PORCINE INNATE ANTIVIRAL IMMUNITY: HOST DEFENSE PEPTIDES AND TOLL-LIKE RECEPTORS

by

YONGMING SANG

M.S., Nanjing Agricultural University, Nanjing, China, 1993
D.Sc., Nanjing Agricultural University, Nanjing, China, 1996

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

Abstract

The immediate antiviral defense residing in the innate immune system of multicellular organisms critically determines the outcome of viral infection. This dissertation presents a study of the "effectors" and "receptors" of porcine innate immunity in infection caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is the most devastating pathogen impacting the swine industry.

In the first investigation, eleven novel porcine host defense peptides (HDPs), β-defensins (pBDs), were identified and characterized. All of these peptides have a consensus β-defensin motif and phylogenetically are similar to orthologs from other species. A differential expression pattern for these 11 newly identified genes was found. For example, *pBD-2* and *pBD-3* were expressed in bone marrow, lung, skin and other lymphoid tissues. *pBD-2* and *pBD-3* were further characterized for their gene structure, and antimicrobial activity of synthetic peptides.

The second study was conducted to evaluate PRRSV-induced differential expression of porcine HDPs and direct antiviral activity of selected HDPs against PRRSV. *In vitro* incubation of PRRSV with synthetic pBD-3 or protegrin-4 (PG-4) significantly inhibited viral infectivity. Using nine protegrin-derived peptides, it was determined that cyclization of PG-4 increased anti-PRRSV activity and mutation of some residues in PG-4 diminished some of the activity. These findings suggest the potential role of porcine HDPs as a group of innate antiviral effectors.

In the third and fourth investigations, porcine Toll-like receptor (TLR) 3 and TLR7 were identified and functionally expressed. Increased expression of TLR3 was observed in PRRSV-infected porcine lungs. Stimulation of porcine alovelar macrophages with poly (I:C), a synthetic TLR3 ligand, increased expression of interferon-β and suppressed PRRSV infectivity. Activation of porcine TLR3 overexpressed in a PRRSV-sensitive cell line, elicited antiviral responses to PRRSV infection. Partial silencing of TLR3 in PAMs resulted in increased PRRSV infection. In summary, these data provide molecular information on porcine TLR3 and TLR7, and their involvement in PRRSV pathogenesis, which may elicit new strategies to prevent this costly swine disease.

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Acknowledgements

In the Spring of 2005, after working in Dr. Blecha's laboratory for 3 years, I decided to pursue another doctoral degree focused on immunophysiology. During the last several years working here, I really appreciate the efficient administration in the Department of Anatomy & Physiology and the pleasant scientific atmosphere established in Dr. Blecha's and Dr. Ross's labs, which are supervised by my two co-major professors. Both of them are the best advisors I have ever met with regard to their open and stimulating instructional style, expertise in animal innate immunity and philosophical insights into scientific research. The process of completing this academic degree has been rewarding, nevertheless, with some obstacles. I am grateful to my two co-major professors and my colleagues in the department for their encouragment whenever I needed it.

I appreciate the substantial help and instructional effort from Dr. Rowland and Dr. Minton, members of my program committee. Many times Dr. Minton helped us in sampling porcine tissues and gave me precious advice within his expertise in animal science and physiology. I have to say that it would have been impossible to carry on this project without the substantial input from Dr. Rowland. He not only gave me professional insight and advice in virology, Dr. Rowland also helped establish the basis for the project to pursue porcine viral immunology.

Many thanks are given to Ms. Danielle Goodband and Dr. Jun Yang, for their friendship and the best ever in maintaining Dr. Blecha's and Dr. Ross's labs as efficient and pleasant scientific "workshops".

The period of this program has been accompanied by the growth of my two boys, Peter and Eric. I am proud of and grateful to my wife, Chuanmin Cheng, for her great effort and patience in managing three boys at home, in addition to her own job responsibilities.

Finally, I would like to express my appreciation to Dr. Anna Zolkiewska, for her willingness to be the chair of my program committee.

CHAPTER 1 - Literature Review: Mammalian Antiviral Innate Immunity

1.1. Introduction

The immune system in higher vertebrates comprises both innate and adaptive immunity (Pancer and Cooper, 2006). Either sensitized by vaccination or evoked latter during infections, adaptive immunity consists of immune responses characterized by the arrest of B cells and T cells to exert pathogen-specific protection *via* secretion of humoral antibodies and activation of cell-mediated cytotoxicity. Conversly, innate immune mechanisms provide immediate frontline protection against infections (Beutler, 2004; Pancer and Cooper, 2006). In previously unvaccinated individuals, it may take 1-2 weeks to establish antibody levels and T cell responses (i.e. adaptive immune responses) post viral infection. Hence, without efficient protection from innate immunity, this time gap could potentially leave viruses unchecked and cause lethal consequences in animals (Pichlmair and Reis e Sousa, 2007; Takeuchi and Akira, 2007). Notably, despite being less specific than adaptive immunity, the promiscuity of innate immune recognition broadly extends its capacity for surveillance of pathogens. This is especially important for viruses, which keep changing their antigenic epitopes specifically recognized by neutralizing antibodies and T-cell receptors (TCR) in the adaptive immune system (Beutler, 2004; Pancer and Cooper, 2006). Evidences from both host and virus indicate that early invoking of appropriate innate immune responses determines the outcomes of viral diseases, whether the infection is controlled or developed into a persistent status resulting in serious diseases (Beutler, 2004; Pancer and Cooper, 2006). For the host *per se*, mutations which cause inefficiencies of the

innate immune response in viral sensing, signaling transduction or expression of antiviral effectors, often significantly elevate susceptibility or mortality to infection of multiple viruses (Akira et al., 2006; Pichlmair and Reis e Sousa, 2007). Virus isolates or species, which have the ability to suppress or evade innate immune surveillance, are likely to cause chronic infection leading to pandemic diseases (Haller and Weber, 2007; Loo and Gale, 2007). The critical role of innate immune cells and components not only obligates to early antiviral activity, but also potentiates the adaptive system for viral clearance (Hoebe et al., 2004; Kabelitz and Medzhitov, 2007; Wen et al., 2008).

1.2. Afferent and Efferent Arms of Innate Antiviral Immunity

Similar to adaptive immunity, the innate immune system also comprises both afferent and efferent arms to discriminate and kill pathogens **(Fig. 1.1)** (Beutler, 2004). This capability is intensified when dealing with viruses, which use host cellular metabolism to facilitate their own life cycles. Although innate immune sensors are diversified to distinguish between viral and cellular molecules, components of the efferent arm are also specialized to convey virus-dependent activity. For example, interferon (IFN)-inducible proteins such as human tripartite motif protein (TRIM)-5α targets capsids of invading retroviruses, and Mx proteins target nucleoproteins of bunya- and orthomyxoviruses (Pichlmair and Reis e Sousa, 2007). In contrast, antibacterial proteins (such as lysozyme and bactericidal/permeability-increasing protein (BPI)) are less diversified and are generally active against most bacteria. Nevertheless, some components in both afferent and efferent arms of innate immunity are "universally" functional against bacteria, fungi, protozoa and viruses; however, only the activity in antiviral immunity will be discussed in this review. To focus on the main theme of this dissertation, examples

related to respiratory viral infections are primarily used. In **Fig. 1.1**, major components of innate antiviral immunity in higher vertebrates, mainly exemplified with that of humans and mice (Beutler, 2004), are illustrated. Like that of adaptive immunity, innate antiviral immunity also consists of humoral and cellular parts, in which each part contains afferent (sensing) and efferent (effector) arms. To some extent, both sensor and effector molecules in the humoral part of innate immunity may have a role in inactivation of viral infectivity. In contrast, the afferent and efferent arms of the cellular part are more specialized, as sensors are not killing but recognizing pathogen-associate molecular patterns (PAMPs) and effector molecules mainly function to inactivate viruses (Pichlmair and Reis e Sousa, 2007; Zuniga et al., 2007). Most steps of the viral lifecycle are limited within cells with short intervals of intercellular dissemination; therefore, innate immune mechanisms for virus determination/inactivation are evolutionally concentrated in the cellular arm. Accordingly, the components of afferent and efferent arms in the cellular part will be mainly discussed in this review. Type I interferons (IFNs) and host defense peptides (HDPs), which are primarily secreted by immune cells into the humoral system after synthesis, will be extensively discussed to highlight their central role as effectors and regulators in antiviral immunity (García-Sastre and Biron, 2006; Klotman and Chang, 2006; Pestka, 2007). In addition, two exceptions listed in **Fig. 1.1** include natural antibodies (NAb) and micro RNA (miRNA). Although NAbs (secreted from B1 cells) belong to the "combinatorial system" adjunctive to specific immunity, they show innate-like characteristics and often work together with complement, a prominent humoral innate immune system, to perceive and to promote killing of viruses or virus-infected cells (Cummings, et al., 2007; Dörner and Radbruch, 2007). Micro RNA and related RNA interference is a prominent antiviral defense mechanism in invertebrates

such as *Drosophila*; the role of miRNA in antiviral immunity of vertebrates is emerging but still controversial (Kumar, 2008).

1.3. Innate Immune Cells

All nucleated cells, to some extent, are capable of mounting innate immune responses upon exposure to viral infection (Beutler, 2004). Nevertheless, a highly differentiated immune system is an evolutionary advantage in higher vertebrates and the specialized immune cells are involved in eliciting and coordinating immune responses (Pancer and Cooper, 2006). Mammalian innate immune cells, which are specialized to fulfill requirements in pathogen recognition, immune surveillance, and/or effector killing, include hematopoiesis-derived granulocytes, natural killer (NK) cells, macrophages and dendritic cells (DCs), as well as epithelial and endothelial cells which line the the cavities/surfaces of stuctures throughtout the body. Other immune cells, such as γδ T cells and CD1d-restricted T cells, exert innate immune function (such as pattern recognition with their restricted T cell receptors (TCR)) as well, but their TCR diverge by rearranging TCR genes and they can also develop a memory phenotype –– the marker events of adaptive immune cells. Thus, they are considered as belonging to adaptive immunity (Beutler, 2004; Pancer and Cooper, 2006; Yamagata et al., 2006). On the one hand, granulocytes, macrophages and NK cells are known for their role as effector cells in engulfing and digesting trapped microorganisms or promoting active death of infected cells (Appelberg, 2007; Ludwig et al., 2006; Takeuchi and Akira, 2007; Vivier et al., 2008). DCs, epithelial and endothelial cells, pertaining to their special anatomical locations, are among the first group of cells contacting the initial load of the virus (Barchet et al., 2005; Hammad et al., 2008; Opitz et al., 2007; Wen et al., 2008); therefore, their ability in viral recognition and immune surveillance are augmented such

as in coordinating subsequent immune responses and linking to adaptive immunity. On the other hand, all innate immune cells are dually functional as "sensors" and "effectors" to pathogens. Even the professional killer cells (eg. neutrophils of granulocytes) are facilitated somehow for immune surveillance with TLRs (Borregaard et al., 2007; Haselmayer et al., 2006), and the professional antigen presenting cells (i.e. cDC) potently inactivate engulfed pathogens through autophagy (Lee and Iwasaki, 2008; Schmid et al., 2006). This kind of functional combination is best illustrated in macrophages, which are a diverse group of professional phagocytes lethal to most trapped pathogens and behave as critical immunoregulatory cells in production of type I IFN and antigen presenting cells after activation **(Fig. 1.2)** (Hashimoto et al., 2007; Kumagai et al., 2007; Randolph et al., 2008). Nevertheless, upon viral infection the overall immune response is dependent on coordination of these immune cells to exert immune surveillance and to produce immune effectors. Quite often, innate immune cells (esp. epithelial and endothelial cells) are initial footholds for viruses to begin infections. Thus, innate immune cells represent an unequivocal platform for examining virus-host interaction, viral recognition, signaling transduction and function of antiviral effectors (Hammad et al., 2008; Opitz et al., 2007; Wen et al., 2008). Innate immune cells are also important because of their situation at the interface of innate and adaptive immunity to shape specific antiviral immune responses **(Fig. 1.2)** (Hammad et al., 2008; Hoebe et al., 2004; Opitz et al., 2007; Wen et al., 2008).

Epithelial cells: Distinct types of epithelial cells and secretory cells cover the body's external and internal surfaces all over. Mucosal surfaces are classified into two categories, type I mucosae are lined by simple epithelia of one cell-layer thickness and type II mucosae are covered by stratified squamous epithelia (keratinocytes) (Holgate, 2007; Iwasaki 2007). The

primary role of the epithelia in type I mucosae, represented by those that cover alimentary, respiratory and most parts of reproductive tracts, is to perform absorptive, respiratory, excretory, and reproductive functions. On the other hand, type II mucosae include those that line skin, cornea, oroesophagus, and lower female reproductive organs (ectocervix and vagina). The epithelia that line type II mucosal surfaces (esp. skin keratinocytes) do not have absorptive or respiratory functions, and primarily serve as a physical barrier with intensified innate immune mechanisms. Clearly, epithelia lining external and internal surfaces are at the first line in contacting with viruses and are frequently used by the virus as initial footholds for infection (Holgate, 2007; Iwasaki 2007; Schleimer et al., 2007; Turner 2006). For example, viruses implicated in various respiratory viral syndromes, including influenza, parainfluenza, enterovirus, adenovirus, respiratory syncytial virus (RSV), rhinoviruses (RV), and coronaviruses, are all capable of infecting airway epithelial cells (Bousse et al., 2006; Fernandez-Sesma, 2007; Guillot et al., 2005; Holgate, 2007; Schleimer et al., 2007; Shi et al., 2007). Therefore, epithelial cells are well equipped for immune surveillance in addition to their primary physiological functions (Schleimer et al., 2007). For example, epithelial cells express most identified pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and RIG-like receptors (RLRs) **(Table 1.1)** (Behera et al., 2002; Fu et al., 1999; Message et al., 2004; Schleimer et al., 2007; Takaoka et al., 2006). In regard to the effector system, all mucosal epithelial cells are covered by a viscous layer of mucus, which is produced by the adjacent mucus-secreting cells and effectively impedes virus access to epithelia. The important effectors produced by epithelial cells after responding to viral infection include IFNs, proinflammatory cytokines/chemokines, nitric oxide (NO) and host defense peptides (HDPs), incompletely listed in **Table 1.1** (Alp et al., 2005; Duits et al., 2003; Klotman and Chang, 2006; Pestka, 2007; Stroinigg et al., 2005; Sun et

al., 2005; Takaoka et al., 2006). Type I IFNs produced by virus-infected epithelial cells in turn induce surrounding cells to express hundreds of IFN-stimulated genes (ISG) including IFNs themselves, which have antiviral potency. These ISGs include the dsRNA-activated protein kinase K (PKR), which reduces cellular activity in translation and transcription, two enzymes, 2', 5'-oligoadenylate synthetase (2'5'OAS) and RNase L, which promote viral mRNA degradation, the myxovirus resistance (Mx) proteins, and RNA-specific adenosine deaminases, which are involved in RNA editing to interfere with genomes of RNA viruses (García-Sastre and Biron, 2006; Sen and Sarkar, 2007; Zuniga et al., 2007). The activation of epithelial cells also produces a variety of proinflammatory cytokines and chemokines which recruit other immune cells including lymphocytes and neutrophils to infection sites for development of overall antiviral immune responses (Holgate, 2007; Message et al., 2004; Schleimer et al., 2007; Shi et al., 2007; Takaoka et al., 2006).

Endothelial cells: Another main route of viral entrance into animal bodies is through blood infection, either *via* arthropod vectors, contaminated needles or blood transfusion (Valbuena and Walker, 2006). The formation of primary and secondary viremia is a critical step for viruses to establish systemic infection. Endothelia, composed of specilizazed epithelial cells that line serous cavities and blood vessels, are among the first cells coming into contact with viruses entering the bloodstream. Rationally, endothelial cells are equipped with both transmembrane and cytosolic surveillance systems capable to sense viral PAMPs **(Table 1.1)** (Opitz et al., 2007; Warke et al., 2003; Chaudhuri et al., 2007; Frantz et al., 2005). Similar to epithelial cells, endothelial cells are likely capable of producing a variety of antiviral effectors in triggering immune protection. However, there are limited studies providing related information in

endothelial cells and there is no direct evidence about the existence of TLR7-9 and cytosolic RLRs in endothelial cells.

Dendritic cells: In higher vertebrates, an additional role of the innate immune system is to evoke specific immunity in preparation for the pathogens escaping from non-specific surveillance (Beutler, 2004; Pancer and Cooper, 2006; Yamagata et al., 2006). If a viral disease is developed, productive adaptive immunity is crucial for hosts to systemically eliminate viruses. In this context, dendritic cells (DCs) represent a primary group of innate immune cells to bridge innate and adaptive immune responses (Iwasaki, 2007; Hammad and Lambrecht, 2008; Wen et al., 2008). DCs develop in bone marrow, exist in blood in an immature state and migrate to peripheral tissues as tissue-specific DCs including skin Langerhans cells and various mucosal DCs underneath the epithelial layers, where they are ready to sample antigens including those from infective viruses (Iwasaki, 2007; Hammad and Lambrecht, 2008; Wen et al., 2008). Two major types of dendritic cells are classified as conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively). cDCs are prominently antigen-presenting cells with high activity for autophagy, a process to uptake the antigen. After activated by antigen-uptake, DCs follow the gradient of chemokines (CCL19/CCL21) migrating to the adjacent lymph nodes, where they undergo a maturation process evidenced by the expression of surface molecules for co-stimulation (CD80/CD86) and antigen presentation (CD1, ICAM-1, LFA-3, and MHC class I and II). Meanwhile, the cells are reshaped with long extensions (dendrites) which facilitate interaction with naïve T cells. Although cDC are important in antigen presentation for training T cells in secondary lymphoid tissues, no direct antiviral role of periphery cDCs has yet been defined in a primary viral infection (Iwasaki, 2007; Hammad and Lambrecht, 2008). The pDCs

are known as natural IFN-producing cells because of their capacity for high-level production of IFN-α, which is essential for inducing a series of antiviral ISGs and establishing an antiviral state in surrounding cells (Barchet et al., 2005; Sen and Sarkar, 2007; Zuniga et al., 2007). To a lesser extent, pDC also produce IL-12, which is thought to influence T cell skewing towards a Th1 response (Barchet et al., 2005). Hence, DCs constitute a special group of innate immune cells with particular function in linking innate and adaptive immunity. For efficient immune surveillance, DCs express a complete repertoire of PRRs including cytosolic RLRs in cDCs and TLRs in all DCs (Takeuchi et al., 2007; Zilliox et al., 2006; Kato et al., 2006; Rodríguez-García et al., 2007; Schlender et al., 2005). However, seldom do viruses infect DCs, it is suggested that cytoplasm-localized receptors in DCs might engage viral PAMPs leaked from the autophagy process (Pichlmair and Reis e Sousa, 2007). In addition, it was reported that activation of antigen-presenting activity in DCs requires TLRs localized in both DCs and stromal cells (eg. epithelial cells) (Sato et al., 2004). Some effectors secreted from surrounding stromal cells may play a role in activation of TLRs in DCs (Hammad and Lambrecht, 2008; Iwasaki, 2007). In this context, human β-defensin-3 (hBD3), a HDP secreted from epithelia, was reported to activate DCs through TLR1 and TLR2 signaling (Funderburg et al., 2007). It is not clear how hBD3 engages the TLR1/TLR2 complex, possibly by serving as an endogenous ligand like murine β-defensin 2 to TLR4 (Biragyn et al., 2002). In another case, the human cathelicidin LL-37 activation of pDCs is through the formation a complex with nucleic acid and presention to TLR9 (Lande et al., 2007). Thus, antiviral surveillance of DCs is possibly composed of receptors in DCs and effectors secreted from infected stromal cells.

Macrophages: Macrophages, similar to dendritic cells, belong to the monocyte lineage of myeloid cells (Randolph et al., 2008). In adult animals, they originate from bone morrow stem

cells, progress through the stages of monoblasts and promonocytes, and differentiate into peripheral monocytes and tissues macrophages. Thus, macrophages are composed of groups of very heterogeneous populations according to their locations, such as blood monocytes, peritoneal macrophages, pulmonary macrophages, Kupffer cells in liver and microglia in brain (Naito, 2008; Randolph et al., 2008; Taylor et al., 2005). Even in the same organ, macrophages are further diversified with regard to different micro-anatomical locations. For example, pulmonary macrophages can be divided into three subgroups in respect to their contacting microenviroments in lung, which are alveolar macrophages (AMs), interstitial macrophages (ISMs), and intravascular macrophages (IVMs) (Naito, 2008; Randolph et al., 2008). AMs are the most abundant pulmonary immune cells in the alveolus located at the interphase between air and lung tissues; substantial numbers of ISMs are detected within the lung stroma despite not easily to be isolated as AMs; and IVMs are mature phagocytes adhering to capillary endothelial cells within lung, which may cover about 16% of lung capillary surface in animal species such as pigs and ruminants (Chitko-McKown and Blecha, 1992).

The ubiquitous presence of macrophages adjacent to the basement membrane of epithelia and endothelia implicates their essential role in immune defenses such as scavenger cells and immunoregulatory cells (**Fig. 1.2**) (Naito, 2008; Randolph et al., 2008; Taylor et al., 2005). In respect to airborne viral infections in lungs, AMs are foremost among the immune cells to be early activated for scavenging and killing mucus-trapped viral particles (or virus infected cells) through phagcytosis. To fulfill this task, AMs are equipped with a variety of surface/internal receptors to detect the antibody/complement-engaged viral particles (by surface Fc or complement receptors) and to recognize viral components by membrane-associated or cytosolic PRRs such as TLRs or RLRs (**Table 1.2**) (Beisswenger et al., 2005; Daffis et al., 2007; Fantuzzi

et al., 2003; Kumagai et al., 2007). AMs are very active killer cells to inactivating trapped viruses with both oxidative and non-oxidative mechanisms and lowering the chance of airborne viruses initializing infections on pneumocytes and pulmonary endothelial cells (Naito, 2008; Randolph et al., 2008; Taylor et al., 2005). Either activated through phagocytosis or PRR recognition of viral components, AMs are active producers of type I IFNs and other proinflammatory cytokines, which lead to antiviral responses including the regulatory loop by type I IFNs and recruitment of other immune cells to infection sites (Kumagai et al., 2007; Takeuchi and Akira, 2007). Normally, AMs represent >90% of immune cells in the bronchoalveolar lavage (BAL) fluid with an increase of granulocytes in BAL fluid indicating infection (White et al., 2007). Recently, AMs, not DCs, were implicated to be the primary IFN-α producer in murine models upon respiratory viral infections (Kumagai et al., 2007; Takeuchi and Akira, 2007). Compared to AMs, ISMs are less phagocytic and more likely to become antigen presenting cells and to secrete cytokines for priming T cells in bronchial lymph nodes. Pulmonary IVMs are important for clearance of pathogenic particles in the lung by their high cytolytic potency (Chitko-McKown and Blecha, 1992). The importance of macrophages in antiviral immune response is shown in that virus escaping the first-line surveillance of tissue macrophages has a much higher chance of escaping from innate immune defenses and causing persistent infections (**Table 1.2**). Quite a few viruses have adapted the ability to directly infect and undermine immune responses (such as production and signaling of type I IFNs) of macrophages. Such as in the cases by HIV-1 and PRRSV, the infected macrophages are functionally compromised in many ways including cytokine production, receptor expression, phagocytosis, and function as accessory cells (Ieong et al., 2000; Luo et al., 2008; Martinelli et al., 2007; Sanders et al., 2007; Thibault et al., 2007). In this regard, virus-infected macrophages

and cell traffic of infected macrophages are kidnapped by the viruses as footholds and "viral reservoirs" for spreading and forming systemic infections (**Table 1.2**).

Natural killer cells: Natural killer (NK) cells and $CD8^+$ cytotoxic T cells are major effector cells with ability to kill malignant cells and virus-infected cells. Although $CD8^+$ T cells belong to the adaptive immune system, NK cells bear no antigen-specific receptors derived from somatic gene recombination (so named "null cells" as well) and are activated prior to antigen sensitization and thus are a part of the innate immune system (Lanier, 2008a; Vivier et al., 2008). Upon viral infection, NK cells are activated through two pathways. The first one is by innate cytokines (eg. IFN-α/β, IL-12, and IL-15) secreted from other cells such as pDCs viral infected cells. The binding of these cytokines to receptors on NK cell surface stimulates NK cells to proliferate (by IL-15), to produce IFN-γ (by IL-12), and to obtain cytotoxic functions (by IFN-α/β) (Lanier, 2008b). The alternative way of NK cell activation is elicited through their surface receptors to detect the changes in virus-infected cells including presence of viral proteins, increase of cell stress proteins and the combination of viral peptides with MHC class I molecules. On human and mouse NK cells, there are three distinct receptor families: human killer-Ig-like receptors (KIRs), murine Ly49 lectin-like receptors, and NKG2 lectin-like receptors found in both species. These receptors can be functionally stimulatory or inhibitory depending on the intracellular signals and cell status elicited following ligand binding. To prevent NK cells from exerting cytoxicity to normal cells, the inhibitory aspect is dominant; seemingly the absence of inhibitor signaling allows activation to kill. Hence, the activation (or inhibition) of NK cells is a fine-tuned balance of stimulatory and inhibitory signals received during interaction of NK and target cells (Lanier, 2008b; Vivier et al., 2008). Several TLRs have been detected in NK cells (**Table 1.2**) (Al-Khatib et al., 2004; Alter et al., 2007; Saikh et al.,

2003), but the question of how TLR-mediated viral molecule recognition contributes to NK cell activation is still open. Treatment of NK cells with viral mimics of the ligands to TLR3, TLR7, TLR8 or TLR9 were reported to activate NK cell cytokine production and cytotoxicity to tumors or virus-infected cells (Alter et al., 2007; Lauzon et al., 2006; Sivori et al., 2004, 2007; Sawaki et al., 2007). In some cases, aggregation of TLRs by their ligands appeared efficient to activate NK cells, but in some other experiments, TLR-engagement had to be combined with existence of pDCs or cytokines such as IL-12 to effectively activate NK cells (Girart et al., 2007). One plausible explanation for this difference is that the status of NK cells varied among samples, such as difference in animal donors and expression levels of different receptors (Sivori et al., 2007).

The overwhelming evidence about the importance of NK cells in antiviral resistance has been well reviewed elsewhere (Biron et al., 1999; Walzer et al., 2005; Golden-Mason et al., 2006; Lee et al., 2007; Lanier, 2008a; Vivier et al., 2008) and can be grouped in two categories. The first group manifests that NK cell-mediated cytotoxicity and cytokine production are stimulated in some viral infections and NK cell deficiencies resulting from genomic defects or purposeful depletion increase host susceptibility to a wide range of virus infections. The incomplete list of viruses reported includes about 30 viruses in 12 virus families (Biron et al., 1999; Lee et al., 2007). The second category of evidence indicates that NK cell receptors, including KIRs, Ly49s and NKG2 in humans and mice, interact with several viral proteins, and this interaction leads to resistance to the particular virus in addition to NK cell activation (Lee et al., 2007). The first effector aspect of NK cells is production of cytokines especially IFN-γ, which exerts direct inactivation activity toward many viruses and enhances antiviral states in adjacent and distal cells. Second, NK cells mediate killing of target cells through releasing and delivering cytolytic

granules, which contain pore-forming proteins of perforin and granzyme, into target cells, in turn triggering a cell death process. Thus, NK cell-promoted death process on target cells could be a "specific" killing mechanism of innate immunity to combat intracellular pathogens like viruses.

Granulocytes: With the presence of cytoplasmic granules and multi-lobed nuclei, granulocytes constitute a major portion (70%) of white blood cells and are the first group of cells migrating to the sites of infection (Hartenstein, 2006). Neutrophils, the primary subgroup of granulocytes, are highly motile phagocytic cells that provide early defenses against infectious microorganisms and orchestrate the inflammatory response (Segal, 2005). Neutrophilic killing was previously thought to be caused mainly by reactive oxygen intermediates generated through oxidase-related mechanisms (NADPH oxidase and myeloperoxidase). This concept is now expanded with the new insights of the following five points (Appelberg, 2007; Segal, 2005). (1) The activity of the membrane-associated NADPH oxidase promotes trans-membrane movement of ions to form an alkaline and hypertonic condition in phagocytic vacuoles for activation of granule contents (Appelberg, 2007; Segal, 2005). (2) The primary neutrophil killing mechanism is the granule polypeptide contents released into phagocytic vacuoles including neutrophil elastase, cathepsin G and antimicrobial peptides (Borregaard et al., 2007). (3) Both NADPH oxidase activity and granule peptide contents (especially the neutral proteases) are required for microbial killing (Appelberg, 2007; Segal, 2005). (4) Neutrophil extracellular traps (NET), formed by activated neutrophils attaching DNA backbones from rapidly dead cells (mainly neutrophils) plus embedded antimicrobial peptides and enzymes, function in filtering and killing pathogens in inflammation sites (Wartha et al., 2007). (5) A series of receptors and

transmembrane proteins have been recently found in secretory vesicles (eg. TLRs and cytokine receptors) and granules (eg. pentraxin 3 in specific granules) of neutrophils (Borregaard et al., 2007; Haselmayer et al., 2006; Hattermann et al., 2007; Lee et al., 2006), and this indicates that neutrophils routinely communicate with the microenvironment and other cells like pDCs in mediation of immune responses (Ludwig et al., 2006). Neutrophils are essential for resistance to bacterial and fungal infections; however, the direct involvement in antiviral infection is not well documented. The emerging role of neutrophils is reflected in antiviral activity of granule antimicrobial peptides, such as human neutrophil α-defensins (HNPs) and non-human primate θ-defensins (Buck et al., 2006; Chang et al., 2004, 2005; Wang W et al., 2003, 2004; Wu et al., 2005). Besides its main activity against parasitic helminthes, emerging evidence indicates another type of granulocyte, eosinophils, also have an *in vivo* protective role against RNA viruses, such as respiratory syncytial virus (RSV) and pneumonia virus of mice (PVM) (Rosenberg and Domachowske, 2001; Rothenberg and Hogan, 2006). Interestingly, eosinophil granules contain abundant eosinophil-associated ribonucleases (EARs) that may degrade single-stranded RNA containing viruses. Consistent with the feature of some innate immune genes (e.g. HDPs), humans and mice have very diverse genomic sequences encoding multiple isoforms of EARs, potentially targeting different species of ssRNA viruses (Rosenberg and Domachowske, 2001; Rothenberg and Hogan, 2006).

1.4. Cell-Based Viral Recognition Mechanisms

1.4.1. Toll-Like Receptors (TLRs) and TLR-Dependent Viral Recognition

Host cells use various receptors to perceive viral infections by recognizing pathogen-associated molecular patterns (PAMPs) and subsequently induce antiviral responses (Akira et al.,

2006; Kabelitz and Medzhitov, 2007; Pichlmair and Reis e Sousa, 2007). Prominent among these receptors are Toll-like receptors (TLRs), which are vertebrate homologues revealed and named after *Drosophila* Toll receptors (Lee and Kim, 2007; Takeuchi and Akira, 2007; West et al., 2006). TLRs are critical for innate immune recognition and for inducing immune responses to most microorganism-caused infections (Beutler, 2004; West et al., 2006). The progress of mammalian genome projects indicates that each mammalian species has approximately 10 TLRs, which are functional for detection of a multitude of molecular ligands derived from various microorganisms as "danger signals" of infections (Gay and Gangloff, 2007; West et al., 2006). Six of these TLRs have been implicated in response to viral infection through sensing viral components (Akira et al., 2006; Pichlmair and Reis e Sousa, 2007; Takeuchi and Akira, 2007). Among them, TLR2 and TLR4 hinged on cell cytoplasmic membranes were found to recognize several viral proteins (Bieback et al., 2002; Kurt-Jones et al., 2000; Rassa et al., 2002); and especially the functional group of TLR3, TLR7, TLR8 and TLR9 was characterized to sense viral nucleic acid, either virus-derived RNA or DNA molecules (Alexopoulou et al., 2001; Diebold et al., 2004, 2006; Forsbach et al., 2008; Gay and Gangloff, 2007; Heil et al., 2004; Hemmi et al., 2002; Lund et al., 2004). Accordingly, these nucleic acid-sensing TLRs are responsive mainly in acidified intracellular compartments including late endosomes and lysosomes, where most viruses undergo a de-coating process during infection (**Table 1.3**) (Pichlmair and Reis e Sousa, 2007). All TLRs belong to a family of class I transmembrane receptors. Each TLR consists of an extracellular domain (ectodomain) to form a ligand-binding structure, a membrane-spanning α-helix to hinge on the membrane, and a cytoplasmic Toll-interleukin receptor (TIR) domain to transduce postreceptor signaling *via* interaction with cytoplasmic adaptor proteins (Lee and Kim, 2007; West et al., 2006).

TLR2 and TLR4: Prominently implicated in perceiving ligands derived from bacteria, fungi and stressed host cells, TLR2 and TLR4 also have been demonstrated to mediate antiviral responses *via* detection of viral proteins (Gay and Gangloff, 2007). In the reports, hemagglutinin (H) protein of wild-type measles virus induced the production of proinflammatory cytokines such as interleukin-6 (IL-6) in a TLR2-dependent manner in both human and murine monocytes. It was shown that wild-type H protein did not induce IL-6 release in macrophages from TLR2-deficient mice, and mutation of a single amino acid (asparagine at position 481 to tyrosine) of wild-type H protein abolished its ability to activate TLR2 (Bieback et al., 2002). Infection by lymphocytic choriomeningitis virus (LCMV) was recently demonstrated to induce the production of chemokines, such as MCP-1, RANTES and TNF-α in a similar TLR2-dependent manner in glial cells of the central nervous system (CNS). In this case, mice deficient in TLR2 or its downstream adaptor proteins (MyD88 and Mal described late) did not produce any of these chemokines upon LCMV infection; however, LCMV induced a similar chemokine response in both TLR3 and TLR4 knockout glial cells (Zhou et al., 2008). On the other hand, TLR4 was found to detect viral fusion protein and to mediate innate immune responses to human respiratory syncytial virus (RSV) (Kurt-Jones et al., 2000). TLR4 is also capable of detecting envelope proteins of retroviruses including mouse mammary tumor virus (MMTV) and murine leukemia virus (MLV) and, thereby, mediating murine B-cell or dendritic cell activation (Burzyn et al., 2004; Rossa et al., 2002). One notable phenomenon is that activation of TLR2/4-dependent signals by viral proteins not only induces immune protection in host cells but also may be exploited by viruses to augment infection through upregulating viral receptors on infected cells,

which were indicated in interactions of TLR2-measles virus and TLR4-MMTV (Bieback et al., 2002; Burzyn et al., 2004).

TLR3: As a set of universal viral PMAPs**,** double stranded (ds)-RNA is produced either as an intermediate of viral replication or as part of the viral RNA genome (Jacobs and Langland, 1996). TLR3 was the first TLR implicated in antiviral responses (Alexopoulou et al., 2001). Besides its high preference for recognition of synthetic RNA analogs (e.g. polyinosinic acid-cytidylic acid (poly(I:C) and especially Ampligen [poly(I)-poly($C_{12}U$)]) (Gowen et al., 2007), TLR3 recognizes viral dsRNAs derived from dsRNA viruses (such as reovirus), ssRNA viruses (such as West Nile virus, respiratory syncytial virus, hepatitis C virus and encephalomyocarditis virus) or DNA viruses (such as herpes simplex virus) (Schröder et al., 2005; Vercammen et al., 2008). Whereas there is no doubt about TLR3 recognition of dsRNA, the immune protective role of TLR3 in viral infection is controversial and dependent on different virus-host cell interactions.

TLR3 is expressed in the respiratory tract, and its expression is markedly upregulated in epithelial cells by infection of either influenza A virus or respiratory syncytial virus (RSV). In wild-type mice, pulmonary upregulation of TLR3 post influenza A infection caused acute pneumonia, whereas TLR3-decifient mice developed less severe pneumonia and had an unexpected survival advantage despite higher viral titer in lungs of TLR3-decifient mice. Thus, murine TLR3 signaling contributes to pulmonary clearance of influenza A virus but meanwhile results in detrimental inflammation (Guillot et al., 2005; Le Goffic et al., 2006). In contrast, TLR3 signaling is necessary to maintain proper immune protection in RSV infection because TLR3-deficient mice accumulate much higher T helper 2 cytokines resulting in mucus

overproduction in lungs, a pathological feature of RSV infection (Groskreutz et al., 2006; Rudd et al., 2006). TLR3 has been shown to be expressed in glial and neuronal cells and proposed to be involved in intrinsic antiviral mechanisms in the central nervous system. During infection with influenza A virus, TLR3 showed a protective role of ameliorating influenza A virus-induced encephalopathy, as symptoms are profoundly developed in the patients with a missense mutation of the TLR3 protein (Hidaka et al., 2006). However, TLR3-dependent inflammatory responses were shown to facilitate West Nile virus (WNV) disruption of the blood–brain barrier and spread from the peripheral system into the brain. Once inside the brain, WNV infection initiated TLR3-independent detrimental responses causing lethal encephalitis (Wang T et al., 2004). In skeletal and cardiac muscle infected by encephalomyocarditis virus, dsRNA released from infected dying cells engaged TLR3 leading to cross-priming of myeloid dendritic cells and activation of cytotoxic T cells. All of this plus IFN-β release from infected cells positively affected the infection by encephalomyocarditis virus (Le Bon et al., 2003). Indeed, compared to wild-type mice TLR3-deficient mice were more susceptible to encephalomyocarditis virus infection and had a significantly higher viral load in the heart (Hardarson et al., 2007). Similarly, dsRNA activation of TLR3 in the female genital tract was implicated in protection against herpes simplex virus type 2 infection (Ashkar et al., 2004). Again, the protective role of TLR3 signaling is controversial in virus-caused liver and kidney diseases. TLR3 upregulation was associated with excessive production of IL-6 in liver infected with a hepatotropic phlebovirus (Gowen et al., 2006). The over-production of IL-6 was detrimental to the liver and associated with viral etiology for some hepatitis infections. In addition, a significant increase of TLR3 mRNA was associated with hepatitis C virus-positive kidney inflammation (i.e. glomerulonephritis) possibly

resulting from stimulation of local synthesis of chemokines including RANTES and monocyte chemotactic protein 1 (MCP1) (Wornle et al., 2006).

Apart from the paradox of TLR3 in response to viral infections, it is clear that TLR3 signaling is critical for mediation of a multitude of viral diseases despite leading to pathological complications in some cases. The importance of TLR3 signaling is shown by viruses which have evolved multiple ways to modulate TLR3 signaling (Schröder et al., 2005; Vercammen et al., 2008). Several viruses, including measles virus, influenza A virus, hepatitis C virus (HCV), RSV, HIV and SIV, augment TLR3 expression and sometimes modulate TLR3 cellular localization (Groskreutz et al., 2006; Sanghavi et al., 2005; Tanabe et al., 2003; Vercammen et al., 2008). This kind of modulation of TLR3 was hypothesized to sensitize cells to subsequent viral or dsRNA exposure, which may skew TLR3 signaling to induce pathological inflammation in addition to antiviral responses (Vercammen et al., 2008). Interestingly, some virus-derived mechanisms, including vaccinia virus A46R (Stack et al., 2005) and A52R (Harte et al., 2003) proteins and HCV NS3/4A protease (Li et al., 2005), directly interfere with components in the TLR3-signaling pathway leading to suppression of IFN synthesis, thereby to allow escaping from the host immune response. On the other hand, a RNA-dependent RNA polymerase NS5B of HCV functioned to induce IFN-β production probably through catalyzing synthesis of dsRNA to engage TLR3 in host cells. This viral "trick" is thought to facilitate maintenance of a low and nonlethal level of HCV, which may distract the host defense system and enable persistent infection (Naka et al., 2006). Therefore, host-beneficial TLR3 signaling is case-dependent. To make the issue more complicated, cellular location of TLR3 is dependent on different cell types, such as localizing on the cell surface of fibroblasts and endosomes in cDCs; and TLR3 location, which affects TLR3 signaling, is modulated by some viruses (Groskreutz et al., 2006; Sanghavi

et al., 2005; Tanabe et al., 2003; Vercammen et al., 2008). Clearly, we have a long way to go before comprehension of the role of TLR3 signaling in virus-host interaction and manipulation of this process to augment host immune responses.

TLR7-9: TLR7, 8 and 9 belong to the same functional subfamily based on similarities in their genomic sequences, cellular locations and interacted agonists. TLR7 and 8 are closely related and colocalized on X chromosomes in some mammalian species including humans, pigs and cattle, which have both functional TLR7 and 8. TLR7 and 9 are mainly expressed in pDCs, neutrophils and eosinophils; in contrast, TLR8 mRNA is highly expressed in cDCs, monocytes and macrophages. Before the identification of viral ligands, murine TLR7 and human TLR7 and TLR8 were found to recognize imidazoquinoline compounds, such as imiquimod (R837) and resiquimod (R848), and guanosine analogs such as loxoribine, which have potent antiviral and/or anti-tumor activities (Akira et al., 2006; Hemmi et al., 2002; Ito et al., 2002). Recent evidence showed that human and murine TLR7 mediates pDC responses to ssRNA viruses including HIV, vesicular stomatitis virus (VSV), Sendai virus and influenza virus (Diebold et al., 2004; Lund et al., 2004) and to genomic ssRNA purified from influenza virions (Diebold et al., 2004; Diebold et al., 2006; Heil et al., 2004). TLR7 engaging activity by these viral ssRNA has been reproduced with synthetic ssRNA oligonucleotides mimicking guanosine (G)/uridine (U) repeats in viral genomes. These U or GU repeats derived from viral RNA genomes were also extensively analyzed as agonists for human TLR8 in PMBCs (Forsbach et al., 2008). In contrast, TLR9 in pDCs detects unmethylated CpG motifs in DNA viruses such as adenovirus, HSV-1 and -2 or murine cytomegalovirus (MCMV). The CpG-containing DNA of HSV-2 engages TLR9 signaling to induce IFN-α production in murine pDCs. TLR9-deficient mice lose resistance to

MCMV infection, suggesting that TLR9 signaling is responsible for antiviral responses by sensing the CpG-containing DNA of DNA viruses (Hochrein et al., 2004; Iacobelli-Martinez and Nemerow, 2007; Krug et al., 2004a, 2004b; Tabeta et al., 2004).

1.4.2. TLR-Mediated Antiviral Signaling Transduction

TLR ectodomains contain multiple leucine-rich repeats forming hosrseshoe-shaped solenoids of ligand-binding structures. Different composition of LRR motifs on each TLR seems to create unique ligand specificities of respective receptors, such as LRR 12 and 20 in TLR3 forming a convex loop suitable for dsRNA binding (Gay and Gangloff, 2007). In order to conform into a functional ligand-binding pocket, two (or three in TLR1/2/6) homogenous or heterogeneous TLR molecules may undergo multimerization upon ligand binding. The intermolecular combination expands the capacity of TLRs to detect numerous PAMPs, and cooperation among dimers may significantly increase ligand specificity/affinity over a mono-TLR. In addition, several membrane-anchored proteins including MD2, CD14, CD36 and dectin-1 have been implicated as co-receptors to TLRs such as TLR2, 3, and 4 and may function in membrane-targeting and enhancing ligand-binding (West et al., 2006) (**Table 1.4**). Ligand-TLR interaction promotes TLR dimerization and formation into a functional complex, which thereby induces conformation changes in the cytoplasmic TIR domains to recruit adaptor proteins. Functional TIR domains are essential for TLR signaling, as site-mutation (e.g. Pro^{712} in murine TLR4) and sequence truncation of TIR domains eliminate the capability of murine TLR3 and TLR4 to recruit downstream adaptor proteins including myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TIR-domain-containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM). Dependent on

different adaptor proteins attached to the TIR domain, the so called MyD88-dependent and – independent (mainly TRIF-dependent discussed herein) signaling transduction pathways are hereby bifurcated respectively leading to cytoplasmic signaling cascades and nuclear responsive gene expression (**Fig. 1.3** and **Table 1.4**) (Gay and Gangloff, 2007; Lee and Kim, 2007; West et al., 2006).

MyD88-dependent signaling: MyD88-deficient mice and cells showed drastically decreased or ablated responses to ligands of TLR2, 4, 7/8, and 9, indicating that these TLRs signal exclusively through the MyD88-dependent pathway. The adaptor proteins like MyD88 contain a C-terminal TIR domain, a sequence similar to TIR domains of TLRs, and a N-terminal death domain. Therefore, the recruitment of MyD88 to a TLR cytoplasmic tail leads to a homotypic association between their TIR domains. The death domain of MyD88, thereby, interacts with the IL-1 receptor–associated kinase-4 (IRAK-4) and recruits and phosphorylates IRAK-1. This promotes IRAK-1 autophosphorylation and further recruits tumor necrosis factor receptor–associated factor-6 (TRAF6) to the MyD88/IRAK-4/IRAK-1 complex. Activated IRAK-1 and TRAF6 subsequently dissociate from the receptor complex and interact with additional molecules including transforming growth factor-β-activated kinase-1 (TAK1), which leads to the activation of c-Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK). The downstream transcription factors including activator protein-1 (AP-1), NF-κB and IRF5 are activated, which translocate into nuclei and promote the transcription of genes encoding proinflammatory cytokines and chemokines such as TNFα, IL-6, IL-8, IL-12 and IL-1β (Lee and Kim, 2007; West et al., 2006). Thus, the current revealed signaling stream of MyD88-dependent pathway flows through: PAMP/TLR → MyD88/IRAK4/(IRAK1)/(TRAF6) → TAK1 →

JNK,IKK,p38 → AP-1, NF-κB, IRF5 → proinflammatory cytokines, where the complex of signaling components are clustered together and separated with slashes and the components in the parentheses may disassociate from the complex to activate downstream signaling. In the case of TLR7/8/9, two signaling streams are bifurcated after TRAF3/TRAF6 joining in the MyD88 complex thus leading to transcription factor activation. These two signaling streams are through PAMP/TLR → MyD88/IRAK4/(IRAK1)/(TRAF6)/(IRF5) → IRF5, AP-1, NF-κB → proinflammatory cytokines, and through PAMP/TLR → MyD88/IRAK4/(IRAK1)/(TRAF3)/IKKα/(IRF7) → IRF3/7 → type I IFNs (Lee and Kim, 2007; West et al., 2006) (**Fig. 1.3** and **Table 1.4**).

TRIF-dependent signaling: The fact that stimulation of TLR3 and TLR4 in MyD88-deficient cells results in IRF3 activation and delayed NF-kB activation *via* TLR4 signaling, lead to the identification of TRIF and TRIF-dependent signaling pathways. Although TLR4 uses this as an alternative pathway, TRIF is the only adaptor protein identified for TLR3 signaling to induce type I IFN production and exert antiviral responses. After association with TIR domains upon TLR3 or TLR4 stimulation, TRIF mediates distinct signaling *via* its N- or C-terminal motifs. Its N-terminal region was indicated to form a complex with two noncanonical IκB kinases, IKKi/IKKε and TRAF family-member-associated NF-κB activator (TANK)-binding kinase (TBK-1). This TRIF-kinase complex promotes appropriate phosphorylation and activation of associated IRF3, which is in turn translocated into nuclei to induce IFN-β production. On the other hand, the C-terminal domain of TRIF may associate with a receptor-interacting protein 1 (RIP1) (Meylan et al. 2004). RIP1 is suggested to link TRIF to TRAF6 and thus to TAK1, and this cascade is indicated to be critical for TLR3-mediated signaling to NF-κB activation which

leads to cytokine production (Lee and Kim, 2007). The two other identified TLR adaptor proteins, TIRAP and TRAM, have been shown to mediate TLR2/4 signaling, but there is a lack of information about their involvement in antiviral responses (Lee and Kim, 2007; West et al., 2006).

1.4.3. TLR-Independent Viral Recognition and Signaling Transduction

Besides TLR-mediated viral recognition mainly in endosomal or lysomal compartments, animal cells also posses a set of viral recognition and signaling mechanisms in cytosol, where most viruses carry out their infectious cycle (Lee and Kim, 2007; Pichlmair and Reis e Sousa, 2007). Four cytosol PRRs including retinotic acid inducible gene I (RIG-I), melanoma differentiation factor-5 (MDA5) and laboratory of genetics and physiology-2 (LGP2), which all recognize virus derived-RNA, and a cytosol dsDNA sensor named DNA-dependent activator of IRFs (DAI), have been identified (Lee and Kim, 2007; Pichlmair and Reis e Sousa, 2007; Takaoka et al., 2007). All of these cytosol receptors perceive distinct molecules of virus-derived nucleic acids and signal the production of IFNs including IFN-α, IFN-β and IFN-λ (Onoguchi et al., 2007; Takaoka et al., 2006, 2007). In addition, a cytoplasmic serine and threonine kinase PKR, previously identified to sense dsRNA and mediate NF-kB activation (Kumar et al., 1994; Williams, 2001), has not been convincingly demonstrated to have a role in induction of IRFs and production of type I IFNs (Honda et al., 2003; Pichlmair and Reis e Sousa, 2007; Smith et al., 2001) (**Fig. 1.4**).

RIG-I, MDA5 and LGP2 have been classified into the RIG-like receptor (RLR) family. RIG-I contains an RNA-binding helicase domain, two caspase activation and recruitment

domains (CARDs) and a C-terminal repression domain (RD). Likewise, MDA5 bears a helicase domain and two CARDs, but it lacks the C-terminal RD. In contrast, LGP2 only has a helicase domain but lacks CARDs (**Fig. 1.4**). Current evidence illustrates an interplay of these cytosol RLRs in signaling IRF activation and IFN production (Pichlmair and Reis e Sousa, 2007). Whereas the helicase domains of these RLRs bear the function of RNA detection and ligand specificity, the CARDs function positively, verses RD negatively, in activation of IRFs and IFN production. In this context, MDA5 recognizes viral dsRNA and induces the production of IFN-α and -β upon overexpression (Saito et al., 2007). The function of RIG-I seems to be achieved by intramolecular domain interactions, showing that the C-terminal RD functions as an internal repressor on exposure of CARD. The agonist interaction with RIG-I helicase domain induces protein multimerization and conformational change which relieves the autorepression of RD on CARD (Saito et al., 2007). In addition, mutated RIG-I with an impaired CARD domain acted as dominant negative proteins to suppress wild type RIG-I in IFN-induction (Yoneyama et al., 2004). Likely, LGP2, which lacks CARD, resembles that of the RIG-I helicase domain to interfere other RLRs signaling IFN induction in some cases (Rothenfusser et al., 2005; Venkataraman et al., 2007; Yoneyama et al., 2005).

Previously, the appearance of all RLRs being capable of recognizing synthetic dsRNA (poly I:C) leads to an ambiguous interpretation of how RIG-I and MDA5 distinguish different virus infections (Lee and Kim, 2007; Pichlmair and Reis e Sousa, 2007). However, it has been found recently that each RLR may have remarkable ligand specificity upon different virus-derived RNAs. Deficiency of RIG-I greatly reduced cell responses to influenza A virus, vesicular stomatitis virus (VSV), Japanese encephalitis virus (JEV), and Sendai virus (SEV), whereas

MDA5-deficient cells lost responsiveness mainly to picornaviruses (e.g. encephalomyocarditis virus (EMCV), Theiler's encephalomyelitis virus, and mengovirus) as well as JEV and SEV (Diao et al., 2007; Gitlin et al., 2006; Kato et al., 2006). This suggests that RIG-I and MDA5 are capable of distinguishing PAMP from different RNA viruses as well as recognizing some common patterns shared by JEV, SEV or poly(I:C). Recently, it was elucidated that the critical determinant for RIG-I stimulation is the presence of triphosphates at the 5' end of RNA, independent of single or double strandedness (Hornung et al., 2006; Pichlmair et al., 2006). Indeed, ssRNA genomes of influenza and VSV or *in vitro* delivered dsRNA which bear 5' triphosphates activate RIG-I (Hornung et al., 2006; Pichlmair et al., 2006). In contrast, genomes of picornaviruses in which no triphosphates are attached at the 5' ends, are weak activators of RIG-I. Compared to RIG-I, it is unknown if there is an extra molecular determinant for MDA5 to sense dsRNA from picornaviruses (Pichlmair et al., 2006; Weber et al., 2006). Besides virus-derived RNAs, there are also dsDNA derived from DNA viruses or bacteria that also induce IFN-α and -β production (Ishii et al., 2006; Stetson and Medzhitov, 2006). Correspondingly, one cytosolic dsDNA sensor has recently been termed as DNA-dependent activator of IRFs (DAI, previously known as the IFN-inducible protein DLM-1 or Z-DNA binding protein 1) (Takaoka et al., 2007). Recent evidence shows that the binding of DAI to dsDNA potentiates signaling of TBK-1 to IRF3, the critical components in the RLR-signaling pathway (**Fig. 1.4**) (Takaoka et al., 2007).

Mainly illustrated in RIG-I- and MDA5-signaling, RLR recognition of ligands leads to activation of their CARD domains through ubiquitination. The activated CARD thereby interacts with IFN-β promoter stimulator-1 (IPS-1), which is a mitochondrion-associated adaptor protein

used by RIG-I and MDA5 but not by LGP2 and DAI (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). IPS-1 contains an N-terminal CARD that interacts with the CARDs of RIG-I and MDA5 through homotypic matching. This results in activation of the C-terminal catalytic domain and initiation of a signaling cascade including activation of the IKK and TBK-1 kinase family which phosphorylate and activate IRF7 and/or IRF3 (Seth et al., 2005; Sun et al., 2006; Xu et al., 2005). The signaling cascades culminate in induction of type I IFN gene expression (Honda et al., 2005). Activated IPS-1 also regulates mitochondria-mediated apoptosis *via* the RIP-1 involved pathway; the process has been implicated in determination of cell death upon viral infections (Festjens et al., 2008; Takahashi et al., 2006). In contrast, current evidence does not support that LGP2 and DAI use IPS-1 to interplay in RLR-signaling, likely they adopt other mechanisms to target the components of TBK-1 and IKK in this cascade (**Fig. 1.4**) (Ishii et al., 2006; Kumar et al., 2006; Pichlmair and Reis e Sousa, 2007; Stetson and Medzhitov, 2006; Sun et al., 2006).

1.4.4. Porcine TLR-Dependent and -Independent Viral Recognition

TLRs are conserved in pigs as indicated by the gene sequences of porcine TLR1-10 (Shinkai et al., 2006a, 2006b; Sang et al., 2008). Porcine TLR-2 and TLR-9 have been identified and demonstrated to be highly expressed in gut-associated lymphoid tissues (GALT) including ileal Peyer's patches and mesenteric lymph nodes (MLN) (Shimosato et al., 2005; Tohno et al., 2005, 2006). The expression of some TLRs and their function in stimulation of IFN production was partially examined in monocytes and monocyte-derived dendritic cells after treatment with synthetic poly (I:C) and CpG oligos (Raymond and Wilkie, 2005). Along with the identification of porcine TLRs, MyD88 has also been studied in TLR2 signaling (Tohno et al., 2007). In addition, the porcine RNA helicase RIG-I has been identified and shown to be differentially

expressed in PRRSV present tissues (Zhang et al., 2000). Despite some evidence from TLR stimulation with agonists of poly (I:C) or CpG oligos, little is known about how TLR or RLR signaling mediates antiviral responses in pigs.

1.5. Antiviral Effectors of the Innate Immune System

Two major groups of innate immune effectors, type I interferons (IFNs) and host defense peptides (HDPs), and their role in antiviral responses will be discussed in this section. Please also refer to a review paper for extensive discussion of porcine HDPs (Sang and Blecha, 2008).

1.5.1. Major Innate Antiviral Cytokines --Type I Interferons

Type I IFNs are a group of innate immune effectors prominent in eliciting antiviral responses. In contrast to type II IFN (IFN-γ alone), type I IFNs comprise multiple subtypes including IFN-α, IFN-β, IFN-ε , IFN-ω, and IFN-κ (Pestka, 2007; LaFleur *et al*., 2001). Humans have multiple IFN-αs, and single members of IFN-β, IFN-ε , IFN-ω, and IFN-κ (Takaoka and Yanai, 2006). Type I IFNs also include IFN-δ (Lefevre *et al*., 1998), -τ and -ζ (limitin) (Oritani *et al*., 2000), which are only detected in pigs or cattle (IFN-δ), ruminants (IFN-τ) and mice (IFN-ζ), respectively (Takaoka and Yanai, 2006) (**Table 1.5**). In pigs, type I IFNs consist of multiple porcine IFN-α, IFN-ω, and IFN-δ like molecules, such as porcine IFN-α which are encoded by as many as fifteen functional genes (Cheng et al., 2006; Sang, unpublished data). In addition, pigs have single gene loci encoding either IFN-β, IFN-ε and IFN-κ (Artursson et al., 1992; Sang, unpublished data). In humans and mice, ubiquitously expressed IFN-α/β are among the most studied subtypes in antiviral responses. Although less extensively

studied, the tissue/cell-specific expressed subtypes, such as IFN-ω in various leukocytes, IFN-δ/ε in female reproductive tissues (Demmers et al., 2001; Lefevre et al., 1998) and IFN-κ in epidermal keratinocytes, are potentially induced by viral infection in these cell types and further confer an antiviral state on uninfected cells (Pestka, 2007; Takaoka and Yanai, 2006).

Type I IFNs, especially IFN-α/β, are central cytokines in antiviral innate immunity. First, almost all known viral recognition and signaling pathways, particularly TLR- and RLR-mediated, lead to activation of genes encoding IFN-α/β in virus-infected cells. Local production of type I IFNs around infection sites comprises a major antiviral barrier to inactivate viruses and limit virus spreading. The direct antiviral activity of type I IFNs has been well documented by the development of various IFN-based antiviral therapies which are effective against many viral diseases including viral hepatitis, HIV and severe acute respiratory syndrome (SARS) (Deutsch and Hadziyannis, 2007; Haagmans and Osterhaus, 2006; Loomba and Liang, 2007; Sulkowski and Benhamou, 2007; Yu et al., 2007). Type I IFNs, produced during the early phase of virus-cell interaction, not only activate antiviral responses by autocrine means, but also diffuse or transmit systemically to induce an antiviral state in surrounding and distal cells. The induction of the antiviral state, which includes suppression of cellular metabolic processes such as protein synthesis and profound expression of genes encoding antiviral products (Haller and Weber, 2007; Zuniga et al., 2007), is critical for developing effective immune protection against viral infections. Type I IFNs collectively induce antiviral responses through a common receptor composed of two subunits, IFNAR-1 and IFNAR-2 (**Table 1.5**). However, the efficacy of induction of antiviral responses appeares different among subtypes and even members belonging to the same subtype, such as human IFN-αs varying in their activity to activate human NK cells and IFN-β showing more potency than IFN-α2 in inhibition of monocyte proliferation (Takaoka

and Yanai, 2006 García-Sastre and Biron, 2006). Functional differences among type I IFNs is related to their diverse affinities and kinetics in their interaction with IFNAR subunits. In addition, differential expression of each type I IFN and receptor subunits in regard to tissue/cell types also contributes to distinct regulation of antiviral responses (Uzé et al., 2007).

The interaction of type I IFNs with their receptors leads to activation of transcription factors of STATs (Signal Transducers and Activators of Transcription protein) by two IFNAR-associated kinases (**Fig. 1.5**). The activated STAT1, STAT2 and IRF9 thereby form an activator complex of IFN-activated trimeric transcription factor, ISGF3, which interact with the IFN-stimulated response element (ISRE) in promoters of IFN-stimulated genes (ISGs) to prompt transcription. Hundreds of ISGs have been revealed with various functions such as direct virus targeting (e.g. MxA, RNase L and RNA deaminases), amplifying antiviral resistance (e.g. PKR, 2'-5'OAS, and type I IFN themselves), and sequestration of cellular metabolic processes to repress virus replication (e.g. PKR-mediated arrest of protein synthesis) (Sadler and Williams, 2007; Sen and Sarkar, 2007). Profiles of most ISGs are overlapping with the responsive genes stimulated by dsRNA or other viral PMAPs, which further indicates an IFN-centered antiviral loop jointly evoked by several antiviral signal pathways (**Fig. 1.5**) (Pichlmair and Reis e Sousa 2007; Sen and Sarkar, 2007).

Type I IFN-centered innate immune reactions are critical for animal antiviral immunity. Whereas deficiency in IFN signaling results in hosts highly susceptible to viral infection, viruses *per se* must overcome IFN defensive systems to obtain successful infection. Thus, it is notable that most notorious viruses have evolved capabilities to evade or subvert the IFN-I system for their own benefit. Extensive reviews (Haller and Weber, 2007; Iannello et al., 2006; Li and Ding, 2006; Loo and Gale, 2007) on this topic indicate that a collection of virus-derived factors may

interfere with major virus recognition signaling pathways leading to IFN production or IFN-stimulated pathways resulting in antiviral gene expression. Examples of hepatitis C virus (HCV) and influenza A virus (IAV) are summarized here to evoke the aspects in PRRSV studies. Several proteins encoded by HCV genome have been implicated in interfering with IFN production or signaling. The non-structural protein NS3/4A of HCV has a protease activity and was implicated in abolishing both RIG-I and TLR3 mediated induction of type I IFNs through cleavage of IPS-1 and TRIF, the adaptor proteins of some RLRs and TLR3 respectively (Li et al., 2005). In chronic HCV infected patients, NS3/4A cleavage of IPS-1 was shown to closely correlate with suppression of ISG expression; conversely, targeting inhibition of NS3/4A protease activity restored IFN signaling (Loo and Gale, 2007). The viral core protein and NS5A protein of HCV have the ability to stimulate the activity of cellular protein phosphotase 2A (PP2A), leading to dephosphorylation of STAT1 (Thimme et al, 2006) and alteration of MAPK and JNK signaling (Loo and Gale, 2007; Thimme et al, 2006). In addition, HCV E2 and NS5A proteins were found to work as inhibitors of PKR. Collectively, HCV exploits multiple strategies to modulate IFN production/signaling thus enhancing the formation of a persistent infection, a major feature of most epidemic viral diseases (Haller and Weber, 2007; Loo and Gale, 2007). In regard to IAV, the virus encodes a NS1 protein to bind dsRNA thus antagonizing dsRNA signaling *via* TLR3 and MDA5. IAV strains with mutated NS1 protein impaired in dsRNA binding activity, resulted in increased cellular IFN response, and had attenuated pathogenicity (Fernandez-Sesma, 2007). Because of its capacity for interaction with dsRNA, NS1 protein of IAV also blocks cellular pre-mRNA processing and impairs antiviral function of several dsRNA-regulated ISG products such as PKR, 2'5'-OAS, and RNaseL. Therefore, NS1 protein is one crucial virulent factor for IAV infection (Fernandez-Sesma, 2007; Haller and Weber, 2007; Loo

and Gale, 2007). It is known that PRRSV also frequently causes chronic infection. Emerging evidence indicates that PRRSV may employ a protease activity of NSP2 to evade host innate immunity (Frias-Staheli et al., 2007; Rowland, 2007). In addition, PRRSV was recently shown to inhibit IFN induction *via* interfering with IPS-1 and TRIF, the adaptor proteins of RIG-I and TLR3-signaling pathways, respectively (Luo et al., 2008).

Besides type I IFNs, the single type II IFN-γ, mainly produced by activated T cells and NK cells, is thought to be much more related to adaptive antiviral immunity (Takaoka and Yanai, 2006 García-Sastre and Biron, 2006). Three novel IFN-λ1 to -λ3, previously known as IL-29, IL-28A and IL-28B respectively, have been recently classified as type III IFNs. Despite exploiting a distinct receptor system (**Table 1.5**) to exert antiviral function, type III IFNs, similar to IFN-α/β, were shown to be up-regulated through the RIG-I signaling pathway during viral infection (Onoguchi et al., 2007). Notably, although several signaling pathways (i.e. TLR-, RLR-, and IFN-signaling) lead to the production of proinflammatory cytokines and type I IFNs, these innate antiviral responses are finely checked. It was recently shown that interaction of protein phosphatase SHP-1 with kinase cascade (e.g. IRAK1) in TLR and RLR signaling pathways provides reciprocal regulation in balancing type I IFN production (**Fig. 1.5**) (An et al., 2008; Miyake et al., 2008). This regulation is crucial, because if unchecked (e.g. by SHP-1 mutation) or distorted (e.g. by chronic viral infection) over-production of type I IFNs or other cytokines results in dangerous immunopathological conditions in hosts such as autoimmune diseases, which is another important immunological issue of type I IFNs (Banchereau et al., 2006; Crow et al., 2007; Tourbah et al., 2007).

1.5.2. Host Defense Peptides in Innate Antiviral Immunity

Host defense peptides (HDPs) are another group of important innate immune effectors (**Fig. 1.1** and **Fig. 1.2**) (Sang et al., 2008 and references therein). Although the antiviral activity of HDPs has been noted for some time (Daher et al., 1986), research in this area has recently intensified (Klotman and Chang, 2006; Lehrer, 2007). Using direct inactivation assays, several recent studies have demonstrated direct effects of LL-37, the only human cathelicidin, in reducing infectivity of herpes simplex virus 1 (HSV-1) and replication of lentivirus (Steinstraesser et al., 2005). Human α−defensins, mainly members of neutrophil α−defensin (HNP) 1 to 4, have been shown to inhibit entry and/or postentry events of viruses including HIV, HSV, IAV, adenovirus, papillomavirus and a rhabdovirus (Buck et al., 2006; Chang et al., 2005; Falco et al., 2007; Furci et al., 2007; Hazrati et al., 2006; Salvatore et al., 2007; Smith and Nemerow, 2008; Wang W et al., 2004). Inhibition HIV by HNPs was related to direct interaction of these peptides to viral envelope glycoprotein gp120 and cellular viral receptor CD4, as well as indirect inhibition of cellular protein kinase C (PKC) signaling and CD4 expression (Chang et al., 2005; Furci et al., 2007). Evidently, the presence of serum neutralized the direct effect of HNP-1 in inactivation of HIV infection suggesting a lectin-dependent property of HNPs; and the indirect suppression of HIV replication in cells by HNP-1 could be reproduced using the treatment of a PKC inhibitor and reversed by a PKC activator (Chang et al., 2005). Similarly, HNP-1 inhibited IAV infection mainly *via* indirect suppression of cellular PKC activity (Salvatore et al., 2007), and HNPs closely interplayed with other antiviral mechanisms such as SP-D at the IAV infection sites (Hartshorn et al., 2006). All six human α−defensins (HNP1-4 and human enteric α−defensins HD5 and 6) were effective at blocking HSV infection, but differ

in their capacities to bind viral envelope and receptor proteins. Hazrati et al. (2006) showed that HNP1-3, and HD5 bound HSV glycoprotein B with high affinity, but had minimal binding to heparan sulfate, the receptor for HSV attachment to cells. In contrast, HNP-4 and HD6 bound heparan sulfate but not glycoprotein B. Through interaction with virion proteins or other membrane-independent mechanisms, HNPs hamper both adenovirus (Smith and Nemerow, 2008) and papillomavirus (Buck et al., 2006) escape from endocytic vesicles thereby blocking virus infection. Leukotriene B4 (LTB4), a potent lipid activator for neutrophils, induced antiviral activity in blood leukocytes. It was shown that, HNPs and LL-37 contributed to the critical part of antiviral activity induced by LTB4 (Flamand et al., 2007; Gaudreault and Gosselin, 2007). In addition, HNPs released from activated neutrophils in turn increase neutrophil uptake of IAV and augment phagocytosis-accompanying oxidative burst (Tecle et al., 2007). Human β-defensins (hBDs), hBD-2 and -3 are increased in airway epithelial cells infected by human rhinovirus (HRV), showing that an increase of hBD2 mRNA correlated with viral titer and the nasal lavages of HRV infected patients had increased levels of hBD-2 protein (Duits et al., 2003; Proud et al., 2004). In addition, an increase of hBD2 production in airway epithelial cells stimulated with IL-17A, a cytokine activator for T cells, was thought to contribute to IL-17A modified anti-HRV response in concert with other cytokines (Wiehler and Proud, 2007). Exposure of primary oral epithelial cells to HIV-1 increased expression of hBD-2 and -3 mRNA 4- to 78-fold, respectively. No hBD-1 upregulation in the epithelial cells by HIV-1 exposure was observed. Consistently, recombinant peptides of hBD-2 and hBD-3 showed dose-dependent *in vitro* inhibition of HIV-1 replication and *in vivo* suppression of HIV intracellular replication (Quiñones-Mateu et al., 2003; Sun et al., 2005). Furthermore, hBD3 was shown to block HIV replication *via* direct interaction with virions and through internalization of cellular receptor

CXCR4 to dampen HIV binding (Feng et al., 2006). In addition, crosslinking of hBD3 with IAV membrane glycoprotein impaired the viral infection by blocking the virus fusion with the cell membrane (Leikina et al., 2005). Most recently, circular θ-defensins, such as retrocyclin (RC)-1 and -2, showed potential anti-HIV activity *via* interaction with the viral envelope protein gp41 (Cole and Cole, 2008; Gallo et al., 2006; Wang W et al., 2003, 2004). RC-1 and its D-amino acid derivative, RC-112, protected human cells from infection by 30 primary HIV-1 isolates (Owen et al., 2004). RC-112 was several times more potent than RC-1, likely because of the resistance to degradation of the D-amino acid-backbone (Owen et al., 2004). In addition, RC-2 has been shown to suppress the infection of several viruses including HSV, IAV, Sindbis virus and baculovirus (Yasin et al., 2004; Leikina et al., 2005). Besides the antiviral activity, cytotoxicity to host cells is also a critical factor influencing the therapeutic potential of HDPs (Hancock and Sahl, 2006). Most of above studies indicate that human cathelicidin LL-37, hBD-2 and -3, and retrocyclins, have no or tolerable cytotoxicity to host cells (Leikina et al., 2005; Sun et al., 2005). However, most α-defensins are toxic to host cells as well as viruses. One porcine cathelicidin, protegrin-1, has shown antiviral activity comparable to LL-37, however, it is cytotoxic to host cells as well (Steinstraesser et al., 2005). To our knowledge, protegrin-1 is the only porcine HDP that has been reported to have antiviral activity, albeit to viruses of no importance to the swine industry. In summary, animal HDPs have the potential to distort virion glycoproteins and lipid membrane in enveloped viruses, and to impede viral fusion with and entry into host cells. Other mechanisms for HDP antiviral activity have also been proposed, including downregulation of viral receptors (eg. hBD3 for CXCR4 of receptor to HIV-1) (Feng et al., 2006), modulation of cellular antiviral signaling (eg. HNP-1 for PKC signaling) (Chang et al., 2005; Salvatore et al., 2007), and potentiation of adaptive immunity (Yang et al. 2004).

1.5.3. Multifunction Property of Host Defense Peptides (HDPs) and Relationship to IFN Signaling

Besides direct inactivation of virions, HDPs have been shown to exert profound immunoregulatory functions that may link to IFN-signaling and production of cytokines including TNF-α, IL-8 and IL-18 (Braff et al., 2005; Yang et al., 2007). For example, human LL-37 mediates IL-8 and IL-18 production in keratinocytes, airway epithelial cells and monocytic cells (Bowdish et al., 2004; Braff et al., 2005; Mookherjee et al. 2006; Niyonsaba et al., 2005; Tjabringa et al., 2003). The process is mediated by p38 and other MAPK cascade possibly involving some Toll-like receptors (TLRs) (Bowdish et al., 2004; Mookherjee et al. 2006). On the one hand, HDPs such as murine β-defensin 14 (mBD14), an ortholog of hBD3, could be induced by dsRNA in dendritic cells (Röhrl et al., 2008); other β-defensins such as hBD2 and hBD3 have been reported to modulate TLR7 expression (Stroinigg and Srivastava, 2005) and to activate dendritic cells *via* TLR1/2 (Funderburg et al. 2007). Prominently, HNP-1 was recently reported to impede a rhabdovirus infection through inactivation of virus particles and induction of a type I IFN response (Falco et al., 2007). Therefore, interplay of HDPs with IFN-signaling is emerging *via* expression of some antiviral HDPs being regulated by TLR signaling and some HDPs at infection sites will directly inactivate viruses and augment IFN production (**Fig. 1.5**). It is noteworthy that both unchecked production IFNs and deviant induction of HDPs may lead to detrimental effects to the host (e.g autoimmune diseases) (Crow et al., 2007; Tourbah et al., 2007; Lande et al. 2007). For instance, abnormally high expressed LL-37 at skin lesions of psoriatic patients acts as a key factor to trigger autoimmune reaction in psoriasis by converting self-DNA into a potent agonist for TLR9 (Lande et al. 2007). Likewise,

higher risk of psoriasis in a subgroup of people was shown to have significant association with aberrantly multiplied genomic copy numbers of β-defensin genes (Hollox et al. 2008). Although it is not known how β-defensin is involved in psoriasis, potential links to IFN-signaling may be logical. Clearly, the beneficial immune reaction of both IFNs and HDPs requires appropriate control of expression.

1.6. Determining Adaptive Immune Responses

Despite arbitrary separation in mammals, innate and adaptive immunity comprise a virtual continuum to interactively protect hosts. Whereas most invading microorganisms are halted at the level of innate immunity, pathogen-specific adaptive immunity is developed in mammals to eliminate the most dangerous pathogens and to mount immune memory to effectively prevent infection in the future (Hoebe et al., 2004; Kabelitz and Medzhitov, 2007). Activation of adaptive immunity in antiviral responses is manifested mainly by elevation of humoral neutralizing antibodies and cytotoxic T cells to target viruses or virus-infected cells. Although still largely unknown, accumulated evidence implicates the importance of innate immune components in shaping adaptive immunity. First, adjuvant and potentiation effects are served by innate immune molecules. The action of innate immune cells (plus humoral innate immune cascades esp. complement cascade (**Fig. 1.1**)) leads to processing of pathogenic molecules and secretion of a multitude of immune regulators including cytokines, chemokines, HDPs and other molecules conveying "activator signaling" (**Table 1.1**) (Hoebe et al., 2004; Kabelitz and Medzhitov, 2007). For antiviral responses, prominent examples are pDCs, which are characterized as natural IFN-α producers (Szabo and Dolganiuc, 2008), and pulmonary alveolar macrophages, which are implicated as primary IFN-α producers in murine models upon

respiratory viral infections (Kumagai et al., 2007). The *in situ* formation of a chemical gradient of chemokines recruits leukocytes to infection sites collectively during the inflammatory process, and elevated IFNs in the peripheral system may signal immune cells in distant lymphoid tissues. Second, engagement and activation of antigen-presenting cells (APCs) are the major players directing adaptive immune cells. Innate immune cells include both professional (i.e. DCs) and non-professional (e.g. macrophages) antigen-presenting cells (APCs). One critical part during an innate immune reaction is engagement of MHC II class moluceles with antigenic peptides and upregulation of co-stimulatory molecules on APCs. The process is associated with directly sensing viral PMAPs by APCs *via* TLRs and stimulated by IFNs produced from other innate immune cells. The activated APCs will migrate to the cortex of lymphoid tissues to educate the differentiation and maturation of T/B cells (Hoebe et al., 2004; Kabelitz and Medzhitov, 2007). Third, B cells and T cells themselves also express innate immune receptors such as TLR receptors. Collectively, human naïve B cells express a low amount of most TLRs; however, abundant TLR9 along with TLR10 is significantly upregulated on activated B cells and expressed at constitutively high levels in memory B cells. TLR9 activation promotes proliferation and differentiation of both naïve and memory B cells (Gerondakis et al., 2007). In T cells, stimulation of TLR2, 3, and 9 was indicated to act as co-stimulatory receptors subsidiary to T-cell receptor (TCR) to enhance proliferation and/or cytokine production of T lymphocytes (McGettrick and O'Neill, 2007). In addition, TLR2, TLR5 and TLR8 may modulate the suppressive activity of a special group T regulatory (Treg) cells (Kabelitz, 2007; McGettrick and O'Neill, 2007).

1.7. PRRSV: An Immunnological Challenge

Porcine reproductive and respiratory syndrome virus (PRRSV), belonging to newly defined arteriviridae in the order of nidovirales, causes an economically significant pandemic viral disease in pigs (Neumann et al., 2005). PRRSV is an enveloped virus, which has a positive-sense, single-stranded RNA molecule that is 14.5 kb containing nine open reading frames (ORF) encoding as many as nine viral proteins including a membrane-spanning (M) protein, nucleocapsid (N) protein, glycoprotein (GP)-5 and non-structural protein (NSP)-2. The primary infection of PRRSV is through alveolar macrophages or other tissue macrophages, followed by spread from the lungs to the rest of the body *via* peripheral circulation (viremia). A second route of PRRSV infection initiates from the reproductive tract by insemination with infected sperm further spreading from sows to newborns. Immunity to PRRSV begins with the interaction between the virus and porcine cells, predominately pulmonary alveolar macrophages (PAMs) and intravascular macrophages of the placenta and umbilical cord (Oleksiewicz and Nielsen, 1999; Riber et al., 2004; Thanawongnuwech et al., 2000). PRRSV frequently causes persistent or repeated infection in susceptible pigs and herds. Pigs vaccinated with one serotype are generally not protected against infection by heterogonous strains indicating high genomic diversity among PRRSV isolates (Labarque et al., 2004). PRRSV infection stimulated much less IFN-α production than did porcine coronavirus or swine influenza virus in the lungs (van Reeth et al., 1999). Different isolates of PRRSV are diverse in their ability to induce IFN-α, IL-10 and IL-12 in lung or PAMs; and a weakened IFN response plus an IL-10 increase may contribute to immune modulation by some viral isolates (Lee et al., 2004; Diaz et al., 2006). Down-regulation of type I IFNs and other early inflammatory cytokines represents one of early consequences from the initial PRRSV-host cell interaction, which probably leads to inappropriate stimulation of antiviral immune responses and results in persistent viral infection. Recent evidence indicates

that PRRSV uses NSP2-derived protease activity to alter host innate immunity (Frias-Staheli et al., 2007; Rowland, 2007), and the virus was also shown to intervene in dsRNA-stimulated signaling leading to weaker IFN induction (Luo et al., 2008). PRRSV infection of monocyte-derived DCs decreases the expression of MHC molecules thus compromising antigen presenting activity of DCs to activate T cells (Loving et al., 2007; Wang et al., 2007). Considering adaptive immunity to PRRSV, paradoxical responses include (1) circulating antibodies to PRRSV are detectable in 1-2 weeks post infection but the antibodies are not efficient at neutralizing the virus (Yoon et al., 1994; Mateu and Diaz, 2007); (2) although early upregulation of IFN-γ production was detected, an increase of IFN-γ-secreting cells (mainly $CD4^{+}CD8^{+}$-T cells) was much weaker and delayed (Meier et al, 2003; Mateu and Diaz, 2007). In summary, optimal immune protection was not achieved to eradiate the virus from infected pigs (Wang et al., 2007; Xiao et al., 2004). Despite significant efforts to identify immunogenic viral epitopes (de Lima et al., 2006; Plagemann, 2006; Zhou et al., 2006) and to develop and optimize vaccines with various adjuvants, effective means to control this disease have not been achieved (Charerntantanakul et al., 2006; Royaee et al., 2004). Therefore, direct investigation and manipulation of IFN-centered antiviral signaling of porcine innate immunity may provide novel strategies to prevent this economically significant swine disease.

1.8. Concluding Remarks

Innate immune mechanisms, comprising receptors, effectors and intervening signaling cascades, protect most species from virus infection. The functional conservation and expansion of innate immune components in higher vertebrates, further indicates the integral role of animal innate immunity in both early protection and overall orchestration of systemic antiviral

responses. The discoveries of type I IFNs and signaling connections to upstream PRRs and downstream IFNARs have opened our understanding about cellular innate immune networks to combat viral infection. Whereas increasing insights in mice and humans will reveal more information about innate immune mechanisms in antiviral responses, it is imperative to extend these findings to other mammalian species. Studies in domestic animals will not only expand our understanding about the repertoire of innate immune receptors and effectors such as IFNs and HDPs, which serve as paratypes for developing novel antimicrobials, but may also directly suggest therapeutic approaches to prevent epidemic viral diseases in animals and zoonotic viral diseases important for both animals and humans. In addition, pigs are increasingly used as animal models for human diseases and for xenotransplantation (Cooper et al., 2007; Lunney, 2007). Thus, direct information about the porcine innate immune system, i.e. the receptors and effectors and regulation over integral immune responses, will be beneficial for developing novel antimicrobial therapies and controlling immune rejection during xenotransplantation.

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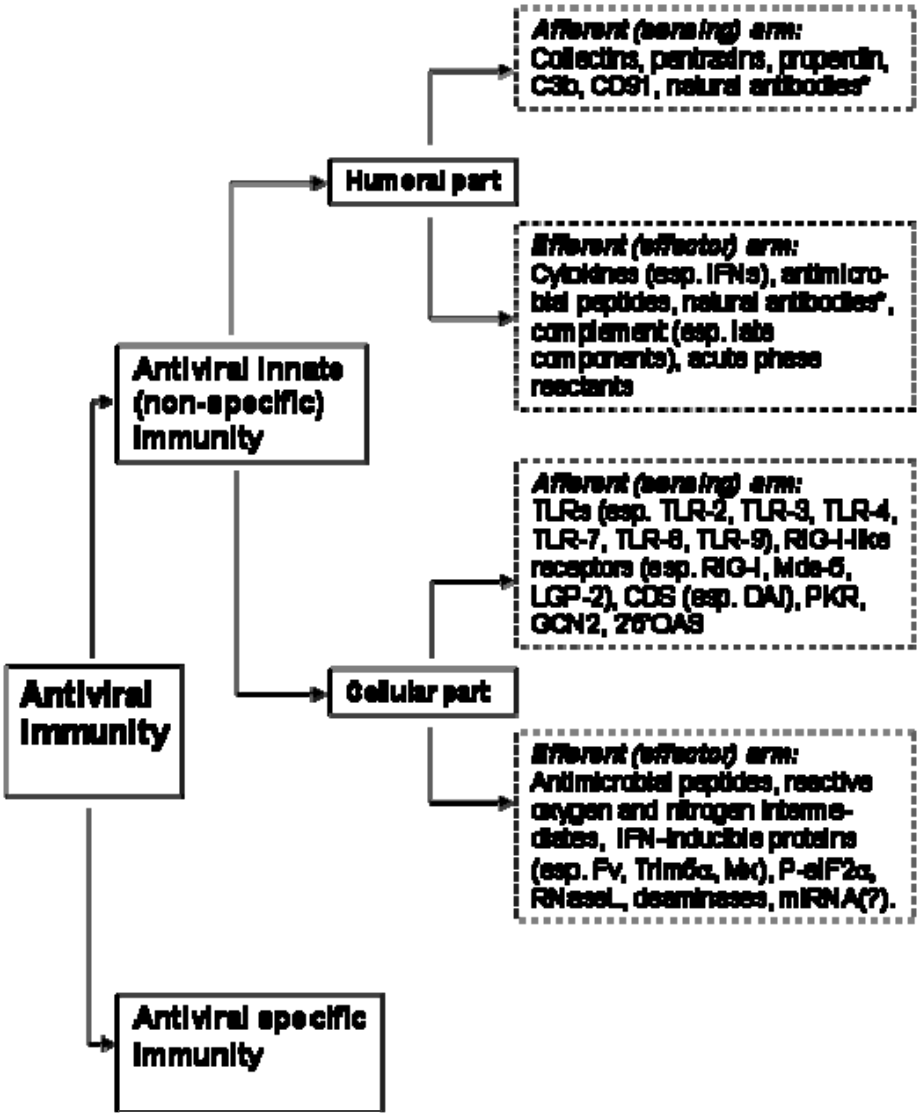

Fig. 1.1. The afferent and efferent arms of the innate antiviral immune response. Lists of potential cellular and humoral components of each arm are illustrated. *Natural antibodies belong to the "innate" part of specific immunity. (Abbreviations: CDS, cytosolic DNA sensor; DAI, DNA-dependent activator of IFN-regulatory factors; GCN2, general control nonderepressible-2; LGP-2, laboratory of genetics and physiology 2; Mda-5, melanoma differentiation-associated antigen 5; 2'5'OAS, 2',5'-oligoadenylate synthetase; PKR, protein kinase R; RIG-I, retinoic acid-inducible gene I; TLR, toll-like receptor; TRIM5α, tripartite motif protein).

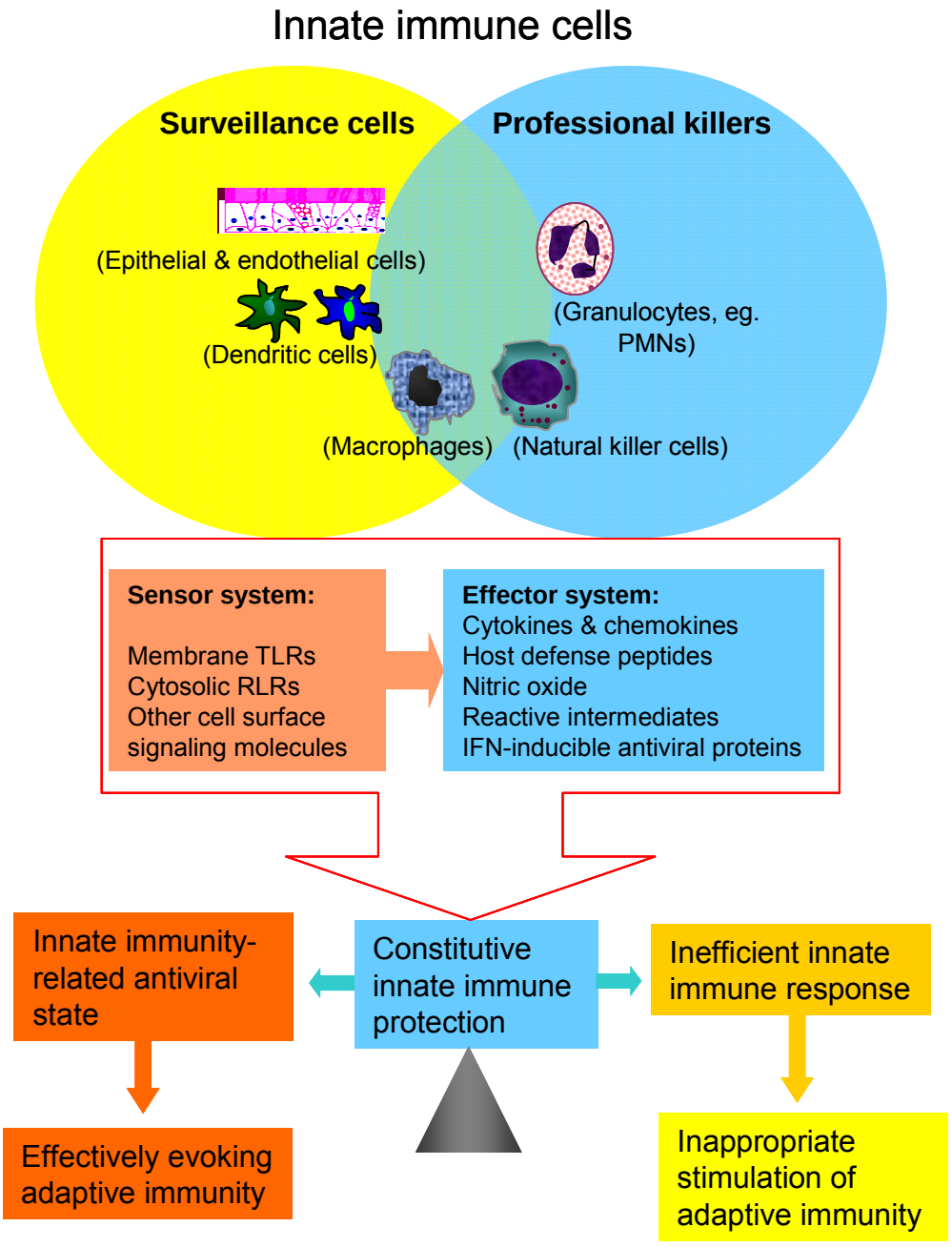

Fig. 1.2. Diagram of innate immune cells and their functional systems in antiviral immune responses. Innate immune cells are arbitrarily distributed by their preference in immune surveillance (yellow circle) or killing (blue circle). The afferent and efferent components interplay to direct innate immune responses and bridge adaptive immunity in antiviral responses. RLR, retinoic acid-inducible gene I-related receptors; TLR, Toll-like receptors.

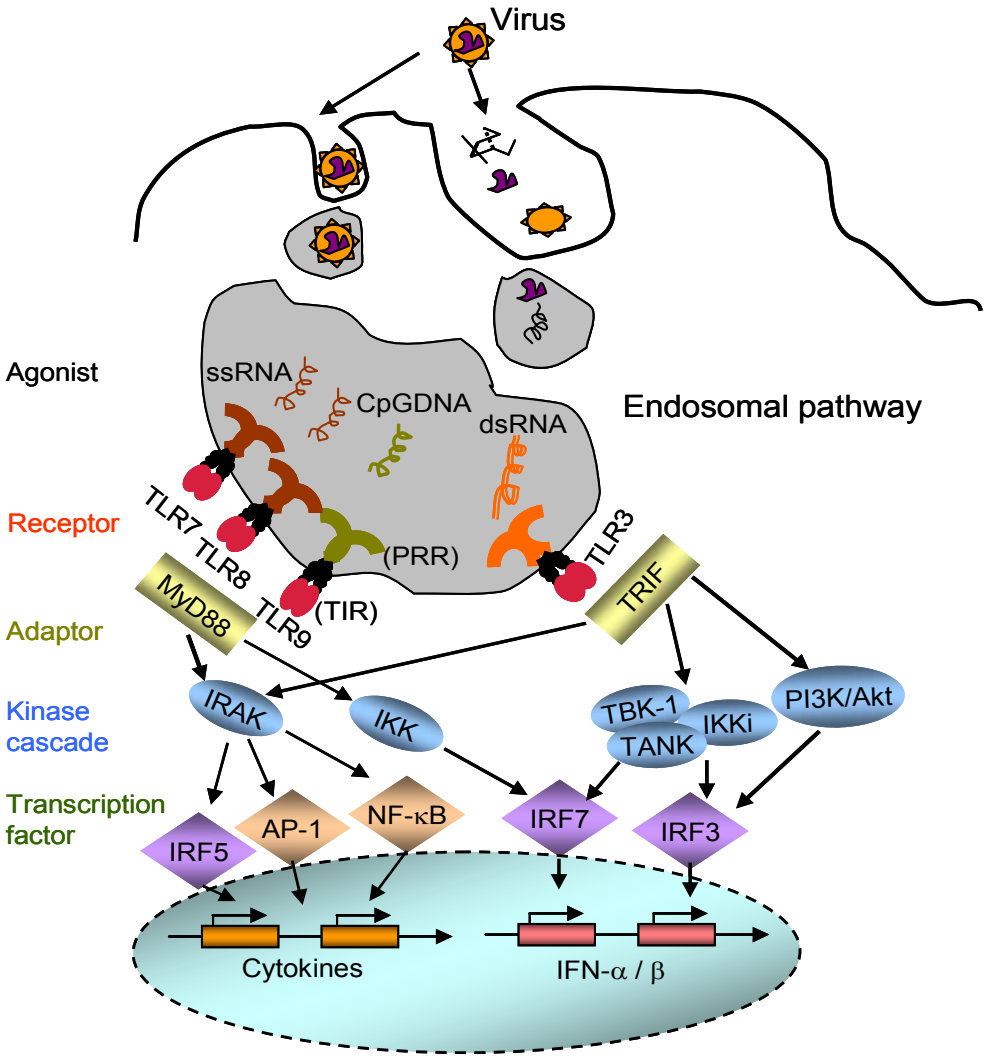

Fig. 1.3. TLR-dependent antiviral signaling. Viral responsive TLRs, mainly TLR3, TLR7-9 are illustrated from agonist recognition, adaptor recruitment, main kinase cascade to induction of transcription factors and gene expression. Please refer the abbreviations from legend of Fig. 1.1 and Table 1.4.

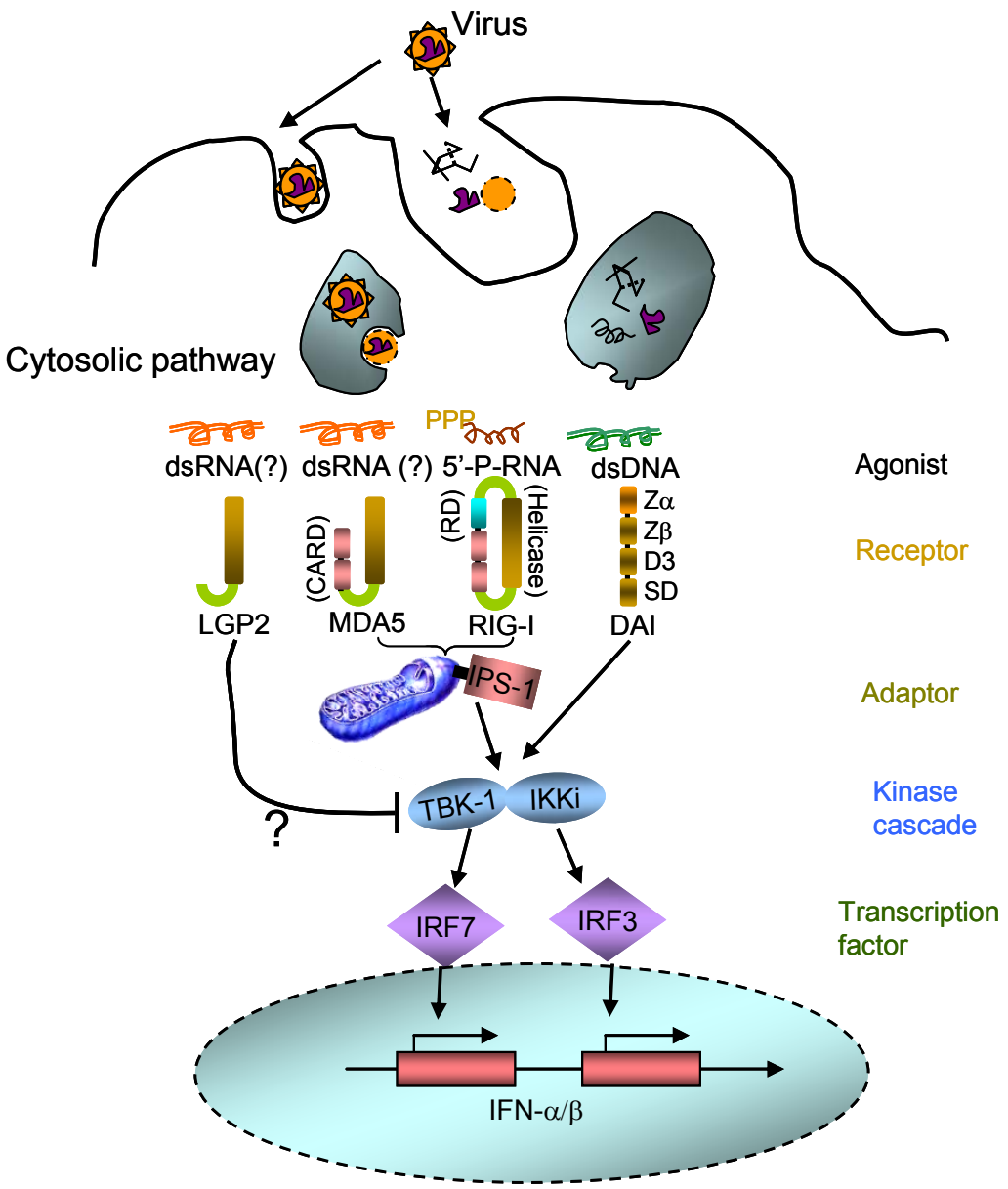

Fig. 1.4. RLR-dependent antiviral signaling. Viral responsive RLRs and their main domains are illustrated with major events of agonist recognition, adaptor recruitment, kinase cascade to induction of transcription factors and gene expression. Please refer the abbreviations from legend of Fig. 1.1 and Table 1.4.

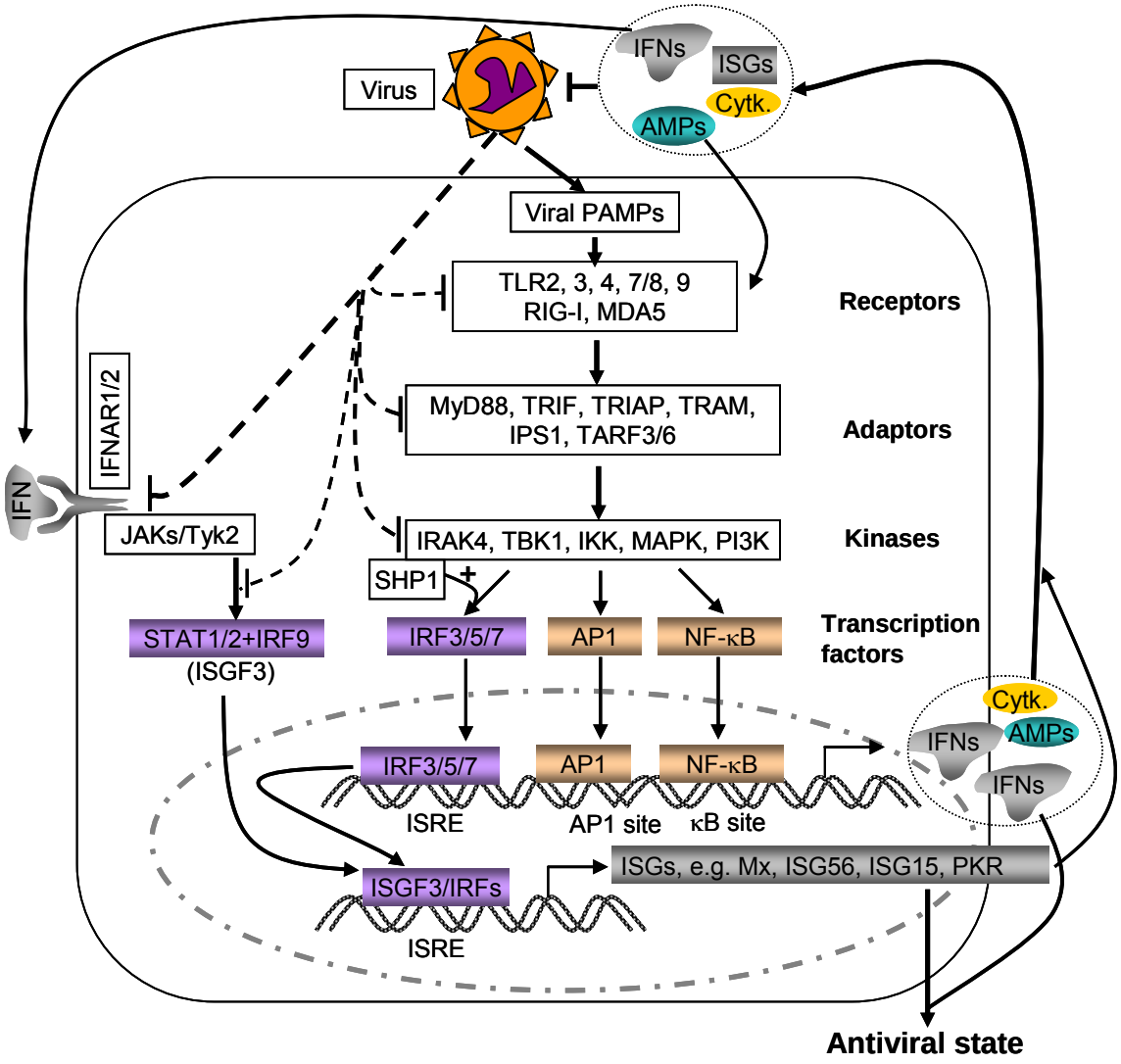

Fig. 1.5. Interplay of IFN-centered innate antiviral signaling. Viral infection is sensed by cell-based receptors such as TLRs and RLRs. Antiviral effectors including IFNs, HDPs and proinflammatory cytokines are shown. The antiviral effectors interact to inactivate viruses and re-modulate signaling leading to IFN expression/signaling loop. The production of IFNs in turn induces the expression of hundreds of ISGs (including IFNs themselves) to establish an antiviral state. Please refer the abbreviations from legend of Fig. 1.1 and Table 1.4.

Table 1.1. Antiviral effectors and receptors in innate immune cells.

Cell types	Viral PRRs	Antiviral effectors	References
Epithelial cells	**TLRs***: TLR-2, -3, -4, -7/-8, and -9 **RLRs**: DAI, RIG-I, Mda-5, PKR	AMPs, type I IFNs, proinflammatory cytokines (such as IL-1α/β, IL-6, IL-11, IL-16, TNF-α, and GM-CSF), chemokines (such as IL-8, RANTES, and MIP-1α), collectins, nitric oxide, IFN-inducible proteins	Behera et al., 2002 Fu et al., 1999 Message et al., 2004 Schleimer et al., 2007 Takaoka et al., 2006
Endothelial cells	**TLRs:** TLR-2, -3, -4, and -9(?) **RLRs**: RIG-I(?), Mda-5(?)	AMPs (?), type I IFNs, IL-8, TNF-α, proinflammatory cytokines, IFN-iducible proteins, nitric oxide	Chaudhuri et al., 2007 Frantz et al., 2005 Opitz et al., 2007 Warke et al., 2003
Dendritic cells (Conventional DC (cDC) and plasmacytoid DC (pDC))	**TLRs:** TLR-2, -3, -4, -7/-8, -9 **RLRs**: RIG-I**, Mda-5**	Autophagocytic activity related (cDC), type I IFNs (pDC), IL-12 (pDC), defensins (immature DC)	Barchet et al., 2005 Kato et al., 2006 Rodríguez-García et al., 2007 Takeuchi et al., 2007 Zilliox et al., 2006
Macrophages	**TLRs**: TLR-2, -3, -4, -7/-8, -9 **RLRs**: DAI, RIG-I, PKR, Mda-5	Oxidative related: reactive oxygen or nitrogen intermediates Nonoxidative related: Phagocytic and endocytic related (esp. pH gradient, lysosomal enzymes), AMPs and overall macrophage antiviral factors (MAFs) (incomplete list including type I IFNs, IL-10, TNF-α, and chemokines of RANTES, MCP-1, MIP-1α and MIP-1β).	Beisswenger et al., 2005 Daffis et al., 2007 Fantuzzi et al., 2003 Kumagai et al., 2007
Natural killer cells	**TLRs**: TLR-2, -3, -4, -8	Two-receptor model promoted granule exocytosis to kill target cells, production of INF-γ, TNF-α, and GM-CSF.	Alter et al., 2007 Saikh et al., 2003
Neutrophils	**TLRs:** TLR-2, -4, TLR-7/-8, -9	Activated killing system including a variety of granule microbicidal proteins, and hypertonic and alkaline condition made by oxidative burst	Borregaard et al., 2007 Hattermann et al., 2007 Lee et al., 2006 Segal, 2005

* Primarily exemplified with features of cells in human or mouse respiratory tracts. TLR, Toll-like receptors; RLR, RIG-I-like receptors including retinoic-acid-inducible protein I (RIG-I), melanoma differentiation-associated protein 5 (Mda-5), DNA-dependent activator of interferon-regulatory factors (DAI), and protein kinase R (PKR).
** Mainly in cDCs not in pDCs.

Table 1.2. Examples of viruses that infect alveolar macrophages.

Hosts/Viruses (Genome, family)	PRRs possibly related*	Mechanism and effect on type I IFN production*	Evading innate immunity?	References
Human respiratory syncytial virus ((-)ssRNA, Paramyxoviridae)	TLR3, TLR4, TLR7, TLR9, RIG-I, PKR	NS1, esp. NS2 gene products inhibit IFN activity	Yes	Groskreutz et al., 2006 Kotelkin et al., 2006 Liu et al., 2007 Schlender et al., 2005, 2006 Tulic et al., 2007
Human (pig) Influenza (Segmented (-) RNA, Orthomyxoviridae)	Multiple TLRs and RLRs in respect to cell types	NS1 gene product inhibit IFN secretion and activity	Yes	Koyama et al., 2007 Seo et al., 2004 Tyner et al., 2005 van Riel et al., 2006
Human severe acute respiratory syndrome ((+)ssRNA, Coronaviridae)	?	A papain-like protease inhibits IFN secretion via interfering IRF3	Yes	Devaraj et al., 2007 Shieh et al., 2005
Human immuno-deficiency virus-1 ((+)ssRNA, Retroviridae)	TLR2, TLR3, TLR4, TLR7, TLR8, TLR9	Suppression via undermining immune function of macrophages	Yes	Ieong et al., 2000 Martinelli et al., 2007 Sanders et al., 2008 Thibault et al., 2007
Parainfluenza/mouse (ssRNA, Paramyxoviridae)	Multiple TLRs and RLRs in respect to cell types	Sendai virus C protein blocks signal trans-duction of IFN	Yes	Bousse et al., 2006 Carey et al., 2007 Kato et al., 2005 Melchjorsen et al., 2005,
Adenovirus/mouse (dsDNA, Adenoviridae)	Some TLRs and RLRs in respect to cell types	Induction	?	Nociari et al., 2007 Tyner et al., 2005 Zhu et al., 2007
Porcine pseudorabies virus (dsDNA, Herpesviridae)	?	Suppressed in PAM, and induction in pDC	?	Iglesias et al., 1992 Vincent et al., 2006
Porcine African swine fever virus, ((+)ssRNA, Flaviviridae)	?	Strain-dependent suppression	Yes	Afonso et al., 2004 Zhang et al., 2006
Porcine circovirus-2 (ssDNA, Circoviridae)	Possible TLR7 and TLR9	Suppression	Yes	Chang et al., 2006 Vincent et al., 2006
Porcine foot-and-mouth disease virus ((+)ssRNA, Picornaviridae)	?	Suppression	Yes	de Los Santos et al., 2006, 2007 Rigden et al., 2002
Porcine arterivirus (PRRSV) ((+)ssRNA, Arteriviridae)	TLR3 and RLRs**	Delayed and weakened	Yes	Luo et al., 2008 Mateu et al., 2007

* Based on information from AMs and other cells; ** Based on studies mainly in this dissertation.

Table 1.3. Viral or synthetic ligands for Toll-like receptors (TLRs) and non-TLR receptors

Receptors	Ligands	Origin of ligands	Reference
TLRs			
TLR2	Hemagglutinin	Measles virus	Bieback et al., 2002
TLR3	Poly (I:C)*	Synthetic compounds	Alexopoulou et al., 2001
	dsRNA	Virus	
TLR4	Fusion protein	Respiratory syncytial virus	Kurt-Jones et al., 2000
	Envelope proteins	Mouse mammary tumor virus	Rassa et al., 2002
TLR7	GU-rich ssRNA	Virus	Heil et al. 2004 Lund et al. 2004
	Imiquimod (R-848)	Synthetic compounds	Hemmi et al., 2002
TLR8	GU-rich ssRNA	Virus	Heil et al. 2004
	R-848	Synthetic compounds	Jurk et al., 2002
TLR9	Unmethylated CpG DNA	Bacteria, virus, yeast, insects	Gay and Gangloff, 2007
Non-TLRs			
RIG-I	5'-PPP-ssRNA/dsRNA	Virus	Yoneyama et al., 2004
Mda5	dsRNA	Virus	Yoneyama et al., 2005
LGP2	dsRNA	Virus	Yoneyama et al., 2005
DAI	dsDNA	DNA viruses	Takaoka et al., 2007

***Abbreviation:** DAI, DNA-dependent activator of IRFs; LGP2, laboratory of genetics and physiology-2; Mda5, melanoma differentiation factor-5; Poly (I:C), polyriboinosinic:polyribocytidylic acid; RIG-I, retinoic acid inducible gene I. Modified mainly from the references of Gay and Gangloff, 2007; Pichlmair and Reis e Sousa, 2007.

Table 1.4. Key components in TLR-mediated antiviral signaling cascades

	TLR2/4	TLR3	TLR7/8/9
Agonists	Viral proteins	Viral/synthetic dsRNAs	Viral ssRNAs or um-CpG DNA
Co-receptors	**?TLR2:** homo-/heterodimers, CD14 *or CD36 **?TLR4:** homo-/heterodimers, MD2, or CD14	Homodimer and CD14	Homodimer
Adaptors	**TLR2:** MyD88, TIRAP **TLR4:** MyD88, TIRAP, TRIF, TRAM	TRIF	MyD88
Protein kinases and associated proteins	**MyD88-dependent (TLR 2 or 4):** IRAK4, TRAF6, IRAK1, TAK1, IKKγ, JNK, p38, ERK **MyD88-independent (TLR4):** RIP1, TRAF6, TRAF3, TBK1/IKKi, TAK1,	**TRIF-dependent:** RIP1, PI3K**,** TBK1/IKKi	**MyD88-dependent:** IRAK4, IRAK1, TRAF3, TRAF6, TAK1, MAPK/IKKα,
Transcription factors	**MyD88-dependent (TLR 2 or 4):** NF-κB, AP-1, IRF5 **MyD88-independent (TLR4):** AP-1, IRF3, NF-κB	IRF3, IRF7, NF-κB	NF-κB, AP-1, IRF7
Responsive genes	Proinflammatory cytokines, NO, HDPs, IFNβ	IFNβ, IFN-inducible genes and proinflammatory cytokines	IFNα, IFN-inducible genes and proinflammatory cytokines,

***Abbreviation:** AP-1**,** activator protein 1; CD14 and CD36, cluster of differentiation cell marker protein14 and 36, co-receptor of some TLRs; ERK, extracellular signal-regulated kinase; HDP, host defense peptide; IFN, interferon; IRF, interferon regulatory factor; IKK, IκB kinase; IRAK, IL-1R associated kinase; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MD2, also lymphocyte antigen 96, a co-receptor of TLR4; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-kappa B**;** NO, nitric oxide**;** p38, p38 MAP kinase; RIP1, receptor-interacting protein1; TAK, TGFβ-activated kinase; TBK, TRAF-family-member-associated NF-κB activator-binding kinase; TIRAP/MAL, TIR domain-containing adaptor or MYD88 adaptor-like protein; TRAF, TNF receptor associated factor; TRAM, TIR-domain-containing adapter molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β. Major references are Gay and Gangloff, 2007; Lee and Kim, 2007; West et al., 2006.

Table1.5. The interferon family members and porcine gene candidates*

Types	Subtypes	Gene locus/ (numbers)[a]	Receptors	Amino acids	Expression pattern
I	IFN-α	1q22-q27 (15 & 2ψ)	IFNAR1/IFNAR2	181-189	Ubiquitous expression
	IFN-β	1q23-q27(1)		186	Ubiquitous expression
	IFN-δ [b]	SSA1 (9)		170	Trophoblasts
	IFN-ε	SSA1 (1)		193	Uterus, ovary
	IFN-ω	SSA1 (5 & 2ψ)		179-190	Leukocytes
	IFN-κ	SSA10 (1)		207	Epidermal keratinocytes (?)
	IFN-τ [c]	N/A			Trophoblasts
	IFN-ζ [d]	N/A			Lymphoid tissues
II	IFN-γ	5p1.2-q1.1 / (1)	IFNGR1/IFNGR2	166	Activated T cells, NK-cells
III	IFN-λ1 (IL-29)	?	IL-28Rα/ IL-10R2	?	?
	IFN-λ2 (IL-28A)				
	IFN-λ3 (IL-28B)				

*Modified from Cheng et al., 2006; Takaoka and Yanai, 2006; and other unpublished data defined from NCBI website: http://www.ncbi.nlm.nih.gov/

a. Pig
b. Found in pigs and cattle only
c. Found in ruminants only
d. Found in mice only

CHAPTER 2 - Bioinformatic and Molecular Analysis of Novel Porcine β-defensins: Emerging Repertoire and Functions

ABSTRACT. β-defensins are a major group of mammalian antimicrobial peptides. Although more than 30 β-defensins have been identified in humans, only one porcine β-defensin has been reported. Here we report the identification and initial characterization of 11 novel porcine β-defensins (pBD). Using bioinformatic approaches, we screened 287,821 porcine expressed sequence tags for similarity of their predicted peptides to known human β-defensins and identified full-length or partial sequences for 11 novel pBDs. Similar to the previously identified pBD1, all of these peptides have a consensus β-defensin motif. A differential expression pattern for these newly identified genes was found. For example, unlike most β-defensins, pBD2 and pBD3 were expressed in bone marrow and in other lymphoid tissues including thymus, spleen, lymph nodes, duodenum and liver. Including pBD2 and pBD3, six porcine β-defensins were expressed in lung and skin. Several newly identified porcine β-defensins, including pBD123, pBD125, and pBD129 were expressed in male reproductive tissues, including lobuli testis and some segments of the epididymis. Phylogenetic analysis indicates that in most cases the evolutionary relationship between individual porcine β-defensins and their human orthologs is closer than the relationship among β-defensins in the same species. Structural modulation and antimicrobial analysis also indicates that they conserve tertiary structure and antibacterial activity. Genomic sequences of pBD2, pBD3, and pBD4 show highly diverse in intron regions and conserved exon regions. These findings establish the existence of multiple porcine β-defensins and suggest that the pig may be an ideal model for the characterization of β-defensin diversity and function.

2.1 Introduction

Antimicrobial peptides are small cationic polypeptides that function as one of the earliest mediators of host defense in many species of insects, plants, and animals (Boman 1995; Boman 2003). In humans and other mammals, defensins are a major family of antimicrobial peptides whose two main subfamilies, α and β, are characterized by β-sheet folds and a framework of six disulphide-linked cysteines (Ganz 2003; Lehrer and Ganz 2002; Schutte and McCray 2002). The α-and β-defensins differ in their cysteine disulphide parings and in the number of amino acids between the six cysteines. For β-defensins, the six cysteines are linked at positions 1-5, 2-4, and 3-6. The canonical sequence represented by human β-defensins usually is X_{2-10}-C-X_6-C-X_{3-4}-C-X_{9-13}-C-X_{4-7}-C-CX_n, where X represents any amino acid residue (Ganz 2003; Patil et al. 2005; Selsted and Ouellette 2005; Yeaman and Yount 2007). The third defensin subfamily, θ-defensins, is structurally unlike α and β-defensins and is expressed in Old World monkeys and orangutans (Nguyen et al. 2003; Selsted and Ouellette 2005).

At least one and usually several defensins have been identified in all mammals that have been studied (Boman 2003; Ganz 2003). However, tissue distribution profiles and existence of subfamily defensins vary greatly even between closely related species. For example, humans have α-defensins in leukocytes and intestinal Paneth cells, and β-defensins are found in many epithelial cells. Mice and rats have Paneth cell α-defensins and epithelial cell β-defensins; however, although mice lack leukocyte α-defensins, rats possess neutrophil α-defensins (Boman 2003; Ganz 2003; Patil et al. 2004; Patil et al. 2005; Yang et al. 2004). Bovine neutrophils have several β-defensins and epithelial β-defensins are expressed in bovine trachea, tongue, and intestine (Diamond et al. 1991; Ganz 2003; Selsted et al. 1993). To date, only one epithelial β-defensin (pBD1) has been identified in pigs and defensins have not been detected in porcine leukocytes (Ganz 2003; Zhang et al. 1998; Zhang et al. 1999). Moreover, there is no evidence

that α-defensins are present in pigs (Ganz 2003).

Although considerable progress has been made in identifying complete defensin repertoires in several species including humans, chimpanzees, mice, rats, dogs, and chickens, (Kao et al. 2003; Patil et al. 2004; Patil et al. 2005; Rodriguez-Jimenez et al. 2003; Schutte et al. 2002; Xiao et al. 2004), information on the complete repertoire of porcine β-defensins is lacking. Because pigs are often used for comparative physiological and immunological studies and because porcine tissues and organs are often used for xenotransplantation, we sought to identify the complete β-defensin profile in pigs. Here we report the identification and initial characterization of 11 novel porcine β-defensins; information that is fundamental to the comparative investigation of β-defensins in innate immunity.

2.2. Materials and Methods

BLAST-based searches. The porcine expressed sequence tag (EST) database was searched with BLASTP and TBLASTN programs (Altschul 1990), using the National Center for Biotechnology Information (NCBI) website tools (http://www.ncbi.nlm.nih.gov/blast/) against the EST collection other than human or mouse (EST_others). Initial queries for the search used amino acid sequences for known human defensins (*DEFB1-31*) (Schutte et al. 2002) and three *HE2/EP2* sequences (Frohlich et al. 2000, 2001) as well as some identified bovine β-defensins (Diamond et al. 1991; Selsted et al. 1993). NCBI default parameters were used in the searches and any potential hits were curated manually.

EST annotation and defining coding regions. Most porcine EST clones represent information defining full-length β-defensin cDNA and thus do not need additional annotation. However, in some cases the ESTs were annotated using the stack-PACK version 2.2 program

(http://www.sanbi.ac.za) as described (Lynn et al. 2003, 2004). Briefly, input ESTs were masked to remove repeat sequences and clustered if they shared more than 100 bp at greater than 96% identity. Representative EST sequences or processed consensus sequences were used to define the coding region with ESTScan (http://www.ch.embnet.org/software/ESTScan.html) and predicted peptides were translated using the Translate program (http://www.us.expasy.org/tools/).

Alignment and phylogenetic analysis. Multiple sequence alignment was performed using the PILEUP program from the Wisconsin Package Software (Accelrys, San Diego, CA). Amino acid sequences selected for alignment were three residues before and several residues after the six-cysteine motif (Schutte et al. 2002). The comparison matrix was set at Blosum 62 with a gap creation penalty of 8 and a gap extension penalty of 2. Phylogenetic and molecular evolutionary analyses were conducted on the most complete peptide sequences using MEGA version 2.1 (Kumar et al. 2001).

Expression analysis by semiquantitative RT-PCR and real-time RT-PCR. Tissues obtained from healthy 5-week-old male crossbred pigs, as previously described (Zhang et al. 1998), were chosen to represent organs of the digestive, pulmonary, and immune systems and included bone marrow, intestine, liver, lung, spleen, thymus, testes, and epididymis. All collection procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. Tissue samples were collected, placed immediately in liquid nitrogen, and stored at −135°C until use. Total RNA was extracted with TRI Reagent (Sigma-Aldrich, St. Louis, MO) after grinding frozen tissues in liquid nitrogen. A one-step RT-PCR was used to detect expression of target transcripts. Briefly, total RNA was treated with RQ1 RNase-free

DNAse I (Promega, Madison, WI) to remove possible genomic DNA contamination. RNA samples (250 ng) were run in a 25-μl RT-PCR reaction mixture with a 0.1 μM concentration of each sense and antisense primer derived from cDNA sequences (**Table 2.1**). Semiquantitative RT-PCR was performed using a one-step RT-PCR kit (Qiagen, Valencia, CA). cDNA synthesis and predenaturation were performed at 50°C for 30 min and 95°C for 15 min to activate the antibody-protected DNA polymerase, and amplification was carried out at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec; final extension was at 72°C for 10 min. For most genes, 32, 35, and 40 PCR cycles were used in different replicates to ensure linear amplification and optimal estimation of relative expression levels. After amplification, 10 μl of each reaction mixture were analyzed by 2% agarose gel electrophoresis, bands were then visualized by ethidium bromide staining in a FluorChem^{TM} digital imaging system (Alpha Innotech Corp., San Leandro, CA), and integrated density values were measured using the digital imaging system. Integrated density values were standardized with values of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and presented as a ratio relative to the expression of *GAPDH*.

For genes reported to show expression patterns similar to their human orthologs, we confirmed their expression profiles in tissues using a SYBR-Green-based real-time RT-PCR system (Qiagen). In brief, real-time quantitative RT-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA) as previously described (Sang et al. 2005) with gene-specific primers as described. DNase-treated total RNA (200 ng) was used in each 25-μl RT-PCR reaction. The RT-PCR cycling conditions were 30 min at 50°C, 95°C for 15 min, followed by 45 cycles of 15 sec at 95°C, 30 sec at 56°C, and 30 sec at 72°C with the optic on to perform fluorescence data collection. Amplicon authenticity was confirmed by sequencing before performing real-time RT-PCR. Threshold cycle (C_t) was determined by exponential product amplification and subsequent

increased fluorescence intensity above background. Relative gene-expression data were normalized against the C_t values of the housekeeping gene (*GAPDH*) and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of control samples with the index defined as 1.000 (Livak and Schmittgen 2001).

Tertiary structure simulation and alignment. Structural models were generated via homology modeling for the various β-defensin peptides with structures from the database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=structure). Initial sequence alignment for each target with its set of templates was effected via the Clustal-W program (Thompson et al., 1994), using the Blosum 30 substitution matrix, a gap-opening penalty of 10 and a gap-extension penalty of 0.1. The resulting alignment and the corresponding three-dimensional peptide structures were then processed via the Modeller program (Sanchez and Sali, 2000) to yield structural predictions for the β–defensin targets. Modeller's default simulated annealing cycles were used for structural refinement. Analysis of the peptide secondary structure and surface characteristics was carried out on the resulting structures via SYBYL 6.9.2 (The Tripos Associates, St. Louis, MO), and secondary structure prediction was validated via the PSIPRED program (McGuffin et al., 2000).

Genomic sequences of pBD2, pBD3 and pBD4. Gene sequences of pBD2, pBD3 and pBD4, including exon and intron regions and ~1-2 kb putative promoter regions before the first exon, were amplified with a PCR-based GenomeWalker™ kit (Clontech, Mountain View, CA). Briefly, porcine genomic DNA (Novagen, Madison, WI) of high quality was digested separately with four different restriction enzymes and ligated to the specially designed adaptors in the kit to make premade libraries. Serial gene-specific primers were designed based on identified cDNA

sequences. Amplicons generated using PCR amplification of target sequences from some libraries were cloned into a TA vector (New England Biolabs, Ipswich, MA) or directly sequenced to obtain a 2-3X coverage, which were used to fuse final gene sequences with a SeqBuilder program in Lasergene (DNASTAR, Inc. Madison, WI).

Antibacterial assays. Two 34- and 36- amino acid peptides, spanning the pBD cysteine-motif of pBD2 and pBD3 respectively, were chemically synthesized (Abgent, San Diego, CA). Peptide preparation and quality control were conducted as described (Sang et al., 2005). For antibacterial assays, a broth microdilution method was used to determine susceptibilities of tested bacteria to peptides of pBDs and canine cBD as positive control (Sang et al., 2005). The bacterial strains used, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19115, and *Staphylococcus aureus* ATCC 10832, were cultured in trypticase soy broth (TSB). Working solutions of peptides (0, 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 μg/ml), were obtained by dilution of 50 μl stocks (in 0.1% HAS plus 0.01% acetic acid) in 96-well plates, in which 25 μl of 30 mM sodium PB (pH 7.4, $[Na^+]$ = 53.49 mM) and 25 μl H_2O and 100 μl of the microbial suspension (10^3 CFU), were previously added. The final sodium concentration for the assays was 15 mM in a total volume of 200 μl/well. Microtiter plates were incubated at 37 ^oC for 2 h in a shaking incubator (100 rpm). To determine minimal inhibitory concentrations (MIC), 100 μl double strength broth was added to each assay well. MICs were defined as the lowest concentration in which the microorganism growth was prevented, as determined by no colony development on TSB agar plates after 24 h of incubation at 37 ^oC in 5 % CO_2.

2.3. Results and Discussion

The first porcine β-defensin, pBD1, was identified based on PCR amplification of tissue RNA with primers generated from bovine lingual β-defensin (Zhang et al. 1998, 1999). We now know that identification of pBD1 was quite fortuitous, because of the 287,821 EST entries generated from various sources today, no pBD1 EST was identified. In contrast, using a bioinformatic approach, we have identified 11 novel porcine β-defensins. Each novel porcine defensin is related to at least one EST clone whose sequence range covers the six-cysteine defensin motif. Porcine β-defensin-2 (pBD2) is highly expressed in many tissues and, has the most (19) EST clones identified, with 12 of these covering the entire open reading frame in their registered cDNA sequences. Similarly, pBD3, pBD4, and pBD129 have abundant EST entries in the database; all are represented by five almost identical ESTs. The other eight pBD candidates are represented by one to three EST clones and cover all or most of the coding regions of putative porcine β-defensins. We did not identify any porcine EST with an α-defensin signature in their translated frames using a similar strategy.

Alignment of the pBD predicted peptides clearly shows that they possess typical β-defensin characteristics, such as the six-cysteine β-defensin motif, spacing patterns of the six cysteines, and representative content of positive-charged residues (**Fig. 2.1** and **Table 2.2**). In addition, the canonical sequence of porcine β-defensins is almost identical to that of human β-defensins. After identifying the novel porcine defensins, we used them to query the Swiss-Prot database using BLASTP and assigned a tentative name for each porcine defensin as suggested by the HUGO Gene Nomenclature Committee, University College, London, UK (http://www.gene.ucl.ac.uk/nomenclature/). For example, pBD125 is most homologous to human DEFB125 and so on. Exceptions to this naming convention are pBD2, pBD3, and pBD4, which we previously identified experimentally, confirmed by bioinformatics searching, and thus were

named serially following pBD1 (Zhang et al. 1998). Most porcine β-defensins show significant similarities to their human counterparts in both sequence length and identity.

Generally, β-defensin precursors consist of less than 80 amino acid residues, which are encoded by two exons (Ganz 2003*;* Yang et al. 2004). This characteristic is found in pBD1, pBD2, pBD3, pBD4, and pBD114. Exceptions to this characteristic are two groups of defensin-like molecules, including some EP2/HP2 gene products (EP2C/D/E) and DEFB25-29. These defensins are longer because of the addition of as many as 20-100 amino acids after the N-terminal leader sequence or at the C-terminus adjacent to the C5 and C6 residues (Schutte and McCray 2002). Candidates representing these two groups of β-defensins also have been identified in the pig and are represented by pBD125, pBD129, pEP2C, and pEP2E (**Table 2.2**). Two other features we compared are related to the six-cysteine motif. As shown in Table 2, almost all porcine β-defensins conserve the number of amino acids between the six cysteine residues and the ratio of positive residues, which contributes to the positive charge of the cationic peptides and relates to antimicrobial activity (Schutte et al. 2002*;* Yang et al. 2004).

Phylogenetic analysis of representative human and porcine β-defensins indicated that they were derived from a common ancestor. This analysis showed that individual porcine β-defensins are in the same sub-branch as their human homolog, except pBD1, which is a more diverse isoform than human β-defensins and has no close human homolog. Furthermore, similarity among each subclass of β-defensins from different species is higher than that between different subclasses from the same species. The phylogenetic relationship between human and porcine β-defensins is supported by the high bootstrap values on the branches, which are based on multiple resampling of the original data. Bootstrap analysis is the most common method for estimating

the degree of confidence in the topology of phylogenetic trees (**Fig. 2.2**) (Kumar et al. 2001*;* Lynn et al. 2004).

To evaluate tissue expression profiles of pBDs and verify the accuracy of the EST sequences, we designed gene-specific primers based on representative EST sequences (**Table 2.1**). Each set of primers was designed from diverse regions on different exons to facilitate specific amplification of target genes and to ensure that PCR products were amplified from cDNA and not genomic DNA. **Fig. 2.3** shows the quantitative data and represents the expression profile of all newly found pBDs, except the EP2E molecule. In general, *pBD2*, *pBD3*, *pBD114*, *pBD125*, and *pBD129* were expressed in multiple tissues, including intestine, spleen, lung, and male reproductive tissues. *pBD2* and *pBD3* are expressed in bone marrow, which suggests that they may represent porcine myeloid β-defensins. *pBD3* is detected primarily in lymphoid tissues including thymus, spleen, and lymph nodes, and there is high-level expression in duodenum, liver, skin, testis, and proximal epididymis. Similar to the β-defensin expression profile in species like rats and humans, all pBDs were detected, to some extent, in male reproductive tissues including lobuli testis and various segments of the epididymis. In particular, *pBD114*, *pBD123*, *pBD125*, and *pBD129*, similar to human *DEFB 125-129*, were differentially expressed in various segments of the epididymis. Real-time RT-PCR data emphasize that the highest expression of *pBD2* and six pBDs, including *pBD3*, *pBD4*, *pBD114*, *pBD123*, *pBD125*, and *pBD129*, were expressed in lung; and at least seven pBDs, including *pBD2*, *pBD3*, and *pBD4*, were expressed in skin (**Fig. 2.4)**. Conversely, *pBD104* and *pBD108* were lower-expressed isoforms. *pBD104* was weakly detected in spleen, liver, and testis, and *pBD108* was weakly expressed in liver and somewhat strongly expressed in the proximal epididymis. All of

our findings relate to constitutive gene expression; however, it is possible that under different conditions such as infection porcine β-defensins may exhibit inducible expression.

The detection of *pBD2* and *pBD3* in bone marrow and the prevalent expression of several pBDs in porcine lung and skin likely relates to their involvement in innate immune responses. Similarly, because adaptive immunity is largely absent in the male reproductive system, the preferential expression of β-defensins in the testis and different regions of the epididymis likely provides an innate immune mechanism for defense of this system. In support of this hypothesis, many testis- and epididymis-specific β-defensins have been found to be antimicrobial and capable of protecting sperm from infections (Frohlich et al. 2000, 2001; Patil et al., 2005; Rodriguez-Jimenez et al. 2003). It is noteworthy that *pBD3* is abundantly detected in lymphoid tissues. The preferential expression of multiple pBDs in thymus suggests the potential for β-defensins linking innate immunity to adaptive immune reactions in lymphoid tissues.

The gene sequences of pBD2, pBD3 and pBD4 were obtained by a PCR-based genome-walking technique (Clonetech). Elucidated gene structures show that each of the three pBD genes has two exons, encoding a signal peptide and most of mature peptide respectively, and one very diverse intron (Schutte and McCray 2002). **Fig. 2.5** shows the proportional diagrams of gene structures of the three pBDs. Whereas pBD3 and pBD4 genes are 2.8 and 5.6 kb long with introns of 0.9 and 4.2 kb respectively, the pBD2 gene is about 10 kb long with an intron of 8 kb. Preliminary prediction shows conserved binding sites for transcription factors such as AP1, NF-κB, and IRFs existing in the putative promoter regions for pBD3 and pBD4 genes (Zhang et al., 1999; Sang unpublished data). We already know that the pBD-1 gene is located on 15q14-q15.1, and the progress of porcine genome project will allow rapid discovery of the chromosomal location of these pBDs. Current information indicates that porcine pBDs genes may resemble to

human orthologs regarding to their genomic cluster numbers and distribution among chromosomes. We know that human β-defensins are clustered in five chromosomal loci (Schutte and McCray 2002; Patil et al., 2005), and the identified 12 pBDs mirror very well in the chromosomal distributions. The top six (pBD-1 to pBD108L) and bottom two (pEP2CL and pEP2EL) in **Fig 1**, reflect the human's on 8p23-p22 locus, pBD125L and pBD129L are similar to DEFB25 and 29, which cluster on 20p13, and the other two, pBD114L and pBD123L reflect two human's defensins on 6p12 and 20q11.1 respectively. No porcine EST was identified to encode a peptide similar to DEFB30 and DEFB31, which were indicated in an ambiguous locus of human chromosome (Schutte et al., 2002; Schutte and McCray 2002; Patil et al., 2005).

Although β-defensins from different species show substantial difference in their primary sequences, they generally are conserved in tertiary structure, which may underlie a common theme of the comparable functions of these molecules (Selsted and Ouellette 2005; Yeaman and Yount 2007). To investigate the tertiary structural information of porcine β-defensins, we have predicted their three-dimensional structures (Molecular Graphics/Modeling Lab, The University of Kansas, Lawrence, KS) (McGuffin et al., 2000). As show in **Fig. 2.6**, pBD1 and pBD2 both exhibit strong homology with a number of other mammalian (human and bovine) β-defensins for which x-ray crystal structures exist. As general properties of mammalian β-defensins, predicted 3D structures of pBD peptides contain a short N-terminal amphipathic alfa-helix and three-stranded twisted antiparallel beta-sheets stabilized by three disulfide bonds (Ganz, 2003; Selsted and Ouellette 2005; Yeaman and Yount 2007). The surface charge modules show that pBD1 has a hydrophobic core and pBD3 has a cationic (positive charged) core (**Fig. 2.6**), which are consistent with their residual composition and likely related to their antimicrobial activity. In contrast, pBD2 has relatively less and an even distribution of hydrophobic/cationic residues,

which may indicate its weak interaction with microbial membranes (Ganz, 2003; Selsted and Ouellette 2005; Yeaman and Yount 2007). The peptides deduced from the 34 and 36 C-terminal residues of pBD2 and pBD3 were synthesized and tested against *E. coli*, *L. monocytogenes*, and *S. aureus.* pBD3 showed similar activity to the positive control (a canine β-defensin) (Sang et al., 2005), and pBD2 is weaker (**Table 2.3**). However, a 37-residue peptide of pBD2 appeared to have increased antimicrobial activity compared to the 34-residue peptide used here (Veldhuizen et al., 2008).

In summary, 11 novel porcine β-defensins have been identified using bioinformatic exploration of the available EST database and confirmed by experimental detection. Tissue expression profiles of these genes indicate some special patterns in comparison with species in which α-defensins have evolved, such as humans and mice. Extended studies with pBD2, pBD3 and pBD4 demonstrate the properties of pBDs in gene structure, tertiary structure and antimicrobial activity. Our findings also support the notion that β-defensins represent the most conserved group of antimicrobial peptides in mammals and suggest that the pig may be an ideal animal model to investigate defensin-related diseases and other physiologic functions of these innate immune molecules.

2.4. References

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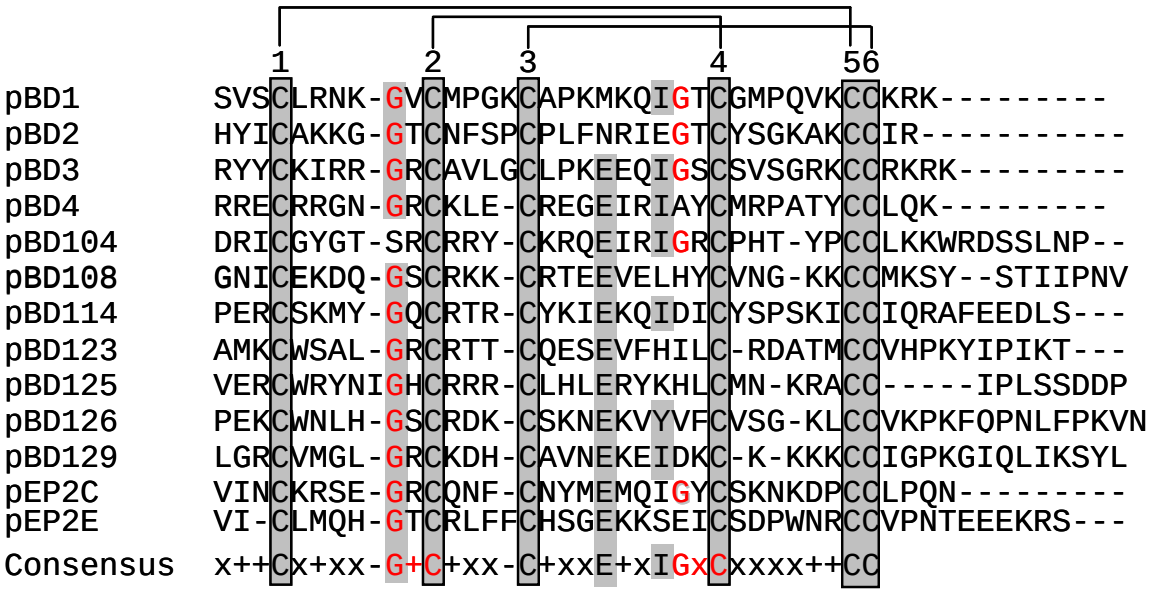

Fig. 2.1. Multiple sequence alignment of porcine β-defensin proteins. Amino acid sequences were predicted from cDNA sequences and aligned with minor manipulations to maximize sequence alignment. The Gly residues of GXC motif(s) are labeled in red. Conserved residues are shaded and the six cysteines are also boxed. The consensus sequence shows cysteines (C), positively charged amino acids (+), and other amino acids if they are represented in more than 50% of all predicted β-defensin proteins.

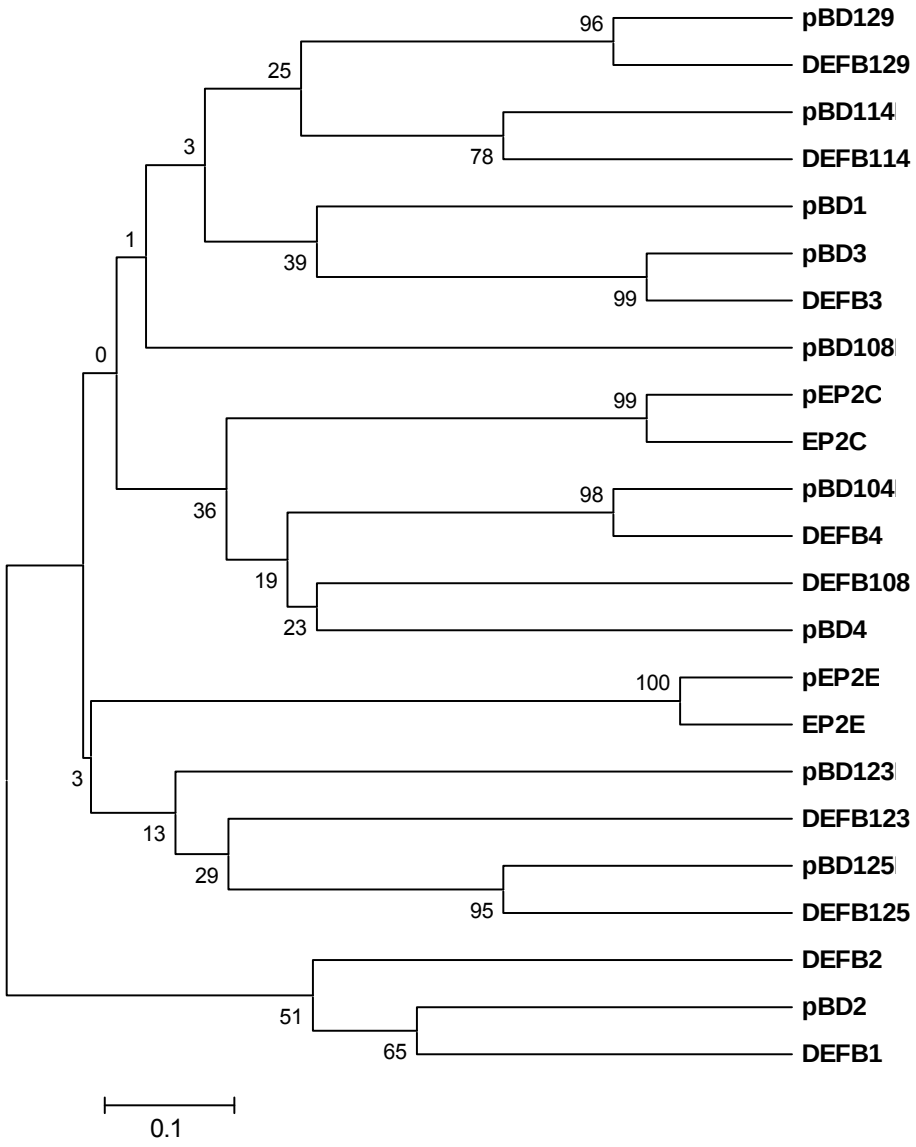

Fig. 2.2. Phylogenetic tree of human and porcine β-defensins. Human β-defensins are DEFBs and porcine β-defensins are pBDs. For branches supported by bootstrap analysis with the percentage of 1000 replications, the percentage is indicated on the branches. The *bar* indicates the *p*-distance. GenBank accession numbers are the same as in Table 2.2.

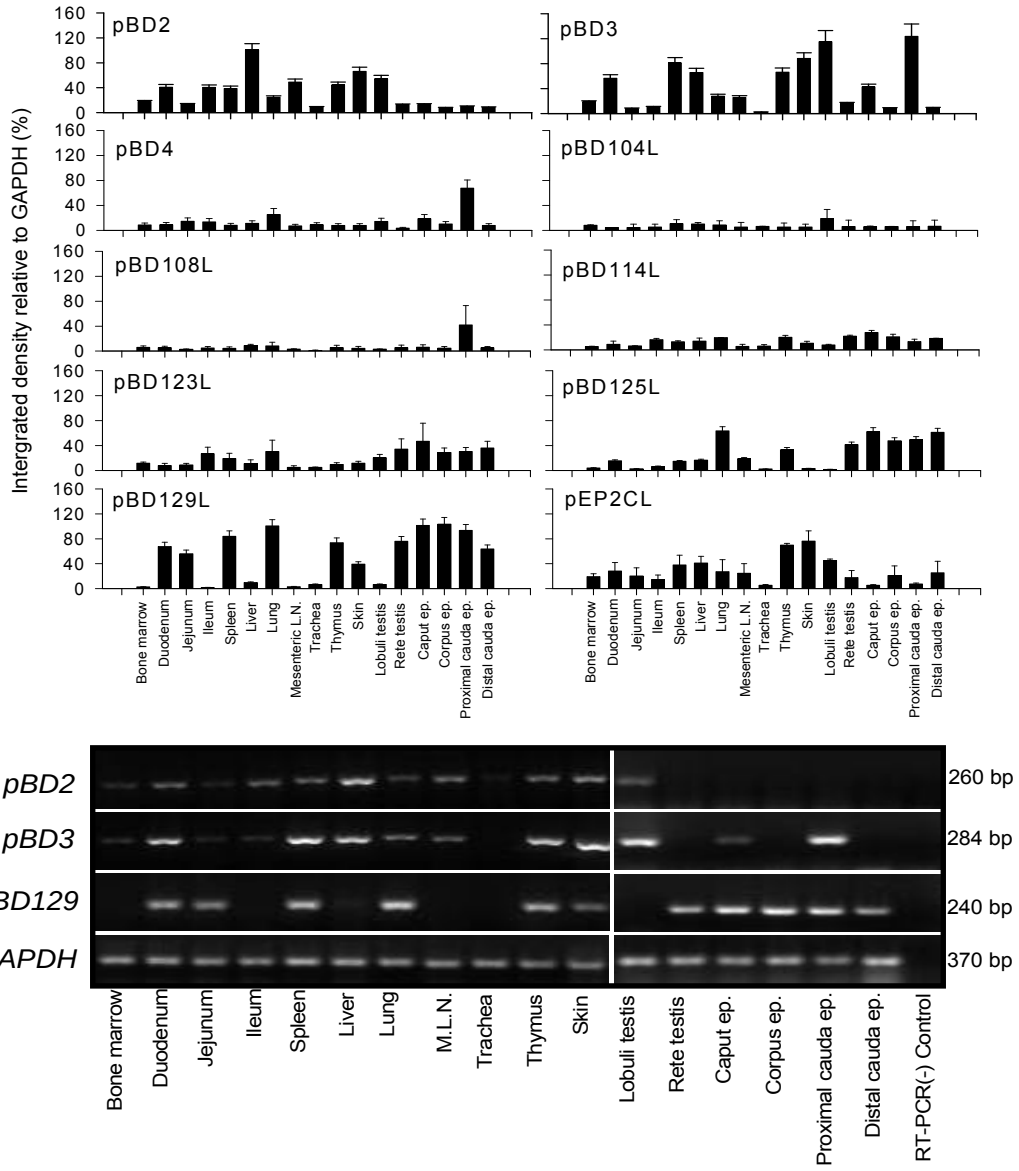

Fig. 2.3. Tissue expression profile of novel porcine β-defensins. (A) Tissues were obtained from healthy 5-week-old pigs and RT-PCR was performed using 250 ng total RNA as template in each reaction. The PCR products were measured and calibrated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described in the *Material and methods* section. Data are means ± SD, *n* = 3, L.N. = lymph nodes; ep. = epididymis. (B) Representative (*pBD2*, *pBD3*, and *pBD129*) RT-PCR products (10 µl/lane) of porcine β-defensins. Amplicons were resolved and stained with ethidium bromide in 1.5% agarose gel. M.L.N. = mesenteric lymph nodes; ep. = epididymis.

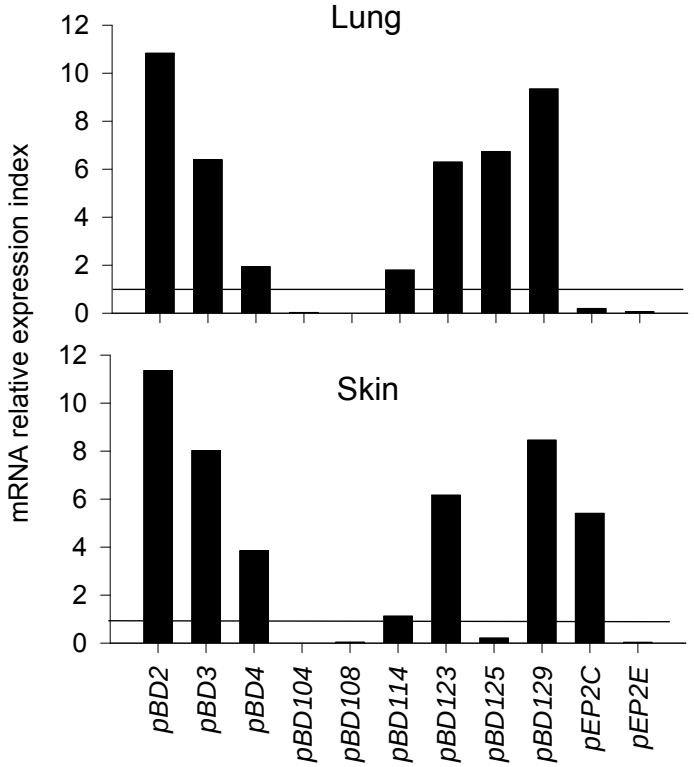

Fig. 2.4. Lung and skin expression of porcine β-defensin mRNA. Real-time RT-PCR was conducted with gene-specific primers using total RNA (200 ng in 25-µl PCR reaction). Relative gene-expression data were normalized against C_t values of the housekeeping gene, *GAPDH*, and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of all β-defensins with the index defined as 1.000 (indicated by the horizontal line).

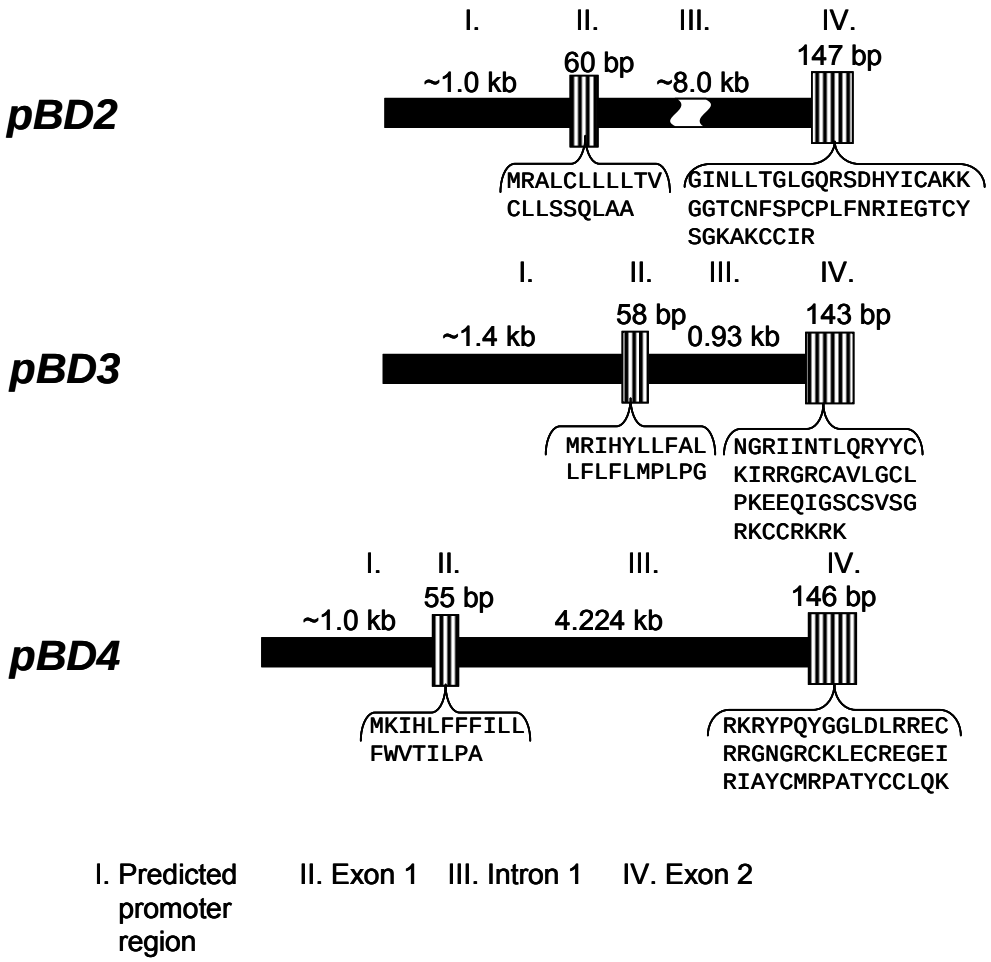

Fig. 2.5. Gene structure schematic of porcine β-defensin (pBD)2, pBD3 and pBD4. Putative promoter regions (solid bars before Exon 1), exons (dashed bars) and introns (solid bars) are proportional to the length of DNA fragments with the exception of the intron in pBD2. The length of each region is labeled above. The translated peptide sequences of each exon is included under the exon.

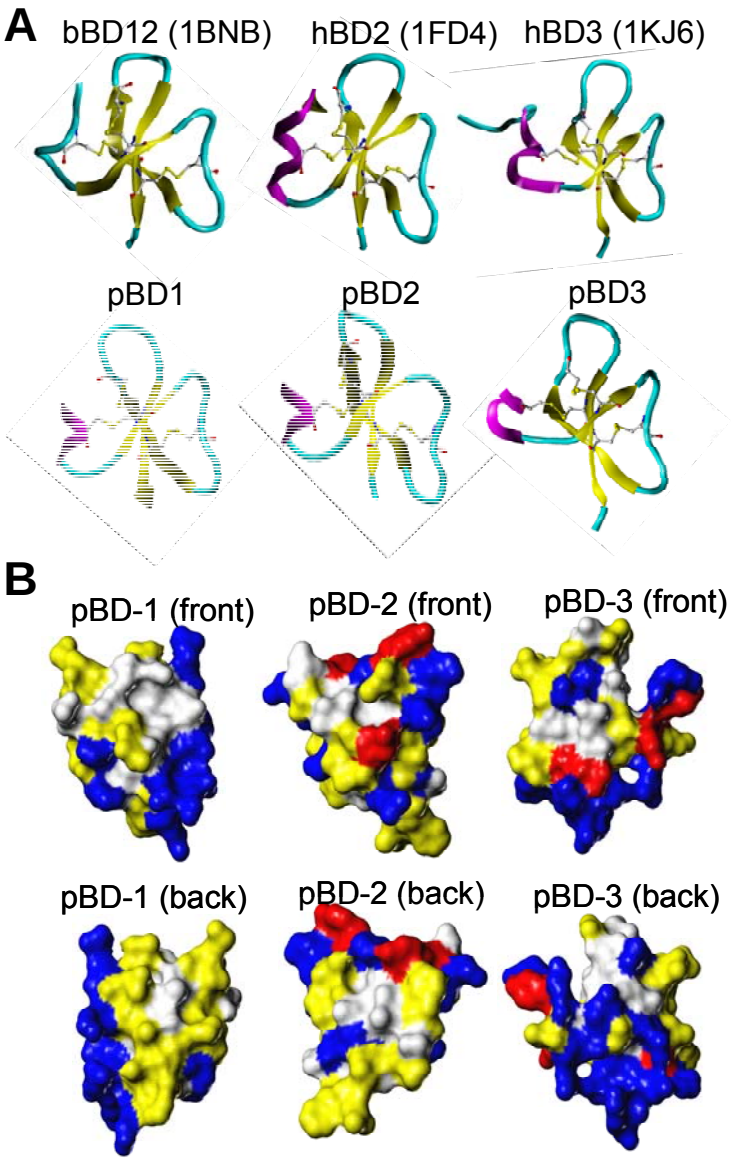

Fig. 2.6. Tertiary structures for porcine β-defensin (pBD) 1-3. (A) Ribbon models of pBD1, pBD2, and pBD3 tertiary structures were generated via homology modeling from various β-defensin templates. The coloring scheme using for the diagrams is purple = helix, yellow = strand/sheet and cyan= coil. **(B)** Surface charge models of pBD1, pBD2, and pBD3. The upper is the front view and the bottom, back view. The color scheme is, red = acidic or mostly negative; blue = basic or mostly positive; yellow = hydrophobic; and white = neutral or nonpolar.

Table 2.1. Primer sequences for RT-PCR and real-time RT-PCR analysis

pBDs	*Primer sequence (5' to 3')*	*GenBank accession number*	*Location in cDNA (nt)*
pBD2		AY506573	
sense	ATGAGGGCCCTCTGCTTGCT	(AW785442)	53-72
antisense	ATACTTCACTTGGCCTGTGTGTCC		312-289
pBD3		AY460575	
sense	CTTCCTATCCAGTCTCAGTGTTCTGC	(CF789126)	200-225
antisense	GGCTTCTGTAGACTTCAAGGAGACAT		508-483
pBD4		AY460576	
sense	GTGGCTTGGATTTGAGGAGAGAGT	(BX672669)	107-130
antisense	AGTGATACACAGGCCTGGAAGGAT		339-316
pBD104		DQ274056	
sense (1)	TCCTTCCACGTATGGAGGCTTGTT	(BX918848)	300-323
antisense(1)	TTACAATACCTCCGGCAGCGAGAA		632-608
sense (2)	AAGACTCCTGTTAGCACCCAGCAT		449-472
antisense (2)	TTACAATACCTCCGGCAGCGAGAA		632-608
pBD108		DQ274057	
sense (1)	GACGATTGTCATTCTTCTGATCCTGG	(BX917425)	33-58
antisense(1)	TAGGTTGACTTGTGGTGCCCGAAA		291-268
sense (2)	GTGAGAAAGACCAAGGATCATGCAG		124-148
antisense (2)	TAGGTTGACTTGTGGTGCCCGAAA		291-268
pBD114		BK005518	
sense (1)	TGTACCTTGGTGGATCCTGAACGA	(BX923414)	95-118
antisense(1)	CGCCCTCTGAATGCAGCATATCTT		221-196
sense (2)	TGTACCTTGGTGGATCCTGAACGA		95-118
antisense (2)	ATTCCTACACCTCTCTGTACTGGTGC		304-279
pBD123		BK005519	
sense (1)	AGCCATGAAGTGTTGGAGTGCGTT	(BX915917)	76-100
antisense(1)	GTACACAGCACATAGTTGCATCCC		177-153
sense (2)	GTGCGTTGGGAAGATGCAGAACAA		93-116
antisense (2)	AACAGGGTAGGGCCAAGAATGAGT		322-298
pBD125		BK005520	
sense (1)	AGCCATGAATCTCCTGCTGACCTT	(BX926653)	32-55
antisense(1)	TGCAGCATGCTCGCTTGTTCATAC		201-178
sense (2)	GTGACCAAAGCTGGCTGGAATGTT		81-104
antisense (2)	TCCTGCTCAGTTCCTGTGCTTTCT		370-347
pBD129		BK005521	
sense	CAAAGACCACTGTGCCGTGAATGA	(BX918362)	118-141
antisense	TTGATGCTGGCGAAAGGGTTGGTA		357-334
pEP2C		BK005522	
sense	CCCTTTCCAGGAACCTGAACCAAA	(BX925543)	184-208
antisense	TGGCTTGTAGGCTCTGGAGAACAA		388-365
pEP2E		BK005523	
sense (1)	TGCCTTATGCAACATGGAACCTGC	(BX919973)	295-318
antisense(1)	AGGTGCTAGAACCACCATTCATCG		445-422
sense (2)	TCCAGACACTTCCCTATGGCCTTT		12-35
antisense (2)	GCCTGCAGGTTCCATGTTGCATAA		322-299

Table 2.2. Properties of porcine β-defensins (pBDs)

pBDs	*GenBank accession number*	*Amino acids*	*ORF verified* [a]	*Six-cysteine spacing pattern* [b]	*H+R+K* [c]	*Main expression features in healthy tissues*
pBD1	AF031666	64	Y	6 4 9 6	9	Airway and oral mucosa
pBD2	AY506573	69	Y	6 4 9 6	8	Liver, intestine[d], lung and bone marrow
pBD3	AY460575	67	Y	6 4 9 6	12	Bone marrow, liver, lung and lymphoid tissues
pBD4	AY460576	67	Y	6 3 9 6	9	Lung and proximal epididymis
pBD104	DQ274056	-	-	6 3 9 5	11	Spleen, liver and testis
pBD108	DQ274057	73	Y	6 3 9 5	10	Liver and proximal epididymis
pBD114	BK005518	69	Y	6 3 9 6	8	Ileum, spleen, liver, lung, and male reproductive tissues
pBD123	BK005519	-	Y	6 3 9 5	8	Ileum, spleen, lung, and male reproductive tissues
pBD125	BK005520	147	Y	7 3 9 5	13	Lung, thymus, and epididymis
pBD129	BK005521	>173	Y	6 3 9 4	11	Epididymis, intestine, spleen, lung , skin
pEP2C	BK005522	>108	Y	6 3 9 6	6	Thymus, skin, testis, and some sections of epididymis
pEP2E	BK005523	85	Y	6 4 9 6	9	Not evident in all tested tissues

a. Open reading frames (ORF) in EST sequence were verified either by computational predication or sequencing after PCR amplification.
b. Numbers of amino acids that separate the cysteine residues (C1-C2, C2-C3, C3-C4 and C4-C5) in the six-cysteine β-defensin motif.
c. Numbers of positively charged residues (H, histidine; R, arginine; K, lysine) in the putative mature β-defensin peptides. Calculations count positive residues between seven amino acids before the first cysteine (C1) and, at most, seven amino acids beyond the last cysteine (C6).
d. Indicates the gene expressed in all three tested sections (duodenum, jejunum and ileum) of intestine.

Table 2.3. Antimicrobial activity of porcine β-defensin (pBD)-2 and pBD-3.

Microorganism	ATCC number	MIC (μg/ml)[c]		
		cBD	**pBD2**	**pBD3**
Escherichia coli (generic)[a]	25922	20	80	30
Staphylococcus aureus	10832	100	150	100
Listeria monocytogenes[b]	19115	10	150	100

[a] Enterobacteriaceae. [b] Non Enterobacteriaceae

[c] The MIC was determined with a broth microdilution method adapted from NCCLS and the canine BD (cBD) was taken as a control (Sang Y. et al. 2005) .

CHAPTER 3 - Porcine Antimicrobial Peptides in Arteriviral Infection: Differential Expression and Inactivation of PRRSV

ABSTRACT. In this report, we evaluated the potential antiviral activity of porcine host defense peptides (HDPs) against porcine reproductive and respiratory syndrome virus (PRRSV); a virus that causes an economically significant pandemic disease in pigs. Porcine alveolar macrophages (PAMs) are primary cells of PRRSV infection. Among twenty-four identified porcine HDPs, most are highly expressed in lungs. However, only β-defensins (pBDs) not cathelicidins were detected in PAMs. In PRRSV-positive lungs of fetal and 2-week-old congentinally infected pigs, gene expression of most HDPs showed no significant upregulation. Expression of pBD-1 and protegrins was down-regulated in PRRSV-infected fetal lungs. In vitro incubation of PRRSV with synthetic pBD-3 or protegrin (PG)-4 at >20 μg/ml significantly inhibited viral infectivity. Using nine protegrin-derived peptides, we determined that cyclization of PG-4 increased anti-PRRSV activity and that substitution of Phe^{14} with Val in PG-4 diminished most of the activity. Consistently, the presence of pBD-3 and PG-4 at 5-40 μg/ml in culture medium suppressed PRRSV titer in PAM cultures. Collectively, these findings suggest a potential role for some porcine HDPs as innate antiviral effectors in PRRSV pathogenesis. Manipulation of porcine innate immune mechanisms with HDPs may be one tactic for preventing this costly pandemic viral disease.

3.1. Introduction

One defense mechanism of an animal's innate immune system is constitutive or inducible production of host defense peptides (HDPs). Many HDPs have broad-spectrum antimicrobial activity with potency against bacteria, fungi, protozoa or/and viruses. Antiviral activity of HDPs was noted in early studies and research in this area has recently intensified (Daher et al., 1986; Klotman and Chang, 2006). Using direct inactivation assays, several studies have demonstrated that LL-37, the only cathelicidin in humans, significantly reduces the infectivity of herpes simplex virus (HSV)-1, adenovirus, *Vaccinia* virus and human immunodeficiency virus (HIV) in some tissues and cells (Bergman et al., 2007; Gordon et al., 2005; Howell et al., 2004, 2006). Constitutive expression of β-defensins and combinations of other HDPs in oral epithelia and vaginal fluids has been shown to form an effective innate immune barrier against HIV infection (Quiñones-Mateu et al., 2003; Sun et al., 2005). Human β-defensin 3 (hBD-3) inhibited influenza virus infection through direct interaction with viron surface glycoprotein preventing virus fusion to the cell membrane, and suppressed HIV entrance into cells by competition for viral co-receptors on immunocompetent cells (Feng et al., 2006; Leikina et al., 2005). Retrocyclins, circular θ-defensins based on human pseudogene sequences, have been characterized for their antiviral activity. Retrocyclin (RC)-1 protected human cells from infection by 30 primary HIV-1 isolates and RC-2 effectively suppressed infection by several viruses including influenza, Sindbis virus and baculovirus (Cole and Cole, 2008; Gallo et al., 2006; Wang et al., 2003, 2004). In addition to antiviral activity, cytotoxicity of a HDP to host cells is a critical aspect for potential therapeutic use (Hancock and Sahl, 2006). Studies have shown that LL-37, hBD-2 and -3, and RCs have limited and tolerable cytotoxicity. However, most α-defensins exert cytotoxicity

to mammalian cells at a concentration that kills virus (Leikina et al., 2005; Sun et al., 2005). A porcine cathelicidin, protegrin (PG)-1, exerted anti-lentiviral activity comparable to LL-37, but had cytotoxicity to host cells as well (Steinstraesser et al., 2005). To our knowledge, protegrin-1 is the only porcine HDP that has been reported to have antiviral activity. To date, about thirty porcine HDPs including 13 β-defensins (pBDs) and 12 cathelicidins have been identified. There are no α-defensins identified in pigs, and it has been suggested that some porcine cathelicidins shch as PG-1 substitute for the functions of α-defensins as in other species (Ganz, 2003).

Few studies have been involved in antiviral activity of porcine HDPs. Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, (+)-ssRNA virus that has been an immunological challenge and a devastating pathogen for the swine industry (Mateu and Diaz, 2007; Neumann et al., 2005). We reasoned that some porcine HDPs may be potential antiviral effectors during PRRSV infection. Accordingly, we examined the expression profile of all identified porcine HDPs in lungs from PRRSV-negative and -positive pigs, and evaluated the direct inactivation activity of a group of synthetic porcine HDPs. Here we report that PRRSV infection displays little upregulation of HDP expression, and that pBD-3 and PG-4 show direct suppression of PRRSV infectivity.

3. 2. Materials and Methods

Virus strains and titration in cell cultures. The North American macrophage-tropic PRRSV strain, SDSU-23983-P6 (P6), was used to infect pigs as previously described (Kim et al., 2002; Rowland et al., 2001, 2003). All animal and virus procedures were approved by the Kansas State University Institutional Animal Care and Use, and Biosafety Committees. MARC-

145 cells, an African green monkey kidney cell line sensitive to PRRSV infection, was used to test PRRSV infectivity and for virus titration. Virus stocks [SDSU-23983-P7 (P7)] collected from supernatant of P6-infected MARC-145 cells were used to infect cell cultures. In addition, a full-length cDNA infectious clone with expression of green fluorescent protein (GFP) in the region of nonstructural protein 2 (Nsp2) of PRRSV (GFP-PRRSV) was used to infect MARC-145 cells to facilitate examination with fluorescent microscopy. The GFP-PRRSV was generated from North American type 1 PRRSV isolate, SD01-08, and maintained growth properties similar to those of the parental virus in cell cultures (Fang et al., 2006). The tissue culture 50% infectious dose ($TCID_{50}$) of P7 and GFP-PRRSV stocks was $10^{7.25}$ and $10^{7.14}$ PFU/ml, respectively. Cells were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell (Rowland et al., 2001, 2003). For titration, MARC-145 cells were cultured in Eagle's minimum essential medium (MEM, ATCC) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 IU penicillin and 100 μg/ml streptomycin, CHEMICON International, Inc., Temecula, CA) in a humidified 5% CO_2, 95% air atmosphere at 37 °C. P7-virus-infected monolayers of MARC-145 cells in 96-well tissue culture plates were fixed with 80% cold acetone and incubated with fluorescent-labeled monoclonal antibodies to PRRSV nucleocapsid protein N (SDOW17, Rural Technologies, Inc., Brookings, SD). PRRSV-positive cells were identified by fluorescent microscopy, or duplicate cell monolayers were fixed with buffered 4% formaldehyde and stained with crystal violet to identify viral plaques (Rowland et al., 2001, 2003).

In vivo infection and tissue collection. Fetal and young pig lung samples from an earlier study were used in which seronegative pregnant females were infected at 85-90 days of gestation

with wild type P6 as described (44, 46). Some animals were allowed to give birth and live-born pigs were euthanized at 14 days of age or fetuses were obtained at 107 and 112 days of gestation. Tissue samples were immediately placed in RNA*later* (Ambion, Inc. Austin, TX) and stored at -20 °C until used (Rowland et al., 2001, 2003).

PAM collection, treatment and infection. Porcine pulmonary alveolar macrophages (PAM) were obtained by bronchoalveolar lavage from healthy 5-wk-old pigs and cultured as previously described (Chitko-McKown et al., 1991; Xiao et al., 2004). Two days before treatments or infection with PRRSV, PAMs were thawed from stocks and plated in 24-well tissue culture plates (7×10^4 cells/well) in supplemented RPMI 1640 medium and cultured in a humidified 5% CO_2, 95% air atmosphere at 37 °C. After one-change of fresh medium 2 h later to remove non-adherent cells, PAMs were cultured with replenished medium containing synthetic peptides of pBD-2, pBD-3 and PR-39 at 0, 5, 10, 20 or 40 μg/ml for indicate time (0, 4, 10 or 24 h). PAMs used for viral infection were infected with PRRSV-P7 at the indicated MOI for 18 h. Supernatants were collected for viral titration on MARC-145 cells, and cell RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO).

RT-PCR and real-time RT-PCR. Analysis of gene expression by RT-PCR and real-time RT-PCR was conducted as previsously described (Chapter 2, Section 2.2, Page 91-92).

Peptide synthesis and preparation. The C-terminal peptides of pBD-2 (34 aa), pBD-3 (36 aa), pBD-3 analogue (36 aa) and PR-39 (39 aa) were chemically synthesized by solid-phase peptide synthesis (Abgent, San Diego, CA). The linear analogue of pBD3 was designed and

synthesized using alanines to replace six cysteine residues in pBD3 peptides. The material was eluted as a single peak by reverse-phase HPLC and the peptide identity was confirmed by mass spectroscopy. The final purity of the peptide is >95%. The eight protegrin-related peptides and RC-100B were prepared at the University of California, Los Angeles, by solid-phase peptide synthesis, using fluorenylmethoxycarbonyl (FMOC) chemistry. The peptides were purified by HPLC, and concentration was determined by measuring absorbance at 280 nm. PG-1, PG-4 and PG-5 were derived from the C-terminal 18 aa (without the last glycine residue) of the endogenous protegrins. The cyclic versions of PG-4 and PG-5 bear end-to-end peptide bonds linking two arginine residues at both ends of the corresponding linear forms. The peptides were oxidized to form two inter-strand disulfide bonds. The other two analogs of PG-4 include PG-4/ F^{14}-V, in which phenylalanine at position 14 is replaced with valine, and cyclic PG-4/^{1}Nal/Chg/Phe^{F5}, which contains 1-naphthy-alanine (^{1}Nal), cyclohexylglycine (Chg), and pentafluorophenylalanine (Phe ^{F5}) instead of residues of GWI. These substitutions were designed to make the analog much more hydrophobic than endogenous PG-4. The three other protegrin-like peptides are PG-307, cyclic PG-307 and PG-303, which were designed based on backbones of PG-1 and PG-5 to improve their antiviral activity and reduce their cytotoxicity. The peptides were lyophilized and dissolved in 0.01% acetic acid at 1 mg/ml (~0.5 mM) as a stock solution and stored at –135 ºC until further use.

Viral inactivation and cytotoxicity assays of HDPs. To evaluate antiviral activity of HDPs, peptides were diluted in 50 μl of FBS-free MEM containing PRRSV (P7) or GFP-PRRSV at 10 X of viral titers used for inoculation (MOI of 1 $TCID_{50}$/cell). Final concentrations of tested HDPs were at 0, 5, 10, 20, and 40 μg/ml, and some peptides were used up to 240 μg/ml. After

incubation at 37 °C for 2 h, 10 μl of the mixture of virus and peptide was added to 90 μl medium of MARC-145 cells cultured in wells of 96-well plates. Most times, HDPs were directly diluted in culture medium containing the virus at MOI of 0.1 $TCID_{50}$/cell and applied to the cells in 96-well plates (Salvatore et al., 2007). The plates were washed with fresh medium after infection for 2 h, and replenished with HDP-containing medium. The infectivity of virus was examined at 24 or 48 h by immunostaining of PRRSV nucleocapsid protein N or detection of fused GFP fluorescence in GFP-PRRSV infected cells. The virus positive cells were photographed using an inverted fluorescent microcopy and fluorescent intensity was quantified with digital image software (AlphaEase FC, FluorChem^{TM}, Alpha Innotech Corp., San Leandro, CA). For evaluation of HDP effects on viral infection in PAMs, viral supernatants were collected from PAMs infected with PRRSV for 18 h in presence of HDPs. The supernatants were diluted 10-fold serially into MARC-145 cell monolayers in 96–well plates. MARC-145 monolayers were fixed with buffered 4% formaldehyde after 48-72 h post infection and stained with crystal violet to identify viral plaques for calculation of $TCID_{50}$/ml (Rowland et al., 2001, 2003).

Cytotoxicity of each HDP was microscopically evaluated or quantified by determining the number of viable cells using a tetrazolium-based colorimetric (MTT) assay (Maher and McClean, 2006).

3.3. Results and Discussion

Differential expression of HDPs in PRRSV-infected lungs and PAMs. To determine if PRRSV influences the expression of HDPs, we evaluated the expression of porcine defensins and cathelicidins in lungs and PAMs exposed to the virus. Lung samples were collected from either 14-d-old piglets or fetuses (107 and 112 days of gestation), which were farrowed by sows

infected by PRRSV at 85-90 days of gestation (Rowland et al., 2001, 2003). PAMs were obtained by bronchoalveolar lavage from healty pigs, and infected with PRRSV in vitro (Xiao et al., 2004). Tissue/cell RNA was extracted and a one-step standard RT-RNA (Qiagen) was run using gene-specific primers (**Table. 3.1**). As shown in **Fig. 3.1**, multiple β-defensins are expressed in lungs of 14-d old pigs and fetuses. pBD-2 and pBD-3 displayed the highest expression levels, following by pBD-1 and pBD-114. No significant stimulation or suppression was found in lungs of 14-d-old pigs; however, lungs from PRRSV-infected fetus showed a decrease in pBD-1 expression. pBD-2 and -3 were also the main defensins detected in isolated PAMs, and slightly downregulated by exposure of cells to PRRSV for 5 hours. Conversely, pBD-114 (along with pBD123 and pBD125, data not shown) was slightly stimulated, but not significantly, in lungs of fetuses from PRRSV-infected sows, and a similar stimulation was also observed in the macrophages exposed to the virus *in vitro*. Weak regulation of innate immune genes appears to be prominent feature in PRRSV infections, such as expression of IFN-α, IL-1 and IL-6 genes (Petry et al., 2007). This was also found for porcine defensin expression in pig lungs. However, pBD expression in fetal lungs was more responsive such as in the case of pBD-1 and pBD-114, indicating that pBD expression in lungs may be differentially responsive to PRRSV infection and dependent on pig development.

Most porcine cathelicidins were expressed in lungs. PR-39, prophenin-2 (PF-2), and protegrin-1-5 (PGs) were expressed at levels two- to fourfold higher than defensins. PR-39 was not influenced by PRRSV infection; however, PF-2 was suppressed in lungs of 14-d-old pigs and stimulated in lungs of fetuses from PRRSV-infected sows. Lungs of fetuses from sows infected with PRRSV had lower expression of protegrins; other cathelicidins, PMAP-23 and PMAP-37 were detected in lungs at low levels; however, PAMP-36 was only detected in fetal lungs (**Fig**

3.1). No porcine cathelicidins were detected in PAMs (data not shown). The high expression of cathelicidins in lungs but not in PAMs is consistent with the literature showing that porcine cathelicidins are not present in macrophages (Zanetti, 2004). The source of cathelicidin expression in lungs probably relates to pulmonary presence of granulocytes. Again, weak, even negative responses of cathelicidin expression may be probably due to suboptimal induction of inflammatory responses during PRRSV infection (Petry et al., 2007; Thanawongnuwech et al., 2004).

Suppression of PRRSV infectivity in MARK-145 cells. Porcine HDPs, including pBD-1, pBD-2, pBD-3, PR-39, PG-1, PG-4, and PG-5, were selected to evaluate anti-PRRSV activity. Two primate HDPs, hBD-3 and RC-100B, which have been shown to be active in suppression of multiple viruses, were also evaluated. As shown in **Fig. 3.2** and **Fig. 3.3**, pBD-3 and PG-4 suppressed PRRSV infectivity in MARC-145 cells. Incubation with either pBD-3 or PG-4 at higher than 20 μg/ml suppressed about 50-80% of viral infectivity when estimated by immunofluoresce intensity using an antibody to PRRSV nucleocapsid protein N. The effects of peptides at the concentrations lower than 10 μg/ml were not significant, and complete elimination of PRRSV positive cells could not be attained even at 240 μg/ml of the peptides (**Fig. 3.3**). Other porcine HDPs, including pBD-1, pBD-2, PR-39, PG-1 and PG-5, showed no significant effects repeatedly at concentrations lower than 40 μg/ml. Of the primate HDPs used, hBD-3 showed activity similar to pBD3 (data not shown), and RC-100B was not active against PRRSV. For most peptides assayed, higher than 80 μg/ml inhibited virus infectivity to some extent; however, these concentrations were not considered to be physiological or due to cytotoxicity to cells (**Fig. 3.4**). In most cases, the two procedures, either peptide pre-incubation

for 2 h with virus in FBS-free medium prior to addition to cells or simultaneous addition to cells in cell culture medium, had similar results in respect to inactivation of viral infectivity (Salvatore et al., 2007). However, slightly better activity of pBD-3 and PG-4 was noted sometimes with the pre-incubation procedure. In addition to above activity assays, which used a wild-type PRRSV isolate (P7), a cell-culture-adapted PRRSV (P136) and a DNA infectious clone GFP-PRRSV (Fang et al., 2006) have also been tested for sensitivity to the listed HDPs. The efficacy of these HDPs in suppression of PRRSV infectivity was consistent among these three PRRSV strains; however, the GFP-PRRSV infectious clone provided a real-time means to examine the HDP effect by GFP fused to viral Nsp2 protein. As summarized in **Table 3.2,** pBD-3 and PG-4 were active in tests against all three PRRSV strains. Incubation cells with PR-39 or RC-100B at >40 μg/ml, inhibited PRRSV and GFP-PRRSV clone infectivity for 20-40% without obvious cytotoxicity. In contrast, PG-1 and PG-5, at concentrations higher than 40 μg/ml, decreased the viral infectivity probably via cytotoxicity to cells thus limited virus replication (**Fig. 3.4**).

Anti-PRRSV activity of protegrin-derived peptides. The nature of pBD-3 and PG-4 in inactivation of PRRSV promoted us to study this activity further. We were especially interested in the characteristics of PG-4. Alignment of the five isoforms of porcine protegrin mature peptides indicates that only four of eighteen residues of PG-4 differ from the other PGs (**Fig 3.5**). Whereas PG-4 has a hydrophobic beta turn at residue 10 to 12 (GWI) and other PGs have arginine-rich polar turns with residues of (R)RRF or (R)PRF. In addition, PG-4 has a Phe (F) at residue 14 and other PGs have Val (V) or Ile (I) at this position. Considering that cyclic PGs may exert more antimicrobial activity, we synthesized cyclic forms of PG-4 and two analogs of PG-4 including PG-4/ F^{14}-V (PG4FV), which Phe^{14} is replaced with Val, and cyclic PG-

4/^{1}Nal/Chg/Phe^{F5} (PG-4NCP), which contains 1-naphthy-alanine (^{1}Nal), cyclohexylglycine (Chg), and pentafluorophenylalanine (Phe ^{F5}) instead of the residues GWI. These substitutions were designed to make the analog more hydrophobic than ordinary PG-4 (**Fig. 3.5**).

We thus evaluated antiviral activity of these protegrin-derived peptides against both wild-type PRRSV and GFP-PRRSV. Examined at 48 or 72 h post infection, the presence of linear PG-4, cyclic PG-4 (PG-4CY) and cyclic PG-4NCP had similar anti-PRRSV activity at 5-20 μg/ml (**Fig. 3.6**). The cyclization of PG-4 (PG-4CY and PG-4NCP) showed some improvement in anti-PRRSV activity comparing to PG-4 at 20 and 40 μg/ml. However, PG-4NCP was no better than PG-4CY. Clearly, cyclic PG-4 with substitution of Phe^{14} with Val^{14} (PG-4FV) has less anti-PRRSV activity than PG-4 and PG-4CY at all test concentrations. We also tested the activity of cyclic PG-5 and three other protegrin-like peptides, PG-303, PG-307, and cyclic PG-307, which were designed based on backbones of PG-1 and PG-5 but modified in position 2 with Trp. Both PG-303 and PG-307 have been shown to be more active against HIV-1 than PG-1 (personal communication, Dr. Lehrer, UCLA). In respect to suppression of PRRSV infectivity, PG-5 had little anti-PRRSV activity at <40 μg/ml; but cyclic PG-5 showed increased anti-PRRSV activity at earlier time of viral infection when tested with GFP-PRRSV (Data not shown). In general, PG-303 and PG307 showed no obvious activity in suppression of PRRSV infectivity. In conclusion, anti-PRRSV activity was shown in PG-4 and its derived peptides, PG-4CY and PG-4NCP in which cyclization and residue-substitution were conducted to improve its viral accessibility and molecular hydrophobicity. Whereas cyclization increased some of viral-suppression activity, changing GWI to NCP did not improve anti-PRRSV activity. In contrast, PG-4F-V (substitution of Phe^{14} with Val) diminished most of the anti-PRRSV activity. This may indicate that the

aromatic side-chain of Phe^{14} defines a stereo-specific interaction with a viral target on the surface of PRRSV virions. Still the hydrophobic beta turn (GWI) in PG-4 may coordinate with Phe^{14} to determine the potential peptide-PRRSV interaction therefore to suppress viral infectivity. The ability of a simple tripeptide motif (GWI) to have marked functional consequences is reminiscent of the RGD motif in integrin studies (Garrigues et al., 2008).

Suppression of PRRSV infection/replication in PAMs. We also evaluated antiviral activity of selected HDPs directly on PAMs. The peptides were co-incubated with PAMs during virus infection for 18 h, and released viruses in the PAM supernatants were titrated on MARC-145 cells. As shown in **Table 3.3**, at concentrations of 5 and 10 μg/ml, pBD3 and PG-4 were most active peptides suppressing PRRSV $TCID_{50}$ comparing to the control. PG-1 and PG-5 also decreased PRRSV $TCID_{50}$ at 10 μg/ml. At the concentrations of 20 and 40 μg/ml, all tested HDPs showed some effects on suppression of PRRSV infectivity for ~1-3 $TCID_{50}$ unit or 10-30% suppression to the control. We interpreted these results arose from the combination effects of direct PRRSV-inactivation activity, such as by pBD3 and PG-4, and indirect induction effects, such as by PR-39, and cytotoxicity such as in the cases of PG-1 and PG-5. In addition, substitution of six cysteine residues of pBD3 did not decrease the antiviral activity in comparison to original pBD3 peptide (Taylor et al., 2008).

In summary, we have evaluated differential expression patterns of porcine HDPs in lungs of PRRSV-infected pigs that indicate a potential weak induction mechanism adopted by PRRSV infection. Selected porcine HDPs were used to suppress PRRSV infectivity in cell culture systems. Although no peptide eliminated PRRSV completely, the porcine defensin pBD-3 and the cathelicidin PG-4 showed promising activity in suppression of PRRSV infectivity. A panel of

peptides derived from pBD3 and protegins were used to determine the critical motifs of the peptides interacting with PRRSV and to improve anti-PRRSV activity. Whereas the cysteine residues were found to not be essential for the antiviral activity of pBD3, the stereo-structure formed by GWI and Phe^{14} residues appears critical for PG-4/PRRSV interaction and provokes further investigation.

3.4. References

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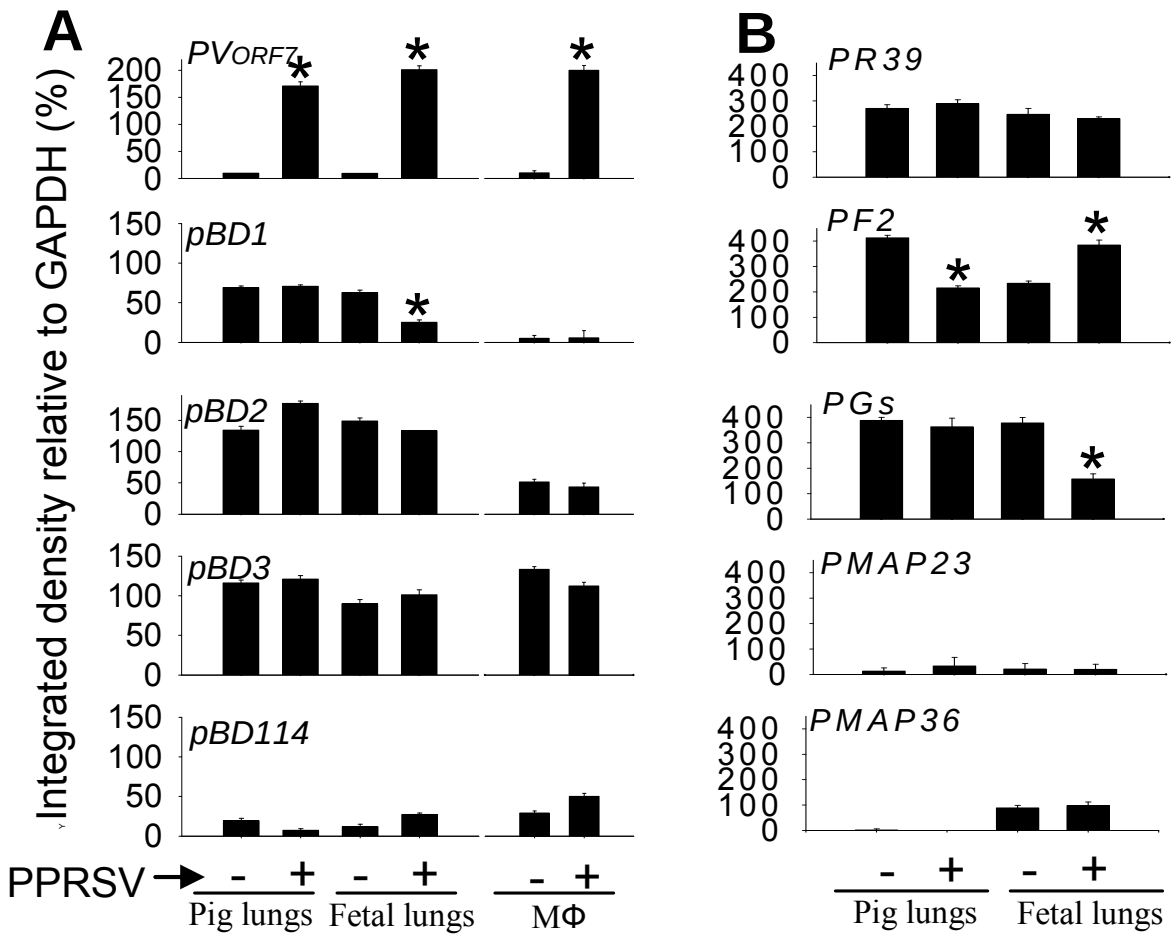

Fig. 3.1. Expression of porcine HDPs in PRRSV-infected lungs and pulmonary alveolar macrophages (PAMs)**.** Fetal and 14-d-old pig lungs from sows infected with PRRSV, and PAMs infected *in vitro* with PRRSV were evaluated for mRNA expression of porcine defensins (**A**) and cathelicidins (**B**). RT-PCR was conducted with gene-specific primers using total RNA of 200 ng in 25 µl volume. The gene-specific amplicons were resolved and ethidium bromide-stained on 1.5% agarose gels and band intensities were quantified using a digital imaging system. * Differential from control $p<0.05$, n=3.

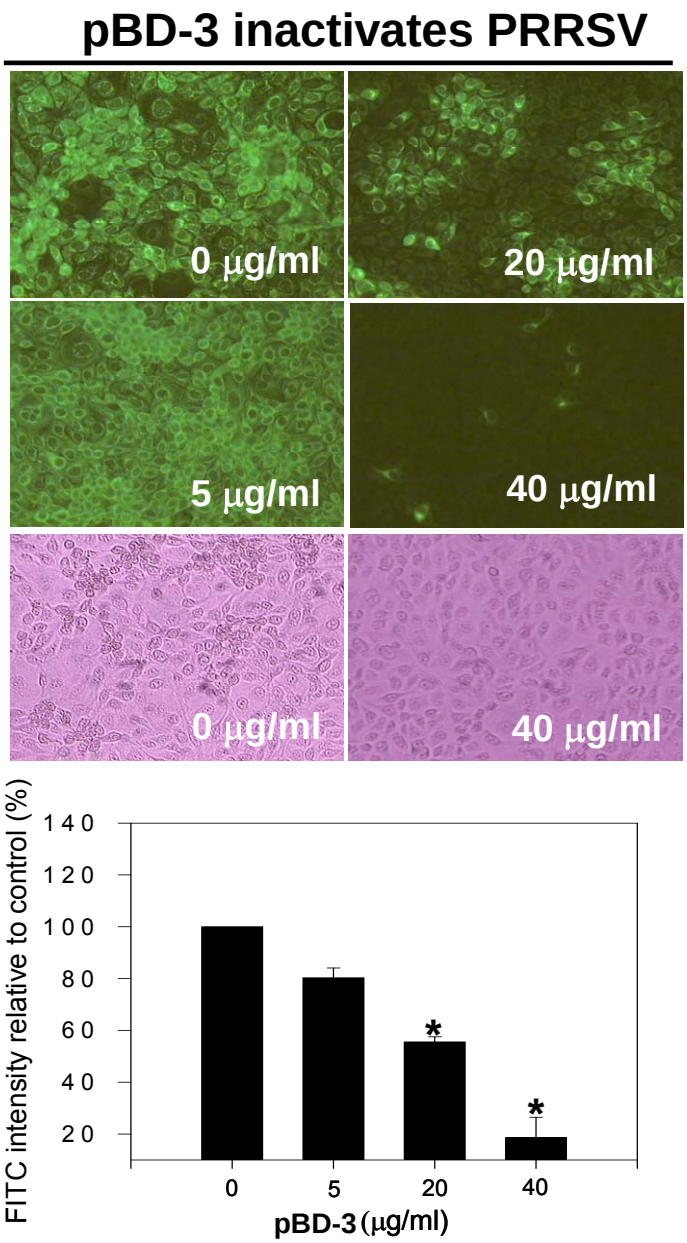

Fig. 3.2. pBD-3 inhibits PRRSV infectivity. PRRSV was incubated with pBD-3 before infection of MARC-145 cells for 2 h and infected cells were cultured in the presence of pBD-3 for 48 h. Top four panels are immunofluorescence of cells detected with a mAb to PRRSV. Bottom two panels are brightfield micrographs. Immunofluorescence intensity of 3-5 randomly photographed areas was measured and standardized relative to the controls using a digital imaging system. * Differential from control $p<0.05$, $n=3$.

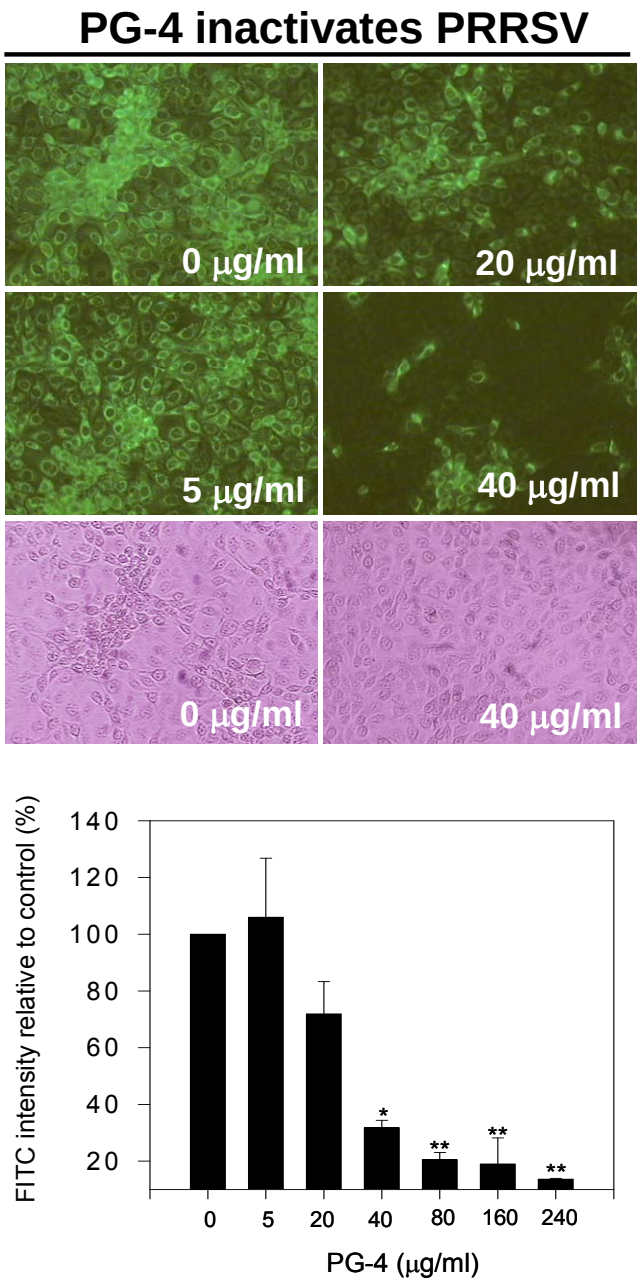

Fig. 3.3. PG-4 inhibits PRRSV infectivity. PRRSV was incubated with PG-4 before infection of MARC-145 cells for 2 h and infected cells were cultured in the presence of PG-4 for 48 h. Top four panels are immunofluorescence of cells detected with a mAb to PRRSV. Bottom two panels are brightfield micrographs. Immunofluorescence intensity of 3-5 randomly photographed areas was measured and standardized relative to the controls using a digital imaging system. * Differential from control $p<0.05$, $n=3$.

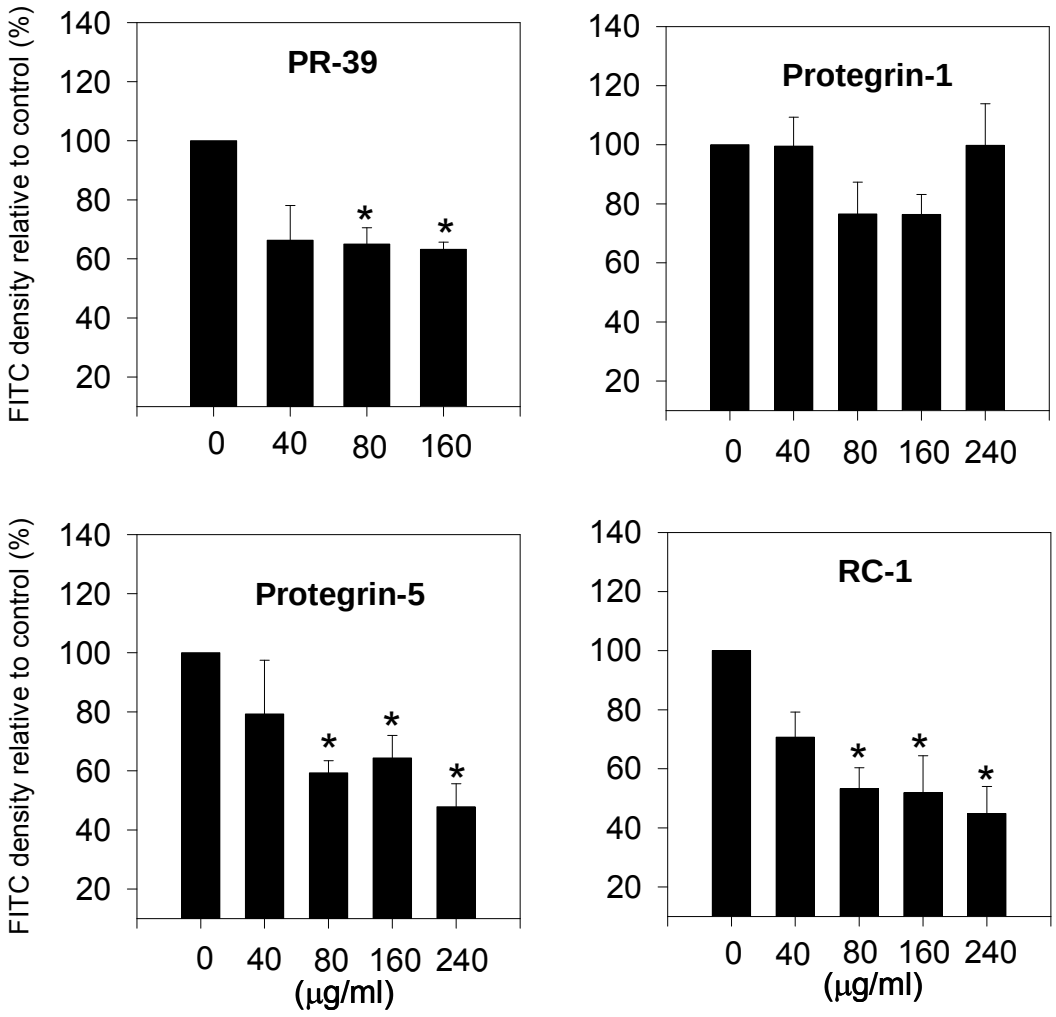

Fig. 3.4. Effects of other HDPs inhibiting PRRSV infectivity. PRRSV was incubated with individual HDP before infection of MARC-145 cells for 2 h and infected cells were cultured in the presence the same HDP for 48 h. PRRSV was then detected using immunofluorescence and quantified as indicated. Immunofluorescence intensity of 3-5 randomly photographed areas was measured and standardized relative to the controls using a digital imaging system. * Differential from control $p<0.05$, $n=3$.

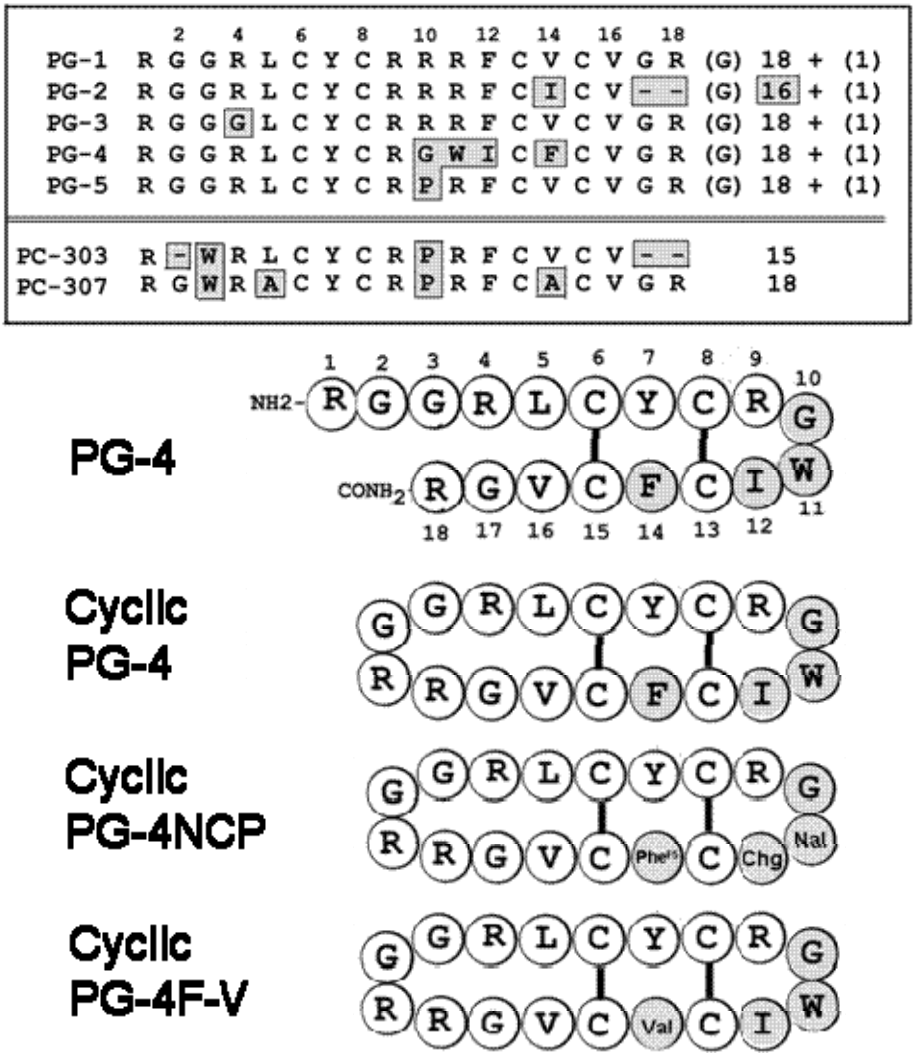

Fig. 3.5. Peptide sequences of porcine protegrins (PGs) and PG-4 derived peptides. Different residues of PG-4 including the aromatic beta turn (GWI) and Phe^{14} (F) are shaded. The designed cyclic forms of PG-4 (PG-4CY) and derived peptides (PG-4NCP and PG-4F-V) with indicated residue changes (residues defined by three letter codes replacing the original residues indicated by one letter codes in original sequence) are shown. The cyclization was catalyzed at the positions mimicking Arg^{1}/Gly^{2} and Gly^{10}/Trp^{11} and synthetic peptides were oxidized to form intramolecular disulfide bonds (black bars). ^{1}Nal, 1-naphthy-alanine, Chg, cyclohexylglycine, Phe^{F5}, and pentafluorophenylalanine.

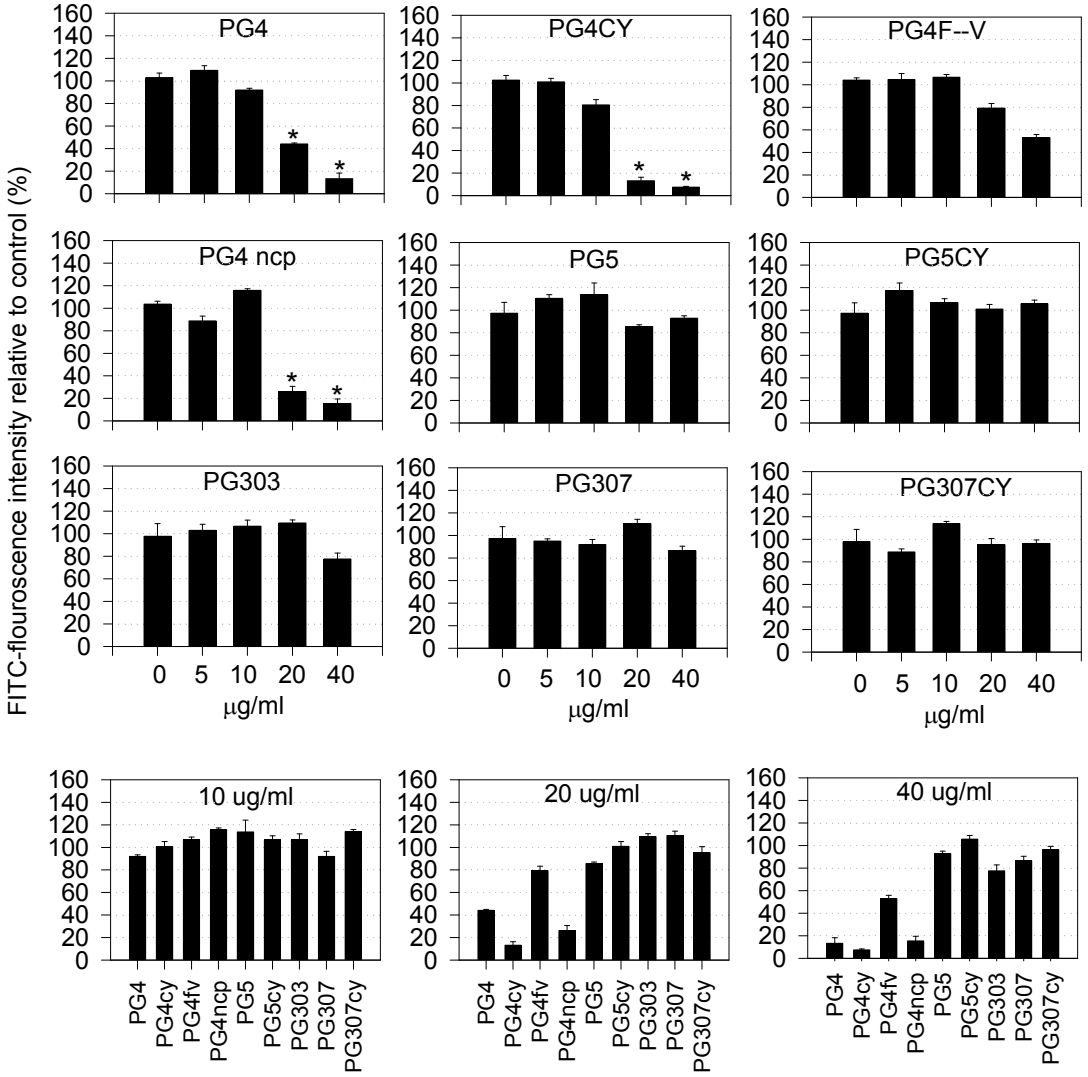

Fig. 3.6. Effects of other PG-4 derived peptides inhibiting PRRSV infectivity. PRRSV was incubated with individual HDP before infection of MARC-145 cells for 2 h and infected cells were cultured in the presence the same HDP for 48 h. PRRSV was then detected using immunofluorescence and quantified as indicated. Immunofluorescence intensity of 3-5 randomly photographed areas was measured and standardized relative to the controls using a digital imaging system. * Differential from control $p<0.05$, n=3.

Table 3.1. Primer sequences for RT-PCR analysis*.

Cathelicidin	*Primer sequence (5' to 3')*	*GenBank accession number*	*Location in cDNA (nt)*
PR-39			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	L23825	295-319
antisense	ATGGGTATGTTATCAGCCACTCCAT		560-534
PF-1/2			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	X75438	280-304
antisense	AAAGGTGGAGGCGGAGGGAACCA		643-621
PMAP-23			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	L26053	291-315
antisense	AAATTTGGGTTTCTGTGGCCGACG		454-431
PMAP-36			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	L29125	291-315
antisense	ACCCAAGGGTATTGAGCCGACAAT		505-482
PMAP-37			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	L39641	291-315
antisense	TCCGACCACGATCACTGAGGAAAT		449-426
PG-1-5			
sense	CGGAGCTGTGTGACTTCAAGGAGAA	X79868	278-302
antisense	TGCCGTCGCAACCGTCATCCT		464-444

* Please refer to **Table 2.1** for the primers for porcine defensins.

Table 3.2. Summary of anti-PRRSV activity of tested HDPs*

Tested HDPs	Direct inhibitory effects	Cytotoxicity at 40 μg/ml
Porcine HDPs		
pBD-1	-	-
pBD-2	-	-
pBD-3	+	-
PR-39	+/-	-
PG-1	-	+
PG-4	+	-
PG-5	+/-	+
Primate HDPs		
hBD-3	+	-
RC-100B	+/-	-

*Based on inactivation of infectivity of three PRRSV strains on MARC-145 cells at the HDP concentration ≤ 40 μg/ml

Table 3.3. Suppression of PRRSV infectivity by HDPs in PAMs.

$TCID_{50}$ Peptides	5 μg/ml	10 μg/ml	20 μg/ml	40 μg/ml
Control	7.4	7.4	7.8	7.4
pBD-2	7.4	7.4	6.6	6.8
pBD-3	6.8	6.6	6.5	4.8[a]
pBD-3ΔC	7.3	6.7	6.8	5.3[a]
PR-39	7.2	7.5	7.4	5.8[a]
PG-1	7.4	6.6	5.8	4.7[a]
PG-4	6.8	6.4	6.3	4.7[a]
PG-5	7.2	7	7	5.3[a]
RC-100B	7	8	7.2	7

a. $p<0.05$ in comparison to controls.

CHAPTER 4 - Molecular Identification and Functional Expression of Porcine Toll-Like Receptor (TLR) 3 and TLR7

ABSTRACT To investigate porcine Toll-like receptors (TLR) responding to viral pathogen associated molecular patterns, the full-length cDNA of porcine TLR3 andTLR7 were identified and characterized. Porcine TLR3 and TLR7 cDNA encode 904- and 1050-amnio-acid polypeptides, respectively. Both porcine TLR3 and TLR7 contain typical functional TLR domains and share about 80% sequence identity to other mammalian orthologues. Tissue expression profiles showed that TLR3 was highly expressed in kidney, duodenum, spleen and liver, and moderately expressed in bone marrow, lung, and skin. Conversely, TLR7 was moderately and constitutively expressed in all tissues evaluated. Stimulation of mammalian cells transfected with porcine TLR3 and TLR7 constructs elicited activation of interferon regulatory factors (IRFs). These data provide molecular and functional information for porcine TLR3 and TLR7, and implicate their role in mediating immune protection against porcine viral diseases.

4.1. Introduction

Toll-like receptors (TLRs) are pathogen recognition receptors that are primary components of the afferent arm of innate immunity (Pichlmair and Reis e Sousa, 2007; Takeuchi and Akira, 2007). Among the more than ten TLRs that have been identified in mammals, four receptors, TLR3, TLR7, TLR8 and TLR9, are involved notably in virus recognition. For example, TLR3 detects double-stranded RNA (dsRNA) formed during viral genome replication or transcription; TLR7 and TLR8 recognize elements of single stranded RNA (ssRNA) found in genomes of RNA viruses; and TLR9 senses unmethylated cytosine-phosphate-guanine (CpG) motifs common to both bacterial and viral DNA (Barton, 2007; Pichlmair and Reis e Sousa, 2007; Takeuchi and Akira, 2007). Unlike TLRs located on the cell surface such as TLR1 to TLR6 and TLR10 to TLR13, virus-sensing TLRs are located mainly in endosomes, which is where viruses undergo decoating during infection (Pichlmair and Reis e Sousa, 2007). Structurally, all identified TLRs contain a ligand-binding, leucine-rich extracellular domain, a transmembrane region, and a conserved Toll/IL-1 receptor (TIR) domain, which transduces perceived signals and induces expression of immune responsive genes (Barton, 2007; Gay and Gangloff, 2007). Prominently in antiviral responses, TLR-mediated signaling pathways activate core transcription factors including nuclear factor (NF)-κB and interferon regulatory factors (IRFs), such as IRF-3 and IRF-7, which subsequently induce the production of type I IFN, a hallmark of antiviral immune responses (Barton, 2007; Kawai and Akira, 2006; Pichlmair and Reis e Sousa, 2007; Severa and Fitzgerald, 2007).

Although several porcine TLRs have been identified (Meier, et al., 2004; Shimosato et al., 2005; Shinkai et al., 2006a; Shinkai et al., 2006b; Tohno et al., 2005; Tohno et al., 2006), identification and studies on porcine TLRs responding to viral pathogen associated molecular patterns are limited. Porcine TLR9 has been identified and shown to be expressed in intestinal

Peyer's patches and expression was stimulated in monocytes and monocyte-derived dendritic cells after treatment with synthetic poly (I:C) and CpG oligonucleotides (Tohno et al., 2006). In addition, a complete cDNA for TLR8 has been identified and deposited in GenBank™, accession number NM 214187. Here, we report the identification and initial characterization of porcine TLR3 and TLR7. Both TLRs were expressed in immune tissues, show 76 to 90% identity to other mammalian orthologues, and conserve the typical TLR domains. Furthermore, gain-of-function experiments showed that both TLRs augment the activation of IRFs. Collectively, these findings provide a molecular foundation to examine the role of porcine TLR3 and TLR7 in mediating immune responses against porcine viral diseases.

4.2. Identification and Phylogenic Analysis of Porcine TLR3 and TLR7

To begin investigating porcine TLR3 and TLR7, full-length cDNAs were obtained using RT-PCR based on a SMART™ RACE cDNA (Clontech, Mountain View, CA) amplification technique (Sang et al., 2005; Sang et al., 2007). Briefly, 'Smart Oligo' was incorporated into the 5'-end of reverse-transcribed cDNA for 5'-RACE analysis. For 3'-RACE analysis, the Smart Oligo was attached to an oligo (dT) primer to yield cDNA that had a complete 3'-UTR, a poly-A tail, and a Smart Oligo sequence extension. The 5'- and 3'-ends of the cDNA fragments were amplified using an Advantage 2 PCR kit (Clontech). Serial gene-specific RACE primers shown in **Table 4.1** were retrieved from the identified cDNA fragments and the consensus sequences derived from alignment of other mammalian orthologues (listed in **Fig. 4.1**). Amplified 5'- and 3'-RACE fragments were cloned and sequenced. The full sequences were generated using the sequence editor program in Lasergene 6 (DNASTAR, Inc. Madison, WI) to fuse the identified cDNA fragments and to remove the overlapped regions. Both porcine TLR3 and TLR7 genes have been registered in GenBank™, accession numbers DQ647698 and DQ647699, respectively.

Porcine TLR3 cDNA has an open reading frame (ORF) of 2712 nt encoding a polypeptide of 904 aa, an approximate 400 bp 5'- UTR, and an approximate 500 bp 3'-UTR. Porcine TLR7 cDNA has an ORF of 3150 nt encoding a polypeptide of 1050 aa, an approximate 120 bp 5'-UTR, and an approximate 400 bp 3'-UTR. Both porcine TLR3 and TLR7 show high similarity to orthologues from other mammalian species with approximately 80% identity in their peptide sequences. Porcine TLR3 or TLR7 cluster most closely to bovine orthologues with identity near or at 90%. TLR functional domains, including the N-terminal extracellular domain, the transmembrane domain, and the TIR domain, were clearly detected in the primary structure of porcine TLR3 and TLR7 (**Fig. 4.1**).

4.3. Expression Analysis of Porcine TLR3 and TLR7

To evaluate tissue expression profiles of porcine TLR3 and TLR7, mRNA expression in tissues from 5-week-old healthy pigs were examined using semi-quantitative RT-PCR (Sang et al., 2005). Total RNA was extracted with TRI Regent (Sigma-Aldrich) directly from cells or from frozen tissues ground in liquid nitrogen. A one-step RT-PCR (Qiagen) was used to detect expression of mRNA transcripts as previously described (Sang et al., 2005; Sang et al., 2007). Briefly, total RNA was treated with RQ1 RNAse-free DNase I (Promega) to remove possible genomic DNA contamination. RNA samples (100-250 ng) were run in a 25-μl RT-PCR mixture with 0.1 μM of each sense and antisense primer derived from cDNA sequences (**Table 4.1**). Semi-quantitative RT-PCR was performed at 1 32, 35, 38, and 40 cycles. After amplification, 10 μl of each reaction mixture was analyzed by 1.5% agarose gel electrophoresis and bands were visualized by ethidium bromide staining using a FluorChemTM digital imaging system (AlphaInnotech Corp., San Leandro, CA). Integrated density values were measured and standardized to values of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase

(GAPDH), and presented as a ratio relative to the expression of GAPDH in the same samples (Sang et al., 2005; Sang et al., 2007). Porcine TLR3 was highly expressed in kidney, duodenum, spleen and liver, and moderately expressed in bone marrow, lung, and skin (**Fig. 4.2**). Conversely, porcine TLR7, was moderately expressed in all tissues evaluated, which included bone marrow, segments of the intestine, spleen, liver, lung, mesenteric lymph nodes, trachea, thymus, kidney and skin.

4. 4. Overexpression of Porcine TLR3 and TLR7 in Mammalian Cells

To examine functional aspects of TLR3 and TLR7, ORFs were amplified from enriched mRNA from porcine macrophages using a high fidelity RT-PCR system (Invitrogen). To facilitate cloning, linkers containing *Hind III* and *BamH I* restriction sites were introduced at their 5' and 3' termini, respectively, by PCR. The inserts were cloned into the expression vector pEGFP-N1 (Clontech). *E. coli* (Topo 10, Invitrogen) transformed with the constructs were selected with kanamycin (30 μg/ml; Sigma), and positive clones were identified and confirmed by sequencing (Sang et al., 2005). Purified plasmids from the positive clones were used to transfect HEK293A cells for expression of enhanced green fluorescent protein (EGFP)-tagged proteins using FuGENE HD transfection reagent (Roche Diagnostics) as previously described (Sang et al., 2005; Sang et al., 2007). Transformation efficiency was determined by EGFP expression using fluorescence microscopy. Expression of EGFP-tagged proteins was confirmed using both RT-PCR and immunoblotting with monoclonal anti-EGFP antibodies (1:8,000; Clontech). In addition, partial ORFs of porcine TLR3 and TLR7 fused to EGFP at their carboxyl terminus were generated. The partial ORFs were designed to express N-terminal extracellular and transmembrane domains (termed TLR3N and TLR7N) after truncation of 3'-cDNA regions encoding C-terminal TIR domains. Thus, these truncated mutants were used as functional

controls that would not induce signal transduction upon perceiving ligands (Kawai and Akira, 2006; Werts et al., 2006). The expression of fused proteins was achieved by transfection of a HEK293A cell line (Invitrogen). Complete and truncated ORFs of TLR3 and TLR7 were expressed into appropriate proteins as predicted. Estimated molecular weights of porcine TLR3 and TLR7 were 103.5 and 120.8 kDa, respectively, and the TLR3N and TLR7N proteins were 24.3 and 28.9 KDa less, respectively, than their intact forms (**Fig 4.3**).

4.5. Functional Induction of Interferon-Related Gene Expression

Two reporter systems were used to characterize the function of porcine TLR3 and TLR7 in activating type I IFN-related gene expression. Two transcription factor genes, IFR-3 and IRF-7, which have been implicated in TLR3 (both) and TLR7 (primarily IRF-7) signaling pathways, were evaluated (Barton, 2007; Kawai and Akira, 2006; Pichlmair and Reis e Sousa, 2007; Severa and Fitzgerald, 2007). Briefly, promoter regions corresponding to human IRF-3 (-779/+1) (Lowther et al., 1999) and IRF-7 (-1123/+575) (Lu et al., 2001) were retrieved from human genomic DNA (Invitrogen) using a high-fidelity PCR kit (Invitrogen). The promoter regions were cloned into a luciferase reporter vector pGL4.14 [luc2/Hygro] (Promega, Madison, WI) through its *Kpn I* and *Hind III* multi-cloning sites. Cloning and plasmid purification procedures were conducted as described above (Sang et al., 2005). HEK293 cells were chosen because they have been shown to express low levels of endogenous TLRs and have been widely used for functional expression of TLR constructs (Invivogen) (Kariko et al., 2004). HEK-293A cells were cultured to 80% confluence and co-transfected with a pair of TLR and IFN related reporter constructs in 6-well culture plates (FuGENE HD, Roche Diagnostics). Twenty-four hours after transfection, the efficiency of transfection, estimated with green fluorescent proteins, was 50-70% in different construct combinations. Cells were then trypsinized at 37˚C for 5 min and

collected by centrifugation at 500 × g for 10 min. After fresh medium was added (2 ml), single-cell suspensions from each well of the 6-well plates were dispersed into wells of 96-well plates at a density of 5000 cells/well. Cells were cultured for 5 h to ensure adherence, and replenished with fresh medium plus agonists for TLR3 (poly I:C at 5 μg/ml, Invivogen), TLR7 (R837 at 5 μg/ml, Invivogen), or vector-type adenovirus (pAd/CMV/V5-DEST-EGFP, Invitrogen) at a MOI of 1. Control wells contained fresh medium plus equal volumes of endotoxin-free water, which was used as the agonist vehicle. Sixteen hours later, cells were lysed and luciferase was detected simultaneously by addition of a Steady-Glo® luciferase assay reagent at 100 μl/well (Promega). After incubation for 10 min at 22˚C, luminescence was measured in a luminometer (Fluoroskan Ascent FL). TLR3 agonist stimulation of HEK293A cells that were co-transfected with TLR3 and reporter constructs for IRF-3 or IRF-7 augmented induction of IRF-3 and IRF-7 promoter activity (**Fig. 4.4A**). TLR7 agonist stimulation in TLR7 co-transfected cells increased IRF-7, but not IRF-3 promoter activity (**Fig. 4.4B**). Adenoviral infection stimulated IRF reporter activity in all TLR3 and TLR7 transfected cells, except in cells transfected with TLR7 plus IRF-3 (**Fig. 4.4 C**).

Collectively, these data provide comparative molecular information for porcine TLR3 and TLR7. Importantly, functional overexpression of porcine TLR3 and TLR7 and subsequent agonist-induced stimulation of IFN-inducible genes in mammalian cells authenticates the molecular information. These findings may be useful in examining the role of porcine TLR3 and TLR7 in mediating immune responses against porcine viral diseases.

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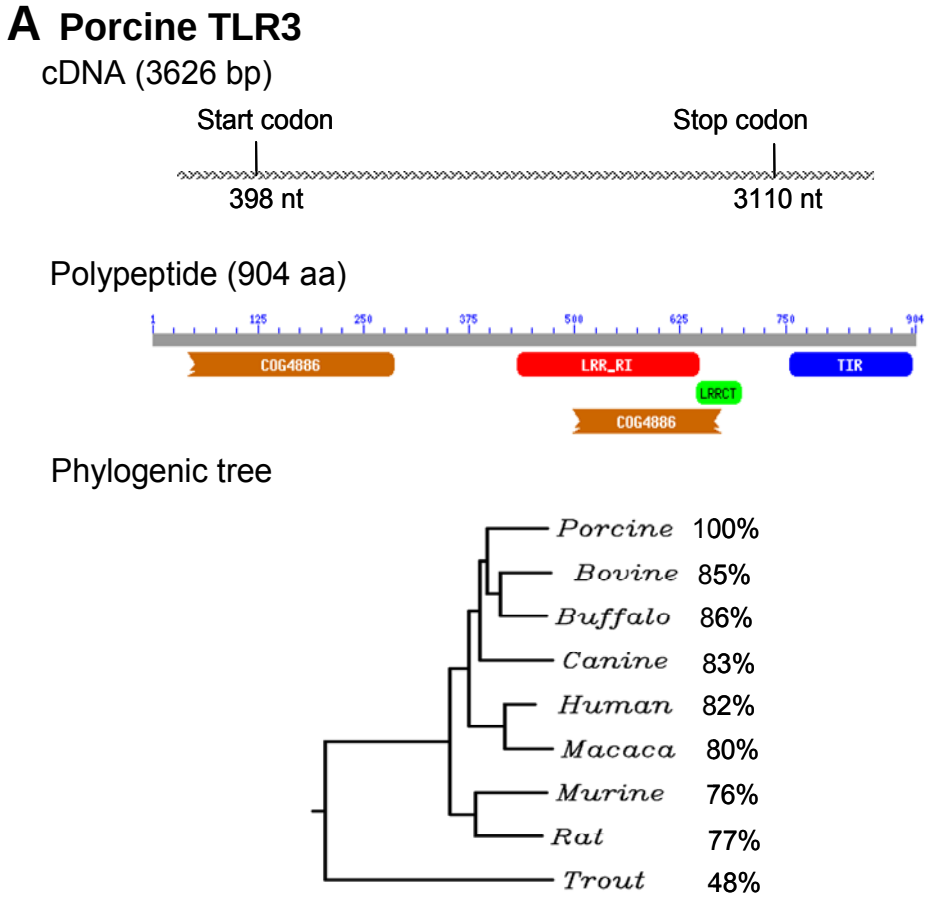

Fig. 4. 1A. Identification and phylogenic analysis of porcine TLR3**.** Schematic diagrams of cDNA and predicted conserved domains in peptide sequences are aligned (LRR_RI or COG4886, leucine-rich repeats (LRRs) domains; TIR, Toll/interleukin-1 receptor homology domain). Gene and protein sequence analyses were conducted with the CDD program at the NCBI website and ClustalW was used for phylogenic analysis. GenBank™ accession numbers of TLR3 homologues are: *Bos taurus*, AJ812026; *Canis lupus*, XM_540020; *Homo sapiens*, BC017954; *Mus musculus*, NM_126166; *Bubalus bubalis*, ABF59103; *Macaca mulatta*, AY864735; *Rat norvegicus,* NP_942086; *Sus scrofa,* DQ647698 and *Oncorhynchus mykiss,* AAX68425

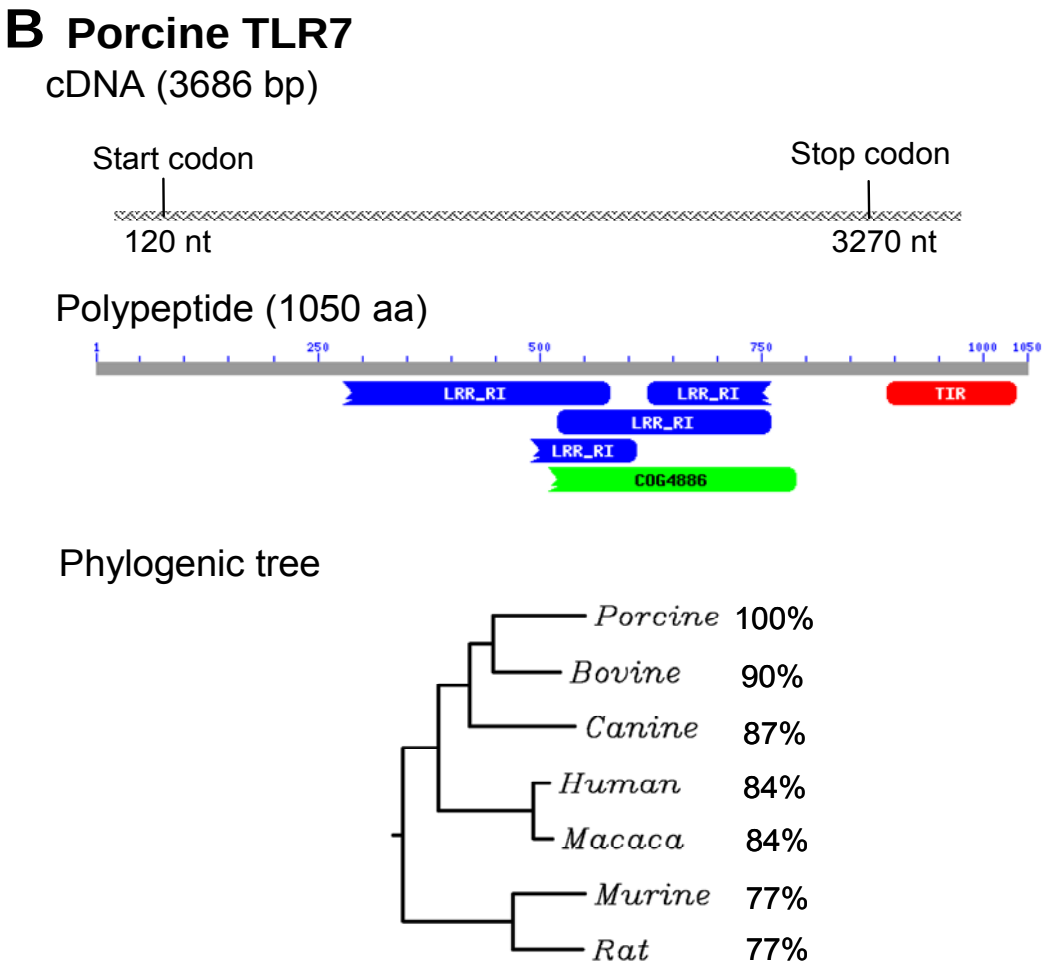

Fig. 4. 1B. Identification and phylogenic analysis of porcine TLR7**.** Schematic diagrams of cDNA and predicted conserved domains in peptide sequences are aligned (LRR_RI or COG4886, leucine-rich repeats (LRRs) domains; TIR, Toll/interleukin-1 receptor homology domain). Gene and protein sequence analyses were conducted with the CDD program at the NCBI website and ClustalW was used for phylogenic analysis. GenBank™ accession numbers of TLR7 homologues are: *Bos taurus*, NP_001028933; *Canis lupus*, NP_001041589; *Homo sapiens*, NP_057646; *Mus musculus*, NP_573474; *Macaca mulatta*, XP_001095269; *Rat norvegicus*, XP_228909 and *Sus scrofa*, DQ647699.

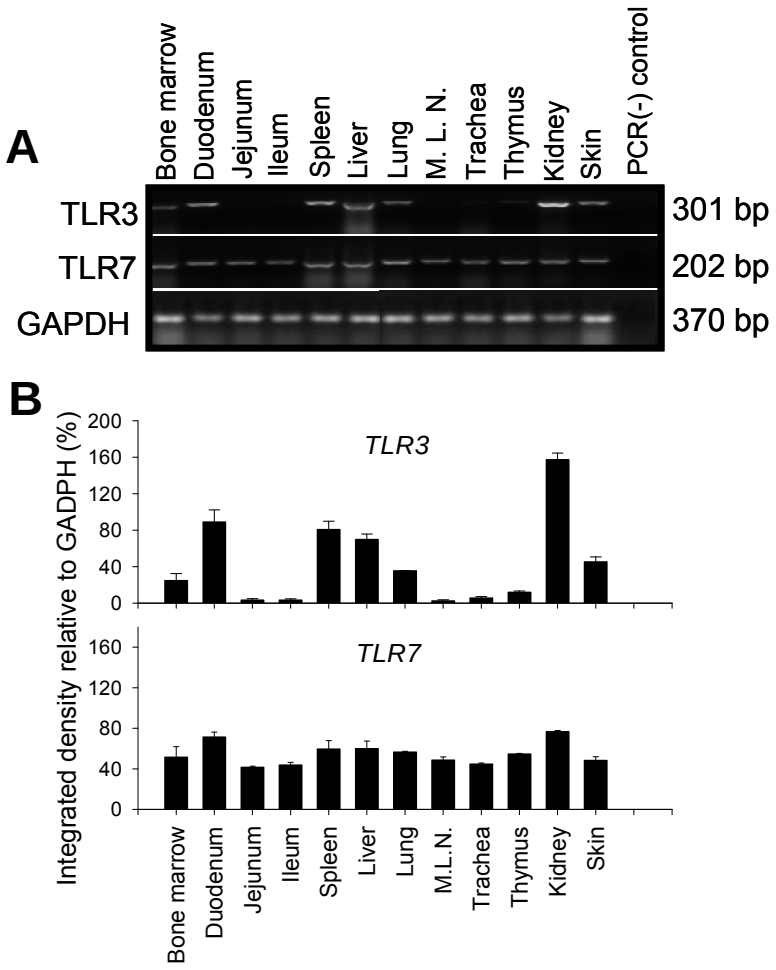

Fig. 4.2. Tissue expression profiles of porcine TLR3 and TLR7. Total RNA (250 ng in 25 µl) was used to conduct one step RT-PCR. **(A)** Gels presented were run with 10 µl of amplicons at 35 cycles for TLRs and 32 cycles for GAPDH. **(B)** Integrated density values of DNA bands were measured and standardized to the housekeeping gene, GAPDH, and presented as a ratio relative to the expression of GAPDH in the same samples. Data are means ± SD, n=3.

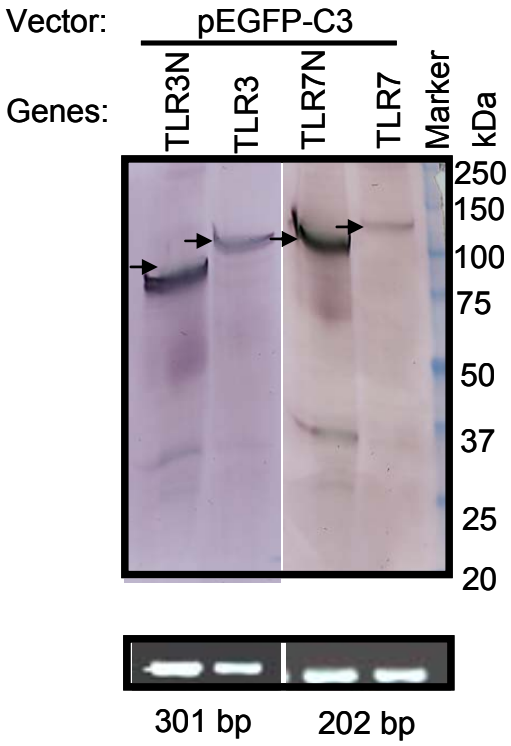

Fig. 4.3. Overexpression of porcine TLR3, TLR7 and corresponding TIR-domain truncated mutants (TLR3N and TLR7N respectively) in HEK293A cells. The overexpressed proteins are fused with EGFP at C-termini. Overexpression of EGFP-tagged proteins was detected using immunoblotting (upper) with monoclonal anti-EGFP antibodies (1:8,000; Clontech) and RT-PCR (bottom) at both protein and RNA levels. The primers used for RT-PCR are listed in Table. 4.1.

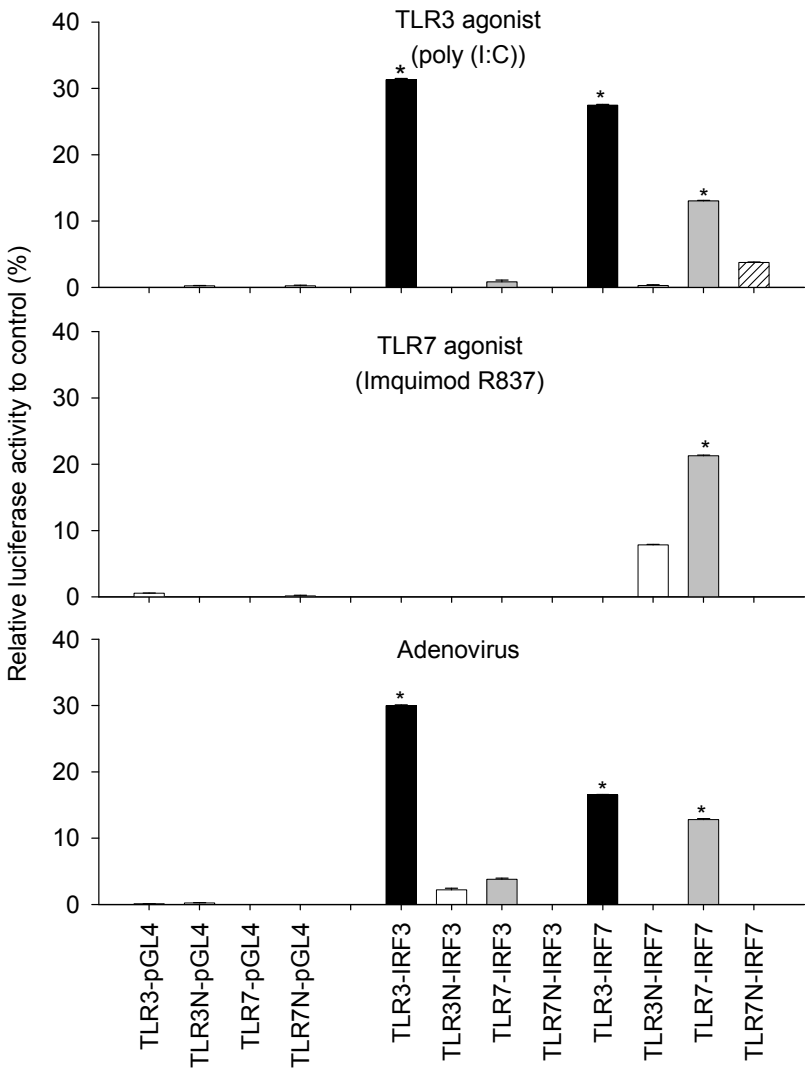

Fig. 4.4. Induction of interferon regulatory factor promoter activity in HEK293A cells transfected with porcine TLR3 and TLR7. Promoter regions of IRF-3 and IRF-7 were amplified and cloned into a luciferase reporter vector pGL4.14 [luc2/Hygro]. Cells were co-transfected with a pair of TLR and reporter constructs as indicated in the X-axis legend. TIR-domain truncated null mutants, TLR3N and TLR7N were used as null tunction controls. Induction of reporter luciferase activity by agonists of **(A)** TLR3 (poly (I:C), at 5 μg/ml) **(B)** TLR7 (R837, at 5 μg/ml) or **(C)** adenovirus was measured and standardized to controls; pGL4 = the empty reporter vector. Data are means ± SD, n=3, 9 *$p<0.05$ in comparison to the controls.

Table 4.1. Primers used for PCR and RT-PCR

Primers	Primer sequences (5' to 3')	accession numbers	Location in cDNA (nt)
TLR3 RACE		DQ647698	
5' RACE-inner	TTGCCGGGAGGTCATCGGATATTT		555-531
5' RACE-outer	AGCTGAGAGAGCTCATTGTGTTGG		741-718
3' RACE-inner	GCACCATGCAGTTCAGCAAGCTAT		2893-2916
3' RACE-outer	CAGGTGCCCTTGAACTTGAAGCAA		2781-2804
TLR3 RT-PCR			
Sense 1	AAGAACTCACAGGCCAGGARTGGA		1731-1754
Antisense 1	AGCCGTGCTAAGTTGTTATGCTGM		2032-2009
TLR3 clone			
Sense	gg*aagctt*ATGTCTACCTACTTATGTACTGTTAGA CAT (underlined part, introduced for cloning)		470-496
Antisense 1	ccggatcccgATGTACTGAATTTCTTGAACCAAG		3132-3109
Antisense 2	ccggatccGCACTGTCTTTGCAGGGTGATGT		2520-2498
TLR7 RACE		DQ647699	
5' RACE-inner	AGTAACAGTTCTGGCCCAGGTAGA		672-649
5' RACE-outer	AGGGCAATTTCCACTTAGGTCCAG		902-879
3' RACE-inner	AAATCCACAGGCTCACCCGTACTT		3173-3196
3' RACE-outer	TCACCCAATTCCTGCTACGATGCT		2778-2801
TLR7 RT-PCR			
Sense	ACAATGATATCGCCACCTCCACCA		1936-1959
Antisense	TGGCCAAGGAGAGAGTCTTCAGAT		2172-2149
TLR7 clone			
Sense	gg*aagcttATG*GCTAGATGGTTTCCTAAAACTCTG		197-220
Antisense 1	gaccgcggTGTCTCTTTGAACACCTGACT		3266-3246
Antisense 2	gaccgcggATGGTTAACCCACCAGACAAG		2522-2502

CHAPTER 5 - Toll-Like Receptor 3 (TLR3) Activation Decreases Porcine Arterivirus Infection

ABSTRACT. Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus that initiates infection in pulmonary alveolar macrophages (PAMs), elicits weak immune responses, and establishes a persistent infection. To understand the role of dsRNA intermediates in eliciting host immunity, we sought to determine if Toll-like receptor-3 (TLR3), a well-known dsRNA sensor, is involved in the regulation of PRRSV infection. TLR3 gene expression was increased in PAMs of congenitally infected 2-wk-old pigs. Stimulation of PAMs with dsRNA increased gene expression for TLR3 and interferon-β, and suppressed PRRSV infectivity. To investigate activation and signaling parameters, expression constructs of wild-type and functional-domain-truncated porcine TLR3 were used in cell transfection studies. When cells that overexpressed porcine TLR3 were stimulated with dsRNA a rapid and robust calcium influx was induced. Moreover, ligand activation of porcine TLR3 expressed in MARC-145 cells elicited an antiviral response to PRRSV. Conversely, transfection of PAMs with small-interfering RNA targeting porcine TLR3 resulted in up to 80% suppression of TLR3 mRNA expression and an increase in PRRSV infectivity. These data provide fundamental genetic and molecular information for porcine TLR3, and implicate its involvement in PRRSV infection; findings that may suggest new strategies to limit this costly pandemic disease.

5.1. Introduction

Although pigs are increasingly used as animal models for human diseases and for xenotransplantation (Cooper et al., 2007; Lunney, 2007), information about porcine receptors that recognize disease agents, particularly viruses, is limited. The porcine arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV), causes an economically significant pandemic viral disease in pigs (Holck et al., 2003; Neumann et al., 2005). This virus is one of the primary pathogens involved in the porcine respiratory disease complex, which causes severe respiratory disease in young pigs (Rossow et al., 2006; Stevenson, 2003; van Reeth, 1997). During late gestation, PRRSV infection results in reproductive failure including aborted, stillborn and weak-born pigs followed by decreases in conception and fertilization rates (Neumann et al., 2005; Rossow et al., 2006; Stevenson, 2003; van Reeth, 1997; Yoon, 2003). Although much has been learned about PRRSV since its initial appearance as "mystery swine disease" in the late 1980s, several aspects of the interaction of PRRSV with the host immune system remain unresolved (Murtaugh et al., 2002, 2003; Neumann et al., 2005; Thacker, 2001).

Immunity to PRRSV begins with the interaction between the virus and porcine cells, predominately pulmonary alveolar macrophages (PAMs), and intravascular macrophages of the placenta and umbilical cord (Oleksiewicz and Nielsen, 1999; Riber et al., 2004; Thanawongnuwech et al., 2000). Recent studies have made progress in identifying a receptor for PRRSV (Calvert et al., 2007; Delputte et al., 2007; Kim et al., 2006; Shanmukhappa et al., 2007); however, how porcine cells interact with PRRSV at early stages of innate immunity is largely unknown. PRRSV is an enveloped, positive-sense, single-stranded RNA virus (Yoon, 2003). During replication, genomic and subgenomic RNAs participate in the formation of

several double-stranded intermediate structures (Yoon, 2003). Interaction between PRRSV and macrophages likely alters the production of cytokines, including interferon (IFN)-α, IFN-β, IFN-γ, and interleukin (IL)-10 (Carter and Curiel, 2005; Charerntanttanakul et al., 2006; Royaee et al., 2004; Thanawongnuwech et al., 2000). Replication of PRRSV in monocyte-derived dendritic cells also leads to suboptimal induction of adaptive immunity (Wang et al., 2007). Although early upregulation of IFN-γ production, activation of NK cells and γδ T cells, and stimulation of protective antibody were found in PRRSV-infected pigs (Olin et al., 2005; Wesley et al., 2006), optimal immune protection was not achieved (Wang et al., 2007; Xiao et al., 2004). Despite significant efforts to identify immunogenic viral epitopes (de Lima et al., 2006; Plagemann, 2006; Zhou et al., 2006) and to develop and optimize vaccines with various adjuvants, effective means to control this disease have not been achieved (Rompato et al., 2006; Charerntanttanakul et al., 2006; Royaee et al., 2004).

Host cells use various receptors to perceive viral infections by recognizing pathogen-associated molecular patterns (PAMPs) and subsequently induce an antiviral response (Kawai and Akira, 2006; Werts et al., 2006). Prominent among these are Toll-like receptors (TLRs). Currently, more than ten TLRs have been identified in humans and mice (Kawai and Akira, 2006; Meylan and Tschopp et al., 2006; Werts et al., 2006). Several TLRs perceive viral PAMPs, including: TLR3, which detects double-stranded RNA (dsRNA) derived from viral replication; TLR7 and TLR8, which recognize single-stranded RNA (ssRNA) fragments derived from viral genomes; and TLR9, which senses unmethylated cytosine-phosphate-guanine (CpG) motifs common to both bacterial and viral DNA. A non-TLR cytosolic receptor, retinoic acid inducible gene I (RIG-I) that was originally thought to recognize dsRNA, binds to 5' triphosphate ssRNA (Hornung et al., 2006). TLR7, TLR8 and TLR9, likely form a functional subgroup within

the TLR family that recognizes viral PAMPs in endosomal or lysosomal compartments (Kawai and Akira, 2006; Meylan and Tschopp et al., 2006). In contrast, the location of TLR3 varies depending on the viral infection and cell type, being expressed intracellularly or on the cell surface (Groskreutz et al., 2006; Meier et al., 2004; Schroder and Bowie, 2005). After perceiving the presence of viral nucleic acids, these TLRs, such as TLR3, mediate the induction of type I IFNs through a signaling pathway involving adaptor proteins, myeloid differentiation factor 88 (MyD88) or Toll/IL-1R domain-containing adapter-inducing IFN-β protein (TRIF), elicit a rapid increase of intracellular calcium influx, activate the intermediate protein kinase cascade, and activate transcription factors including NF-κB and interferon regulatory factor (IRF)-3 and IRF-7. Although several porcine TLRs have been identified (Meier et al., 2004; Shimosato et al., 2005; Shinkai et al., 2006; Tohno et al., 2005, 2006), identification and detailed studies on porcine TLRs responding to viral PAMPs are limited. We have recently reported the molecular identification and functional expression of porcine TLR3 (Sang et al. 2008). Here we report the involvement of porcine TLR3 in PRRSV infection. Our findings show that activation of porcine TLR3 signaling is important in stimulating effective responses to PRRSV infection, a property that may be exploited by the virus to avoid eliciting effective immune responses and may suggest new strategies to limit this costly pandemic disease.

5.2. Materials and Methods

Virus strains and titration. The North American macrophage-tropic PRRSV strain, SDSU-23983-P6 (P6), was used to infect pigs as previously described (Kim et al., 2004; Rowland et al., 2001, 2003). All animal and virus procedures were approved by the Kansas State

University Institutional Animal Care and Use, and Biosafety Committees. MARC-145 cells, an African green monkey kidney cell line sensitive to PRRSV infection, was used to test PRRSV infectivity and for virus titration. Virus stocks [SDSU-23983-P7 (P7)] collected from supernatant of P6-infected MARC-145 cells were used to infect cell cultures. The tissue culture 50% infectious dose ($TCID_{50}$) of P7 stocks was $10^{7.25}$/ml. Cells were infected at a standard multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell. This MOI was chosen because quantities of the SDSU-23983 virus greater than 0.1 MOI do not enhance infectivity of MARC-145 cells (Rowland et al., 2001). For titration, MARC-145 cells were cultured in MEM supplemented with 8% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 IU penicillin and 100 μg/ml streptomycin, Chemicon International, Inc., Temecula, CA) in a humidified 5% CO_2, 95% air atmosphere at 37 °C. Monolayers of MARC-145 cells in 96-well tissue culture plates were fixed with 80% cold acetone and incubated with fluorescent-labeled monoclonal antibodies to PRRSV nucleocapsid (N) protein (SDOW17; Rural Technologies, Inc., Brookings, SD). PRRSV-positive cells were identified by fluorescent microscopy.

PRRSV infection and tissue sample collection. Fetal and young pig lung samples from an earlier study were used in which seronegative pregnant females were infected at 85-90 days of gestation with wild type P6 as described (Rowland et al., 2001, 2003). Infection of late gestation females is a standard model for the study of fetal and congenital PRRSV infection. For the pigs that survive birth, the outcome is productively infected pigs with more severe disease (Rowland et al., 2001). Animals were allowed to give birth and live-born pigs were euthanized at 14 days of age or fetuses were obtained at 107 and 112 days of gestation. Tissue samples were

immediately placed in RNA*later* (Ambion, Inc. Austin, TX) and stored at -20 °C until used (Rowland et al., 2001).

PAM collection and culture. Porcine pulmonary alveolar macrophages (PAMs) were obtained by bronchoalveolar lavage from healthy 5-wk-old pigs (Chitko-McKown et al., 1991; Xiao et al., 2004). Lungs were lavaged with PBS and recovered lavage fluid was centrifuged at 400 × g for 15 min. After washing with PBS, cells were resuspended in culture medium (RPMI 1640, 10% fetal bovine sera, 5 mM HEPES, 1 mM glutamine, antibiotic-antimycotic, and 50 mg/ml gentamicin; Invitrogen Life Technologies). Cells were cultured in T75 flasks for 2 h in a humidified 5% CO_2, 95% air atmosphere at 37 °C. Adherent PAMs were collected by washing with cold (4 °C) PBS, counted and cryopreserved until used.

Cell cultures and treatments. Two days before treatments or infection with PRRSV, PAMs were thawed from stocks and plated in 24- or 48-well tissue culture plates (7×10^4 or 4×10^4 cells/well) in supplemented RPMI 1640 medium and cultured in a humidified 5% CO_2, 95% air atmosphere at 37 °C. After one-change of fresh medium 2 h later to remove non-adherent cells, PAMs were cultured for 16 h with replenished medium containing the TLR3 ligand, synthetic dsRNA (polyinosinic-polycytidilic acid; Invivogen, San Diego, CA) at 5 μg/ml. This concentration was chosen after dose-response tests using ranges of 1-25 μg/ml. Supernatants were collected for interferon assays and RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO). Cells in duplicate wells were infected with PRRSV-P7 and replenished with fresh medium containing the indicated stimulators for 18 h. PAMs were directly stained for PRRSV N protein with a monoclonal antibody (SDOW17) and visualized with

TRITC-conjugated goat anti-mouse IgG. The PRRSV-positive cells were examined and counted using fluorescent microscopy.

RT-PCR analysis. Total RNA was extracted with TRI reagent (Sigma-Aldrich) from cells or from frozen tissues ground in liquid nitrogen. Real-time and one-step RT-PCR assays (Qiagen) were used to detect expression of mRNA transcripts. Briefly, total RNA was treated with RQ1 RNase-free DNAse I (Promega) to remove possible genomic DNA contamination. For real-time RT-PCR a SYBR-Green-based assay (Qiagen) was performed on a SmartCycler (Cepheid, Sunnyvale, CA) as previously described (Sang et al., 2005, 2006). Relative gene-expression data were normalized against Ct values of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of control samples with the index defined as 1.000 (Livak and Schmittgen, 2001). Semi-quantitative RT-PCR was performed using one-step RT-PCR (Qiagen) at 32, 35, 38, or 40 cycles. RNA samples (50-250 ng) were run in a 15 or 25-μl RT-PCR reaction mixture with 0.1 μM of each sense and antisense primer derived from cDNA sequences (Sang et al., 2008). Generation of PCR primers, PCR data acquisition and standardization were conducted as described (Sang et al., 2005, 2006, 2008).

Interferon bioassay. Concentrations of type I IFN were measured using a bovine kidney cell line (MDBK) stably expressing a human MxA-promoter (Fray et al., 2001) in a luciferase reporter vector pGL4.14 [luc2/Hygro] (Promega, Madison, WI). Luminescence from the activated luciferase in IFN-treated cells was measured with a Steady-Glo® luciferase assay system (Promega) according to the manufacturer's instructions. Standard curves were calculated

using recombinant human IFN-β (R&D Systems) at a concentration range of 0-1000 U/ml, and regression analysis between luminescence intensity and IFN concentration was conducted with an exponential growth model (SigmaPlot 9.0, Systat Software, Inc.).

Construction and expression of porcine TLR3 and TLR3N cDNAs. Full-length cDNA of porcine TLR3 was obtained using RT-PCR and rapid amplification of cDNA ends as described (Sang et al., 2006, 2008). In addition, the 3'-terminal region of TLR3 at 612 nt long, which is predicted to encode the cytoplasmic TIR/IL-1 domain, was truncated using PCR to create mutated TLR3, i.e., TLR3N. Purified plasmids were used to transfect HEK293A cells for expression of EGFP-tagged proteins using Fugene 6 transfection reagent (Roche Diagnostics) as described (Sang et al., 2008). Transformation efficiency was determined by enhanced green fluorescent protein (EGFP) expression using inverted fluorescence microscopy. After transformation (24 h), cells were collected and transformants were sorted by fluorescence-activated flow cytometry (FACSVantage SE; Becton Dickenson). Overexpression of control EGFP and EGFP-fused TLR proteins was confirmed by both RT-PCR and immunoblotting. For immunoblotting, proteins were extracted with a mammalian protein extraction reagent (Pierce), separated on 4-20% precast sodium dodecyl sulfate (SDS)-polyacrylamide gels (Pierce) and transferred onto polyvinylidene difluoride blots. EGFP and EGFP-fused proteins were then detected with monoclonal anti-EGFP antibodies (1:8,000; Clontech) and visualized using a color development reaction catalyzed by alkaline phosphatase-conjugated secondary antibodies (Sang et al., 2005).

MARC-145 cells were transfected with pEGFP-TLR3 or pEGFP-TLR3N constructs using FuGENE® HD transfection reagent (Roche Diagnostics). Cells were cultured in 24-well plates or

8-well chamber slides (Electron Microscopy Sciences, Hatfield, PA), and transfection was performed according to the manufacturer's instructions. After transfection (24 h), cells (>20 % of EGFP positive cells) in different wells were treated with dsRNA (poly (I:C), 5 μg/ml), or incubated with monoclonal antibodies to EGFP (Clontech, Jl-8, 10 μg/ml) for 30 min then crosslinked with goat anti-mouse IgG (5 μg/ml, Sigma). Cells were then infected with PRRSV (P7) for 48 h (Kim et al., 2002; Rowland et al., 2003), and collected for RNA extraction and detection of viral RNA replication using RT-PCR. Cells in chamber slides were fixed and permeablized with Fix/Perm solution (BD Biosciences). Slides were then treated with a monoclonal antibody (SDOW17) to PRRSV N protein and treated with TRITC-conjugated goat anti-mouse IgG. After counterstaining nuclei with Hoechst 33342, the slides were examined using fluorescent microscopy for EGFP (transformed TLR3 or TLR3N) and TRITC (PRRSV). The influence of porcine TLR3 overexpression in mediating PRRSV infectivity was evaluated by comparison with TLR3N transfected and normal MARC-145 cells.

Intracellular calcium measurement. TLR3-mediated calcium flux in transfected HEK293A cells was measured (1) using the calcium dyes Fluo-3-AM and Fura Red-AM (Molecular Probes). HEK293A cells transfected with pEGFP-C3 constructs were sorted (45) and suspended at 5×10^6/ml in 1X Hanks' balanced salt solution (HBSS) with Fluo-3 and Fura Red (2.6 μM Fluo-3 and 5.5 μM Fura Red). Cells were incubated at 37 °C for 30–45 min, washed once with 1X HBSS and resuspended at 1×10^6/ml. Aliquots (250 μl) were warmed to 37 °C prior to measurement on a FACSCalibur flow cytometer (Becton Dickenson) equipped with argon and Red diode lasers. Fluo-3 was detected at 530/30 nm and Fura Red at 610/20 nm. Cells were analyzed at a rate of ~1,000 events/s. After establishment of the baseline for about 20 s,

dsRNA was added to achieve a final concentration of 10 μg/ml. Recording commenced after replacing the tubes with the stimulator and continued for up to 204 s. Fluorescence signals of Fluo-3 and Fura Red were collected and the data were processed using flow cytometry analysis software (WinList 5.0, Verity Software House, Inc. Topsham, ME) to obtain the geometric mean fluorescence intensity every 2 s. The ratio of fluorescence intensity of Fluo-3 to Fura Red was plotted against time using SigmaPlot 9.0 (Systat Software, Inc. San Jose, CA).

Confocal microscopy. Confocal laser microscopy was used to determine the cellular localization of the EGFP-tagged TLR3. Briefly, slides with >20% EGFP-positive cells were fixed and examined 24 h after transfection. Media were removed and cells were washed once with Dulbecco's phosphate buffered saline (DPBS; pH 7.4) and 1 ml of 4% paraformaldehyde/DPBS was added to each well. Cells were fixed for 30 min at 22 °C, nuclei were counterstained with Hoechst 33342 (0.12 μg/ml), and cells were rinsed twice with DPBS. Slides were mounted with ProLong® Antifade solution (Molecular Probes), and examined with a confocal laser scanning microscope (LSM 510, Zeiss).

siRNA. Gene-specific siRNA for porcine TLR3 was designed (*siRNA Target Finder* http://www.ambion.com/techlib/misc/siRNA_finder.html) and matched to position 1088-1108 in the TLR3 cDNA sequence (5'-UGUCAAACUGAGCCCCAGUtt-3', GenBank™ accession number, DQ647698). Sense and antisense sequences of the siRNA were synthesized (Qiagen). To facilitate the selection of efficient transfection reagents, an Alexa Fluor-488 (AF 488)-labeled scrambled siRNA (Qiagen), which was designed to not suppress any mammalian gene, also was used. PAMs were seeded in a 24-well plate at 7×10^4 cells/well/0.5 ml, and transfected with

different reagents after 24 h according to the manufacturer's instruction. Transfection efficiency was estimated using the AF 488-labeled scrambled siRNA and evaluated by inverted fluorescence microscopy after 24 h. The transfection reagent, HiPerfect™ (Qiagen) was used for transfection of TLR3 siRNA into PAMs. Twenty-four hours after siRNA transfection, cells were infected with PRRSV for an additional 24 h. Cells in different wells were collected for RNA extraction at 24 and 48 h, or fixed for PRRSV immunostaining as described. Infection (%) was obtained by calculation of PRRSV-positive cells in ~500 randomly examined cells in each treatment.

Statistical analysis. Data are presented as means ± SD. Experimental data were analyzed by Student's *t*- test and statistical significance was denoted at $p < 0.05$.

5.3. Results

Expression of porcine TLR3 in PRRSV-infected lungs and PAMs. Our initial approach in studying porcine TLR3 aimed to determine if it was expressed and regulated in PRRSV-infected animals and cells. Indeed, porcine TLR3 was expressed in porcine lungs, and PAMs. Upon PRRSV infection, expression of TLR3 mRNA was increased up to threefold in lungs of infected 2-wk-old pigs from pregnant females that had been exposed to PRRSV in late gestation; however, TLR3 mRNA expression was not altered in fetuses from PRRSV-infected sows or in PAMs infected with PRRSV *in vitro* (**Fig. 5. 1**). The absence of upregulated TLR3 in PAMs and fetuses does not reflect a lack of infection as PRRSV replication was clearly evident (**Fig. 5.1**).

TLR activation induces anti-PRRSV activity in PAMs and MARC-145 cells. Engagement of TLR3 with dsRNA caused a clear decrease in PRRSV infectivity in PAMs, indicated by almost no PRRSV-positive cells in the dsRNA-treated PAMs (**Fig. 5.2**). In addition, the influence of TLR3 activation in MARC-145 cells on PRRSV infectivity also was evaluated. Consistent with the PAM data, dsRNA treatment suppressed PRRSV-infectivity in MARC-145 cells. The effective concentration range of dsRNA tested on MARC-145 to suppress PRRSV infectivity was 0.5 to 25 μg/ml. More than 60% infectivity of PRRSV was inhibited by dsRNA at concentrations higher than 2.5 μg/ml, with the optimal suppressive dose at 5-10 μg/ml (data not shown).

PRRSV infection and dsRNA treatment increases TLR3 and IFN-β gene expression and IFN-β activity. Treatment of PAMs with dsRNA for 6 h significantly increased expression of TLR3 mRNA three- to fourfold (**Fig. 5.3A**). The increase in TLR3 mRNA expression was accompanied with a similar increase in IFN-β gene expression; however, IFN-α was not altered (**Fig. 5.3A**). Similar to the gene expression data, dsRNA increased the production of type I IFNs in PAMs as early as 5 h after treatment and the activity was equivalent to 40- and 158-fold increases in IFN-β at 5 and 10 h, respectively (**Fig. 5.3B**). PRRSV-infected PAMs exhibited delayed and lower IFN-β activity compared to cells treated with dsRNA. Supernatants from PRRSV-treated PAMs, had 30- and 60-fold increases in IFN-β activity at 10 and 24 h, respectively, compared to controls.

Overexpression of porcine TLR3 and its truncated mutant. We recently reported the molecular identification and functional expression of porcine TLR3 (Sang et al., 2008). Using

those findings to facilitate gain-of-function studies, we expressed whole or partial ORFs of porcine TLR3 fused to EGFP at their amino termini. The partial ORFs were designed to express N-terminal extracellular and transmembrane domains after truncation of the 3'-cDNA regions encoding the C-terminal TIR domains (named TLR3N). Thus, this truncated mutant was designed as a functional control that lacks the ability to induce signal transduction upon perceiving ligands (Kawai and Airka, 2006; Werts et al., 2006). Expression of fused proteins was first achieved by introducing TLR3 plasmid constructs into HEK293A cells. Shown in **Fig. 5.4A** are the expressed whole and truncated proteins encoded by the whole and truncated ORFs of TLR3. Estimated molecular weight of porcine TLR3 is 103.5 kDa and TLR3N is 24.3 kDa less than its intact forms.

Location and expression of functional porcine TLR3 in HEK293A cells. Before examining potential antiviral responses mediated by cells overexpressing porcine TLR3, we first identified its location and functional properties in HEK293A cells. The transfection efficiency of HEK293A cells was consistently >50% with lipid-formulated reagents (Fugene 6 or HD, Roche). Cells treated with dsRNA displayed a small but authentic proportion of porcine TLR3 that localized on the cell surface (**Fig. 5.4B**). Importantly, porcine TLR3 expressed in HEK293A cells was biologically relevant as cells stimulated with dsRNA (10 μg/ml) exhibited a rapid and robust calcium influx (**Fig. 5.4C**). Before stimulation, cells transformed to express EGFP, EGFP-TLR3N and EGFP-TLR3 all had similar basal levels of calcium influx.

Activation of porcine TLR3 suppresses PRRSV infectivity. To evaluate the role of TLR3 in mediating anti-PRRSV activity, MARC-145 cells, an established cell line sensitive to PRRSV

infection, were transfected with EGFP-TLR3, or EGFP-TLR3N constructs; control cells were mock transfected. Treatment of cells with dsRNA suppressed PRRSV infectivity in transformed and non-transformed cells (**Fig 5.5A**); a finding that likely resulted from activation of endogenous TLR3. To address this issue, anti-EGFP antibody plus secondary antibody was used to aggregate surface-expressed N-terminal EGFP fused to TLR3 in transformed cells (de Bouteiller et al., 2005). Similar to dsRNA, aggregation of surface-expressed porcine TLR3 with anti-EGFP antibody also significantly decreased PRRSV infectivity. This response was specific as antibody aggregation of TLR3 was only able to induce an antiviral response in EGFP-TLR3 transformed cells, not in mock- or EGFP-TLR3N-transformed cells (**Fig. 5.5A**). When PRRSV replication was evaluated by fluorescent microscopy, similar results were obtained, i.e., cross-linking TLR3 with the anti-EGFP significantly decreased PRRSV infectivity (**Fig. 5.5B**). TLR3 in transfected cells colocalized with PRRSV N protein in cytoplasmic areas with some PRRSV detected with EGFP-TLR3 near the cell surface (**Fig. 5.5C**).

Silencing porcine TLR3 decreases IFN-β mRNA expression and increases PRRSV infectivity in PAMs. To further investigate TLR3 involvement in PRRSV infection in PAMs, siRNA was used to silence endogenous TLR3. Optimal transfection conditions were established with an AF-488-labeled control siRNA (Qiagen) (Zhang et al., 2005). At 50 h after transfection, approximately 80% of cultured PAMs loaded with siRNA were successfully transfected as estimated with the control AF-488 siRNA. Transfection with siRNA for TLR3 decreased endogenous TLR3 mRNA expression 50-70% at 24 h, and 80% at 48 h (**Fig. 5.6A**). When compared to mock and scrambled siRNA-transfected cells, suppression of TLR3 in PAMs by

gene-specific siRNA caused six to eightfold increases in PRRSV-positive cells at 20 h after infection (**Fig. 5.6B**).

5.4. Discussion

This study provides experimental support for three new findings concerning the involvement of TLR3 in porcine viral infections. First, it provides the first molecular and functional characterization of porcine TLR3; data that are critical for comparative studies using porcine models for infection and immunity. Second, it shows that activation of TLR3 produced significant antiviral responses to PRRSV and decreased replication of this economically significant porcine virus. Third, it shows that cells with mutated or diminished TLR3 function exhibit increased PRRSV infection. These findings suggest that TLR3 is important for effective innate immunity to PRRSV.

Similar to other mammals, pigs have an extensive repertoire of TLRs and, at the amino acid sequence level, generally show about 80% similarity to TLR orthologs from other mammalian species (Meier et al., 2004; Sang et al., 2008; Shimosato et al., 2005; Shinkai et al., 2006; Tohno et al., 2005, 2006). Here we provide the first functional characterization of porcine TLR3, an innate immune receptor well characterized for its function in perceiving viral molecules. TLR3 recognizes dsRNA originating from viruses or host cells. Other viral sensing TLRs, TLR7, TLR8 and TLR9 recognize viral ssRNA and unmethylated CpG DNA in viral genomes (Kawai and Akira, 2006; Meylan and Tschopp, 2006; Schroder and Bowie, 2005). When we examined viral TLR gene expression in response to PRRSV infection in porcine lungs and PAMs, we found that TLR3 mRNA expression was increased in lungs of young pigs but not in lungs of fetal pigs or

PAMs. The absence of upregulation of TLR3 in the fetal pig may reflect downregulation of in utero TH1 responses or the immaturity of the fetal immune system. Expression of other TLR mRNA was not significantly influenced by PRRSV infection (data not shown). Several possibilities might explain the finding that PRRSV infection selectively influenced the expression of these viral TLRs. First, these innate immune receptors may be constitutively expressed in various tissues, and thus are not susceptible to significant upregulation. Second, an increase in TLRs elicited by PRRSV infection may have occurred at an earlier time than when the samples were collected. Third, it is possible that PRRSV has a mechanism to mitigate upregulation of TLRs.

Because PAMs are a primary cell type for PRRSV infection, we examined expression of porcine TLR3 in PAMs and evaluated the influence of activating TLR3 on innate viral protection. Poly (I:C), a synthetic dsRNA TLR3 ligand, induced significant effects in PAMs yielding protective responses against PRRSV infection. Porcine TLR3 and IFN-β were significantly increased by poly (I:C) treatment, which suggests that TLR3 was involved in the stimulation of IFN-β expression and the subsequent suppression of PRRSV infection. After extensively comparing poly (I:C)-induced cytokine profiles in porcine PAMs and peritoneal macrophages, Loving et al. (2006) concluded that dsRNA induced cytokine expression in PAMs mainly *via* mediation of TLR3 and in peritoneal macrophages *via* PKR. Similar to our findings in PAMs, treating MARC-145 cells with poly (I:C) also suppressed PRRSV infection, suggesting that poly (I:C) stimulated similar antiviral responses at least partially *via* TLR3 in MARC-145 cells (Luo et al., 2008).

To evaluate the contribution of the TLR3 pathway in anti-PRRSV responses, we conducted gain-of-function and loss-of-function studies. In gain-of-function experiments, EGFP-tagged

porcine TLR3 was functionally expressed in both HEK293A cells and MARC-145 cells. Porcine TLR3, when overexpressed in MARC-145 cells, colocalized intracellularly with the virus nucleocapsid (N) protein (Fig. 5C). During virus replication and after transfection with PRRSV N gene constructs the N protein traffics through the nucleoplasm and accumulates in the nucleolus, where it forms an association with nucleolar proteins, such as fibrillarin (Dongwan et al., 2003; Rowland et al., 2003). Translocation of N protein across the nuclear pore complex is via a classic nuclear localization signal sequence (NLS). A second, nucleolar localization signal (NoLS) domain is involved in accumulating the protein in the nucleolus. The localization of expressed EGFP-TLR3 is intriguing. Analysis of the porcine TLR3 peptide sequence by the web-based program PSORT (Nakai and Kanehisa, 1992) identifies a NLS domain, PLCKRFK, beginning at amino acid 825. Another possibility for the accumulation of TLR3 in the nucleus/nucleolus is the co-translocation of TLR3 with N protein. The formation of a non-covalent interaction with N and subsequent sequestration in the cytoplasm could represent a novel strategy for the prevention of newly synthesized TLR3 from reaching the cell surface. This property of TLR3 during virus infection deserves further investigation (Pei et al., 2008).

Selective activation of transfected EGFP-TLR3 using antibody aggregation resulted in protective activity against PRRSV infection. Because only about 10% of the cells had EGFP-TLR3 located on the surface and thus available for cross-linking by antibodies, the cells may exploit a communication pathway to transmit and enhance the signal from stimulated cells that have activated TLR3, perhaps analogous to the cross-priming function of TLR3 in dendritic cells to cytotoxic T cells (Schulz et al., 2005). Overexpression of the null mutant, TLR3N, only slightly enhanced PRRSV infectivity in MARC-145 cells. We reason that this finding likely resulted because PRRSV attains nearly saturated infectivity in MARC-145 cells. The loss-of-

function study using siRNA to TLR3 confirmed that TLR3 plays an irreplaceable role in perceiving dsRNA in PAMs and probably in inducing expression of IFN-β. Importantly, silencing endogenous TLR3 caused a clear increase of PRRSV infectivity; further implicating the importance of TLR3 in antiviral responses to PRRSV. In addition to TLR3, PKR, RIG-I and melanoma differentiation-associated gene-5 (Mda-5) also have been implicated as dsRNA sensors (Gowen et al., 2007). During preparation of this paper, a study reported that PRRSV suppresses IFN-β production primarily by interfering with the RIG-I signaling pathway and partially by suppression of TLR3 signaling in MARC-145 cells (Luo et al., 2008). It is unknown whether PRRSV adopts a similar mechanism to escape innate immunity in porcine cells such as PAMs. Our data, generated mostly in PAMs, show that TLR3 signaling is important for PAM antiviral responses to PRRSV. It is possible that the TLR3-signaling pathway could be augmented in response to RIG-I signaling attenuation by PRRSV. Further investigation of the signaling pathways mediated by TLR3, RIG-I-like receptors, and Mda-5 in innate immune responses to PRRSV is needed.

Taken together, these findings suggest that activation of porcine TLR3 stimulates significant protective activity against PRRSV infection. The TLR3-mediated responses include production of type I IFNs. Because production of type I IFNs has been reported to be compromised during PRRSV infection (Oleksiewicz and Nielson, 1999; Riber et al., 2004; Royaee et al., 2004), it is tempting to speculate that this virus may possess mechanisms to evade TLR3 activation in host immune cells. This possibility warrants further investigation.

5.5. References

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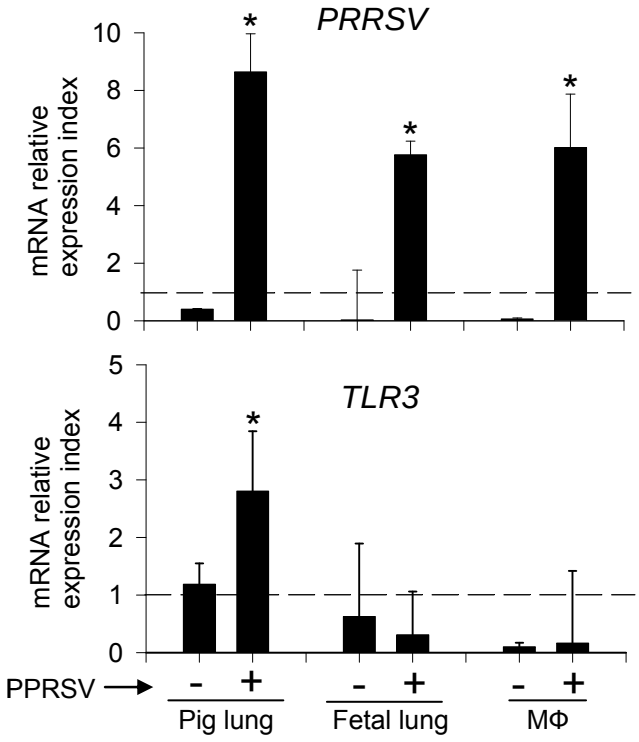


FIG. 5.1. Expression of porcine TLR3 in PRRSV-infected lungs and alveolar macrophages. Lungs from fetal and 14-d-old pigs from pregnant females infected with PRRSV, and alveolar macrophages infected *in vitro* with PRRSV for 16 h were evaluated for mRNA expression of TLR3. Real time RT-PCR was conducted with gene-specific primers using total RNA (200 ng in 25-µl PCR reaction). Relative gene-expression data were normalized against Ct values of the housekeeping gene, GAPDH, and the relative index ($2^{-\Delta\Delta Ct}$) was determined in comparison to the average expression levels of control samples with the index defined as 1.000 (indicated by the horizontal line). Data are means ± SD, n=3. *Different from control, $p < 0.05$.

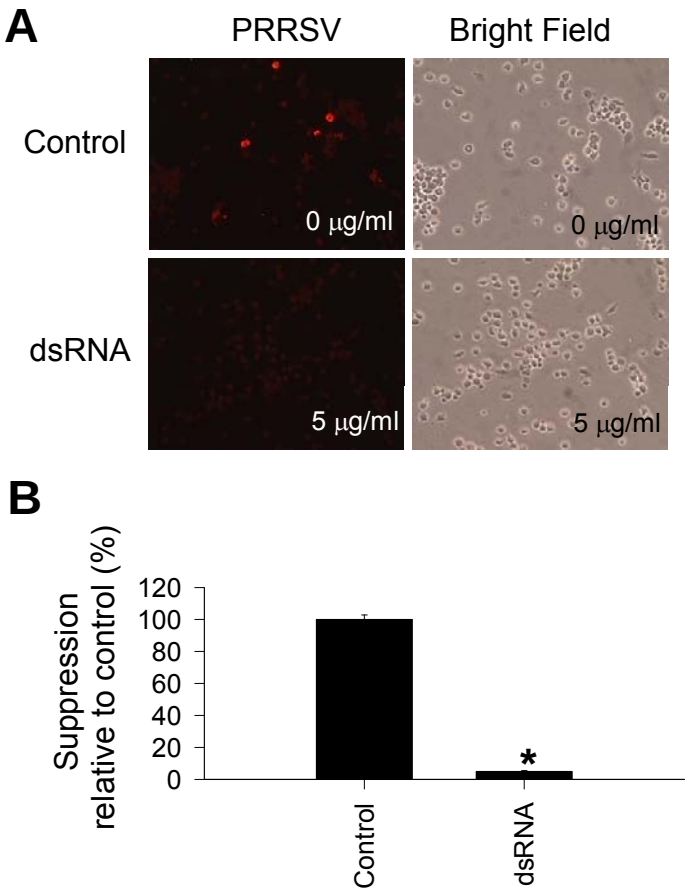


FIG. 5.2. TLR3 ligand, dsRNA, decreases PRRSV-infection in porcine alveolar macrophages (PAMs). PAMs were cultured in 48-well plates, treated with dsRNA and infected with PRRSV. Eighteen hours after infection, PAMs were stained for PRRSV N protein with a monoclonal antibody and visualized with TRITC-conjugated goat anti-mouse IgG. The PRRSV-positive cells were examined using fluorescent microscopy. Data are means ± SD, n=3 of 500 cell counted fore each repeat.*Different from control, $p < 0.001$.

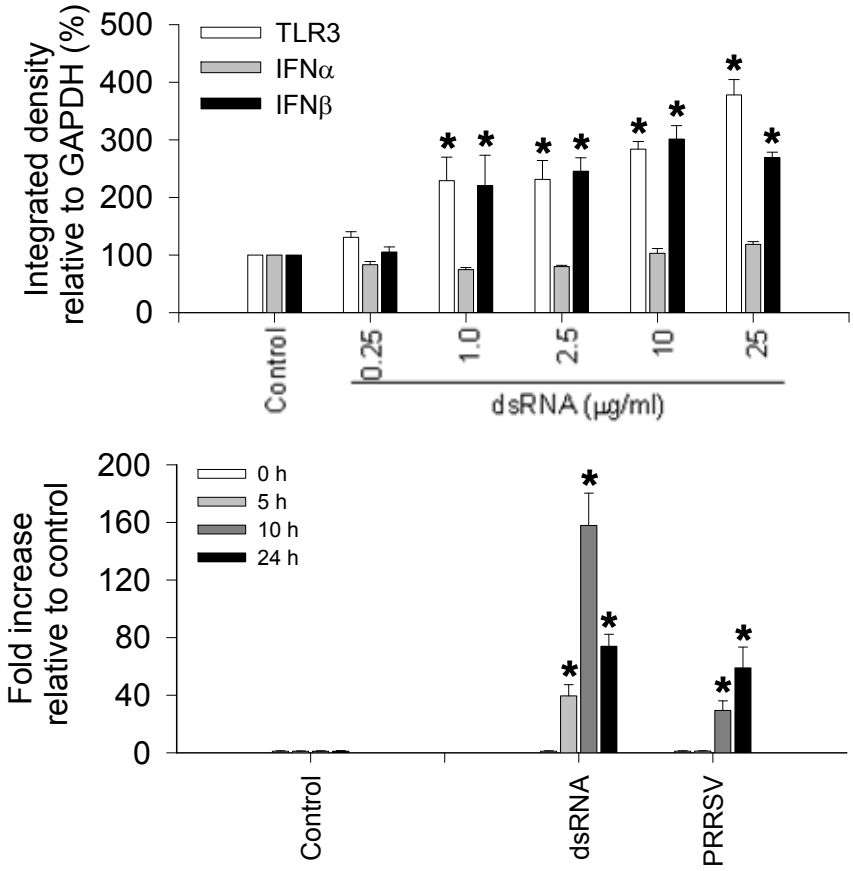


FIG. 5.3. Stimulation of TLR3 and type I IFN expression by synthetic dsRNA, poly (I:C). (A) PAMs were cultured in 48-well plates and treated with dsRNA for 6 h and cells were collected for RNA detection with RT-PCR. (B) Bioassay of porcine type I IFNs in PAM supernatants after stimulation with the TLR3 ligand, dsRNA and infection with PRRSV. Data are activity equivalent to IFN-β. Data are means ± SD, n=3. *Different from control, $p < 0.05$.

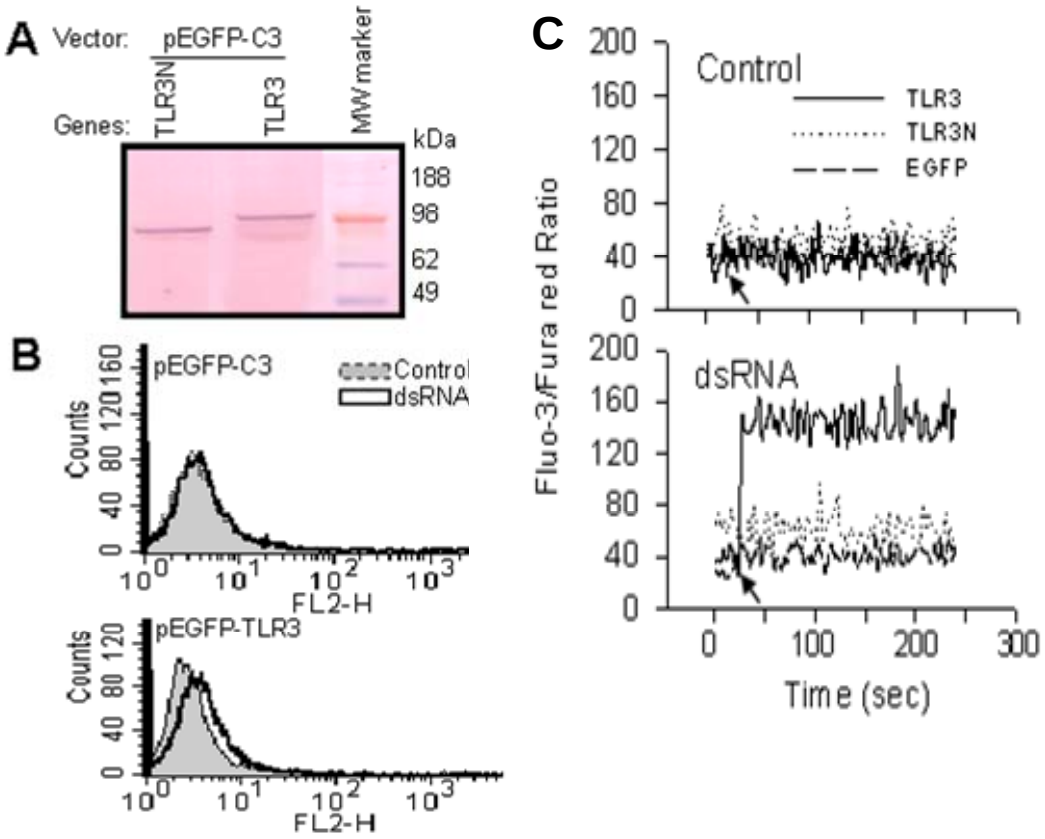
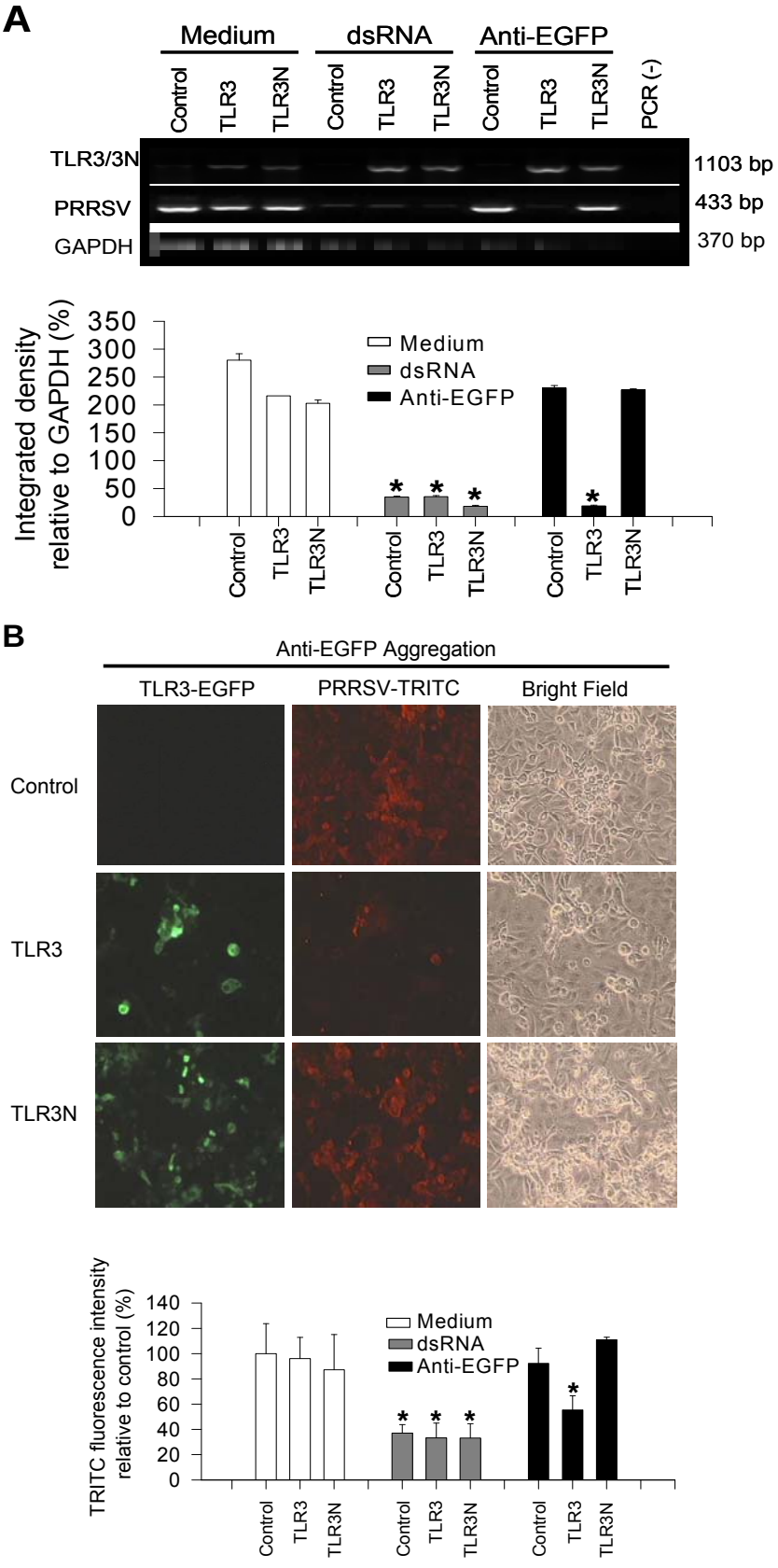


FIG. 5.4. Expression of functional porcine TLR3 in HEK293A cells. (A) Immunoblot of EGFP-fused TLR3 and TIR-domain truncated TLR3 (TLR3N) in lysates of cells transfected with pEGFP-C3 constructs. Cell lysates (50 μg) were resolved on a SDS-PAGE and immunoblotted. MW, prestained molecular mass markers. (B) Cell surface translocation of EGFP-tagged porcine TLR3 was stimulated by dsRNA (poly (I:C), 10 μg/ml) in transformed cells. Surface located EGFP-TLR3 was labeled with anti-EGFP mAb and detected with phycoerythrin (R-PE)-conjugated goat anti-mouse IgG. The ratio of R-PE positive cells (FL2) in EGFP-positive cells (FL1) was quantified by flow cytometric analysis. (C) Stimulation of calcium influx in TLR3-transformed HEK293A cells. Cells were loaded with calcium dyes Fluo-3 and Fura Red, stimulated with dsRNA (poly (I:C); fluorescence was detected with FL1 and FL2 detectors of a flow cytometer in the EGFP-positive cells, and the fluorescence ratio of Fluo-3/Fura red was analyzed with WinList 5.0.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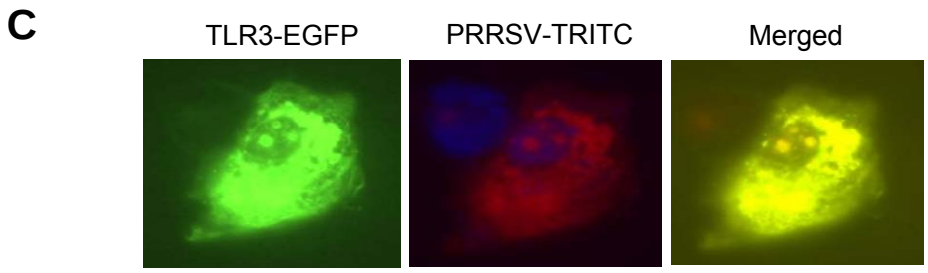

FIG. 5.5. Activation of porcine TLR3 suppresses PRRSV infectivity in MARC-145 cells. (A) MARC-145 cells were transformed with pEGFP-TLR3 or -TLR3N constructs in 24-well plates. Control and transformed cells were infected with PRRSV and stimulated with dsRNA (poly (I:C), 5 μg/ml) or anti-EGFP mAb (10 μg/ml) plus goat anti-mouse IgG (5 μg/ml) from 24 h after transfection. Total RNA was extracted from cells collected from individual wells and TLR3, TLR3N, and PRRSV mRNA expression was detected using RT-PCR. (B) Cells were treated as in (A) but cultured in 8-well chamber slides. Cells were fixed with 4% paraformaldehyde in PBS at 48 h (24 h after PRRSV infection) and examined after immunostaining of PRRSV with a mAb (SDOW17) to viral nucleocapsid protein and detected with TRITC-conjugated goat anti-mouse IgG. Representative images from the group treated with anti-EGFP antibodies are shown. PRRSV fluorescence data for all treatments are shown below the images. (C) Colocalization of EGFP-tagged porcine TLR3 with PRRSV in MARC-145 cells. Samples were prepared as in (A) and examined by confocal microscopy. The left image is EGFP-TLR3, the middle image is TRITC-labeled PRRSV, and the right image is a merged image of green (TLR3) and red (PRRSV) fluorescence. Nuclei were stained with Hoechst 33342 as shown in the middle image. Data in panels A and B are means ± SD, n=3. *Different from control, $p < 0.05$.

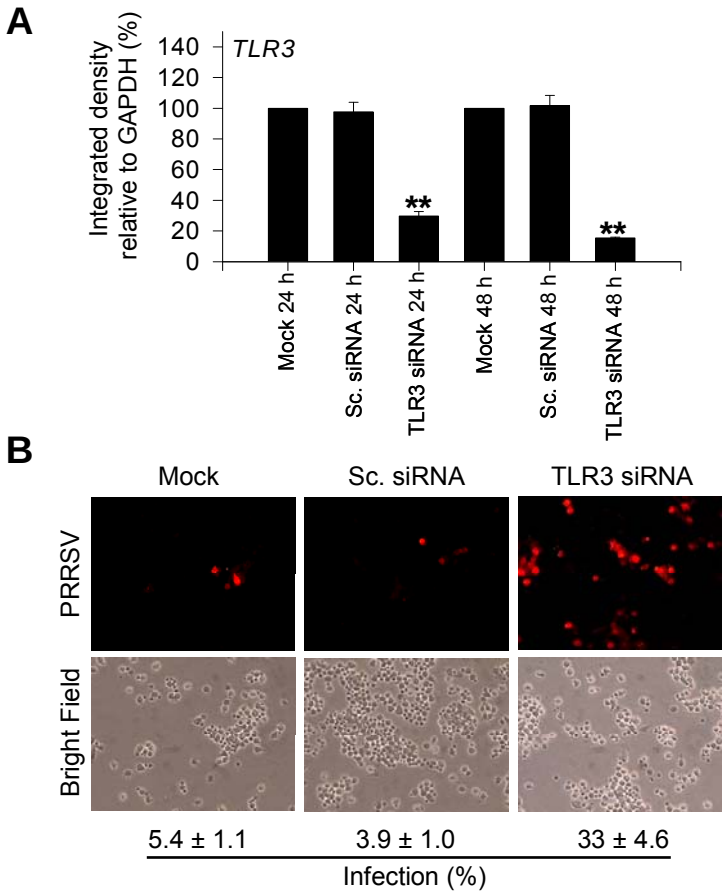

FIG. 5.6. Silencing TLR3 increases PRRSV infectivity in porcine alveolar macrophages (PAMs). (A) PAMs were cultured in 24-well plates and transfected with, siRNA dissolving buffer (Mock), Alexa Fluor 488 (AF 488)-labeled scrambled (sc) siRNA or gene-specific siRNA to porcine TLR3 (TLR3 siRNA) for 48 h. Cells in individual wells were collected for RNA extraction at 24 h and 48 h. Gene expression of TLR3 and GAPDH was assayed using RT-PCR and quantified as described in Fig. 5. Data are means ± SD, n=3; different from mock-transfected cells, $^{**}p < 0.01$. (B) Silencing TLR3 increases PRRSV infection. PAMs were transfected with siRNA and infected with PRRSV at 30 h after transfection. Twenty hours after infection, PAMs were fixed and immunostained for PRRSV as in Fig. 5. Infection (%) was obtained by calculation of PRRSV-positive cells in ~500 randomly examined cells in each treatment.

CHAPTER 6 - OVERVIEW AND FUTURE PROSPECTS

The general background and idea for this dissertation on innate antiviral immunity was reviewed in Chapter 1. This short chapter is written to summarize the studies in this dissertation and to highlight the theme of our project about innate antiviral immunity. Innate immunity is the first line of protection against infectious microorganisms. Early antiviral immune responses residing in innate immunity consist of (1) hindering viral entrance, (2) viral recognition and signal transduction, and (3) innate cell activation and synthesis of innate effector molecules to inactivate viruses. The evolution of adaptive immunity in vertebrate animals expands the ancient innate immunity into a more intricate defense system. Recent studies have highlighted the role of innate immune components in triggering adaptive immune defense. This dissertation focused on the second and third events of the innate immune cascade by functional characterization of some newly identified porcine innate molecules in a porcine viral disease model caused by PRRSV. Named “mystery disease” or “blue-ear disease” when it first appeared in the mid 1980's, PRRSV is the most devastating pathogen impacting the swine industry and a challenge for immunological studies and disease control. To date, few studies have reported on the role of porcine innate immunity in defense against PRRSV infection.

Host defense peptides (HDPs), also known as antimicrobial peptides (AMPs), represent a large family of multifunctional innate immune effectors. Besides the well-known antimicrobial activity against a broad-spectrum of microorganisms, the new name reflects their role in immunomodulation, anti-endotoxins, wound healing and anti-cancer involvement. The antiviral activity of HDPs has been known for about two decades and was highlighted recently in effects against a series of human RNA and DNA viruses including herpes simplex virus (HSV), influenza virus and HIV. However, few studies have been conducted on porcine HDPs against porcine viral diseases. In Chapters 2 and 3, we focused on studies designed to complete the porcine HDP profile, and initially characterized their antiviral activity against PRRSV. Previously, only one porcine defensin had been identified. Using the combinational techniques of bioinformatics and molecular biology, we identified and characterized 11 novel porcine

defensins as described in Chapter 2. In Chapter 3, we evaluated the anti-PRRSV activity of eight selected HDPs which included porcine β-defensin-3 (pBD-3) and protegrin-4 (PG-4). Data showed that pBD-3 and PG-4 can effectively reduce PRRSV infectivity in a cell culture system. These findings demonstrate phylogenic conservation of multiple porcine β-defensins and suggest a potential role of porcine HDPs as a group of innate antiviral effectors.

Another indication of the antiviral role of HDPs is their tissue distribution. HDPs are mostly synthesized or stored in internal and external mucosa, epithelial cells, skin cells, some leukocytes and intraepithelial immune cells. These tissue sites are often used by viruses to initiate their infection. Thus, upon viral presence at these sites, immediate innate recognition and well-regulated expression of innate effectors (e.g. IFNs and HDPs) critically determine the effectiveness of local innate immune defense before the ramp-up of systemic adaptive immunity. Therefore, our other efforts in innate antiviral immunity have focused on the characterization of innate immune receptors, which function in perceiving viral molecules and mediating synthesis of innate immune effectors. TLRs are a main group of innate immune receptors. Particularly, TLR3, TLR7, TLR8 and TLR9 have been characterized for their role of recognizing viral nucleic acid and conveying antiviral signaling to mediate the expression of IFNs and probably HDPs. In Chapter 4, we cloned the full-length cDNA of porcine TLR3 and TLR7, examined their expression at RNA and protein levels, and functionally overexpressed them to show their capability in regulation of transcription factors of IRF. In Chapter 5, we concluded that porcine TLR3 is involved in anti-PRRSV innate immune responses. The conclusion was supported by the following evidence. First, PRRSV infection upregulated the expression of TLR3 in porcine alveolar macrophages (PAMs) and treating PAMs with TLR3 ligands, a synthetic dsRNA, suppressed PRRSV infectivity. We further demonstrated this by molecular manipulation of the TLR-3 gene in a cell culture system. Specific activation of porcine TLR3 overexpressed in MARC-145 cells elicited antiviral responses to PRRSV infection. Transfection of PAMs with small-interfering RNA targeting porcine TLR3, resulted in 50-80% suppression of TLR3 expression and consequently increased PRRSV infectivity. In addition, porcine TLR3 is suggested to mediate cell responses including IFN-β production in anti-PRRSV process. In summary, Chapters 4 and 5 provide molecular information about porcine TLR3 and TLR7, and their mediation in PRRSV pathogenesis, which may provide new strategies to prevent this costly swine disease.

Studies included in this dissertation represent an initial exploration for the potential of applying innate immune mechanisms to aid in the prevention of an epidemic viral disease. The presented results were promising, but more questions were raised, such as:

- What combinations of HDPs are most effective for controlling PRRSV in the lung, the primary infection site of PRRSV?
- What are the mechanisms by which some HDPs inactivate PRRSV?
- Besides TLR3, are other TLRs or non-TLR receptors involved in mediation of PRRSV pathogenesis?
- What molecular patterns of PRRSV are recognized by TLR3 or other TLR?
- In addition, considering the connection between TLRs and HDPs, how does viral recognition TLRs regulate HDP expression in PAMs or other innate immune cells?
- PRRSV has been described for its ability to prevent optimal immune responses in pigs, thus PRRSV infection mostly results in a weak immune response and a persistent infection. Is it possible that PRRSV targets the components in TLR-mediated signaling transduction and prevents optimal immune responses?
- Could PRRSV infection be controlled through modulating innate immune components including TLRs and HDPs?

We welcome these questions, and will absolutely incorporate them into our future investigation in the area of innate antiviral immunity.

Appendix A - PubMed Links and Photocopies of Publisher's Permission for Reproducing Published Materials

All related publications during graduate studies

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Publisher: Springer
Date: Apr 5, 2006
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Author: Yongming Sang, Jun Yang, Chris R. Ross, Raymond R.R. Rowland and Frank Blecha

Publication: Veterinary Immunology and Immunopathology

Publisher: Elsevier

Date: 30 April 2008

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