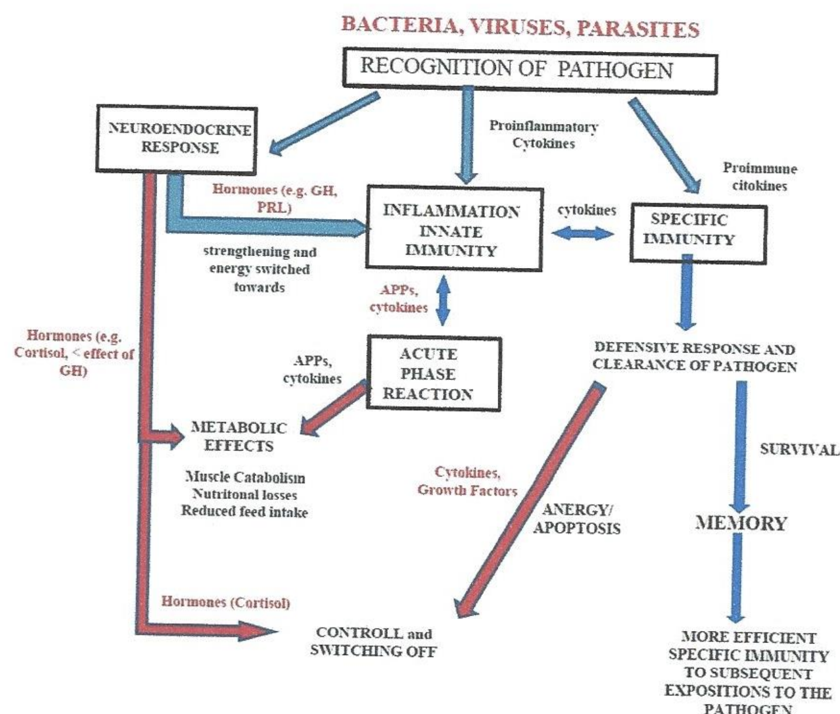


Figure 4. Summary of the drivers of the immune response (Innate immunity, inflammation, specific immunity and neuroendocrine response).

MEASURING THE IMMUNE RESPONSE TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)

3.1 Effect of PRRSV infection on innate immunity

In order to measure the effect of PRRSV on innate immunity, it is important to recognize the early signals necessary to substain the innate response following pathogen recognition. In fact, most data suggest that PRRSV induces a **reduced production of type 1 IFNs** (IFN-alfa and beta) through inhibition of the RIG-I pathway, associated with a **high production of IL-10**. Further studies have shown that the largest proteins in PRRSV play a role in the reduction of IL-1beta and TNF-alfa and, in addition, negatively affect type1 IFN by NF-kB signalling pathway. The **IFN-gamma response to the virus is also poor**.

CD163 is a member of the SRCR (Scavenger receptort cystein-rich) family which is strongly associated with the development of the innate response to infection. CD163 expression on macrophages increases after maturation and activation, and is also regulated by the TLR signal, resulting in IL-6 and IL-10 production (Weavewr et al. 2007). CD163 I is a receptor for PRRSV and a correlation between its expression and PRRSV infectivity has been reported. The role of CD163 in the pathogenesis of PRRSV infection is interesting in that its expression can be upregulated by IL-10 (Patton et al. 2009).

Lee et al. (2014) reported that different PRRSV isolates have differing capacity to induce IFN-alfa production. The **reduction of IFN-alfa** is correlated with significant **inhibition of NK cell activity**, independently of the number of these cells in the blood (Lunney et al., 2016). Early production of IL-4 could be involved in some aspects of PRRSV - mediated inhibition of the innate response.

It has been reported that diverse strains of PRRSV can **modulate differently the expression of TLR**, particularly TLR3. Furthermore, PRRSV infection of monocyte derived Dendritic Cells (moDC) enhance the production of IL-1 Receptor Antagonist (IL-1 RA), an intrinsic inhibitory mechanism to control inflammation (Nedumpoun et al. 2016).

Higly Pathogenic (HP) PRRSV isolates appear to stimulate a higher secretion of cytokines. Particularly, the infection of porcine alveolar macrophages (PAM) induces an overexpression of IFN-alfa, IL-8. Compared to "normal" strains, HP PRRSV are also stronger inducers of TLR3, 7, 8 expression (Zhang et al. 2013) and of several cytokines like IL-1beta, TNF-alfa, IL-6 and IFN-gamma

Reduction of early INF-alfa production is one of the key mechanism for viral persistence. The reduced aptitude of a Modified Live Vaccine to compromise the early mediators of the innate immune response is a target for an efficacious vaccine. Furthermore, another important mechanism for **PRRSV persistence is the increased production of IL-10** due to the ability of the virus to interact with the dendritic cell, directly interfering with the induction of acquired immunity. This mechanism was recently correlated to the role of viral proteins N. The measurement of **TNF-alfa in association with IL-10 is theoretically a tool for the evaluation of the efficiency of the innate immune response in PRRSV infection** but these are not applicable nor useful in field conditions. Current research is aimed at evaluating other early mechanisms of evasion of innate immunity such as the modulation of early recognition signal (TLR) expression in PAM and dendritic cells or the evaluation of anti-inflammatory signals, such as IL-1 receptor antagonist or PD-L1 and FoxP3.

3.2 Effect of PRRSV infection on the acquired immunity

PRRSV induces suppression of inflammatory and antiviral cytokines (type 1 INF, IL-1, IL-8, IFN-gamma) causing **dampened acquired immunity**.

In fact, even if an early humoral immune response can appear after 7–9 days post infection (dpi), these anti-N protein antibodies (measured by ELISA) are non-neutralizing and do not correlate with protection. So the prompt production of **ELISA antibodies** soon after infection is useful for diagnostic purposes but not for studying the immune response and consequent protection against infection. Moreover, these non-NA ELISA antibodies are thought to act as a "Trojan Horse", enhancing the internalization of the virus in macrophages as a result of opsonisation. This phenomenon is called **antibody-dependent enhancement** (ADE). *(Lopez OJ, Osorio FA., 2004)*. Conversely, **serum neutralizing antibodies (NAbs)** appear only after approximately 4 weeks and they are not associated with viral clearance (immunological protection) (Yoon et al, 1994; Loving et al, 2015). Furthermore, NAbs are produced and detectable in association with viremia and viral persistence at the tissue level. However, high titres of NAb could transfer passive protection by both protecting passively iperimmunized sow to challenge infection and preventing transplacental PRRSV infection of the fetus, thus conferring sterilizing immunity to both sow and piglets *in utero* (Osorio et al, 202; Lopez et al., 2007).

Experiments on passive transfer in piglets have shown significant differences compared to the results obtained in gestating sows. High concentrations of passive antibodies (required end-point titer of 1:32) are needed to provide sterilizing immunity in piglets. Conversely, low titres of passive transferred NAbs (required end-point titer of 1:8) can protect gestating sows. These results are likely the consequence of **differences in susceptibility** and overall capacity for PRRSV **replication and persistence in adult sows *versus* piglets.** It is thought that piglet target cells (macrophages) are much more permissive to PRRSV replication and consequently the viral load in tissues is higher in young pigs than in sows. So, in younger pigs, a vaccine may need to elicit higher titers of NAb to be effective (Loving et al.,2015). The potential mechanisms responsible for **delayed NAbs** include (*a*) glycan shielding effects of N-linked glycosylation in GPs (Ansari et al., 2006); (*b*) presence of an immunodominant decoy epitope in GP5 upstream of the neutralizing epitope (Ostrowski M. et al., 2002); (*c*) antibody-dependent enhancement (ADE) of viral entry into target cells (Cancel-Tirado SM. Et al., 2004) (*d*) suppression of innate immune responses (Sang Y et al, 2011); and (*e*) prevention of normal B cell repertoire development (Butler et al., 2014).

Either natural exposure or vaccination provides only limited protection against secondary challenge. Protective levels of NAbs usually requires multiple vaccinations or repeated infections and cross-protection (heterologous) can be limited (*Vu HL et al., 2011; Zhou L.* et al., 2012). Importantly, PRRSV viremia can be controlled even in the absence of detectable NAbs.

Antigen-specific **lymphocyte proliferation** is first detected at four weeks post-infection (PI), peaks at 7 weeks PI, and declines after 11 weeks PI. Experiments that utilized blocking antibodies to specific porcine leukocyte antigens demonstrated that CD4+ T-cells were the main effector cells proliferating (*Bautista E.M., Molitor T.W., 1997*). **Changes in PRRSV-specific T cell responses** following infection have been assessed by the **IFN-gamma ELISpot assay** which gives a measure of the number of NK cells, helper T cells and/or cytotoxic T cells producing IFN-γ. Using IFNγ ELISpot assays, Xiao et al. (2004) demonstrated that PRRSV-specific T cells in blood were observed as early as 2 weeks post-infection, with no significant difference in these T cells in lymphoid tissues, with no apparent correlation of tissue viral levels and PRRSV-specific T-cell frequencies and with wide variation over time and among animals (Xiao Z. et al., 2004)

When the IFNγ-secreting CD8+T-cell (**Cytotoxic T-Lymphocytes - CTL**) response was evaluated, a late and low virus-specific response was observed (Ferrari L. et al., 2013)

There is limited data on the role of CD8+ cytotoxic T cells (CTLs) in controlling primary PRRSV infection, and anti-PRRSV-targeted CTLs have been detected only after clearance of the virus from blood (Costers S. et al., 2009). Temporary depletion of CD8+ T cells at the time of infection did not lead to an increase in infection, suggesting that cytotoxic T cells do not have a functional role in the control of acute infection (Lohse L., et al., 2004). Similarly, CTL activity was not detected against PRRSV-infected macrophages until after viremia was cleared. Memory CTL proliferation was observed at 14 days after infection but CTL activity was not detected until 49 days after infection (Costers S. et al., 2009).

The role of **memory T cells** in anti-PRRSV immunity has not been studied extensively. It has been reported that a recall response that is mainly dependent on CD4+ cells and SLA-II, is present from 4 weeks after infection and remains for more than 3 months (Lopez Fuertes et al., 1999).

The majority of studies evaluating T cell responses to PRRSV have investigated the response during the acute infection rather than following clearance of viremia. During infection, it is assumed that mainly T effector cells are detected as opposed to memory cells. The distinction between effector versus memory T cells is difficult in pigs due to the lack of phenotypic markers and functional characterization of individual T cell populations. Clearly, the evaluation of vaccine immunogenicity and efficacy warrants the measurement of PRRSV-specific memory T cells; however, memory T cells have not been clearly characterized in pigs.

PRRSV infection is reported to **increase the frequency of putative Foxp3+ T-regulatory cells (Tregs)** producing TGF-β (Silva-Campa E. et al., 2012) and to **promote the secretion of IL-10**, inducing a strong **immunosuppressive response**, resulting in **delayed onset of a Th1 immune response** (Diaz I., et al., 2005; Johnsen CK et al., 2002, *Dwivedi V.*, et al., 2011; *Wongyanin P, et al., 2012)*

As summarized by the above-mentioned studies, **dysregulated expression of immune molecules** following PRRSV infection results in weakened adaptive immunity.

The proportion of pig showing ELISA antibodies soon after vaccination is variable, ranging from 30 to 80%, very seldom 100%. Neutralizing antibodies are detectable after 3-4 weeks' post-vaccination with a high interindividual variability. After natural or experimental infections, non-NAbs promptly increase and 100% of the animals are positive within a few days (week). The rise of the S/P value in ELISA is not correlated with clinical and immunological protection, so the information given by these serological data is limited to the diagnostic setting.

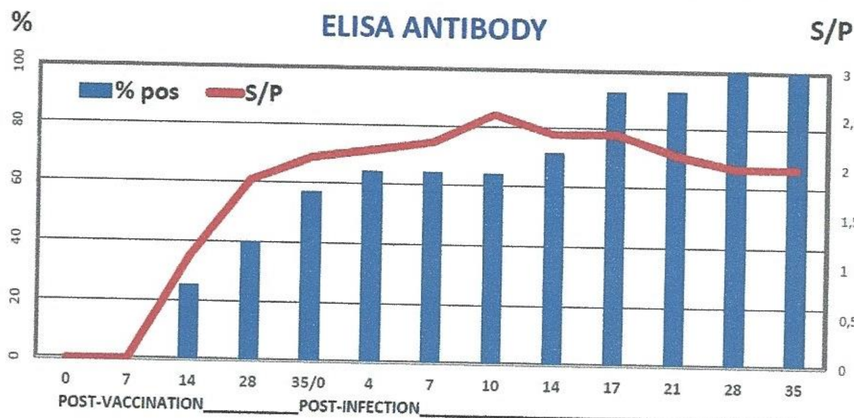


Figure 5. Time course of the ELISA antibodies following vaccination and natural exposure to PRRSV.

The measurement of NAbs is not routinely performed and limited to research activity. To note that NAb titres depend on the cross reactivity between two isolates, the one infecting the animal and the one used in the test. Variability can occur and therefore these antibodies are not valuable for evaluating the immune response and the degree of protection and cross-protection. Measurement of cell mediated immunity is based on the ELISpot assay [enumeration of the INF-gamma Secreting Cells (SC) specific to PRRSV]. In naïve animals, both post vaccination and post-infection, the number of IFN-gamma SC starts to increase from 3-4 weeks onward. The course of the specific SC response is rather erratic with a tendency to increase over time.

As mentioned above for the ELISA antibodies, a boosting effect is detected after infection with a prompt and more intense increase of these cells. It is worth mentioning that both the antibodies and the cellular response feature an interindividual variability. Animals can be classified as "low" and "high" responders. These divergent responses to vaccination may be the result of the host's genetic background (Ait-Ali T. et al., 2016).

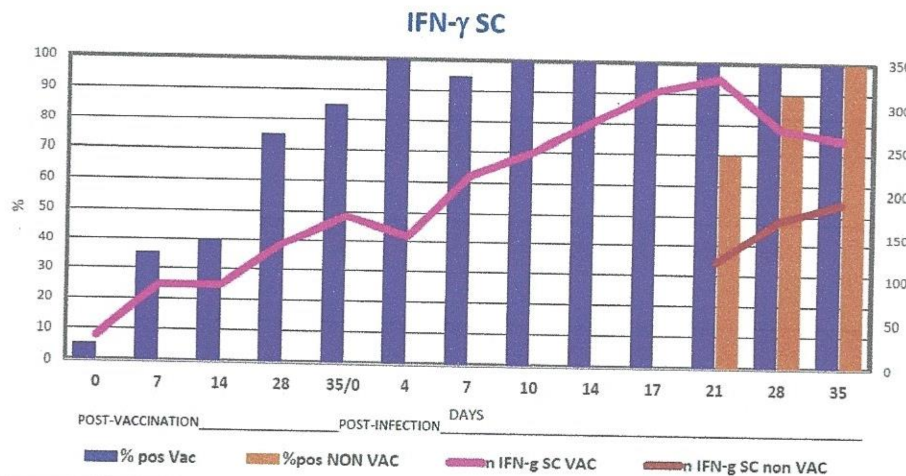


Figure 6. PRRSV-specific IFN-γ secreting cells (SC) following vaccination and post-exposure to natural infection.

MEASURING THE IMMUNE RESPONSE TO PORCINE CIRCOVIRUS 2 (PCV2)

Effect of PCV2 infection on the innate immunity

The internalization of PCV2 has been demonstrated for both conventional blood DCs and plasmacytoid DCs. The persistence of PCV2 in DCs, hoeever, does not cause loss of viral infectivity or cell death, but actually helps the virus to evade the host immune system and facilitates the transmission of PCV2 by reducing the first signal of the innate immune response. In fact, PCV2 does not replicate in these cells, but accumulates within them. **PCV2-induced impairment of DC function** does not require viral replication and is mediated by the viral DNA, with a minimum concentration of double strained (ds) DNA (replicative form) necessary to mediate such inhibition. Furthermore, the infection of pDC **reduces the production of IFN-alfa and TNF-alfa** and impairs the maturation of myeloid DCs. PCV2-mediated inhibition of natural IFN-producing cells appears to have a broad spectrum and can affect responses **induced by Toll-like receptors TLR7 and TLR9** agonists and by several other pig viruses. This would suggest that PCV2 modulation of the innate immune response may render the pigs more **susceptible to secondary or concurrent viral and bacterial infections.** PCV2 interaction with immune cells and with the lymphoid system appears to play an important role in the pathogenesis of PCV2 infection. The **increase of IL-10** and the **suppression of the innate cytokine** pattern play a major role in the pathogenesis of PCV2 and Porcine CircoVirus-Disease (PCVD). The whole PCV2 virus and some fragments of the genome are able to induce the suppression of cytokine production. Conversely, the Virus Like Particles (VLPs) do not show this suppressive effect.

4.2 Effect of PCV2 infection on acquired immunity

The lymphoid tissues and immune cells are the primary target of PCV2 infection that alters the **cytokine responses** in infected pigs. Several studies have shown that anti-inflammatory and pro-inflammatory cytokines are differently regulated in PCV2 infection, depending on the lymphoid tissue considered. **IL10 plays a particularly important role in the immune dysregulation upon PCV2 infection.** (***Ramamoorthy S, Meng XJ. 2009; Darwich L, Mateu E. 2012; Darwich L. et al., 2003)*** Pigs which are subclinically infected with PCV2 also develop a transient raise of IL-10 response during the peak of viremia (***Darwich L.*** et al., ***2008).***

PCV2-infected pigs have a significantly **decreased number of CD8+ and CD4+CD8+ double-positive cell** subsets compared to PCV2-negative pigs, which further indicates that PCV2 infection impairs the host immune response. Nevertheless, both neutralizing antibodies and cell mediated immunity contribute to the protection against PCV2 infection (*Darwich L, Mateu E., 2012).* PCV2 infection induced virus-specific neutralizing antibodies at approximately 21 days postinoculation (10-28 days post inoculation) (*Beach NM. Et al., 2010; Allan GM, Ellis JA. Lekcharoensuk P. et al., 2004).* Low NAb titers have been related to increased virus replication and development of PCV-Sistemic Disease. It is worth mentioning that some animals develop a **humoral response lacking NAb** or NAb develop later than non-NAb.

In gnotobiotic pigs experimentally infected with PCV2, neutralizing antibodies specific for PCV2 and IFN-gamma gene expression in PBMCs were detected, although the onset of adaptive immunity varied among the infected pigs. In fact, the absence of neutralizing antibodies is an important factor for increased PCV2 replication and protection against the development of PCVD (*Meerts P. et al., 2005; Meerts P. et al. 200).*

IFN-gamma SC develop specifically in response to PCV2 infection and may contribute to viral clearance in pigs *(Fort M. et al., 2009; Martelli P. et al., 2011)* and depletion of CD4+CD8+ T cells has been shown to weaken the virus-specific IFN-gamma responses (*Steiner E et al., 2009).* Therefore, IFN-γ appears to be an important immunological player. The contribution of cell-mediated responses in vaccine-induced protection is pivotal, because the effect of PCV2 antibodies is titre-dependent and the sole induction of a humoral response might not guarantee full protection against PCV2 infection (Blanchard et al., 2003; Opriessnig et al., 2009; Fort et al., 2009b).

After the onset of infection, non vaccinated pigs show a marked increase in IFN-gamma SC associated with a reduction of viremia. The IFN-gamma SC responses are also related to viral replication. Thus, in pigs in which PCV2 viral load is low or absent, as occurrs more frequently in vaccinated animals, the number of these cells is rather low or is maintained at residual levels as a consequence of the primary activation by vaccination (ranging from 40 to 60 IFN-gamma SC on average).

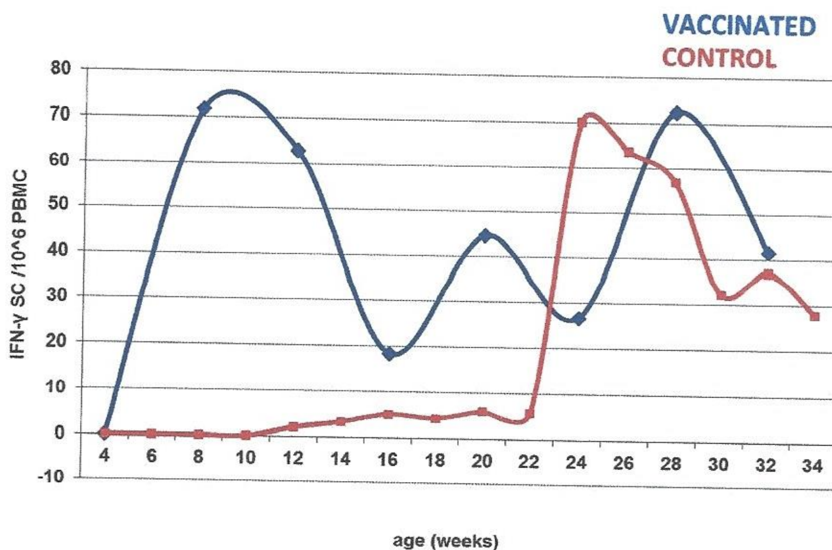
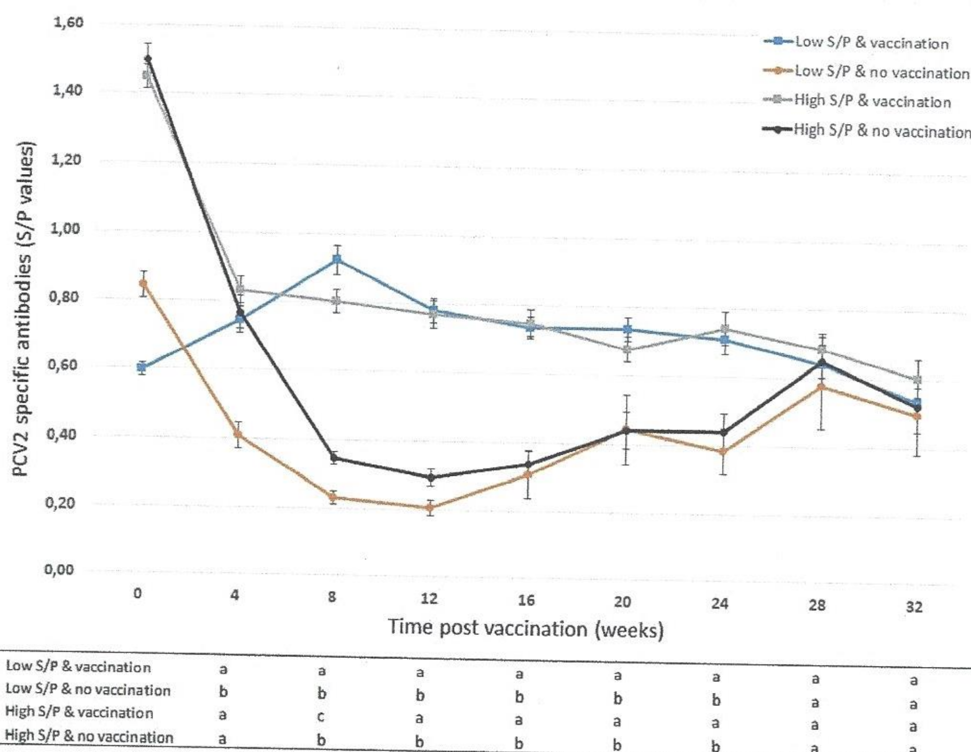


Figure 7: INF-gamma SC response after vaccination and natural infection.



Low S/P & vaccination	a	a	a	a	a	a	a	a
Low S/P & no vaccination	b	b	b	b	b	b	a	a
High S/P & vaccination	a	c	a	a	a	a	a	a
High S/P & no vaccination	a	b	b	b	b	b	a	a

Figure 8. Course of the serologic response to PCV2 (Mean ± STD anti-PCV2 ELISA antibodies) according to the levels of maternal derived antibodies (MDA) at vaccination and PCV2 infection (viremia) occurred approximately at 20 weeks of age. Overall, the animals were divided based on a low (≤0.9) or high (≥1.2) S/P value at 4 weeks of age when vaccinated animals were inoculated with the vaccine ("HIGH S/P vac" and "LOW S/P vac" groups) while unvaccinated animals were inoculated with the adjuvant alone ("HIGH S/P NV" and "LOW S/P NV" groups) (Martelli et al., 2016 -modified).

MEASURING THE IMMUNE RESPONSE TO MYCOPLASMA HYOPNEUMONIAE

Upon infection with Mycoplasma hyopneumoniae (Mh), immune response is both innate and adaptive. The innate response involves the 'mucociliary system', cytotoxicity by macrophages and neutrophils, increase of T-cell and NK cells, as well as the enhancement of the expression of cell receptors that trigger the complement cascade.

The adaptive immune response involves opsonization, antibody production (systemic and local) and cell-mediated immune stimulation. However, the immune response to M. hyopneumoniae acts as a "double edge sword" helping in protection and also playing a role in the exacerbation of lesions induced during mycoplasmal disease. Furthermore, TLR2 plays an important role in *M. hyopneumoniae*-activated inflammatory responses in porcine alveolar macrophages (Muneta et al., 2003). Mycoplasma lipopeptide-induced cytokine production is impaired in TLR2-deficient macrophages of mice (Takeuchi et al., 2000). Therefore, activation of *M. hyopneumoniae*-induced inflammation is thought to be mainly mediated by TLR2.

Alveolar macrophages and lymphocytes stimulated by *M. hyopneumoniae*, produce **pro-inflammatory cytokines** that are responsible for lung lesions and lymphoid hyperplasia (Rodriguez et al., 2004), suggesting the involvement of the immune response in the development of lesions. In general, mycoplasmas are capable of evading the host's natural defenses. It is known that some pathogenic species use their genetic machinery to alter surface antigens, thus diverting the host immune response and allowing for chronic infection (Razin et al., 1998).

Substantial lymphoid cell infiltration occurs around the airways following infection with M. hyopneumoniae, thanks to the key role of macrophages. Indeed, macrophages are recruited as a result chemo-attraction induced by the release of immunogenic proteins and mediators during M. hyopneumoniae infection. Lymphoid hyperplasia of the bronchus-associated lymphoid tissue progressively leads to obliteration of the bronchiole's lumen and atelectasis of the alveoli.

Helper T-cells are the most numerous subset in the lymphoid infiltration. Cytotoxic T-cells (CTL) are also detected in association with the specific lesions. Upon activation, Th1-cells are responsible for activating and increasing the phagocytic and cytotoxic activity of macrophages. T-cells and the overproduction of IL-1 and IFN-gamma worsen the host mediated tissue damage. Therefore, mycoplasmal pneumonia is not only dependent of the injury caused by ciliostasis and exfoliation of the epithelial cells, but also damage caused by the overexpression of some immune functions. The activation of humoral immunity leads to the production of antibodies with increases in IgG and IgA in the tracheobronchial secretions, lungs and serum of infected pigs. Predominantly, IgG participates in the opsonization of the microorganisms and in the phagocytosis by alveolar macrophages, while IgA provides local immunity interfering with the adherence of M. hyopneumoniae.

Despite the immune mechanisms of the host, the microrganims persist in the respiratory tract of the pig (lack of immunological protection). In fact, phagocytosis by alveolar macrophages is severely impaired upon M. hyopneumoniae infection.

The serological response to M. hyopneumoniae after infection or vaccination is measured by ELISA tests. Commercial kits have low sensitivity. Serology is appropriate to determine the status of a population rather than of an individual pig or for vaccine compliance. Antibodies are first detected in serum of pigs by ELISA at 3-6 weeks post-exposure. Antibody levels following vaccination with a bacterin may vary depending on the vaccine, the infectious status of the pigs and the serological assay (Erladson et al, 2005). No correlation between the level of vaccine-induced antibodies and protection has been observed (Thacker et al., 1998).

In a vaccinated population, an increase of the ELISA S/P values as well as an increase of the proportion of seropositive pigs is commonly observed following challenge infection. In fact, active infection elicits a prompt secondary antibody response (booster effect).

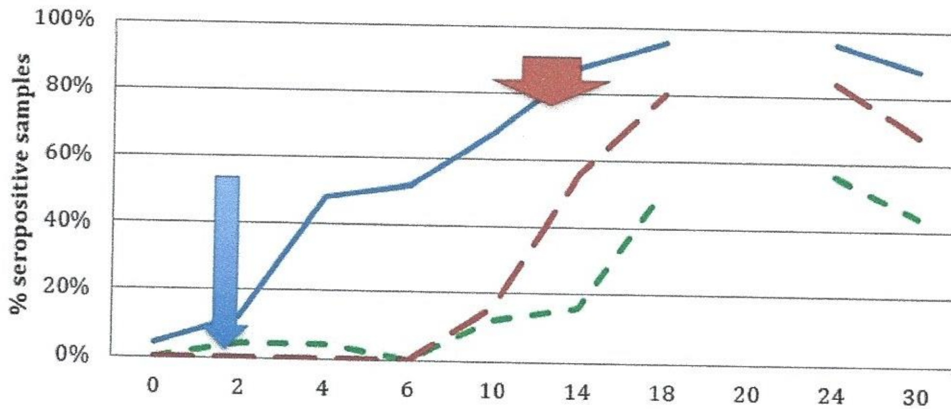


Figure 9: Time course of ELISA positivity following vaccination (blu arrow) and after the onset of infection (red arrow). Legenda: blu line= vaccinated (vaccine A); red dotted line= vaccinated (vaccine B); green dotted line= negative control.

The ELISpot assay is used to measure the specific cell mediated immune response to the microorganism. The number of specific IFN-gamma Secreting Cells is usually low although marked differences between vaccines have been demonstrated.

Importantly, as mentioned above, the humoral immune protection to M. hyopneumoniae is due to local immune function, markedly of production of IgA. The quatification of IgA in the BAL fluid is a direct measure of local immunity. Laborious sampling and lack of repeatability (variability of quantity and location of the samples) make this technique impractical and rarely used in the field. BAL fluid sampling is commonly used for research purposes or as tracheal swabs for diasgnostic purposes. Moreover, no commercial kits for the determination of the IgA are available, thus the measurement of this class of Immunogluobulins is not currently standardized.

References

Available upon request