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The porcine innate immune system: An update

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ABSTRACT

Over the last few years, we have seen an increasing interest and demand for pigs in biomedical research. Domestic pigs (*Sus scrofa domesticus*) are closely related to humans in terms of their anatomy, genetics, and physiology, and often are the model of choice for the assessment of novel vaccines and therapeutics in a preclinical stage. However, the pig as a model has much more to offer, and can serve as a model for many biomedical applications including aging research, medical imaging, and pharmaceutical studies to name a few. In this review, we will provide an overview of the innate immune system in pigs, describe its anatomical and physiological key features, and discuss the key players involved. In particular, we compare the porcine innate immune system to that of humans, and emphasize on the importance of the pig as model for human disease.

Contents

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1. Introduction

Pig (*Sus scrofa domesticus*) was domesticated more than 9000 years ago in multiple areas of the world including the Near-Tigris Basin- and the Far East (Barrios-Rodiles et al., 2005; Epstein and Bichard, 1984). Pigs arrived in America with Hernando de Soto around 1539. Over the last few years the demand for pigs in biomedical research has considerably increased, particularly with the implementation of transgenesis and the release of the swine genome assembly 10.2 (Sscrofa10.2) (Dawson et al., 2013; Groenen et al., 2012). The porcine immune system has been shown to be very similar to its human counterpart in terms of anatomy, organization, and response (Dawson, 2011; Dawson et al., 2013; Rothkotter, 2009). However, to our knowledge there is no comprehensive review focusing specifically on the innate arm of the porcine immune system. With the current review we aim at presenting the main features of the porcine innate immune system and at comparing it to its human counterpart. We start with a brief section highlighting the relevance of the pig model, and then describe the main anatomical particularities of its innate immune system. After we focus on the principal structures involved in the triggering of innate response, we present the main cellular and molecular players of both humoral and cellular responses, with the exception of cytokines, which already have been presented in other reviews.

2. Relevance of the pig as a large animal model to study the innate immune system

Pigs are of proven relevance for use in the study of human health priorities such as obesity (Brambilla and Cantafora, 2004), cardio-vascular disease (Boluyt et al., 2007), nutritional studies (Puiman and Stoll, 2008), and communicable diseases (Meurens et al., 2012) as pigs have anatomical (Swindle et al., 2012) and physiologic (Kuzmuk and Schook, 2011) characteristics comparable to humans. Due to their long-standing domestication (Barrios-Rodiles et al., 2005), there is a substantial amount of information regarding their husbandry making them a particularly attractive animal model. As a litter bearing species with an average litter size of twelve and a gestation of 114 days, swine allow for models involving familial, gender, and sibling matching. This relatively short gestational period affords the opportunity to enrol and follow to farrowing a reasonable number of pregnant sows. Postnatally, piglets grow rapidly reaching sexual maturity around 6 months of age (Reiland, 1978). The pig model enables clear discernment of maternal and foetal contributions to immune responses due to the epitheliochorial placenta (three maternal layers and three foetal layers) (Kim, 1975), which prevents the transfer of maternal cells and immunoglobulins (Igs) into the embryo/foetus. Moreover, placenta and endometrium are easily separated allowing the interactions between each foetus and the uterus to be easily identified and analysed. Importantly, each foetus' placenta can be matched permitting correlation of placenta-specific differences with differences in the foetuses. Further, it has been shown that pig immune responses resemble humans for 80% of analysed parameters whereas mice are similar in less than 10% (Dawson, 2011; Dawson et al., 2013). Although genetically modified mice have played a prominent and extremely valuable role in basic research, the results obtained from mice often failed translation into clinically relevant applications in humans. Moving beyond the mouse into the pig animal model should allow us better translation from the laboratory into clinical research. For instance, because of its comparable size to humans, the pig may be a better model than the mouse for modelling radiation or photodynamic tumour therapy (Adam et al., 2006). Indeed, in this species scaling of energy dose and tumour size is easier. Therefore, the pig has definitely many advantages as an animal model. Some of them are briefly presented in Table 1.

Table 1
Pros and cons for the use of the pig model.

- All season breeding species
- Large litter size (10–12 piglets/litter), 24–36 piglets/year
- Gestation (115 days)
- Short generation interval (12 months)
- Lifespan (10–20 years)
- Omnivorous like humans
- Size, similar to humans, especially miniature Hanford pig
- Various surgical procedures can be performed and many samples can be collected
- Availability, outbred and inbred, more than 500 breeds
- Breeding conditions are very standardized
- High genome and protein sequence homologies with humans
- More tools available
- Cheaper and ethically more acceptable than primates
- Required bigger and more expensive facilities than mouse
- Still some differences with humans
- Still limited access to inbred pigs for basic research
- Less close to humans than monkeys
- More expensive than mice
- Less tools than mice
- Ethically a little bit less accepted than mice
- Genome still not fully annotated
- Pig research community smaller than its mouse counterpart

2.1. Anatomical particularities of the porcine immune system

Since a comprehensive review on that specific subject has already been published (Rothkotter, 2009), only the main anatomical particularities of the porcine immune system will be briefly presented in this section. The porcine thymus is similar to the thymus of other mammals (Rothkotter, 2009; Swindle et al., 2012) acting as a primary lymphoid organ principally involved in primary T cell development (Sinkora and Butler, 2009). Its' size and surgical accessibility has enabled numerous studies about thymic lymphocyte immigration and emigration (Sinkora and Butler, 2009). Lymphoid tissues associated to the oral cavity and upper respiratory tracts are anatomically similar in pigs and humans. For example, the human Waldeyer's ring most closely resembles the anatomical arrangement of the porcine lymphoid tissues in the nasopharynx (Horter et al., 2003). Unlike mice where these organs are absent, pigs possess palatine and pharyngeal tonsils. Their spleen is tightly attached to the stomach by the short gastric blood vessels and therefore is not as pedunculate as it is in species like dog. Because of its similarities with human spleen, pigs have been used extensively to assess the impact of splenectomy (Reihner and Brismar, 1995; Rothkotter, 2009). The porcine peripheral and mucosa-associated lymph nodes have a specific structure that is called inverted (Rothkotter, 2009), a general organisation that is shared with the rhinoceros, the dolphin, and the elephant. The lymph nodes lack a larger medullary area and are preferentially composed of cortical areas and paracortex and the majority of the lymphocytes exit in the blood directly. The reason for developing and flourishing with an inverted lymph node structure still remains unclear and calls for further research. Lymphoid tissue is also present in the small intestine wall as single follicles or as aggregated lymphoid follicles named Peyer's patches (PP) in reference to Johan Conrad Peyer (Schaffhausen, Switzerland, 17th century). Two types of PPs are described in pigs; jejunal (JPP) and ileal PPs (IPP). Unlike the Bursa of Fabricus (chicken) and IPP in sheep (Gerber et al., 1986), porcine IPP are not primary lymphoid organs (Butler and Sinkora, 2013; Sinkora et al., 2000b) as they are neither required to maintain the systemic B cell pool nor are they a site of B cell lymphogenesis.

3. Pattern recognition receptors and inflammation

Pattern recognition receptors (PRRs) are evolutionarily conserved, germline-encoded host sensors mainly expressed on innate immune cells such as dendritic cells, macrophages, and neutrophils (Kumar et al., 2011). Recent data provide evidence that they also play a role in cells from the adaptive immune system such as T cells. These findings are summarized in other reviews and will not be covered in this section (Michallet et al., 2013; Reynolds and Dong, 2013). PRRs detect pathogen-associated molecular patterns (PAMPs) expressed by pathogens as well as commensal flora. PRRs include different classes of cellular receptors: The Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NODs)-like receptors (NLRs), the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), membrane C-type lectin receptors (CLRs), and DNA receptors (cytosolic sensors for DNA) (Kumar et al., 2011). Additionally, PAMPs can also be bound by soluble PRRs such as surfactant proteins (SPs) and mannan-binding lectins (MBLs).

3.1. Toll-like receptors

Toll-like receptors are the best studied PRRs and to date twelve functional TLRs are known in mice and ten in humans. TLR1-10 are also known in swine and therefore, this section focusses on these TLRs. TLRs are type I membrane glycoproteins that recognize extracellular or endosomal/endolysosomal PAMPs via leucine rich repeats (LRRs). They can form homodimers (TLR3-5, 7, 9), heterodimers (TLR1 and TLR6 with TLR2) or complex with other factors (i.e. TLR4 can complex with myeloid differentiation factor 2 -MD2- and CD14) to sense pathogens. After ligand binding, TLRs initiate downstream signalling cascades through a cytoplasmic Toll/interleukin-1 receptor (TIR) domain depending on the recognized pathogen and its location (Kumar et al., 2011). Extracellular PAMPs (e.g. LPS, lipopeptides or flaggelin) from bacteria, fungi and protozoa are recognized by TLRs expressed on the cell surface such as TLR1, 2, 4, 5 and 6 followed by downstream signalling through the myeloid differentiation primary response gene 88 (MyD88) to translocate nuclear factor kappa B (NFκB) to the nucleus and enable the production of inflammatory cytokines. TLR4 can also signal through TIR-domain-containing adapter-inducing interferon-β (TRIF) and the interferon regulatory factors (IRF) 3/7 to induce type I interferon (IFN) production to combat pathogens. TLR10 can be expressed both on the cell surface as well as intracellularly in epithelial cells. Signalling is thought to involve MyD88. Although the specific ligand for TLR10 is so far not determined (Uenishi and Shinkai, 2009) a novel study from Regan et al. found TLR10 involved in sensing and responding to the cell-invasive pathogen *Listeria monocytogenes* (Regan et al., 2013).

TLR3, 7, 8, and 9 are classically but not exclusively (Ioannidis et al., 2013) expressed intracellularly and mainly recognize nucleic acids from viruses and intracellular bacteria in endosomes or endolysosomes. Signalling through TLR3 involves the TRIF and IRF3/7 pathway and induces type I IFNs while TLR7 and 9 signal through MyD88 and can activate further downstream IRF7, as well as NFκB to induce the production of both type I IFNs and pro-inflammatory cytokines (Kumar et al., 2011). Details on the individual TLR ligands and signalling pathways are summarized in Table 2.

Interestingly, TIR domains are not only expressed by eukaryotic cells but were also found in various bacteria. Bacterial pathogens may express TIR domains to manipulate TLR signalling and/or to limit the NFκB expression thereby decreasing the innate immune response (Patterson and Werling, 2013).

In swine, cDNA analysis and sequencing discovered a significant homology of porcine TLRs with their human counterparts (~80% for TLR1, 3, and 10; 85% for TLR7; and 73% for TLR8) and that they share typical functional TLR domains (Sang et al., 2008; Shinkai et al., 2006a; Zhu et al., 2008). Studies on the genetic diversity of TLRs including single-nucleotide polymorphisms (SNPs) identified pronounced differences between wild boars and domestic pigs (Bergman et al., 2010) but also between different pig breeds with several clusters of variation supporting the documented history of pig breed domestication (Palermo et al., 2009). Furthermore, SNP studies located 'hotspots' of variation in various functional domains. Many SNPs were shown to be located in the LRR domain but these SNPs were biased in the analysed animals so breeding did not seem to have an impact on the heterogeneity of TLRs (Shinkai et al., 2006b). In addition, three missense mutations were identified in the extracellular domain with one of them shown to be important for flagellin recognition. SNPs have implications in various human diseases and although this relation has not be described for swine so far, analysis of polymorphism may provide a useful tool in the selection of disease-resistant animals in future (Yang et al., 2013).

In conjunction with these genetic studies, porcine TLR expression patterns and function have been analysed. The porcine intestinal epithelial cell line IPEC-J2 has been shown to express TLR2, TLR4, and TLR9 (Burkey et al., 2009) and is a valuable tool for the study of these porcine TLRs. *In vivo*, TLR2 has been shown to be expressed in the porcine thymus, spleen, IPPs, mesenteric lymph nodes (MLNs), and palatine tonsil (Wilson et al., 2007) as well as being expressed to a lower level on columnar membranous

Table 2
PAMP recognition and responses by PRRs.

PRR	Main ligand	Ligand binding domain	Signalling domain	Signalling pathway	Main immune response	Main target pathogen	References
TLRs							
TLR 1	Triacylglyceride	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	Gram-positive bacteria & fungi	Kumar et al. (2011)
TLR 2	Peptidoglycans & Zymosan	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	Gram-positive bacteria & fungi	Kumar et al. (2011)
TLR 3	dsRNA	LRR	TIR	TRIF – IRF3/7	Type-1 IFN	RNA viruses	Kumar et al. (2011)
TLR 4	LPS	LRR	TIR	MyD88 – NFκB & TRIF – IRF3/7	Proinflamm. cytokines & type-1 IFN	Gram-negative bacteria	Kumar et al. (2011)
TLR 5	Flagellin	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	Flagellated bacteria	Kumar et al. (2011)
TLR 6	Diacylglyceride	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	Gram-positive bacteria & fungi	Kumar et al. (2011)
TLR 7	ssRNA	LRR	TIR	MyD88 – NF-κB & MyD88 – IRF7	Type-1 IFN (& proinflamm. cytokines)	Endosomal bacteria and viruses	Kumar et al. (2011)
TLR 8	ssRNA	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	RNA viruses	Kumar et al. (2011)
TLR 9	Unmethylated CpG DNA	LRR	TIR	MyD88 – NF-κB & MyD88 – IRF7	Type-1 IFN (& proinflamm. cytokines)	Endosomal bacteria and viruses	Kumar et al. (2011)
TLR 10	(*Listeria monocytogenes*?)	LRR	TIR	MyD88 – NF-κB	Proinflammatory cytokines	(*Listeria monocytogenes*?)	Kumar et al. (2011)
NLRs							
NLRA (CIITA) subfamily							
CIITA	Unknown	LRR	AD	NF-κB, MAPK and IRFs	Regulates MHC-II antigen presentation	Unknown	Kobayashi and van den Elsen (2012) and Lupfer and Kanneganti (2013)
NLRB subfamily							
NAIPs	Flaggelin, Rod proteins in T3SS	LRR	BIR	Inflammasome	Secretion of IL-1β and IL-18, pyroptosis	Bacteria	Lupfer and Kanneganti (2013)
NLRC subfamily							
NOD1	Peptidoglycans (DAP-type)	LRR	CARD	NF-κB, MAPK and IRFs	Proinflamm. cytokines & type-1 IFN	Gram-positive bacteria	Kumar et al. (2011)
NOD2	Peptidoglycans (mDP)	LRR	CARD	NF-κB, MAPK and IRFs	Proinflamm. cytokines & type-1 IFN	Gram-positive bacteria	Kumar et al. (2011)
NLRC4 (IPAF)	Flaggelin, Rod proteins in T3SS	LRR	CARD	Inflammasome	Secretion of IL-1β and IL-18, pyroptosis	Bacteria	Kumar et al. (2011)
NLRC3	Unknown	LRR	X	Inhibits NF-κB activation	TLR signaling attenuation	Unknown	Schneider et al. (2012)
NLRC5	Unknown	LRR	X	NF-κB, MAPK and IRFs	Regulates MHC-I antigen presentation	Unknown	Kobayashi and van den Elsen (2012)
NLRX1	Viral RNA	LRR	X	NF-κB, MAPK and IRFs	TLR signaling attenuation	RNA viruses	Hong et al. (2012)
NLRP (NALP) subfamily							
NLRP1 (Nalp1)	("Lethal factor" of *Bacillus anthracis*)	LRR + FIIND + CARD	PYD	Inflammasome	Secretion of IL-1β and IL-18, pyroptosis	(*Bacillus anthracis*, *Toxoplasma gondii*)	Ewald et al. (2014)
NLRP (Nalp)2-9,11-14	Various but often unknown	LRR	PYD	Inflammasome &/or inhibition of inflammation	Secretion of IL-1β and IL-18, pyroptosis &/ or TLR signaling attenuation	Various but often unknown	Lupfer and Kanneganti (2013)
NLRP10 (Nalp10)	Unknown	No LRR	PYD	Inflammasome and IL-1β independent	Inflammasome and IL-1β independent	(*Candida albicans*) –> fungi	Joly et al. (2012)
RLRs							
RIG-1	dsRNA in the cytoplasm	RNA helicase	CARD	NF-κB & IRF3/7	Proinflamm. cytokines & type-1 IFN	RNA viruses	Szabo and Rajnavolgyi (2013)
MDA5	dsRNA in the cytoplasm	RNA helicase	CARD	NF-κB & IRF3/8	Proinflamm. cytokines & type-1 IFN	RNA viruses	Szabo and Rajnavolgyi (2013)
LGP2	dsRNA in the cytoplasm	RNA helicase	No CARD	Influences RIG-1 & MDA5 activity	Uncertain, either activating or inhibiting RIG-1 & MDA5	RNA viruses	Szabo and Rajnavolgyi (2013)
Selected CLRs							
Dectin-1	β-glucan	CRD	ITAM	Syk – Card9-complex and/ or inflammasome	Proinflammatory cytokines incl. IL-1β + TH1 & TH17 support	Fungi	Hardison and Brown (2012) and Lepenies et al. (2013)
Dectin-2	Mannose-based structures	CRD	ITAM	Syk – Card9-complex and/ or inflammasome	Proinflammatory cytokines incl. IL-1β + TH1 & TH17 support	Fungi	Hardison and Brown (2012) and Lepenies et al. (2013)

Table 2 (*continued*)

PRR	Main ligand	Ligand binding domain	Signalling domain	Signalling pathway	Main immune response	Main target pathogen	References
Mincle	Mannose-based structures	CRD	ITAM	Syk – Card9-complex and/ or inflammasome	Proinflammatory cytokines incl. IL-1β + TH1 & TH17 support	Fungi	Hardison and Brown (2012) and Lepenies et al. (2013)
MR	Mannose-based structures	CRD	Unknown	Unknown	Cytokine production, T-cell costimulation and differentiation	Fungi	Hardison and Brown (2012) and Lepenies et al. (2013)
DC-SIGN	Mannose-based structures	CRD	Unknown	Raf-1	Cytokine production, T-cell costimulation and differentiation	Fungi	Hardison and Brown (2012) and Lepenies et al. (2013)
DNA sensors							
DAI	DNA	D3 region, Zα+Zβ	Unknown	NF-κB & IRF3/IRF7	Proinflamm. cytokines & type-1 IFN	DNA viruses and intracellular bacteria	Wang et al. (2008)
AIM2	DNA	HIN200	PYD	Inflammasome	Secretion of IL-1β and IL-18, pyroptosis	DNA viruses and intracellular bacteria	Warren et al. (2010)

(M) cells and innate immune cells such as macrophages and epithelial cells but not on peripheral blood lymphocytes (Alvarez et al., 2008; Tohno et al., 2006). Reduction of TLR3 expression via RNA interference has been shown to trigger an increase in porcine reproductive and respiratory syndrome virus (PRRSV) replication which suggests that TLR3 may play an important role in the porcine innate antiviral response (Miller et al., 2009; Sang et al., 2008). TLR4, 5, and 9 are up-regulated on monocytes and dendritic cells (DCs) after stimulation with various TLR ligands like lipopolysaccharide (LPS), lipoteichoic acid (LTA), and cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) (Raymond and Wilkie, 2005). Alves et al. studied the role of TLR7 and MyD88 in porcine DCs by lentiviral-mediated RNA interference to reduce the expression of TLR7 and MyD88 (Alves et al., 2007). In both knockdown models, IL-6 production was substantially decreased after stimulation with the TLR7-ligand imiquimod (R837) (Alves et al., 2007). Porcine TLR7 and 8 could be found intracellularly and mainly in the endoplasmatic reticulum. Their stimulatory activity was analysed in transfected Cos-7 and HEK-293 cells using an NFκB reporter assay. In contrast to humans, imiquimod could not only activate TLR7 but also TLR8 in the pig, showing altered receptor specificity across species (Zhu et al., 2008). Calzada-Nova et al. found an enhanced production of various cytokines (IFN-α, IFN-γ, IL-2, IL-6, IL-8, IL-12, and TNF-α) after plasmacytoid DCs (pDCs) were stimulated with both imiquimod and the TLR9 ligand CpG ODN (Calzada-Nova et al., 2010). CpG ODN additionally increased the expression of chemokine receptors on monocyte derived DCs (MoDCs) and blood DCs (bDCs) (Auray et al., 2010) as well as altering the general DC morphology of cultured sorted plasmacytoid (pDCs) compared to unstimulated cultures (Calzada-Nova et al., 2010). A more detailed study including different CpG ODNs revealed that the three classes of CpG ODN (A-C) induce different cytokines (Dar et al., 2010). All three CpG ODN classes trigger up-regulation of the IFN-γ inducible protein 10 (IP-10) suggesting its use as a biomarker for TLR9 immune activity induced by CpG ODNs in pigs (Dar et al., 2010).

Beyond the basic characteristics and functions of TLRs, their role in various viral, bacterial, and fungal diseases have been investigated. PRRSV infection increased the expression of TLR3, 4, and 7 as well as the production of the pro-inflammatory cytokines IL-1β, IL-6, TNFα, and IFN-γ in tracheobronchial lymph nodes (Miguel et al., 2010). However, activation of TLR3, but not TLR4, could decrease the infectivity of PRRSV in porcine alveolar macrophages (PAMs) (Miller et al., 2009). A comparison of high (HP) and low pathogenic (LP) PRRSV strains revealed that relative to LP strains, HP PRRSV strains suppress ERK phosphorylation at early time points and impair LPS- and poly I:C-stimulated TNF-α release (Hou et al., 2012). HP PRRSV strains also lead to high serum levels of pro-inflammatory cytokines but lower IL-10 levels compared to LP PRRSV strains (Zhang et al., 2013a). The stronger inflammation induced by HP PRRSV likely contributes to increased tissue damage and clinical signs. Furthermore, HP PRRSV strains have decreased amounts of SARM1, a TIR-domain containing adaptor protein involved in TLR signalling transduction (Zhou et al., 2013) which may decrease the ability to mount an immune response against PRRSV. The African swine fever virus (ASFV) also inhibits poly I:C-mediated NFκB activation via TLR3 and the IFN-β promoter at the level of TRIF (de Oliveira et al., 2011). Hüsser et al. showed that TLR3 is involved in sensing classical swine fever virus (CSFV) but not food and mouth disease virus (FMDV), vesicular stomatitis virus (VSV), and influenza A virus (IAV) (Hüsser et al., 2011).

The role of TLRs in porcine bacterial diseases was analysed in Salmonella and hog cholera vaccines. Burkey et al. found that pigs infected with *Salmonella enterica* subspecies *enterica* serovar Typhimurium (commonly called *Salmonella* Typhimurium) showed increased TLR2 and 4 expression 24 and 48 h later, *in vivo* (Burkey

et al., 2009). *In vitro*, the porcine jejunum epithelial cell line IPEC-J2 showed increased expression of TLR1, 2, 3, 4, 6, and 9 but the porcine ileum cell line IPI-21 expressed more TLR8 and 10 after stimulation with LPS from *S.* Typhimurium (Arce et al., 2010). Li et al. used a plasmid encoding the pig IL-6 gene and eleven CpG motifs in combination with chitosan nanoparticles as an adjuvant to boost the porcine immune system against an attenuated classical hog cholera vaccine. Inclusion of the adjuvant plasmid was shown to increase the T cell frequency, the amount of antibody as well as the serum levels of IL-2, IL-6, and IFN-γ (Li et al., 2011). Thus, they showed a possible role for TLR ligands as adjuvants in vaccines against pig bacterial diseases. TLR expression has been also assessed in various types of cells such as alveolar macrophages in response to many other important porcine bacterial pathogens including *Streptococcus suis* (de Greeff et al., 2010).

Fungal pathogens are also able to manipulate TLR signalling (Seeboth et al., 2012). T-2, a fungal toxin, was shown to decrease the production of the inflammatory mediators IL-1β, TNF-α, and nitric oxide (NO) in PAMs in response to LPS (via TLR4) and synthetic diacylated lipoprotein FSL-1 (via TLR2/6). This reduced pro-inflammatory response was associated with a decline of TLR mRNA expression. Interestingly, the activation of TLR7 by single-stranded RNA (ssRNA) was not modulated by T-2 toxin pre-treatment. These data suggest that fungal pathogens might decrease pattern recognition of pathogens and therefore interfere with the initiation of an effective immune response (Seeboth et al., 2012).

3.2. Nucleotide-binding oligomerization domain (NODs)-like receptors

The NLR family is the largest group of PRRs and they detect PAMPs within the cytosol. Nearly all of its members have a C-terminal leucine-rich repeat (LRR) responsible for the detection of diverse PAMPs, a central NOD (NACHT) domain able to induce oligomerization upon PAMP detection. Different N-terminal domains divide the NLRs into different subfamilies: NLRA (MHC class II transactivator -CIITA-) has an acid transactivation domain, NLRB has a baculovirus inhibitor of apoptosis protein repeat (BIR), NLRC has a caspase-recruitment domain (CARD) (this subfamily includes NLRX1, NLRC3, and NLRC5 which contain a CARD-related X effector domain) and NLRP has a pyrin domain (PYD). Upon NLR-ligand binding to the distinct N-terminal domains, specific signalling cascades are triggered to determine the immunological response (Benko et al., 2008; Kumar et al., 2011; Zhong et al., 2013). NLRs are able to induce an inflammatory response in two ways. On one hand, NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4 (IPAF), and the NLR family, apoptosis inhibitory protein (NAIP), recruit protease caspase-1 and activate inflammasomes. Inflammasomes promote the processing and maturation of the inflammatory cytokines IL-1β and IL-18 and induce pyroptosis – an inflammatory form of cell death. On the other hand, NOD1, NOD2, NLRP10, and NLRX1 induce the transcription of inflammatory cytokines through NFκB, mitogen-activated protein kinases (MAPKs) and IRFs (Zhong et al., 2013). In contrast, NLRC5 and CIITA seem to be master regulators of MHC-I and MHC-II expression, respectively, and thereby strongly influence antigen presentation (Kobayashi and van den Elsen, 2012). Details on the individual NLR ligands and signalling pathways are summarized in Table 2.

In swine, NOD1 and NOD2 were cloned and functionally characterized (Tohno et al., 2008a,b). Porcine NOD (poNOD) 1 and 2 have an 83.8% and 81.6% amino acid identity with human NOD2, respectively (Tohno et al., 2008a,b). Both poNODs were expressed in numerous tissues. In newborn piglets, poNOD1 expression was shown to be highly elevated in MLN and the oesophagus while poNOD2 expression was elevated in MLN and the spleen. In adult pigs, poNOD1 expression remained highly elevated but poNOD2 expression was much more dispersed among various tissues. Furthermore, TLR and NOD ligands as well as immunobiotic lactic acid bacteria strongly increased poNOD1 and 2 expressions in the gut-associated lymphoid tissues (GALT). For functional analysis of poNODs, Tohno et al. transfected HEK-293 cells with poNOD sequences. Transfected HEK293 cells expressed both poNODs as an intracellular membrane-bound molecule and poNOD2 also in the cytoplasm. Upon stimulation with gamma-D-glutamyl-meso-diaminopimelic acid, meso-diaminopimelic acid and meso-lanthionine for poNOD1, and muramyl dipeptide (MDP) for poNOD2, both transfected HEK-293 cells responded with the activation of NFκB (Tohno et al., 2008b). These data demonstrate the potential of poNODs in the innate immune response against bacterial infections. Furthermore, Jozaki et al. found a reduced MBL binding capacity for a SNP in the poNOD2 hinge region emphasizing the role of PRR polymorphism studies in selection for disease resistance in pig production (Jozaki et al., 2009).

3.3. Retinoic acid-inducible gene (RIG)-I-like receptors

RIG-1, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) are the three members of the RLR family and they are responsible for the detection of RNA viruses within the cytoplasm. They bind double-stranded RNA during the replication cycle of RNA viruses by an RNA helicase domain and, upon ligand binding, the CARD domains in RIG-1 and MDA5 associate with the adaptor protein IPS1. This association induces a signalling cascade to activate the expression of inflammatory cytokines and type-I IFNs through NFκB and IRF3/7 and leads to the induction of an antiviral state in virus-infected cells. The role of LGP2 is still controversial (Szabo and Rajnavolgyi, 2013). LGP2 lacks a CARD domain and was therefore thought to have a negative regulatory role in RNA virus sensing but recent data showed that LGP2$^{-/-}$ mice showed a decreased production of IFNs upon RNA virus infection suggesting a positive role of LGP2 in RNA virus detection (Szabo and Rajnavolgyi, 2013). In pigs, an induction of LGP2 has been observed in alveolar macrophages and lung explants in response to influenza virus (Dobrescu et al., 2014). Details on the individual RLR ligands and signalling pathways are summarized in Table 2.

Hüsser et al. described the role played by RIG-1 and MDA-5 (and TLR-3) in porcine RNA virus infections (Hüsser et al., 2011). They analysed their recognition of CSFV, FMDV, VSV, and IAV and used small interfering RNA to knockdown RIG-1 and MDA-5 expression in the porcine epithelial cell line porcine kidney 15 (PK15). These knockdown cell lines were then analysed for their IFN-β responses after stimulation with the various RNA viruses. Their results indicated that VSV and IAV are largely detected by RIG-I with a minor contribution of MDA-5 while CSFV is sensed by MDA-5 and RIG-I (and TLR3). The response to FMDV-infection seems to be solely mediated by MDA-5 (Hüsser et al., 2011). The lack of RIG-1 response upon FMDV-infection is supported by a study from Wang et al. who demonstrated that FMDV is able to inhibit the activation of RIG-1 (Wang et al., 2011). Additionally, FMDV dampened the ubiquitination of TANK-binding kinase 1 (TBK1), TNF receptor-associated factor 3 (TRAF3), and TRAF6. By suppressing these key signalling molecules of the type I IFN response, FMDV might counteract the host antiviral innate immune response (Wang et al., 2011).

3.4. DNA sensors

While TLR9 senses DNA in the endosome, DNA sensors like the DNA-dependent activator of IFN-regulatory factors (DAI; also DM1 or ZBP1) screen the cytoplasm for the presence of DNA (Kumar et al., 2011). DAI-DNA binding triggers type-I IFN production via TBK1/IKKi. Additionally, after DNA transcription into double

stranded RNA by polymerase III, the RLR RIG-1 can be activated by the presence of DNA to induce type-I IFNs (Kumar et al., 2011). Unfortunately, the role of DNA sensors in pigs has not yet been investigated in details. Information about the individual DNA sensors and signalling pathways are summarized in Table 2.

3.5. Membrane C-type lectin receptors

Membrane C-type lectin receptors (CLRs) are a large receptor family including collectins, selectins, lymphocyte lectins, and proteoglycans which recognize PAMPs by a carbohydrate-recognition domain (CRD) (Hardison and Brown, 2012; Lepenies et al., 2013). They are involved in the immune response against bacteria, viruses, and parasites but their central role is to induce immunity to fungal pathogens. Therefore, several CLRs such as Dectin-1 sense β-glucan and mannose-based structures of the mannan part of the fungal cell wall (DC-specific intercellular adhesion molecule-3-grabbing non-integrin -DC-SIGN-, Dectin-2, MBL, macrophage inducible C-type "calcium-dependent" lectin -Mincle-, surfactant protein-A and D). Binding occurs in a Ca^{2+} dependent manner and PAMP recognition either induces phagocytosis only or can also activate signalling pathways in an immunoreceptor tyrosine-based activation motifs (ITAM)-dependent, immunoreceptor tyrosine-based inhibitory motifs (ITIM)-dependent or ITAM/ITIM-independent way. ITAM-dependent signalling can recruit and activate the spleen tyrosine kinase (Syk). Syk can mediate inflammasome activation and/or signal through protein kinase C (PKC)δ and the Card9 complex to induce the production of various inflammatory cytokines and stimulate and direct cellular immune responses mainly towards T-helper 1 (Th1) and Th17. Contrary, ITIM-dependent signalling can downregulate the production of inflammatory cytokines. The mannose receptor (MR) is an example for ITAM/ITIM-independent signalling but the mechanisms involved are so far unknown (Hardison and Brown, 2012; Lepenies et al., 2013). Details on the individual CLR ligands and signalling pathways are summarized in Table 2.

Several porcine CLRs have been identified such as CD69, CD205, CD207 (Langerin), CLEC4G, DC-SIGN, and Dectin-1. With the exception of the Ca^{2+}-dependent binding of DC-SIGN, detailed mechanisms of PAMP recognition or signal transduction including the immunological responses are missing in swine (Flores-Mendoza et al., 2010, 2012; Huang et al., 2009; Huang and Meng, 2009; Nfon et al., 2008).

3.6. Surfactant proteins

Surfactant proteins (SP) were discovered by their essential role in reducing surface tension at the air–liquid interface of the lung. It is known that two hydrophobic SPs (SP-B and SP-C) are in charge of this lung stabilizing function. Pig serves as a donor of these important surfactants and as an animal model to study their function (Parra et al., 2013) and their possible medical applications as such lung transplantation (Inci et al., 2013), lung emphysema (Bruun et al., 2013), and neonatal acute respiratory distress syndrome (ARDS) (Hilgendorff et al., 2008). SP-A and SP-D play important roles in the lung host defence. SP-A and -D belong to the C-type lectins and recognize bacterial, viral, and fungal PAMPs via a CRD. They form oligomers to bind and opsonize microorganisms to increase their aggregation, phagocytosis, and lysis. Furthermore, SP-D binding can facilitate the phagocytosis of apoptotic cells (Pastva et al., 2007). The pig is frequently used to study the role of SPs in innate immune responses. Hillaire et al. demonstrated the potent antiviral activity of porcine SP-D against various influenza A viruses including H1N1 (Hillaire et al., 2011). Therefore, SP-D seems to be a promising novel antiviral agent against pulmonary human diseases (Hillaire et al., 2011). Furthermore, SNPs in SP-A seems to correlate with common porcine diseases and might thereby serve as a genetic marker of impaired innate immune function (Keirstead et al., 2011).

3.7. Mannan-binding lectins

Mannan-binding lectins MBL-A and -C have a cysteine-rich N-terminal region and they recognize PAMPs via a C-terminal CRD, an intermediate collagen-like domain which enables MBLs to form a trimer triple-helix (Juul-Madsen et al., 2006). In serum, MBLs are complexed with MBL-associated serine proteases (MASPs), and a small MBL-associated protein (sMAP) which bind PAMP and then activate the complement cascade by triggering the lectin activation pathway of complement (Bergman, 2011). The effect of this cascade will be discussed in detail in the section "The complement system" below. Porcine MBLs were isolated, cloned, and functionally analysed and cDNA analysis revealed a 64.9% identity with human MBL (Agah et al., 2001). Low serum concentrations of MBL-A and -C are responsible for increased susceptibility to bacterial and viral pathogens in humans as well as in pig (Juul-Madsen et al., 2011; Lillie et al., 2007). Serum MBL concentration was found to be highly heritable in some although not all breeds (Juul-Madsen et al., 2006). Additionally, SNPs in both MBLs may be correlated with decreased serum concentrations in pigs (Bongoni et al., 2013; Lillie et al., 2007) suggesting that MBLs levels may be a possible target for pig breed selection to improve disease resistance.

3.8. Summary

While some PRRs such as TLRs are well-studied in the pig, many others lack even basic knowledge in this species. The examples mentioned above emphasize the potential of porcine PRRs to improve porcine health and resistance to various pathogens. Due to the high sequence homology shared by PRRs among species, synthetic/purified PAMPs used in murine or human studies stimulate also innate receptors in swine like peptidoglycan (PGN), lipoteichoic acid (LTA → TLR2), poly(I:C) (→TLR3) (Auray et al., 2010), lipopolysaccharide (LPS → TLR4) (Islam et al., 2012; Uddin et al., 2012), imiquimod (→TLR7/8) (Calzada-Nova et al., 2010), CpG ODN (→TLR9) (Auray et al., 2010; Calzada-Nova et al., 2010; Dar et al., 2010), and muramil dipeptide (MDP → NOD) (Jozaki et al., 2009). This high level of homology may facilitate research on the other PRR members and might encourage the porcine research community to widen our knowledge on these important factors in the innate immune response against all kinds of pathogens.

4. Humoral innate response

The humoral innate immune system is comprised of soluble plasma components that include, but are not exclusive to, complement, natural antibodies, acute phase proteins, and antimicrobial peptides as well as proteins/peptides involved in the contact system (Shishido et al., 2012). These innate molecules play a role in prevention and control of disease which 'complements' cellular innate immunity.

4.1. The complement system

The complement cascade is an ancient immunological system responsible for the recognition and elimination of microorganisms, apoptotic cells, and infected or cancerous cells as well as mediation of inflammation processes. This complex system is activated through three independent pathways using >30 soluble- and membrane-anchored proteins that together constitute a proteolytic

cascade (Shishido et al., 2012). It ultimately leads to C3 convertase cleaving C3 to produce C3b. Next, C3b promotes cleavage of C5 by C5 convertase to produce C5b which is followed by the initiation of the common terminal complex (Shishido et al., 2012). These processes result in the formation of the membrane attack complex (MAC) which lyses host or pathogenic cells by formation of pores in the cellular membrane. C3b opsonised pathogens are recognized by complement receptor 3 (CR3) expressed on neutrophils and macrophages. Complement receptor 2 (CR2) on the B cell recognizes cleavage products which triggers induction of antibody production (Molina et al., 1996). As well, C3a and C5a released from their respective convertases are potent anaphylatoxins. Many components of the complement system also participate in organ development, trimming of unused neuronal connections, cell migration/morphogenesis, and regeneration/cell-signalling (Leslie and Mayor, 2013).

The pig has been used as an animal model to study the response of the complement cascade in human diseases (Abu-El-Haija et al., 2012; Dalle Lucca et al., 2012; Fernandes et al., 2012). Using pig as a model to study cystic fibrosis (CF) (Rogers et al., 2008), large-scale transcript profiling revealed that the pancreas in foetal and newborn CF pigs exhibited significantly increased expression of genes whose products play a role in inflammation, fibrosis and the complement cascade compared to age matched, non-CF pigs (Abu-El-Haija et al., 2012). Other studies showed that activation of the complement system has been associated with tissue injury after hemorrhage and resuscitation (Dalle Lucca et al., 2012). In a porcine model of hemorrhagic shock, administration of recombinant human C1-esterase inhibitor reduced tissue damage and cytokine release and improved metabolic acidosis (Dalle Lucca et al., 2012). In pigs, complement mediated damage and immunosuppression are key features of post-weaning multi-systemic wasting syndrome (PMWS) (Fernandes et al., 2012). When large scale transcriptional analysis was compared between mediastinal lymph nodes from healthy pigs and pigs naturally affected by PMWS, affected animals had increased relative abundance of mRNAs coded by a large set of genes involved in the inflammatory responses (e.g. cytokines, acute phase proteins, and respiratory burst proteins) (Fernandes et al., 2012). However, the level of CR1 mRNA was decreased suggesting an altered complement system (Fernandes et al., 2012). This decreased CR1 gene expression may contribute to increased complement deposition and secondary infections by impairing phagocytosis (Fernandes et al., 2012).

Because the complement system interacts with other mechanisms of innate and adaptive immunity to enhance host defence (Song et al., 2000), it is being studied to determine which components and their corresponding genes are candidates to improve the general immune response. Research groups sequenced porcine C3 (Wimmers et al., 2003), C5 (Kumar et al., 2004), and mannose-binding lectin genes (Phatsara et al., 2007), and screened them for polymorphisms in a population of crossed Duroc and Berlin miniature pigs (DUMI). C3c and haptoglobin (HP) serum levels were measured before and after vaccination against *Mycoplasma hyopneumoniae* (Mh), Aujeszky disease virus (ADV), and PRRSV. Wimmers et al. determined that the individual variation in the acute-phase response to vaccinations observed within the DUMI resource population depended on the C3 genotype for C3c levels but that serum HP levels was not associated with C3 genomic variation (Wimmers et al., 2003). Four polymorphic sites were detected on the C5 gene, one of which was segregating in the DUMI population in three genotypic patterns: *AA*, *AC*, and *CC* (Kumar et al., 2004). Classical and alternative complement activities, C3c and HP levels, and antibody titres against Mh and ADV were measured in the resource population (Kumar et al., 2004). The homozygote *AA* animals were found to be significantly different from the other two genotypes with respect to alternative and classical complement activation and genotype *CC* was found to be significantly different from the other genotypes for C3c and HP levels (Kumar et al., 2004). No significant difference could be seen between genotypes for antibody titres against vaccinations (Kumar et al., 2004). SNPs of porcine MBL1 and MBL2 (Phatsara et al., 2007) genes were found and genotyped. MBL1 genotypes differed in C3c serum concentration suggesting potential impact on complement activity. Further, linkage analysis identified a quantitative trait locus for C3c serum level close to the position of the MBL genes which may indicate that porcine MBL2 and MBL1 could be possible functional and positional candidate genes for complement activity (Phatsara et al., 2007). These data suggest that genes for components of the complement cascade may be highly polymorphic and therefore, it may be possible to breed animals with advantageous complement activation to promote natural resistance to microorganisms (Wimmers et al., 2003). In a follow-up study, this research group wished to determine whether they could identify any links between polymorphisms, differential basal expression of porcine terminal complement components (TCC) genes (C6, C7, C8a, C8b, and C9), and disease resistance (Wimmers et al., 2011). Lung lesions were recorded as a parameter of health and the transcript levels of TCC genes from lung tissue were obtained from German Landrace, Piétrain, Hampshire, Duroc, Vietnamese Potbelly, and Berlin miniature pig (BMP) breeds (Wimmers et al., 2011). The analysis revealed thirty SNPs indicating considerable polymorphism as well as differences in the levels of expression among various complement genes (Wimmers et al., 2011). The majority of SNPs were located in conserved regions on the gene promoters which may indicate that they affect transcription factor binding. In summary, the pig is an attractive model to study complement cascade and further studies must be performed to determine whether animals with particular complement gene expression variations may be more resistant to disease.

4.2. The contact system

There is considerable cross-talk between the complement and contact cascades. Coagulation factors XI and XII, and plasma kallikrein are the three serine proteases that comprise the contact system, along with the non-enzymatic co-factor high molecular weight kininogen (HK) (Amara et al., 2008; Colman and Schmaier, 1997; Hamad et al., 2012). Both factor XIIa and kallikrein activate the complement cascade independently of known complement initiators, and activated members of the contact cascade such as factors Xa, Xia, and plasmin can cleave C5 and C3 to produce C5a and C3a (Amara et al., 2008; Hamad et al., 2012). Further, complement inhibitor C1INH also inhibits coagulation factor XIIa indicating considerable overlap between the two pathways (Amara et al., 2008; Colman and Schmaier, 1997; Hamad et al., 2012). When in contact with negatively charged surface, the contact system is activated and coagulation factor XII is then cleaved to form factor XIIa which in turn initiates the coagulation cascade. Upon cleavage of prekallikrein to kallikrein, bradykinin (BK) is released (Shishido et al., 2012). BK binds its receptor and induces vasodilation, neutrophil chemotaxis, and vascular permeability, as well as triggers the release of pro-inflammatory mediators such as histamine, prostaglandins, leukotrienes, and cytokines, the latter mostly derived from polymorphonuclear cells (Renne, 2012). In circulation, kallikrein, factor XII as well as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) bind plasminogen which recognizes and binds to fibrin blood clots. These kinases cleave plasminogen and release plasmin, a potent extracellular protease which mediates, among other things, degradation of protein matrices involved in resolution of blood clots, embryogenesis, tumor invasion, wound healing, and angiogenesis (Castellino and Ploplis, 2005). Studies in pig showed that plasmin activates matrix metalloproteinases (MMPs) at sights of trauma enabling the degra-

dation of all components of extracellular matrix and suggesting its involvement in remodelling (Salo et al., 2008).

4.3. Naturally occurring antibodies

Naturally occurring antibodies (NAbs) are germline-encoded antibodies with restricted epitope specificities and they are produced by B1 lymphocytes in primary lymphoid organs (Shishido et al., 2012). Furthermore, NAbs mainly target cellular debris and they bind pathogens with low affinity. The broad reactivity of individual NAbs allows rapid recognition and protection from pathogens never encountered before. Additionally, they can also modulate the adaptive immune response by interacting with B cells, T cells, and DCs (Ochsenbein et al., 1999). The VH genes of NAbs show little somatic hypermutation in human infants, rodents or swine (Butler et al., 2009, 2011, 2006). In most species, the majority of NAbs are IgM isotype but NAbs can also include IgG and IgA isotypes (Engelhardt et al., 2002a). NAbs are potent initiators of the complement cascade and they improve immunogenicity through enhanced antigen-trapping in secondary lymphoid organ and promotion of opsonisation (Michael et al., 1962; Ochsenbein et al., 1999; Shishido et al., 2012).

4.4. Acute phase proteins

Acute phase proteins (APPs) are blood proteins which are rapidly induced in response to inflammation caused by tissue injury, infections or stress (Gonzalez-Ramon et al., 1995; Lampreave et al., 1994). The two major APPs in pigs acute phase protein (pig-MAP) and HP (Gonzalez-Ramon et al., 1995; Lampreave et al., 1994) show 10-fold increased concentration in response to inflammation induced by oil injection, surgical trauma, stress, and acute bacterial or viral infections (Asai et al., 1999; Carpintero et al., 2007; Gonzalez-Ramon et al., 1995; Heegaard et al., 1998; Lampreave et al., 1994). For instance, pigs infected with *Actinobacillus pleuropneumoniae* showed rapid induction of HP, C-reactive protein, pig-MAP, and serum amyloid A (Heegaard et al., 1998). Interestingly, apolipoprotein A-I (ApoA-I) concentration decreased two to five folds in pig serum after sterile inflammation induced by turpentine injection or after infection with *A. pleuropneumoniae* or *S. suis* (Carpintero et al., 2005). Pigs infected with ASFV or ADV showed elevated pig-MAP and decreased ApoA-I levels suggesting that the latter is a negative acute-phase protein (Carpintero et al., 2007). Stress due to transport or change in diet have also been shown to induce the concentration of pig-MAP and HP (Eurell et al., 1992; Piñeiro et al., 2007; Pineiro et al., 2007). Peripheral blood mononucleated cells (PBMCs) from pigs injected with a TLR9 agonist showed consistently increased production of IP-10 suggesting that IP-10 may be a potential biomarker for CpG ODNs activation in pigs (Dar et al., 2010). Pig-MAP shows homology with human serum protein PK-120 which is a putative substrate for kallikrein indicating again how the humoral innate immune system pathways show significant overlap (Gonzalez-Ramon et al., 1995; Nishimura et al., 1995).

4.5. Host defence peptides/antimicrobial peptides

Host defence peptides (HDPs), also known as antimicrobial peptides, are expressed in epithelial cells and phagocytes, stored in secretory granules, and released via regulated exocytic pathways at mucosal surfaces and at sites of infection (Dybvig et al., 2011; Sang and Blecha, 2009). Many HDPs are naturally antibacterial, antiviral, and antiparasitic and mediate their antimicrobial effects by peptide-mediated membrane disruption (Bowdish et al., 2006; Shai, 1999). HDPs promote recruitment of neutrophils, monocytes, and mast cells causing release of nitric oxide, macrophage phagocytosis, and mast cell degranulation. Furthermore, HDPs stimulate tissue and wound repair, promote angiogenesis, and can also suppress the stimulated release of pro-inflammatory cytokines (Bowdish et al., 2004; Garlapati et al., 2009; Hancock and Scott, 2000; Iimura et al., 2005; Mookherjee et al., 2006; Nijnik et al., 2009; Yu et al., 2007). Thus, HDPs provide a functional linkage between the innate and adaptive immune systems (Dybvig et al., 2011).

Mammalian HDPs consist of two peptide families: cathelicidins and defensins. Cathelicidins are small, cationic, mostly α-helical, amphipathic molecules synthesized with a highly conserved cathelin propeptide sequence (Gallo et al., 1997; Sang and Blecha, 2009; Zanetti, 2004). Cathelicidins are expressed in the skin and mucosal surfaces and are found in their proteolytically processed mature form at mucosal surfaces and in most body secretions including sweat, breast milk, and saliva (Murakami et al., 2002; Phadke et al., 2005). Defensins are cationic cystine-rich peptides comprised of three families: α-, β-, and θ-defensins. They are extremely diverse in sequence and structure, the majority are $\leqslant$5 kDa in size, cationic at neutral pH, and amphipathic (Fahlgren et al., 2004). They are either constitutively expressed or induced by bacterial products or pro-inflammatory cytokines (Starner et al., 2005; Sumikawa et al., 2006; Vora et al., 2004). Current annotation of the porcine immunome has demonstrated expansion of the cathelicidin and type 1 IFN gene families (Dawson et al., 2013) and, like that of cattle, the pig genome does not contain any identifiable α-defensins (Choi et al., 2012). Studies in our laboratory have revealed that porcine β-defensin 1 (pBD-1), a porcine defensin homolog of human BD-2 (hBD-2), is absent in the upper respiratory tract in neonatal pigs (Elahi et al., 2006). The expression of pBD-1 was detected in all analysed tissues in 4- to 5-week-old piglets. Broncho-alveolar lavage (BAL) specimens collected from piglets older than 4 weeks of age and chemically synthesized pBD-1 displayed strong antimicrobial activity against *Bordetella pertussis, in vitro*. Furthermore, *in vivo* treatment of newborn piglets with only 500 μg of pBD-1 at the time of challenge conferred protection against infection with *B. pertussis* (Elahi et al., 2006). Thus, there is evidence that pBD-1 expression is developmentally regulated in piglets, and that its absence in newborns may contribute to their increased susceptibility to *B. pertussis*.

HDPs may play a role in wound healing and the resolution of infection. *Staphylococcus aureus*-infected wounds present a major complication experienced by diabetic patients (Hirsch et al., 2009). In the diabetic Yorkshire pig animal model, excisional wounds transduced with an adenoviral vector coding for hBD-3 and seeded with *S. aureus* showed that overexpression of hBD-3 significantly impeded bacterial growth of seeded *S. aureus* and induced wound closure (Hirsch et al., 2009).

In summary, the humoral innate immune system consisting of the complement system, the contact system, natural antibodies, acute phase proteins, and HDPs produce soluble proteins or factors which attempt to contain and/or kill microbes that invade the body. The components which make up this system are pre-formed and immediately released when a microbe is sensed offering a swift response to infection.

5. Cells of the innate immune response

Many cell types are involved in the innate immune response. In this section we present the best known cellular types and we highlight their main characteristics in the pig model.

5.1. Epithelial cells

Epithelial cells constitute epithelia, the first barriers to pathogen entry. They play a crucial role in the initiation of the innate

immune response to protect the different mucosae (genital, intestinal, and respiratory) and the skin. Porcine epithelial cells respond to various pathogens with production of TNF-α and IFN type I (α and β) and III (λ) which block pathogen replication. They also produce chemoattractant/pro-inflammatory cytokines, including interleukin 1 (IL-1), IL-6, IL-8, chemokine (C-X-C motif) ligand 1 (CXCL1), and granulocyte–macrophage colony-stimulating factor (GM-CSF) involved in the recruitment and the activation of immune cells. As well, the epithelial cells of humans, mice, rats, and dogs express major histocompatibility complex II (MHC class II) and are thought to be capable of presenting antigen directly to T cells (Bland, 1988; German et al., 1998). In contrast, MHC class II expression is reported to be absent in the intestinal epithelium of swine (Wilson et al., 1996) and in the porcine intestinal columnar epithelial cell line IPEC-J2 (Schierack et al., 2006). However, endothelial cells which lie below the basement membrane of the intestinal vessels show strong expression of MHC class II antigens (Wilson et al., 1996). The specialized epithelial cells covering the dome area of porcine PP (M cells) are mantled by densely packed, long, thick, and irregularly-shaped microvilli (Torres-Medina, 1981) which can be identified in swine with anti-cytokeratin 18 antibody (Gebert et al., 1994; Pabst and Rothkotter, 1999). Even though not strictly related to the innate immune system, epithelial cells are involved in the active transport of polymeric Igs such as IgA and IgM across the epithelial barrier which is mediated by polymeric immunoglobulin receptor (PIGR).

Various epithelial cell lines such as porcine jejunal and ileal cell line IPEC-1 (Gonzalez-Vallina et al., 1996), porcine jejunal cell line IPEC-J2 (Berschneider, 1989), porcine intestinal epithelial cell IPI-2I (Kaeffer et al., 1993), porcine kidney 13 (PK13, ATCC CRL-6489), PK15 (ATCC CCL-33), and newborn porcine trachea (NPTr) cells (Ferrari et al., 2003) are available. When cultivated on an appropriate support IPEC-1 and IPEC-J2 cell lines can develop a phenotype similar to the one observed in primary epithelial cells (Bruel et al., 2010; Zakrzewski et al., 2013). Recently it has been demonstrated that the supplementation of culture media with porcine serum instead of bovine serum could drastically improve the phenotype developed by IPEC-J2 cells making them more similar to primary intestinal cells (Zakrzewski et al., 2013).

5.2. Mast cells

Mast cells are tissue resident immune cells that are best known for their involvement in IgE-mediated immune reactions. They can be divided into tissue mast cells and mucosal mast cells depending on their location and function. Mucosal mast cells are key players in maintaining homeostasis at the mucosal surfaces, and are involved in the mucosal response to intestinal pathogens, food-borne allergies and probiotics. Upon binding of immunoglobulines via surface Fc-Receptors, mast cells release a variety of immune mediators, including histamines, leukotrienes, prostaglandins and heparin, all of them needed for recruitment and activation of pro-inflammatory immune cells. To this end, mast cells are equipped with a panel of high affinity Fc-Receptors, in particular FcE- and FcG-Receptors, that ensure rapidly binding and release of these molecules. Secretion of these mediators can also be directly induced via complement, peptides, toxins, bacteria, and parasites (Marone et al., 1997). In pigs, very little is known about the phenotype and the distribution of mast cells throughout the body. More than twenty years ago, Xu et al. described the morphology of porcine mast cells from various organs in one to six month-old pigs using histochemical staining techniques (Xu et al., 1993). Ten years later, Rieger et al. established methods to reliably stain mast cells for mast cell counting (Rieger et al., 2013) and nitric oxide production by porcine mast cells of the autonomic ganglia, nerves and renal vessels was described by Vodenicharov (2008) and Vodenicharov et al. (2005). Martinez et al. published a case report of a pig with multiple cutaneous mast cell tumors (Martinez et al., 2011). This type of cancer is rarely seen in pigs, and usually more frequent in dogs and cats. Femenia et al. reported a novel mast cell line derived from hematopoetic progenitors to further investigate the response to nematodes such as *Ascaris suum* and others (Femenia et al., 2005). Interestingly, a trypsin-serin proteinase from porcine mast cells was shown to mediate post-translational proteolytic cleavage of influenza hemagglutinins and other RNA viruses, indicating that mast cell tryptase in pigs might be a trigger of pneumotopic infections in pigs (Chen et al., 2000).

Enrichment of the sow diet with linseed oil induced changes in the postnatal development of the piglet ileum, including altered mast cell regulation (Boudry et al., 2009). The role of mast cells in the context of soybean allergies was described by Sun et al. who found that the levels of mast cells and the intensity of histamine release was greatly triggered by glycinin, the main storage protein of soybeans (Sun et al., 2008). Mast cells are also becoming frequently recognized as mediators of stress-related intestinal disorders. For example, early weaning of piglets, as often proposed by the industry, can cause stress gastrointestinal dysfunction in pigs. Moeser et al. and Smith et al. found that much of this dysfunction is mediated by mast cells and their products, and that the mast cell stabilizer drug sodium cromoglycerate can ameliorate some of these effects (Moeser et al., 2007; Smith et al., 2010). Also, supplementation with L-arginine can greatly enhance intestinal barrier function and maintain intestinal integrity after LPS challenge (Zhu et al., 2013).

5.3. Macrophages

Macrophages differentiate from hemopoietic progenitors either directly or via circulating monocytes (Geissmann et al., 2010) after cell migration to the different compartments of the body. This migration is controlled by various adhesion molecules like integrins, immunoglobulin-superfamily members, selectins, and others. In the tissues, macrophages are able to adapt to their local microenvironment, develop different phenotypes, and reside as Kupffer cells (liver), Langerhans cells (skin) or alveolar macrophages (lung) (Gordon, 2002). Recently, it has been demonstrated in mice that some tissue macrophages derived from non-hematopoietic stem cells (Geissmann et al., 2010; Guilliams et al., 2013; Hashimoto et al., 2013; Schulz et al., 2012). Differentiated residential macrophages can comprise 10–15% of the total cells in tissue. They provide a first line of defence against pathogens mainly via antigen uptake and presentation as well as by the production of inflammatory cytokines (Fairbairn et al., 2011; Murtaugh and Foss, 2002). Macrophages facilitate humoral immunity, allergic and anti-parasite responses. They can also like alveolar macrophages (Hussell and Bell, 2014) dampen the immune response through anti-inflammatory cytokines such as IL-10 and TGF-β and participate to tissue repair.

Gordon and Martinez divide the macrophage life cycle in four different stages: differentiation, priming, activation, and resolution (Gordon and Martinez, 2010). In the differential stage, the balance of macrophage colony-stimulating factor (MCSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), retinoic acid, and lipoproteins can impact the future phenotype and function of the mature macrophage (Gordon and Martinez, 2010). In the next phase, macrophage priming is accomplished by the different cytokine milieus during their continuous recruitment into tissue. This priming has a substantial impact on their response following stimulations and to their role in the innate immune response (Gordon and Martinez, 2010). The presence of IFN-γ leads to macrophage activation in the "classical way" ("M1"), whereas the presence of IL-4 and/or IL-13 leads to macrophage activation in the "alternative

way" ("M2") (Dalton et al., 1993; Stein and Keshav, 1992). After recognition of microbes by macrophage PRRs or via opsonic stimulation via antibody complexes, a mature macrophage becomes fully competent to fulfil its role in the innate immune response.

"Classical activation" via IFN-γ, produced mainly by activated Th1 cells or natural killer (NK) cells, leads to a pro-inflammatory, antimicrobial activity by the macrophage and the promotion of a Th1 response (Fairbairn et al., 2011). IFN-γ priming was shown to promote classical activation of macrophages (Charley et al., 1990) and viral pathogen resistance in pigs (Lunney et al., 2010). Activation of macrophages through the "alternative pathway" via IL-4 and/or IL-13 leads to promotion of a Th2 and antibody-mediated immune response. The role of IL-4 in porcine macrophages was mainly analysed in combination with GM-CSF in the induction of monocyte-derived DC (moDCs) and it triggered decreased expression of inflammatory markers CD14 and CD18, and increased expression of T-cell co-stimulation molecules such as MHC class II and CD80/86 (Swiggard et al., 1995). Several other cytokines may be involved in the fine-tuning of macrophage activation. At the end of infectious process, anti-inflammatory cytokines increase and the macrophage can undergo its final maturation step that Gordon and Martinez termed "resolution". In this stage, the pro-inflammatory potential is suppressed and the macrophage is involved in debris clearance and general repair functions (Gordon and Martinez, 2010).

Activation of porcine macrophage by PRRs stimulation (Fairbairn et al., 2011; Jungi et al., 1996; Zelnickova et al., 2008) revealed a major similarity between porcine and human macrophages which contrast with studies carried out in mice. Specifically, LPS stimulation of murine macrophages in combination with IFN-γ leads to the induction of NO synthase (NOS2, iNOS) triggering the antimicrobial response. In contrast, human and porcine (Fairbairn et al., 2011; Jungi et al., 1996; Zelnickova et al., 2008) macrophages produce indoleamine 2,3-dioxygenase (IDO) instead of NOS2 upon LPS activation leading to production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and IL-12 (Fairbairn et al., 2011). IL-1β and IL-12 promote expansion, differentiation, and survival of T cells (Fairbairn et al., 2011). In addition, they are able to induce IFN-γ production by T and NK cells which starts a pro-inflammatory feedback loop and even stronger IL-1β, IL-12, and CD80/86 levels to prevent the development of tolerance. As well as LPS, cholera toxin (CT) is able to increase IL-1 and CD80/86 expression by macrophages and might be a promising adjuvant to boost the innate immunity (Foss et al., 1999; Murtaugh and Foss, 2002).

The various phases of macrophage differentiation and activation lead to a complex and diverse phenotype of macrophages. Additionally, they can differentiate from shared myeloid progenitors which make them difficult to identify and segregate phenotypically from other mononuclear phagocytes such as myeloid DCs. In mice, CD11b, CD18, CD68, F4/80 and Fc receptors are common markers for the phenotypic definition of macrophages (Murray and Wynn, 2011) but this cannot be directly transferred in other species (Fairbairn et al., 2011). In swine, macrophages are defined as $CD172a^{+}CD1^{+/-}CD4^{-}CD11R1^{-}CD14^{+/-}CD16^{+}$ MHC class $II^{low}CD80/86^{low}$ (Summerfield and McCullough, 2009a). Furthermore, CD163 is selectively expressed on monocytes and macrophages and therefore a useful marker to define this subset (Fairbairn et al., 2011). This molecule is also a receptor used by the PRRSV to infect this important immune cell subset (Calvert et al., 2007).

PRRSV is the most economically significant viral pathogens for the pig industry world-wide and causes reproductive problems and pneumonia (Chand et al., 2012). Infection leads to a significant shift in the gene expression of PAMs. PRRSV strongly induces IFN-β, but not IFN-α, expression (Sang et al., 2011). Additionally, the anti-inflammatory cytokine IL-10 is up-regulated within twelve hours indicating that PRRSV is able to induce the "resolution" stage very early after infection of macrophages (Genini et al., 2008).

Unfortunately, many other viral pathogens like porcine circovirus 2 (PCV2) and ASFV and bacterial pathogens like *A. pleuropneumoniae* and *Chlamydia suis* also replicate in macrophages altering their function and negatively impacting swine welfare and economic returns to swine producers (Fairbairn et al., 2011; Opriessnig et al., 2008; Schautteet and Vanrompay, 2011).

PCV2 is the primary causative agent of the multi-factorial disease PMWS, which is detrimental to pig production (Kekarainen et al., 2010). Symptoms include wasting, dyspnoea and growth retardation and affect mainly pigs between 6 and 18 weeks of age (Segales et al., 2005). Transcriptional analysis of the response of PAMs on PCV2 infection via microarray and quantitative PCR revealed that PCV2 is able to prevent apoptosis in PAMs and to induce the expression of pro-inflammatory cytokines mainly through TLR1 and TLR9 pathways. Elevated secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-8 could be further validated by ELISA (Li et al., 2013b). ASF is a haemorrhagic disease and ASFV replicates in macrophages causes their swelling and rounding, increased phagocytic and secretory activity as well as stronger proliferation (Gomez-Villamandos et al., 2013). These pro-inflammatory changes in the macrophage phenotype and function play a key role in ASF pathogenesis which is characterized by pulmonary oedema, haemorrhage, and lymphoid depletion. Furthermore, macrophages are involved in ASFV replication by hemadsorption and cell necrosis at the end of the replication cycle (Gomez-Villamandos et al., 2013). *A. pleuropneumoniae* belongs to the most economically significant bacterial pathogens in pig production world-wide and causes lung lesions, pneumonia and a strongly impaired animal welfare. Infection of macrophages induces their rapid influx into the lung and the local induction of pro-inflammatory cytokines (Baarsch et al., 2000). Transcriptional profiling indicates that *A. pleuropneumoniae* can circumvent the host immune factors and survive within macrophages by active evasion of the host defences e.g. by urease activity or cell surface sialylation (Klitgaard et al., 2012).

C. suis infections have a minor impact on swine health and economics of swine production but its role in various reproductive problems are not precisely determined and might be underestimated (Schautteet and Vanrompay, 2011). Moreover, due to the earlier mentioned high homology with humans including similar immune response pathways in macrophages and the susceptibility also for the human pathogen *Chlamydia trachomatis*, the pig is a suitable large animal model for human chlamydial infections. Chlamydial infection of epithelial cells and macrophages promotes IL-1 production and plays a central role by the induction of a strong Th1 response leading to the production of IFN-γ which is essential for pathogen clearance (Darville and Hiltke, 2010).

In addition to its role in various infections, porcine macrophages may be involved in xenograft rejection and their blocking "could be effective approach to downregulate human anti-pig cellular immune responses" (Plege and Schwinzer, 2010).

In conclusion, porcine macrophages are an important innate immune cells since they provide one of the first lines of defence against infections, are targets of and significant players against various viral, bacterial, and parasitic pathogens. As well, they may have an important impact on human health as a model cell subset as well as in xenograft transplantation.

5.4. Dendritic cells

Dendritic cells play a major role in sensing infections and danger through PAMPs and damage-associated molecular pattern molecules (DAMPs), in antigen transport from the site of entry at the skin and the mucosa to the lymphoid tissue, as well as in the

regulation of tolerance *versus* immunity. Consequently, DCs plays a major role in orchestrating the immune response. In mouse and man, DCs are recognized to represent a heterogeneous family of cells with specialized functions. DCs can be subdivided into plasmacytoid DCs (pDCs) which have a main function in sensing nucleic acid, and classical DCs (cDCs) which are specialized in antigen presentation. cDCs consist of two distinct subsets, one more specialized in cross-presentation and stimulation of cytotoxic T lymphocyte responses through MHC class I, the other more specialized in stimulating Th responses through MHC class II. All DC subsets have a different phenotype in mouse and man as well as in other species studied but they have not yet been completely characterized phenotypically and functionally for each species. In addition, the phenotype of DC subsets is known to be organ-specific further complicating the issue of DC identification. For instance, monocyte-derived DCs are ontogenetically and functionally distinct from the above *bona-fida* DCs, as their name indicates. Only with the help of studies on the ontogeny of the DC family and transcriptomics, which has made significant progress through studies in the murine system, it is now possible to characterize the DC subtypes (Merad et al., 2013; Miller et al., 2012). Sets of genes and transcription factors specifically expressed in DCs, subsets of DCs, and other haemotopoietic lineages such as monocytes and macrophages have been identified. Selected transcription factors tightly regulate the development of particular DC subsets in a specific manner (Merad et al., 2013; Reizis, 2012; Shortman et al., 2013).

Considering that porcine DCs have been recently reviewed in detail (Summerfield and McCullough, 2009b) we will only give an overview of the topic and focus on important pitfalls in the identification and classification of DCs in the pig for the present review. Porcine blood-derived pDCs have been described as $CD4^{+}CD172a^{low}CD123^{+}CD135^{+}$ cells, which express low levels of CD8, CD16, CD45RA, and MHC class II but are negative for CD14. Porcine cDCs have a similar phenotype but lack CD4, CD123 and have high levels of MHC class II. Thus, in the blood DC can be differentiated from monocytes by expression of CD135 (Flt3) and their lack of CD14. The phenotypic and functional differentiation of the two subsets of cDCs is not yet available in the pig, although based on several markers such as CD1, CD16 and CD172a at least two subsets of cDCs can be defined (Guzylack-Piriou et al., 2010; Summerfield et al., 2003; Summerfield and McCullough, 2009b). It is evident from several other studies that a $CD172a^{-}$ subset of DCs probably exists in the blood, the skin, lymph nodes and the pseudo-afferent lymph of skin, and intestinal duct (Bertho et al., 2011; Bimczok et al., 2005; Guzylack-Piriou et al., 2010; Marquet et al., 2011).

Despite this knowledge, a major problem in the porcine immune system remains the clear discernment of DCs from other cells such as macrophages and even B cells. While DC identification is clear in the blood, we have found that in lymphoid tissues the phenotypic definitions are only partially valid and that the function and phenotype of DCs, macrophages and B cells do overlap. In fact, most porcine 'DC' markers are also expressed on many other leukocyte populations. For instance, CD1 is expressed at high levels on thymocytes, B cells (Pescovitz et al., 1990), and can also be found on monocyte-derived macrophages (unpublished data). CD11R1 (corresponding to human CD11b) which has been used to identify subsets of DCs in combination with CD172a in mucosal tissue (Bimczok et al., 2005), is also found on subsets of monocytes, macrophages, and neutrophils (Dominguez et al., 2001). High levels of CD4 are a hallmark of pDCs in the pig. However, in addition to its expression on Th cells, this marker is also expressed on a subset of cells co-expressing CD14 and CD172a representing an unknown lineage, presumably of myeloid origin (Guzylack-Piriou et al., 2010; Summerfield et al., 2003). This phenotype is much more frequent in the lymphoid tissue as compared to the peripheral blood, making the identification of pDCs simply based on CD4 and CD172a expression difficult or impossible in tissue other than blood (Summerfield et al., unpublished data). Furthermore, considering the expression of CD11R1 on myeloid cells, the employment of this marker to identify DCs is currently not appropriate, even if combined with CD172a, which is also widely expressed on myeloid cells. Also, expression of MHC class II, CD80 and CD86 can be misleading. In the pig, activated macrophages, memory Th cells and cytotoxic T cells (although the levels on T cells are lower) express high levels of MHC class II, CD80, and CD86. CD14 is typically expressed on cells of the myeloid lineage including monocyte-derived DCs (albeit the expression can be low). Although, it is not expressed on blood DCs, its expression on lymphoid tissue and non-lymphoid tissue in the pig needs to be determined.

In the skin, at least four subsets of putative DC have been described. In the epidermis, Langerhans cells have been identified as $CD172^{+}CD1^{+}CD207^{+}$ (Nfon et al., 2008). In the dermis a population of $CD172a^{-}CD163^{-}CD207^{-}CADM1^{+}$ and a $CD172a^{+}CD163^{-/low}$ $CD207^{+}CADM1^{-}$ population was found. The observation that these two subsets efficiently migrate in the lymph supports their DC origin. In addition, a $CD172a^{+}CD163^{+}CD207^{-}CADM1^{-}$ population which also expressed CD206 and CD209 as well as high levels of CD16 was described (Marquet et al., 2011). The latter is denominated as a DC population by the authors, probably of monocytic origin. Whether these cells are DCs or macrophage needs clarification. Taken together, the characterization of the porcine DC cell system in various lymphoid and non-lymphoid tissues is only partially sufficient to identify these cells phenotypically. Of course with microscopic studies, morphological and spatial aspects are helpful but rarely provide a proof of cell identity such as with Langerhans cells in the epidermis. It is therefore necessary to define precisely which combinations of markers permit the clear differentiation from other cell types such as macrophages and how the different DC populations can be discriminated. Transcriptomic analysis such as those performed with murine cell by the Immunological Genome Consortium (Miller et al., 2012) should be undertaken and an effort in generating new reagents for the pig is required.

5.5. Neutrophils

Polymorphonuclear neutrophils (PMNs or neutrophils), the major acute innate specialized phagocyte, act in the first line of defence against various pathogens including bacteria and fungi (for a review see Kumar and Sharma (2010)). Since their discovery by Elie Metchnikoff in the 1880s (see Kaufmann (2008)), neutrophils are also considered as potent inflammatory cells potentially causing massive collateral damage of host tissues. Neutrophils can kill pathogens intracellularly by phagocytosis and extracellularly through degranulation and neutrophil extracellular traps (NETs) formation (Brinkmann et al., 2004; Kumar and Sharma, 2010). These structures are able to trap various pathogens and to kill them extracellularly by release of their antimicrobial peptides such as neutrophil serine proteases (NSPs), i.e. neutrophil elastase (NE) cathepsin G (CatG) and proteinase 3 (Pr3) (Kumar and Sharma, 2010). Only a few reports describe the role of porcine PMNs in the host response to microorganisms. Panyutich et al. demonstrated the bactericidal activity of pig PMNs against *L. monocytogenes* (Panyutich et al., 1997). These authors showed that secretion and elastase-mediated activation of proprotegrins accounted for much of the stable microbicidal activity of PMN secretions against *L. monocytogenes*. Since then, the bactericidal activity of porcine PMN secretions has been reported (Scapinello et al., 2011) and it has been revealed that

the bacteria *S. aureus* and *Pseudomonas aeruginosa* induce porcine NETs secretion (Brea et al., 2012). Porcine PMNs (swine cluster 1, SWC1$^+$ or CD52, and SWC8$^+$ cells (Piriou-Guzylack and Salmon, 2008)) display the same morphology as those of humans with a round shape and rough surface (Clapperton et al., 2005) but are smaller in size, have lower granularity and a higher activation threshold (Brea et al., 2012). Additionally, differences in PMN morphology and function have been reported between pig breeds. PMNs from Meishan pigs, are smaller and less efficient in phagocytising *Escherichia coli* than PMNs of Large White pigs (Clapperton et al., 2005). Moreover, porcine PMNs behaved similarly to human blood PMNs in response to A23187 with the same morphological changes, no DNA secretion and exposure of the three proteases at the cell surface (Brea et al., 2012). The close structural homology between porcine and human NSPs explains their immunological cross-reactivity and indicates closer substrate specificity between pig and human NSPs than observed with mouse NSPs (Kalupov et al., 2009).

5.6. Basophils and eosinophils

Basophils and eosinophils are white blood cells of the immune system expressing CD172a as others myeloid cells (Piriou-Guzylack and Salmon, 2008) and they can be differentiated from neutrophils by the lack of expression of surface antigen 2B2, which is selectively expressed on mature neutrophils (Perez et al., 2007). Basophils are the least common of the granulocytes and they are associated with many inflammatory reactions, particularly in the context of allergic symptoms and the host defence against parasites. Their large basophilic cytoplasmic granules contain heparin and histamine. Moreover, they express on their cytoplasmic membrane receptor for IgE immunoglobulins and prostaglandin D2 receptor. Antagonism of this prostaglandin receptor is known to inhibit their activation as well as eosinophil chemotaxis and Th2 cytokine production (Stebbins et al., 2012). Pigs sensitized to Ascaris *suum* eggs showed significantly greater histamine release from basophils compared to non-sensitized pigs (Uston et al., 2007). Thus, basophils play a role in the control of the mechanisms associated with allergy and asthma.

Eosinophils are involved in the response against multicellular parasites and certain infections in vertebrates (Magyar et al., 1995). Their cytoplasmic granules, appearing brick-red after coloration with eosin, are rich in histamines, eosinophil peroxidase, ribonuclease, deoxyribonucleases, lipase, plasminogen, and others proteins. All these molecules are released in the environment after degranulation. They are toxic for parasites but also host tissue. Transcriptional profiling of swine lung tissue and hilar lymph nodes after experimental infection with Actinobacillus *pleuropneumoniae* showed induction of many genes such as GM-CSF whose increased protein expression can stimulate stem cells to differentiate into granulocytes such as basophils, eosinophils and neutrophils (Yu et al., 2013; Zuo et al., 2013). miRNAs are evolutionarily conserved short single stranded RNAs that can inhibit gene expression at the post-transcriptional level playing an important role in the control of inflammation and immune response. One study showed that a specific miR-155 SNP was significantly associated with basophil percentage and absolute eosinophil value in pigs suggesting that miR-155 may have, a previously unknown, role in immune function (Li et al., 2013a).

Pig eosinophils can be isolated using discontinuous Percoll gradient to separate the granulocyte fraction and by a negative magnetic activated cell separation with mouse anti-pig CD16 antibody (Masure et al., 2013). Several publications presented data related to eosinophils, especially in the context of parasitic infections with *A. suum* (Masure et al., 2013), *Trichuris suis* (Steenhard et al., 2007), and *Toxocara canis* (Sommerfelt et al., 2006).

5.7. Natural killer cells

Natural killer (NK) cells in mice were initially identified as cells that spontaneously kill tumor or virus-infected cells but do not belong to the B- or T-cell lineage (Herberman et al., 1975; Kiessling et al., 1975). Similar, early studies in swine showed that non-B non-T cells have a spontaneous lytic activity against NK-susceptible targets like the MHC class I lacking cell line K562 as well as transmissible gastroenteritis virus or ADV infected target cells (Cepica and Derbyshire, 1986; Martin and Wardley, 1984; Yang et al., 1987). Due to the lack of specific markers recognizing only NK cells in swine, the identification and phenotypic description of this porcine lymphocyte population was rather difficult. In contrast to other species where NK cells are described as large granular lymphocytes, porcine NK cells morphologically resemble small to medium sized cells that lack granules (Denyer et al., 2006; Yang et al., 1987). Nevertheless, porcine NK cells lack the T-cell receptor CD3 as well as T-cell co-receptors like CD4, CD5 and CD6 and were characterized by a uniform expression of CD2, CD8α, CD16, CD45RC as well as the effector molecule perforin (Denyer et al., 2006; Gerner et al., 2009; Pauly et al., 1996; Pintaric et al., 2008; Saalmuller et al., 1994). A minor fraction of NK cells express swine leukocyte antigen-DR (SLA-DR) and this molecule can be further up-regulated after *in vitro* stimulation (Pintaric et al., 2008). More recently the characterization of the activating receptor NKp46 in swine by novel monoclonal antibodies led to additional insight into the phenotype of porcine NK cells. In contrast to other species wherein NKp46 is considered as a pan-NK marker (Walzer et al., 2007), NKp46 is not expressed on all porcine NK cells (Mair et al., 2012). Three distinct porcine NK-cell subsets could be defined on the basis of their NKp46 expression: NKp46$^-$, NKp46$^+$ and NKp46^{high} cells (Mair et al., 2012, 2013). Whereas NKp46$^-$ and NKp46$^+$ NK cells are very similar in their phenotype, NKp46^{high} cells differ in their expression pattern of various NK-associated markers. Although it was postulated earlier that all porcine NK cells express CD8α (Denyer et al., 2006; Pescovitz et al., 1988; Saalmuller et al., 1994), more recent data show that the NKp46^{high} NK-cell subset is rather associated with a CD8α$^{dim/-}$ phenotype (Mair et al., 2013). The recent identification and characterization of SWC2 molecule as porcine CD27 revealed that all porcine NK cells express this marker (Reutner et al., 2012). Although NKp46$^-$ and NKp46$^+$ NK cells express CD27 at relative low density, NKp46^{high} NK cells show an increased expression of this TNF-receptor family member (Mair et al., 2013). So far, information on other NK-associated receptors on porcine NK cells is limited due to the lack of monoclonal antibodies. The activating receptors NKp30 and NKG2D together with its adaptor proteins DAP10 and DAP12 were investigated on mRNA level on enriched or sorted NK cells (Denyer et al., 2006; Toka et al., 2009a,b). More recently it could be shown that NKG2D was very uniformly expressed on the three NKp46-defined NK-cell subsets, whereas NK cells with higher NKp46 expression seem to express lower levels of NKp30 (Mair et al., 2013). During the evolution of mammals, two families of inhibitory NK-cell receptors that recognize classical MHC class I molecules developed: killer cell immunoglobulin-like receptors (KIR) and the Ly49 lectin-like receptors. In swine, one gene of each family was detected so far. A single KIR gene encoding for a predicted inhibitory receptor was identified in the porcine leukocyte receptor complex (Sambrook et al., 2006). Additionally, also a single Ly49 gene was identified, but due to a mutation in a highly conserved cysteine, which is involved in disulphide-bridge formation, the expressed protein may not be functional (Gagnier et al., 2003).

A vast heterogeneity in NK-cell numbers could be observed in swine, ranging from 1% to 24% of blood lymphocytes (Denyer et al., 2006; Mair et al., 2012). Comparable to other species, porcine NK cells can be found in many anatomical locations. In lymphatic

organs the highest frequency can be found in spleen (2–15%), whereas lower frequencies were observed in lymph nodes, tonsils, and thymus (<3%). In the bone marrow NK cells were barely detectable (Denyer et al., 2006; Mair et al., 2012). In contrast, non-lymphatic organs like the liver and lung harbor high frequencies of NK cells that can make up to 15–40% of lymphocytes in these organs (Mair et al., 2012). Likewise, the high abundance of NK cells in non-lymphatic organs was observed in mouse and human (Gregoire et al., 2007). Furthermore it was reported that porcine NK cells were recruited to the uterus during early pregnancy, thus indicating a special role of NK cells at the maternal-foetal interface also in swine (Engelhardt et al., 2002b). In regard to the NKp46-defined NK-cell subsets also a vast heterogeneity between individual animals could be observed. While some animals show higher frequencies of NKp46$^+$ NK cells, others only have minor numbers within the CD3$^-$CD8α^+ NK population (Mair et al., 2012). In regard to anatomical distribution NKp46$^-$ and NKp46$^+$ NK cells can be found in all organs analysed, but NKp46^{high} NK cells are more abundant in lymphatic as well as non-lymphatic organs and are only found in very low numbers in the blood (Mair et al., 2012). A possible explanation for this may be a differing expression pattern of chemokine receptors for migration and/or recruitment of NK cells. First evidence thereof may be the observed elevated expression of the chemokine receptor CXCR3 on cells belonging to the NKp46^{high} subset in spleen (Mair et al., 2013). Up to date, no lineage markers and therefore no information about NK-cell ontogeny are available for porcine NK cells. Nevertheless, some information about the phenotype and distribution of porcine NK cells in blood in the first six month of life was recently described in more detail (Talker et al., 2013). Like in adult pigs, heterogeneity in NK-cell number between individual animals could also be observed in young piglets. Total NK-cell numbers increased after birth and declined after a peak at weeks 5–7, resulting in a relatively low, but stable plateau phase. Therefore, levels of NK cells seem to vary with age and higher levels can be observed in younger pigs (Denyer et al., 2006; Talker et al., 2013). Phenotypically, extracellular markers like CD2 and CD8α were already detectable on NK cells of newborns, whereas SLA-DR expression started only after three months of age (Talker et al., 2013). Additionally, porcine NK cells were already distributed into NKp46-defined subsets from birth on and no age-related changes in the pattern of the NKp46-defined subsets could be observed. Interestingly, all NK cells of newborns already expressed the effector molecule perforin indicating that porcine NK cells seem to be functional and competent for cytolytic activity at very early time-point in life (Talker et al., 2013). This parallels findings in NK cells of human and mice, where perforin$^+$ NK cells can be found in cord blood of newborns and in the neonatal liver (Rukavina et al., 1998; Wu et al., 2012). However, it was reported that NK cells of newborn pigs are not competent to kill susceptible target-cell lines like K562 (Yang and Schultz, 1986).

The two main functions of NK cells are their lytic activity and production of cytokines such as IFN-γ. Porcine NK cells stimulated with different cytokines including IL-2, IL-12, IL-15, IL-18, and IFN-α increased the expression of CD25 and perforin and induced the production of IFN-γ in porcine NK cells (Pintaric et al., 2008; Toka et al., 2009b). Resting porcine NK cells showed only low cytolytic activity against susceptible target-cell lines or infected cells, but this lytic activity could be enhanced by stimulation with different combinations of the above mentioned cytokines (Pintaric et al., 2008; Toka et al., 2009b). Similar effects on NK-cell function and effector molecule production could be observed after stimulation of NK cells with TLR agonists. TLR7, TLR8 as well as TLR9 agonists showed either direct effect on NK cells that were shown to express TLR7 and TLR8 on mRNA level (Toka et al., 2009c), or via indirect effect by cytokines produced by activated accessory bystander cells (Dar et al., 2008; Toka et al., 2009c).

In human as well as in mouse, distinct subsets of NK cells exist that possess different functional properties (Cooper et al., 2001; Hayakawa et al., 2006). Likewise, the three NKp46-defined NK-cell subsets in swine show different capacities to produce cytokines and have distinct degranulation properties. NKp46 expression levels seem to be correlated with IFN-γ and TNF-α production. Therefore, NKp46^{high} NK cells produced the highest levels of these cytokines after activation, whereas NKp46$^-$ cells produced the lowest levels (Mair et al., 2012, 2013). Although both, NKp46$^-$ as well as NKp46$^+$ NK cells had similar cytolytic properties towards susceptible target-cell lines (Mair et al., 2012), NKp46$^-$ NK cells only showed a reduced degranulation capacity after triggering of activating receptors CD16 and NKp46 compared to the NKp46$^+$ and NKp46^{high} NK cells (Mair et al., 2013).

Since NK cells play important roles in the defence against viral, parasitic, and bacterial pathogens (Lodoen and Lanier, 2006), porcine NK cells were investigated as to the immune response against various infectious agents. Increased NK-cell numbers were observed during *Isospora suis* infection (Worliczek et al., 2010), whereas reduced numbers were found after PRRSV and PCV2 infections (Nielsen et al., 2003; Shi et al., 2008). Additionally, NK cell function may be influenced by infections. Thus, a decrease in cytolytic activity of porcine NK cells could be observed after ASFV infection (Norley and Wardley, 1983) as well as in PRRSV (Cao et al., 2013; Dwivedi et al., 2012; Jung et al., 2009; Renukaradhya et al., 2010) and porcine respiratory corona virus infections (Jung et al., 2009; Renukaradhya et al., 2010). Moreover decreased lytic function and reduced numbers of IFN-γ producing NK cells were found following FMDV infection (Toka et al., 2009a).

In summary, a more detailed picture on phenotypical and functional characteristics of porcine NK cells has evolved during the last years, indicating similarities but also peculiarities compared to the knowledge on human and murine NK cells. Undoubtedly, due to its unique position as a large animal model, the pig still holds much promise for further discoveries in NK-cell biology.

5.8. Gamma delta T cells

In all vertebrates, T cells can be separated into two populations on the basis of the expression of different forms of the T-cell receptor (TCR): $\alpha\beta$ T cells and $\gamma\delta$ T cells. $\gamma\delta$ T cells are evolutionary highly conserved immune cells (Hayday, 2000). Depending on their tissue distribution and respective TCR recombination, $\gamma\delta$ T cells can perform different functions including (i) protective immunity against extracellular and intracellular pathogens, (ii) tumor surveillance, (iii) modulation of innate and adaptive immune responses, (iv) tissue healing and epithelial cell maintenance and (v) regulation of physiological organ function (Bonneville et al., 2010). Moreover, activation of $\gamma\delta$ T cells is not only transmitted via the TCR but also by receptors associated with NK cells or myeloid cells, like NKG2D, NKp46 or TLRs (Bonneville et al., 2010; Correia et al., 2011). Hence, $\gamma\delta$ T cells display features of both, the innate and adaptive immune system and are therefore covered in this review.

Their frequency and distribution varies greatly among different species. In most adult animals and humans, $\gamma\delta$ T cells represent only a small proportion (1–5%) in the blood and peripheral organs but are enriched in epithelial surfaces (Carding and Egan, 2002). Together with ruminants and chicken, pigs are $\gamma\delta$ – "high" species in which $\gamma\delta$ T cells are strongly enriched in the blood (approximately 18–47%) and can constitute up to 85% of total lymphocytes (Takamatsu et al., 2006). In conventional pigs, substantial $\gamma\delta$ T cell proportions can also be found in spleen (18–57%), within liver-residing lymphocytes (22–37%) and to a lower extent in thymus (5–31%) (Sedlak et al., 2014). Intermediate proportions are present within lymphocytes residing in lungs (~15%) and the intestine

(~10%). Lower frequencies have been reported for the bone marrow (~7%), tonsils (~5%), and lymph nodes (average 5–6%) (Sedlak et al., 2014; Stepanova and Sinkora, 2012).

In regard to TCR-diversity in pigs, the existence of 28 Vδ, 6 Dδ, 4 Jδ and 1Cδ gene segment has been reported (Uenishi et al., 2009), while to date no information is available about the germline encoded gene segments of the γ-chain. It was also shown that the porcine δ-chain repertoire has the potential of an enormous recombinatorial diversity – greater than that described for humans and mice. Similar to human and mice, the porcine TCR δ repertoire is diverse in young piglets and becomes oligoclonal and compartmentalized in 2- to 5-year old pigs at mucosal and extraintestinal sites, suggesting that intestinal γδ T cells are shaped by selection over time (Holtmeier et al., 2002). Knowledge on the expression of other receptors involved in the activation of innate immune cells is only fragmentary in the pig. However, a subset of porcine γδ T cells may express the activating natural killer receptor NKG2D, commonly present in NK cells, $CD8^+$ αβ T cells and γδ T cells similar to humans, mice, and ruminants (Takamatsu et al., 2006).

A phenotypical discrimination of γδ T cells in swine was achieved by differences in CD2/CD8α expression, demonstrating the existence of $CD2^-CD8\alpha^-$, $CD2^+CD8\alpha^+$ and $CD2^+CD8\alpha^-$ γδ T cells. In terms of their distribution, $CD2^-$ and $CD2^+$ γδ T cells are enriched at distinct anatomical sites. $CD2^-$ γδ T cells clearly dominate in blood and liver, while $CD2^+$ γδ T cells preferentially reside in spleen, thymus, and lymph nodes (Hirt et al., 1990; Yang and Parkhouse, 1996; Sedlak et al., 2014). In regard to their function, it was suggested, that the $CD2^-CD8^-$ subset is mostly composed of naive cells, whereas $CD2^+CD8^-$ cells may represent an effector/memory subset and $CD2^+CD8^+$ probably present terminally differentiated cells (Stepanova and Sinkora, 2012). More recently, Stepanova et al. showed that CD2 expression determines two independent γδ T-cell lineages (Stepanova and Sinkora, 2013).

Similar to other species, porcine γδ T cells are the earliest detectable T-cell subset in the thymus. As early as day 40 of gestation, the first mature $CD3\varepsilon^{hi}$ γδ thymocytes appear and subsequently migrate into the periphery (mature $CD3\varepsilon^{hi}$ αβ thymocytes were observed 15 days later). Compared to αβ T cells, γδ T cells mature faster and can develop without any $CD3\varepsilon^{lo}$ or TCR-$\gamma\delta^{lo}$ transitional stage (Sinkora et al., 2000a, 2005). The CD2/CD8α-defined γδ T-cell subsets have been identified already in the thymus and probably originate from a common γδ T-cell precursor ($CD4^-CD2^+CD8^-CD1^+$ $CD45RC^-$; (Sinkora and Butler, 2009)). After birth, circulating γδ T cells strongly increase until 19–25 weeks of age, indicating an important role during adolescence. However, the ratio of the $CD2^+$ and $CD2^-$ subset in blood remains relatively stable during this time period (Talker et al., 2013).

As mentioned above, γδ T cells show a high variability in their functional capacities. In humans and ruminants, γδ T cells have been shown to play a vital role early in mycobacterial infections (Meraviglia et al., 2011; Plattner and Hostetter, 2011). Mycobacterial phosphoantigens were identified as potent stimulators of human Vγ9δ2 cells (Chen, 2013). In swine, enhanced *in vitro* proliferation and IFN-γ production to mycobacterial antigens following *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccination has been detected as well as memory-like functions of porcine γδ T cells at an early age were suggested (Lee et al., 2004). This is in accordance with humans, where Vγ9δ2 γδ T cells in *Mycobacterium tuberculosis* infections initially produce IFN-γ and TNF-α and show memory characteristics indicated by a marked expansion in blood, lungs and intestinal tissue after a secondary BCG inoculation (Meraviglia et al., 2011).

For human Vγ9δ2 T cells it could be shown that phosphoantigens like isopentenyl pyrophosphate derived from the mevalonate pathway serving in isoprenoid biosynthesis in eukaryotic and prokaryotic cells) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP, derived from alternative isoprenoid biosynthesis pathway used in most eubacteria) are potent stimulators for proliferation and cytokine production (Morita et al., 2007). However, porcine γδ T cells did not respond on isopentenyl pyrophosphate or HMBPP stimulation in proliferation assays, regardless of the presence of antigen presenting cells or IL-2 [Sedlak, *unpublished results*]. These results indicate that, similar to cattle and mice, porcine γδ T cells most probably use alternative ways to sense bacterial pathogens.

Further, γδ T cells were identified as the main source of IL-17 after pulmonary BCG infection in mice. IL-17 production by γδ T cells has also been confirmed in naïve mice and humans but has to be elucidated in cattle (Plattner and Hostetter, 2011). Recently, we identified the porcine $CD2^-$ γδ T cell subset as the main IL-17 producers within total γδ T cells, which mainly co-produced TNF-α, following polyclonal stimulation. Within $CD2^+$ γδ T cells an enrichment of IFN-γ and IFN-γ/TNF-α co-producing cells was identified (Sedlak et al., 2014). Moreover, *in vitro* stimulation of FACS-sorted $CD2^+$ γδ T cells with IL-2 and the macrophage-derived cytokines IL-12 and IL-18 induced strong IFN-γ production. Lower amounts of IFN-γ were also produced by sorted $CD2^-$ γδ T cells after both stimulation strategies. Moreover, CD2 was up-regulated within the $CD2^-$ γδ T cell subset after combined ConA, IL-2, IL-12 and IL-18 stimulation (Sedlak et al., submitted for publication). These results indicate that porcine γδ T cells have a high degree of plasticity in regard to phenotype and cytokine production profile, slightly reminiscent of that what is observed for human γδ T cell lineages, that are considered to acquire distinct Th1-, Th2-, Th17-, T_{FH} and T regulatory-like effector functions (Caccamo et al., 2013).

Cytolytic activity by porcine γδ T cells has been described in some studies. Olin et al. observed enhanced natural killer (NK) cytotoxic activity of porcine γδ T cells against K562 target cells prior to mycobacterium vaccination, whereas, following *M. bovis* BCG vaccination this cytolytic activity was enhanced. Moreover, memory-like cytolytic responses against mycobacterium-infected autologous monocytes as target cells were demonstrated for 3-week-old, BCG-vaccinated pigs (Olin et al., 2005b). $CD2^+CD8^+$ γδ T cell clones were observed to be capable to kill MHC-compatible as well as MHC-incompatible target cells and further, $CD8^{lo}$ γδ T cells were demonstrated to show cytotoxicity in a CD3-redirected lysis assay (de Bruin et al., 1997; Yang and Parkhouse, 1997). These findings are somewhat in contrast to the observation that circulating γδ T cells in swine do not express perforin, even after IL-2 stimulation (Denyer et al., 2006).

Further, the functional aspects of porcine γδ T cells in the context of viral infections or viral antigen stimulation was investigated. *In vitro* stimulation with FMDV vaccine antigen stimulated purified γδ T cells from FMDV-naïve pigs to produce mRNA of IL-1, IFN-α and GM-CSF *in vitro* (Takamatsu et al., 2006). Somewhat different to this, γδ T cells isolated from the blood of PRRSV-infected gilts showed a higher proliferation and IFN-γ production compared to γδ T cells from non-infected animals (Olin et al., 2005a). The infection of gnotobiotic pigs with human rotaviruses (HRV) indicated low frequencies of TLR2, TLR3, and TLR9 expressing γδ T cell isolated from the ileum during the first 5 days following infection. Further, an increase in IFN-γ producing $CD2^+$ γδ T cells derived from spleen and ileum was observed 2 days after infection (Wen et al., 2012).

Human γδ T cells are not only considered to play important roles in protective immunity through cytokine secretion and cytotoxic activity but also can act as professional antigen presenting cells (APCs) (Brandes et al., 2005). In cattle, cultured γδ T cells were able to present soluble antigens after changing their phenotype, but this was not true for freshly isolated bovine γδ T cells (Collins et al., 1998). In swine, CD2 and/or CD8 positive γδ T cells co-expressed surface molecules associated with APCs (MHC class II, CD80/CD86, CD40 and CD31) and showed OVA uptake within 20 min

(Takamatsu et al., 2002), indicating phagocytic activity of porcine γδ T cells.

In summary, the data available so far, indicate that porcine γδ T cells show a similar degree of functional plasticity as reported for human and murine γδ T cells. However, due to their high frequency in blood, especially during adolescence, the pig is an attractive model to study the role of these cells further, especially in regard to their unique combination of features of the innate and the adaptive immune system.

6. Tools

6.1. The porcine toolbox

The pig toolbox has considerably increased in the last few years. Recent advances in the availability of genomic (Dawson et al., 2013; Groenen et al., 2012), transcriptomic (Tsoi et al., 2012) and proteomic (Trigal et al., 2013; Verma et al., 2011) data have made the analysis of porcine physiology at the molecular level cost effective and comprehensive. The porcine genome sequence has been published with the most recent build (Sscrofa10.2) including all 17 autosomes as well as both X and Y chromosomes and encompassing over 26,000 genes (Groenen et al., 2012). Rapid advances in the understanding of the porcine immune system at a genetic level have been made following the long awaited publication of the full genome. Curated annotation has associated more than 1300 genes to the porcine immunome (Dawson et al., 2013). In 2009, a high quality bacterial artificial chromosome map of the genome was done and more than 375,000 single nucleotide polymorphism were identified (Ramos et al., 2009).

6.2. Porcine stem cells and transformed cells

While the development of transgenic porcine models is still in its infancy, the tools to generate induced stem cells (Cheng et al., 2012; Wu et al., 2009) and to perform nuclear transfer (Park et al., 2001) exist and have been successfully applied (Aigner et al., 2010). Induced pluripotent stem cells (iPSC) derived from fibroblasts have been generated (Esteban et al., 2009; Ezashi et al., 2009). Existing relevant models include for instance antibody and B cell deficient swine (Mendicino et al., 2011), CFTR disrupted cystic fibrosis pigs (Rogers et al., 2008), and additional models are likely forthcoming following since the recent publication of the full genome (Groenen et al., 2012). Several transgenesis techniques were used successfully such as pronuclear DNA microinjection, sperm-mediated gene transfer, lentiviral gene transfer, and somatic nuclear transfer (Aigner et al., 2010). Transformed porcine cells have been developed which, when returned to the isogenic host, produced large tumours similar to their human counterparts (Adam et al., 2006). The cells provide a robust cancer model for preclinical studies interested in large tumour treatment. So far, using somatic cell nuclear transfer more than eight knocked out and two knock ins pigs have been produced (Aigner et al., 2010; Walters et al., 2012).

6.3. Gnotobiotic pigs and databases

Gnotobiotic piglets are delivered via caesarean section and then isolated in a pathogen-free environment (Butler et al., 2009). Because isolators allow most environmental influences to be controlled by the experimenter, gnotobiotic pigs offer a unique model to study mucosal immune systems under controlled settings. As of the writing of this review, over 500 papers have been published using gnotobiotic piglets. Gnotobiotic pigs allow researchers to study many enteric diseases which affect humans and pigs such as rotavirus (Yuan et al., 1996), *Cryptosporidium parvum* (Widmer et al., 2000), disseminated candidiasis (Andrutis et al., 2000), entero-hemorragic *E. coli* (Brady et al., 2011), *S.* Typhimurium (Martins et al., 2013a,b; Meurens et al., 2009) and acute gastritis from *Shigella dysenteriae* Type I (Jeong et al., 2010). The gastro-intestinal tract of germ-free piglets colonized with human fecal microbiome better approximate the human gastro-intestinal tract than the mouse gastro-intestinal tract (Che et al., 2009; Zhang et al., 2013b). With these pigs, important discoveries can be made studying how microbial dysbiosis may contribute to diseases such as asthma, diabetes, and irritable bowel syndrome and we can acquire a deeper knowledge of the interactions between commensal flora and the innate and adaptive immune systems in the gut. The number of antibodies available to detect pig proteins is increasing. Considering all the suppliers and the different conjugations available >17,000 pig-specific antibodies are available (www.biocompare.com) for ELISA applications and >8000 antibodies for flow cytometry. In the pig model, there is also the advantage that many antibodies directed against human markers cross-react with their counterparts. Several databases exist and present porcine immunological tools including antibodies and real-time PCR primers. The database established by Dr. Dawson (USDA, Beltsville, USA) named "*Porcine Immunology and Nutrition Database*" is found at http://199.133.11.115/fmi/iwp/cgi?-db=PINdb&-loadframes. Information on swine leukocyte antigen is deposited in the immune polymorphism database (http://www.ebi.ac.uk/ipd/). Additionally, in the NetMHCpan (http://www.cbs.dtu.dk/services/NetMHCpan/) it is possible to predict cytotoxic T cell epitopes.

6.4. Microarrays and kinome array

Microarray tools are also available for the pig model and Agilent Technologies sells a porcine gene expression microarray with 43,803 60-mer oligoprobes (www.genomics.agilent.com/article.jsp?pageId=1508&_requestid=430140) and Affymetrix sells a system with 23,937 probes assessing the expression of 23,256 transcripts, which represents 20,201 genes (Freeman et al., 2012). http://www.affymetrix.com/estore/browse/products.jsp?productId=131488#1_1). Proteomics approaches are also available and the pig proteome database (http://www.peptideatlas.org/) covers roughly 20 tissues for more than 15,000 peptides. Nevertheless, mapping of the pig proteome is still limited although it progressed rapidly in the last few years. New peptide arrays to analyze pig kinome, i.e. the set of protein kinases in porcine genome, are also under development (unpublished data) opening the door to new exciting studies.

6.5. Grants

Computerized retrieval of information on scientific projects (CRISP) search (1999–2003) reveals that NIH-sponsored research from over 20 institutes and centers supported over 2500 separate grants using the pig.

7. Conclusion

There is an increasing interest in the pig model which we predict will continue to expand, especially with the development of new porcine models enabled by the improvement of cell-based transgenic techniques and the increasing interest for the model in the scientific community. Moreover, the ongoing annotation of the porcine genome allows us to have a clear idea of which gene have to be targeted and/or modified to model human diseases. Soon, it is going to be possible to determine the presence of single or multiple copies of a specific gene. The identification and the

Table 3
Particularities of the pig innate immune system: a few examples.

- Ten different TLRs (TLR1-10) like in humans
- TLR7 and TLR9 restricted to pDCs and not expressed on cDCs as it is the case in mice
- Great diversity for interferon type I (39 genes *versus* 19 in humans). Particularly α, δ, and Ω
- No induction of NOS2 in response to LPS like in humans
- More cathelicidin genes than in humans (10 *versus* 1)
- No alpha-defensins in pigs
- Duplication of IL-1β gene (IL-1β and IL1-βL)
- Duplication of 2′,5′-oligoadenylate synthetase 1 (OAS1) gene (OAS1 and OAS1L)
- Duplication of CD163 gene (CD163 and CD163L)
- Substantial proportion of NKp46$^-$ NK cells
- Very high frequency of γδ T cells in the blood of adolescent animals

determination of the specificities of porcine immune system are progressing (Table 3). These new data will undoubtedly and significantly stimulate basic and applied comparative immunology. In recent years, a growing interest for epigenetics has emerged. The involvement of epigenetics in the porcine innate immune response is still at its infancy but there are first results indicating a significant role of epigenetics in the variation in porcine host-pathogen interactions observed between animals (Gomez-Diaz et al., 2012; Tarakhovsky, 2010). There is no doubt that the next few years are going to be very exciting in the field of pig research.

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