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Current transcriptomics in pig immunity research

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Abstract Swine performance in the face of disease challenge is becoming progressively more important. To improve the pig's robustness and resilience against pathogens through selection, a better understanding of the genetic and epigenetic factors in the immune response is required. This review highlights results from the most recent transcriptome research, and the meta-analyses performed, in the context of pig immunity. A technological overview is given including wholegenome microarrays, immune-specific arrays, small-scale high-throughput expression methods, high-density tiling arrays, and next generation sequencing (NGS). Although whole genome microarray techniques will remain complementary to NGS for some time in domestic species, research will transition to sequencing-based methods due to cost-effectiveness and the extra information that such methods provide. Furthermore, upcoming high-throughput epigenomic studies, which will add greatly to our knowledge concerning the impact of epigenetic modifications on pig immune response, are listed in this review. With emphasis on the insights obtained from transcriptomic analyses for porcine immunity, we also discuss the experimental design in pig immunity research and the value of the newly published porcine genome assembly in using the pig as a model for human immune response. We conclude by discussing the importance of establishing community standards to maximize the possibility of integrative computational analyses, such as was clearly beneficial for the human ENCODE project.

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Introduction

Anticipating the need to efficiently feed an estimated 9 billion people, pig farms have significantly increased in size, which also unfortunately increases the risk of disease incidence. A better understanding of the immune system in swine is essential, because susceptibility to infectious diseases has great influence on pig performance (Mellencamp et al. 2008; Boddicker et al. 2012). Selection for improvement in economical traits such as feed conversion and prolificacy is already routine practice, and improvement of immune capacity through selection is gradually catching up (Edfors-Lilja et al. 1998; Uddin et al. 2011; Lu et al. 2012). In addition, pigs are an important biomedical model for humans as they share great similarity in anatomy, physiology, genetics, and genomics, including many genes in the immune system (Dawson 2011; Dawson et al. 2013), which is very helpful when modeling human immune responses and diseases (Lunney 2007; Fairbairn et al. 2011; Kapetanovic et al. 2012; Meurens et al. 2012). Moreover, pigs have recently been genetically modified to serve as an improved model for human disease (Suzuki et al. 2012). For more genetically modified pig models for human diseases, we refer the reader to the reviews of Ross and Prather (2011) and Walters et al. (2012).

At the genomics and transcriptomics level, the pig is a very appealing model since one can extrapolate more easily to human due to the high homology in gene sequence and

chromosomal structure (Groenen et al. 2012). The immune system components of the pig genome (immunome) have become the best-annotated genes in the current genome (Dawson et al. 2013). This improved pig genome annotation will greatly aid creation of biomedical models. When the cause of a medical condition in humans is found, the corresponding mutation(s) can be genetically engineered in pigs to mimic the disease, and (novel) drug therapies can be tested (Walters et al. 2012). Differences in genotype associated with a different immune response phenotype in pig can easily be examined with the use of the porcine 60 K high-density SNP chip (Boddicker et al. 2012; Lu et al. 2012; Wang et al. 2012a). The last decade witnessed a steep increase in the amount of transcriptomic pig immune response data using whole porcine genome microarrays. Meanwhile, custom-made arrays enriched in annotated genes of the pig immune system were used to address specific research questions (Gao et al. 2010). In addition, novel techniques are surfacing in studies focusing on the porcine immune response transcriptome. High-density oligonucleotide tiling arrays and Next Generation Sequencing (NGS) have been further broadening the knowledge of the pig's immunome (Mockler et al. 2005; Morozova and Marra 2008). These techniques are able to identify and quantify known and unknown transcripts and can be used to extend recent efforts made on the pig genome annotation (Gao et al. 2012). The understanding of the function of diverse miRNAs will profit immensely from NGS, and papers on the porcine miRNAome are being published (Li et al. 2010a; Sharbati et al. 2010), as surely soon will follow on epigenetic changes such as DNA methylations or histone modifications. Several research groups have graciously provided information to our review on their recently published and unpublished studies, including whole genome microarrays to RNA-seq, miRNA-seq, RIP-ChIP, and some of those methylation and histone modification studies (Table 1).

The aim of this review is to summarize the use and results of recent transcriptomic research in pig immunity, identify current needs in the field, and anticipate future areas of progress. An overview will be given of studies using whole genome microarrays and immune-specific arrays, small-scale high-throughput expression methods, high-density tiling array, and NGS (Table 2; Fig. 1). Experimental design concerns and studies using the newest sequencing techniques and epigenetic tools together with a systems biology approach to interpret the data will also be discussed. For this review, we searched the Entrez Pubmed database, using keyword search terms such as pig transcriptomics, pig immunity, microarray, immune-specific array, high-throughput qPCR, RNA-seq and miRNA-seq, and systems biology. In addition, references from the articles obtained by this method were checked for additional relevant material. The ArrayExpress database from EBI was consulted to create Supplementary Table 1, using *Sus* scrofa as organism and filtered on 'Array assay' as technology.

Whole genome microarray studies, the start of global high-throughput transcriptomics

The range of genes assayed with whole genome microarray depends on the array used (Table 2; Fig. 1). The first porcine whole genome array available, the Qiagen-NRSP8 array, was a result of collaboration between Qiagen-Operon and the USDA-NRSP-8 Swine Genome community (Zhao et al. 2005). Until then, human arrays were used to tackle porcine transcriptomic issues, or swine arrays were developed for a specific research question, as described in the next section. The Pigoligoarray was a second-generation porcine 70-mer oligonucleotide array (Steibel et al. 2009). Currently, the most well known are commercially available microarrays such as the Porcine Gene Expression Microarray of Agilent and the GeneChip Porcine Genome Array of Affymetrix (Tuggle et al. 2010; Huang et al. 2011; Zhou et al. 2011; Bao et al. 2012). Freeman and colleagues recently introduced the Affymetrix Snowball microarray, which is more comprehensive than the first Affymetrix chip in coverage both for transcript structure and for the transcriptome as a whole, containing more than double as many probes (Freeman et al. 2012). Details and examples of these whole genome arrays are shown in Table 2. An overview of pig immunity experiments submitted to the ArrayExpress database is given in Supplementary Table 1.

Since global porcine microarray studies have been extensively described earlier (Tuggle et al. 2007, 2010), we will limit this section by highlighting the importance of using microarrays in a systems biology approach; we do provide a comprehensive table on all porcine immune-related microarray experiments submitted to ArrayExpress (Supplementary Table 1). Several microarray studies have been conducted to study host response to porcine reproductive and respiratory syndrome (PRRS) using commercial or more specific pig annotated microarrays (Bates et al. 2008; Genini et al. 2008a; Ait-Ali et al. 2011; Wysocki et al. 2012). Badaoui et al. (2013) recently illustrated how the information of multiple PRRS studies could be used simultaneously to gain insight on host response to PRRSv challenges. They collected all publicly available microarray data covering multiple porcine immunology studies and including many different breeds, tissues, pathogens, and array platforms. The data of 779 general immune response arrays were assembled, and separate meta-analyses for differential expression were performed using these 779 arrays as well as a subset of 279 arrays specifically

Table 1 Recently published and unpublished transcriptomic and epigenomic studies in pig immunity, contributed by community members

Laboratory institution	Contact info	Type of analysis tool	Tissues/cell types surveyed	Research focus	Publication status
USDA ARS NADC	M. Bandrick (meggan.bandrick@ars.usda.gov) and T. Stanton (thad.stanton@ars.usda.gov)	RNA-seq	Globin depleted blood, lymph node, duodenum, jejunum, ileum, colon	To study gene (especially as related to immune function) expression patterns between tissues	In preparation
Parco Tecnologico Padano	S. Botti (sara.botti@tecnoparco.org) and B. Badaoui (bouabid.badaoui@tecnoparco.org)	RNA-seq	Porcine alveolar macrophages (PAMs)	To study the transcriptome profile of PAM infected in vitro with low virulent (Lylestad) and high virulent (Lena) PRRSV strains. We quantified the expression levels of genes, isoforms, alternative transcript starting sites and we highlighted a complex transcriptional and post-transcriptional genes regulation during PAM infection	Badaoui et al. (2014)
Humboldt-Universität Berlin	G. A. Brockmann (gudrun.brockmann@agrar.hu-berlin.de)	RNA-seq and miRNA-seq	Mesenteric lymph nodes (Jejunum, Ileum), Peyer's Patches (Jejunum, Ileum)	To study effects of probiotics and zinc on immune cells	In preparation
University of Nebraska	D. C. Ciobanu (dciobanu@unl.edu)	Affymetrix GeneChip Porcine Array	Whole blood	To study differences in gene expression between pigs that display differences in growth and viremia following experimental infection with PCV2b	In preparation
University of Cordoba	J. J. Garrido (ge1gapaj@uco.es)	Affymetrix GeneChip Porcine Array	Neutrophils	To study the transcriptomic response of porcine neutrophils to Salmonella typhimurium and Salmonella choleraesuis	In preparation
INRA—GABI laboratory, France	E. Giuffra (elisabetta.giuffra@jouy.inra.fr)	RNA-seq, Small RNA-seq, RIP-Chip	Trigeminal ganglia	To study the role of miRNAs in Pig:pseudorabies virus interaction during latency	In preparation
University of Alberta	L. L. Guan (lelou.guan@ualberta.ca)	RNA-seq and miRNA-seq	Whole blood	To study the role of miRNAs in in response to *Salmonella Typhimurium*	(Bao et al. 2014; Kommadath et al. 2014)
The Roslin Institute, University of Edinburgh	D. Hume (david.hume@roslin.ed.ac.uk)	RNA-seq and CAGE	Macrophages from lungs and bone marrow	To study gene expression differences in response to lipopolysaccharide (LPS)	In preparation
University of Copenhagen	H.N. Kadarmideen (hajak@sund.ku.dk)	RNA-seq	Porcine subcutaneous fat from obese and lean pigs	To study transcriptomic profiles to elucidate differential and co-regulation and to integrate with 60 k SNP data to detect eQTLs, biological pathways and biomarkers for obesity and metabolic phenotypes in a porcine model	In preparation & WCGALP 2014 Conference paper

Table 1 continued

Laboratory institution	Contact info	Type of analysis tool	Tissues/cell types surveyed	Research focus	Publication status
USDA ARS NADC	L. Miller (laura.miller@ars.usda.gov)	Digital Gene expression Tag profiling (DGETP)	Porcine Trancheobronchial lymph nodes (TBLNs)	Comparative transcript expression snalysis in tracheobronchial lymph nodes of PRRSV-, PCV-2, PRV and SIV-Infected pigs	Miller et al. (2014)
Kansas State University	Y. Sang (ysang@vet.k-state.edu) and F. Blecha (blecha@vet.k-state.edu)	RNA-seq	Polarized macrophages	To profile signature genes and gene response pathways for antiviral regulation in monocytic cells	Sang et al. (2014)
Iowa State University	C. K. Tuggle (cktuggle@iastate.edu)	RNA-seq	Globin depleted blood	To study gene expression differences between residual feed intake extremes in response to endotoxin	In preparation
University of Bonn	M. J. Uddin (judd@itw.uni-bonn.de)	RNA-seq	Porcine dendritic cells	To study the gene expression of DCs in response to PRRSV challenge in vitro	In preparation
University of Bonn	M. J. Uddin (judd@itw.uni-bonn.de)	Affymetrix GeneChip Porcine Array	Porcine PBMCs	To study the transcriptomic profile of porcine PBMCs in response to PRRSV vaccine in vivo	Qu et al. (2014)
University of Bonn	M. J. Uddin (judd@itw.uni-bonn.de)	Epigenetic study: DNA methylation, HDAC activity, Histone acetylation and miRNAs study	Porcine monocyte-derived dendritic cells (MoDCs) and spleenic dendritic cells (SDCs)	To study the epigenetic modulation of TLR4 in response to lipopolysaccharide (LPS)	In preparation
University of Bonn	M. J. Uddin (judd@itw.uni-bonn.de)	Epigenetic study: DNA methylation, HDAC activity, Histone acetylation and miRNAs study	Porcine alveolar macrophages (PAMs)	To study the epigenetic modulation of CD14 in response to lipopolysaccharide (LPS)	In preparation
Leibniz-Institute for Farm Animal Biology	K. Wimmers (wimmers@fbn-dummerstorf.de)	Affymetrix GeneChip Porcine Array	Peripheral blood mononuclear cells (PBMCs)	To study transcriptomic response of PBMC of pigs with divergent humoral immune responses and coping behavior	In preparation

Table 2 Transcriptomic and epigenomic tools, their advantages and disadvantages, and interesting examples with regard to immune response research in swine

Specific tool	Advantage	Disadvantage	Example	Example analysis
Whole genome array				
Qiagen-NRSP8 13297 probes	Whole genome screening	Restricted to probes available on chip and limited to the chip's annotation	Zhao et al. (2005)	Array was validated in porcine liver, muscle, uterine endothelium and brain stem
Pigoligo 20400 probes			Steibel et al. (2009)	Array was validated in porcine liver, muscle, small intestine and lung
Affymetrix 23937 probes			Huang et al. (2011)	Pathways mediated by IFNγ, TNF, and NFκB are upregulated due to *S.* typhimurium infection
Agilent 43803 probes			Bao et al. (2012)	18 genes were differentially expressed after infection with enterotoxigenic *E. coli* (ETEC) with F18
Snowball 47485 probes			Freeman et al. (2012)	Array was validated in 62 porcine tissue/cell types and expression correlation analysis revealed specific clustering
Immune-specific array				
Macroarray 63 probes	Ideal for specific interest focus on immunity genes	Restricted to probes available on chip and limited to the chip's annotation. The chips are of use primarily to the specific research question asked	Ledger et al. (2004)	An upregulation of several immune genes is observed after PMA/ionomycin stimulation of PBMCs
Peyer's Patches array 2400 probes	Specialized tool for specific tissue		Dvorak et al. (2006)	A differential expression has been found in Peyer Patches after stimulation with Salmonella, LPS + Cholera toxin and PMA
Jejunum array 3468 probes	Specialized tool for specific tissue		Niewold et al. (2005)	ETEC upregulates 220 genes in 4-week-old and only 80 genes in 12-week-old pigs present on the array
Immune array 2880 probes	Specialized tool for pathogen infection		Zhang et al. (2006)	Macrophages stimulated with an African Swine Fever Virus change the expression of 125 genes on this array
Immune array 373 probes	Specialized tool for pathogen infection		Skovgaard et al. (2010)	Inoculation with *Actinobacillus* pleuropneumonia reveals a differential expression of 53 genes in the liver
Qiagen + SLA/PrV	Whole genome screening, but with focus on immunity genes in the SLA region and simultaneous data on two species (pig and pseudorabies virus)		Flori et al. (2008)	After 2 hpi a viral gene encoding a TAP inhibitor is upregulated, after 4 until 12 hpi porcine genes involved in SLA presentation are downregulated, showing a mechanism used by the virus to escape the porcine immune system
SLA-RI/NRSP8-13 K	Whole genome screening, but with focus on immunity genes, in and outside the SLA region		Gao et al. (2010) and Solinhac et al. (2011)	PBMCs stimulated with LPS an PMA/ionmycin were examined. While stimulation with LPS triggers a general inflammation response, PMA/ionomycin stimulation favors a T cell activation over a B-cell response

Table 2 continued

Specific tool	Advantage	Disadvantage	Example	Example analysis
Tiling array				
Affymetrix tiling array Agilent tiling array Nimblegen tiling array	No prior assumptions (previous annotation not necessary) and thus possible to refine annotation	Use of a very large number of probes, no whole genome re-sequencing possible with this technique	Gao et al. (2012)	With the use of 386620 probes, 97 genes in the SLA region were found to be differentially expressed between control PBMCs and PBMCs stimulated with PMA/ionomycin
Digital gene expression				
Illumina Genome Analyzer platform	Whole genome sequencing (High-throughput SAGE) with tag at all sequences with a CATG recognition site, no prior assumptions	Restricted to detection of transcripts containing CATG recognition sites and the Tag should be long enough to be unique, otherwise results are ambiguous. Isoforms cannot be detected	Xiao et al. (2010a, b)	Lung expression profile was examined in control or PRRSv-infected pigs (both Highly Pathogenic (HP) and US strain infected) at 4dpi. An average of 6.1 million tags per library was obtained. 4520 significantly differentially expressed genes were found in the HP and 5430 in the US PRRSv-infected animals. Important down-regulated genes are SPI IFN and IFNα and CD163 was upregulated
mRNA-seq				
The SOLiD^{TM}system, Applied Biosystems	Whole genome sequencing, no prior assumptions and splice variants can be detected	With polyA tail library, possible loss of non polyadenylated mRNA sequences and depletion of highly abundant genes might be necessary	Miller et al. (2012)	Trachea-bronchial lymph nodes expression profile was examined in control or PRRSv-infected pigs (both Highly Pathogenic (HP) and US strain infected) at 13dpi. The RNA-Seq showed 5.6 million reads for the control, 4.3 million reads for the HP and 3.5 million for the US PRRSv-infected animals. 632 differentially expressed genes were found to be significant for the HP and 633 for the US PRRSv-infected animals. Among the top ten, upregulated genes are RETN, S100A8, S100A9, and S100A12
Illumina HiSeq PacBio Roche 454 life sciences		Without polyA tail library, depletion of rRNA is necessary		
miRNA-seq				
Same tools as used for mRNA-seq	Sequencing of all miRNAs, no prior assumptions and splice variants can be detected	Examines only miRNAs. Their influence on mRNA still has to be tested	Chen et al. (2011a) and Endale Ahanda et al. (2012)	Inverse expression values were found between miRNAs measured by miRNA-seq and their target mRNAs (measured by mRNA-seq)

from PRRS experiments (Badaoui et al. 2013). To find PRRS-specific expression responses, they eliminated differentially expressed genes common to these two meta-analyses. Other meta-analysis studies, examining the immune response to *Salmonella* typhimurium, combine microarray information with data such as serum cytokine measurements or microbiota differences. The results of these meta-analyses performed on PRRS or *S*. typhimurium will be discussed in the section "Overall value of transcriptomics in important infectious swine diseases."

In addition, whole genome microarrays were used to study pig response to *Haemophilus* parasuis infection by Zhao et al. (2013). In one of their earlier studies, a genome-wide Affymetrix array experiment revealed 931 differentially expressed genes between 3 non-infected and 3 *H*. parasuis-infected pigs in spleen at 7dpi (Chen et al. 2009). Among them were *RETN*, *S100A8, S100A9*, and *S100A12*, all important innate immunity genes marking inflammation. Chen et al. (2011b) reexamined the raw data of this first experiment and used a more robust GeneChip RMA (GCRMA) normalization (Wu and Irizarry 2004), and an improved annotation using ANEXdb (Couture et al. 2009). A network analysis revealed that the *pS100A8/pS100A9-CASP3-SLC1A2* pathway played an important role in *H*. parasuis infection, and *CEBPB* may act as a transcription factor of the two S100 family members (Chen et al. 2011b). Later, Zhao and co-workers describe a systems biology approach starting from the same Affymetrix data. With the use of the KEGG and reactome databases, 1,999 transcripts from the Affymetrix chip were flagged as immunogenes. With this reduced dataset, exploratory analyses such as a principal component analysis (PCA) and a geneset enrichment analysis (GSEA) were conducted. With this PCA/GSEA method, they came to a core set of 16 differentially expressed genes, indicative of an *H*. parasuis infection (Zhao et al. 2013). To construct the immunologically important topology involved in an *H*. parasuis infection, the C_3NET algorithm was used (Altay and Emmert-Streib 2010). Several networks were created, and the largest network had the complement gene *C1R* as hub; although *C1R* was not differentially expressed on the microarray, it was predicted to play a crucial role in the immune defense. With this immunome-focused method, more subtle differences could be pulled out, and much new information was obtained as compared to the earlier whole genome array analysis (Zhao et al. 2013).

The immune-specific array, a focused microarray

Besides using commercially available or custom-made transcriptome-wide microarrays, or examining only immunogenes on those arrays, arrays focusing only on the genes involved in the porcine immune response are also used (Table 2). In the past, several of these arrays were constructed to specifically focus on key immune genes (Ledger et al. 2004), to explore gene expression in a specific immune tissue (Dvorak et al. 2006; Machado et al. 2005; Niewold et al. 2005), or to examine host interactions with a specific pathogen (Zhang et al. 2006; Skovgaard et al. 2010). More information on these studies is given in Table 2. Next to these small-scale arrays, other immune-specific arrays were made using an existing whole genome array and adding probes examining immune-related genes. In a study by Flori and co-workers, the Qiagen-NRSP8 array was extended by adding probes for Pseudorabies Virus RNAs as well as probes for transcripts from the porcine Major Histocompatibility Complex, also called the Swine Leukocyte Antigen (SLA) complex, referred to as the Qiagen + SLA/PrV in Fig. 1. This array was the first to simultaneously examine viral transcripts and porcine immune transcripts (Flori et al. 2008). Gao and colleagues used the NRSP8-13K chip enriched with SLA genes and immunity genes outside the SLA complex and called it the SLA-RI/NRSP8-13K chip (Gao et al. 2010). In the future, when using microarrays to examine gene expression patterns, whole genome arrays or the extended versions of them are clearly preferred. When the objective of the study is to examine only a relative small number of genes, other techniques such as Fluidigm Digital Array or Nanostring (described in more detail below) can be performed. These low cost, user-friendly, and rapid approaches make the small-scale in-house printed arrays redundant.

Upcoming sub-genomic-scale, high-throughput methods: Fluidigm and NanoString

Even though qRT-PCR is often used as the routine method to confirm microarray and RNA-seq findings, it has been used in many pig immune studies as a primary means to measure expression levels of a dozen or more immune genes (Borca et al. 2008; Lastra et al. 2009; Islam et al. 2012a, b; Uddin et al. 2012; Martins et al. 2013a, b; Uddin et al. 2013), together with suitable housekeeping genes for normalization (Cinar et al. 2012, 2013). Although it is a very accurate and sensitive method, standard qRT-PCR has the major drawback that only a few genes in a few samples can be examined at once as compared to high-throughput methods (Fig. 1). Further, significant amounts of money and time are spent controlling sample and assay variation by using appropriate—and preferably multiple—housekeeping genes and validating PCR efficiencies or correcting for them by using standard curves. The use of high-throughput qRT-PCR methods, such as the Fluidigm Digital Array, can help overcome these issues.

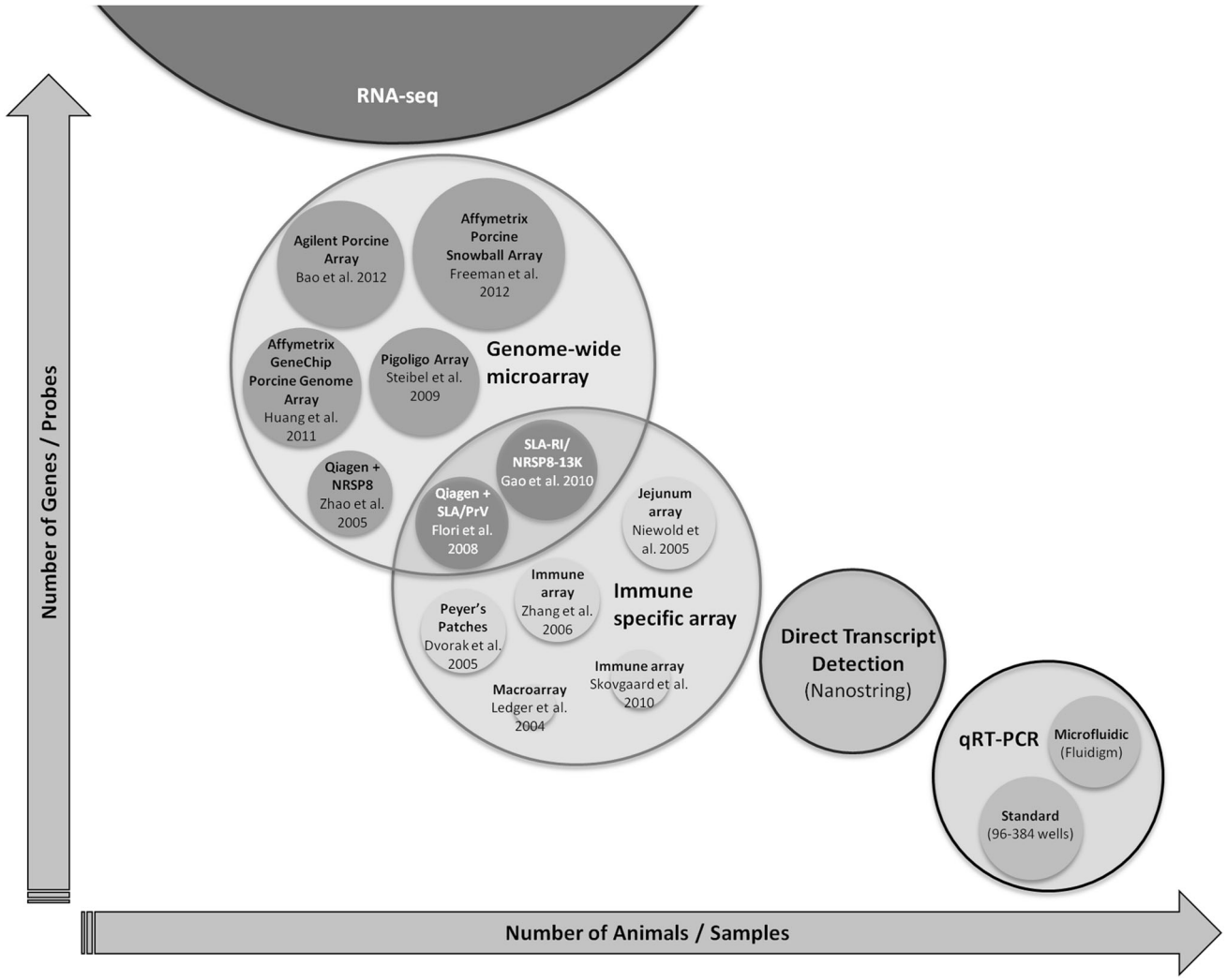

Fig. 1 Different transcriptomic tools used in porcine immune response studies, graphed with respect to the size of the dataset and the depth of the analysis possible. Nanostring has not yet been used in porcine immune response studies

The Fluidigm Digital Array integrated fluidic circuit (Spurgeon et al. 2008) is an example of a nanofluidic chip through which up to 9,216 qRT-PCR reactions can be performed at once, which represents 96 genes tested on 96 samples (Ramakrishnan et al. 2013) (Fig. 1). Beyond sheer speed of analysis, an important advantage of nanotechnology is that a much smaller amount of RNA per sample is necessary to perform 96 assays. In livestock, the first Fluidigm experiments have been published only recently, as a stand-alone expression study or as a validation of a microarray/RNA-seq experiment (Robic et al. 2011; Skovgaard et al. 2013; Sorg et al. 2013; Pilcher et al. 2014). In a H1N2 influenza virus study, Skovgaard and co-workers used Fluidigm to examine the expression of several pattern recognition receptors, IFN and IFN-induced genes, cytokines, and acute response protein genes after 24 hpi, 72 hpi, and 14 dpi. They expanded their study by including data on differentially expressed miRNAs and mRNAs in the lungs and saw several miRNAs potentially targeting the differentially expressed mRNAs (Skovgaard et al. 2013).

Another high-throughput method uses the NanoString nCounter system by which color-coded probes are counted to digitally measure gene expression (Fig. 1). This non-amplification method can be seen as a small-scale, hybridization-based platform. Per gene of interest, two sequence-specific probes are needed, a capture probe that has an affinity tag such as biotin to capture the gene on a surface, and a reporter probe with a multiple-fluorescent tag code that acts as a unique detector (Geiss et al. 2008). Its strength lies in the non-enzymatic approach, with no reverse transcriptase and no PCR amplification. This approach essentially eliminates primer–primer interactions, and up to 800 transcripts can be measured at a time. The

technique is not described in livestock species thus far. However, with the possibility to use degraded RNA samples such as RNA from formalin-fixed paraffin-embedded tissue samples, this method could become a very useful expression measuring tool.

High-density oligonucleotide tiling array for gene expression and genome annotation

High-density oligonucleotide tiling arrays have diverse genomic applications, such as transcriptome mapping and quantification. In general, tiling arrays are similar to microarrays as they both utilize oligonucleotide probes. However, through utilization of large numbers of probes, tiling arrays can span large genomic sequences without being restricted to annotated sequences (Gao et al. 2012). Thus, tiling arrays may contain probes covering the entire genome in an unbiased manner (Liu 2007), and such partially overlapping probes can be used to find expressed genes, previously annotated or not. Gao and co-workers used a Nimblegen tiling array (386,620 probes) for the analysis of the transcription map of the SLA complex (Gao et al. 2012). Ninety-seven genes were found to be differentially expressed between un-stimulated Peripheral Blood Mononuclear Cells (PBMCs) and PBMCs stimulated with Phorbol Myristate Acetate (PMA)/ionomycin. These results are in nearly complete agreement with their previous experiment using the SLA-RI/NRSP8-13K chip. Furthermore, with the tiling array, they were able to confirm and refine the previous annotation of the SLA genes (Gao et al. 2012).

Next generation sequencing

Transcriptome sequencing

RNA-seq is a recently developed method enabling the examination of the complete set of transcripts in a cell using a sequencing by synthesis approach (Wang et al. 2009). With this technique, mRNA is converted into a library of cDNA fragments, and at one or both ends of these fragments, adaptors are ligated. Each fragment is read in a highly parallel manner, and typical read lengths are between 30 and 700 bp, and go up to an average of 4,000 bp and more with the PacBio RS sequencer (Ferrarini et al. 2013). The resulting reads are aligned to a reference genome of the organism examined, and a genome-wide transcription map can be made describing the structure and enumerating the expression level of each gene. De novo assembly of a transcriptome is also possible if the reference genome is not available or poorly annotated (Martin and Wang 2011); integrated approaches are also possible.

RNA-seq has already proven to be a cost-effective way to investigate the sequence of all mRNA transcripts in a specific tissue and/or for a specific physiological condition. Not only is the sequence obtained together with the quantitative level of transcripts, but also splice variants can be detected, which is a major advantage in contrast with techniques such as microarrays. Moreover, with RNA-seq, there is very low, if any, background noise, and no transcript specific primers or probes are required, so no prior knowledge is needed (Wang et al. 2009). This latter attribute is important when working with livestock species for which genomic sequence assemblies are incomplete (Bauersachs and Wolf 2012). As such, Esteve-Codina and colleagues reported hundreds of un-annotated protein coding genes in the porcine genome while studying the gonad transcriptome (Esteve-Codina et al. 2011). However, projects costs are influenced by the research question asked. If the goal is to examine general expression pattern differences in well-annotated, moderately expressed genes, sequencing does not have to be very deep, and samples can be multiplexed in one lane. For novel transcripts or genes with a low expression level, higher coverage is needed, and costs will increase (The ENCODE Consortium Project 2011a). One may also consider depleting highly abundant genes of little interest, such as alpha and beta globin in a blood sample, prior to sequencing in order to improve detection sensitivity (Choi et al. submitted).

In 2010, Xiao and collaborators performed a 3' tag digital gene expression (DGE) analysis of the porcine lung transcriptome on pigs infected with the PRRS virus (Xiao et al. 2010a, b). This DGE technique can be seen as a predecessor of RNA-seq. Whereas RNA-seq will sequence all transcripts containing poly-A^+ tails, DGE detects those transcripts containing CATG recognition sites because the transcripts are 'tagged' through digestion of cDNAs at a *Nla*III restriction site (Hong et al. 2011). With DGE, only a portion of the transcript is analyzed instead of the nearly full transcripts as seen with RNA-seq (Wang et al. 2009). Both techniques offer similar estimates of gene expression levels but RNA-seq has the advantage to be able to provide information about transcript structure and consequently can detect splice variants (Hong et al. 2011). Xiao and colleagues sequenced lung RNA libraries of control non-infected pigs ($n = 3$), PRRS-affected pigs necropsied at 4dpi ($n = 3$,) and PRRS-affected pigs necropsied at 7dpi ($n = 3$) after infection with the classical North American PRRSv type (NPRRSv) (Xiao et al. 2010a) or the highly virulent PRRSv (HPRRSv), typically found in Asia (Xiao et al. 2010b) and found 4,520 (HPRRSv) and 5,430 (NPRRSv) differentially expressed genes. For both types of PRRSv, a higher expression of anti-apoptotic genes and a

lower expression of pro-apoptotic genes could be seen as a viral strategy for replication and spread (Xiao et al. 2010a, b). As such, suppressed expression of *short type I interferon* (*SPI IFN*) and *IFNα*, both important innate anti-viral genes, was detected. Additionally, there was an upregulation of *CD163* noted, which could indicate an increase in internalization of PRRSv since a positive correlation is described between the expression of this PRRSv receptor gene and PRRSv infectivity (Patton et al. 2009). Patton and colleagues described that treatment with modulators such as lipopolysaccharide (LPS) or IL10, affecting cells to express CD163, as a consequence also affected the susceptibility of the host for PRRSv, thereby increasing or decreasing viral infectivity (Patton et al. 2009).

Miller and colleagues examined gene expression profile data obtained by SAGE tag analysis of trachea-bronchial lymph nodes of sham- or PRRSv-infected pigs at 13dpi. Infection occurred with both highly pathogenic HPRRSv ($n = 8$) as well as North American type NPRRSv ($n = 7$) (Miller et al. 2012). The HPRRSv strain showed a more altered gene expression profile with higher fold change differences relative to the controls than the NPRRSv, indicating the increased pathogenicity of the HPRRSv. Among the top 10 genes that were upregulated were three *serum amyloid A2* acute-phase isoforms, *resistin* (*RETN*), and three S100 calcium binding proteins, *S100A8*, *S100A9*, and *S100A12* (Miller et al. 2012). Jiang et al. (2013) used the GO, KEGG, and REACTOME databases to analyze the data on NPRRSv infection further and identified six biological system categories affected by PRRSv, including cellular processes, genetic information processing, environment information processing, metabolism, organismal systems, and human diseases (Jiang et al. 2013). In a large-scale PRRSv infection study aimed at finding genes controlling variation in immune response outcomes, Boddicker and colleagues identified a region on SSC4, containing SNP marker WUR10000125, was strongly associated with both weight gain and PRRS viral load for 21 days post-infection (Boddicker et al. 2012, 2013). Eisley and co-workers performed an RNA-seq analysis of all genes in this WUR10000125 region through comparing blood RNA from 8 pairs of littermates with one of two different WUR10000125 genotypes. They identified a strong candidate gene differentially expressed between favorable and unfavorable genotypes (Eisley et al. 2014).

Several other immune-related RNA-seq studies are forthcoming (Table 1). Their objective is to examine viral or bacterial immune responses in pig macrophages, dendritic cells, lymph nodes, globin depleted whole blood, and tissue samples from the gastrointestinal tract. Undoubtedly, the knowledge of pig immune responses at the transcriptome level will greatly benefit from these recently published and to-be-published studies.

miRNA-seq

Next to exploring the "traditional" transcriptome, miRNAome analyses are also progressing in the pig (McDaneld 2009; Liu et al. 2010). MicroRNAs (miRNAs) are small non-coding RNAs that can regulate gene expression through degrading or interfering with the respective mRNA sequence, often at the 3' un-translated region (3'UTR) of the gene. During the last few years, high-throughput sequencing has lead to a steep rise in discovering novel porcine miRNAs (Xie et al. 2011). Moreover, NGS can facilitate distinguishing between miRNAs and other small RNA fragments, and thus, NGS is the most promising technique for exploring the miRNAome. Criteria to distinguish true miRNA from other RNA fragments are listed in Kozomara and Griffiths-Jones (2011). miRNA sequences and predicted targets for all important livestock animals can be found in miRBase, a comprehensive miRNA information database (Griffiths-Jones et al. 2006). With regard to immunological responses in pigs, recent studies have reported that porcine miRNA can intricately engage itself in host-virus interaction networks (He et al. 2009; Loveday et al. 2012; Guo et al. 2013). Conversely, miRNAs expressed by the viral pathogen can promote a favorable host cell environment for enhanced viral replication by targeting porcine mRNAs (Skalsky and Cullen 2010). An example of this involving response to Pseudorabies virus was published by Anselmo and co-workers. Using NGS, they analyzed both viral and host miRNA expressions in infected dendritic cells, and identified 5 viral miRNAs, 156 known porcine and 27 new porcine miRNAs (Anselmo et al. 2011). Another group used NGS techniques to analyze miRNA expression profiles in Pseudorabies virus infected porcine epithelial cell lines (Wu et al. 2012). Eleven miRNAs were detected in the viral genome, and 209 known and 39 novel miRNAs assigned to the porcine genome were also found. Wu and colleagues mainly focused on the viral miRNAs, which were associated with regulation of viral gene transcription but also proposed to control gene expression in the host of genes annotated for immune processes, viral replication, cell death, as well as other processes. Podolska and colleagues compared the miRNAome in necrotic and visually unaffected pieces of lungs from piglets infected with *A*. pleuropneumoniae and found 169 conserved and 11 candidate novel miRNAs. Twenty-nine were significantly up- or down-regulated between necrotic and unaffected tissue (Podolska et al. 2012). Timoneda and colleagues noted differences in miRNA expression between Aujeszky's disease or suid herpesvirus type 1 (SuHV-1) virus-infected and mock-infected animals, as well as differences when looking at a virulent strain of SuHV-1 compared to an attenuated one (Timoneda et al. 2014).

mRNA-seq and miRNA-seq: a powerful combination

Toward the elucidation of miRNA-RNA interactions, Endale Ahanda and colleagues analyzed the 3'UTR variants of all genes of the SLA region by analyzing RNA-seq data. In this way, SNPs in miRNA target sequences, potentially impacting gene expression, could be revealed. To investigate the co-expression between mRNA and miRNA, mRNA-seq and miRNA-seq data from an earlier study looking at liver, longissimus dorsi, and abdominal fat were used (Chen et al. 2011a; Endale Ahanda et al. 2012). Negative correlation between expression levels of miRNAs and their predicted target genes could be found, which suggested that the prediction algorithms used were reliable (Endale Ahanda et al. 2012).

Since miRNAs can play an important role in host–pathogen interactions (Scaria et al. 2006), Gao and colleagues looked by means of miRNA-seq at host miRNAs that could target PRRSv transcripts. Deep sequencing was performed on PAMs inoculated with a mock dose or with PRRSv. The resulting data were mapped against all known miRNAs listed in the current miRBase. miRNA target prediction revealed that one miRNA family, the miR-181 members, seemed to suppress PRRSv replication in vivo at the early stage. One miR-181c target is the 3'UTR of CD163 mRNA, which encodes an important PRRSv receptor. miR-181c is able to downregulate CD163 expression and thus interferes with viral attachment and penetration (Gao et al. 2013). Similar results were seen for other miR-181 members.

There is also good evidence that expression profile differences with regard to an *S. typhimurium* infection may be partially controlled by miRNAs. Huang and co-workers found that miR-155 was decreased in persistently shedding (PS) pigs in comparison with low shedding (LS) pigs (Huang et al. 2011) after an *S. typhimurium* infection. miR-155 targets transcription factors CEBPB and SPI1, which in turn control the expression of important immunogenes (Adamik et al. 2013; Wei et al. 2014). Bao and co-workers specifically investigated potential miRNA-mRNA regulatory interactions occurring during challenge with *S. typhimurium*. mRNA-seq and miRNA-seq data were collected on whole blood samples of LS and PS animals (Bao et al. unpublished). They found 37 and 24 miRNAs up- and down-regulated at 2 dpi when looking at LS and PS pigs together. Several of them were thought to be involved in innate and adaptive immune responses. They also discovered 3 miRNAs to be higher expressed in LS than in PS pigs at day 2, which could be interesting candidates for biomarkers for selection toward low shedders. Bao and colleagues subsequently used the sequence-based miRNA target prediction software miRanda to propose miRNAs–mRNA regulatory relationships associated with the *S. typhimurium* infection (Bao et al. unpublished). Ye and colleagues searched for factors controlling susceptibility for enterotoxigenic *E. coli* with fimbria 18 (ETEC-F18) in intestinal tissue in the Sutai pig and found 58 differentially expressed miRNAs, and after examining regulatory networks with differentially expressed mRNAs that are target of one of those miRNAs, 12 of them were shown as hubs for an enriched list of differentially expressed immune-related genes (Ye et al. 2012). Several other porcine miRNAome studies are to be published soon focusing on the impact of miRNAs after bacterial or viral infections (Table 1).

Experimental design

In addition to different expression measurement techniques to examine pig immune responses, various experimental designs to study immunity have been used in these studies. First, there are in vitro versus in vivo studies. Whereas in vitro studies are performed outside the living organism, and thus can be more controlled, in vivo studies usually better reflect the underlying biology. Choices can be made to compare challenged and non-challenged or vaccinated versus non-vaccinated pigs, and whenever possible preferably littermates are chosen for such comparisons. To reduce genetic background variation even more, treated and untreated tissue within animal can be compared, as seen with the small intestinal segment perfusion (SISP) technique (Hulst et al. 2013). One can also choose to challenge all animals with a specific pathogen, and not using separate uninfected animals as controls, but contrast high and low responders, as was done in the *Salmonella* experiments (Uthe et al. 2009; Huang et al. 2011; Knetter et al. 2014). Less controlled, but perhaps more realistic, are studies performed after an on-farm outbreak, comparing healthy and diseased pigs or low and high responders (Serao et al. 2014).

Another key variable concerns the type of samples to be collected. Most pig immune studies conducted to identify host response to common porcine pathogens or to immune response stimulators such as LPS or PMA/ionomycin described in this review provide gene expression data from a single tissue or isolated cell type, and this at a limited number of times post-infection/stimulation. To study specific components of immune response, it is clear that dissection and analysis of primary or secondary immune tissue are required. In human and mouse studies, significant effort has been taken a step further—the analysis of the transcriptome of highly specific cell types. Such samples are isolated on the basis of cell surface marker expression. The parameters for cell selection and isolation are often complex, utilizing a multifactorial list of cell surface markers to identify a highly refined cell population

(Novershtern et al. 2011; Shay and Kang 2013). Reports on, and options for, specific cell subsets are limited in swine (Genini et al. 2008a; Kapetanovic et al. 2012) and are mostly due to the relative lack of immune-targeted reagents critical for such detailed cell phenotyping.

Examining the whole blood transcriptome has several advantages, including ease of collection and repeated sampling of the same individual during response to a stimulus, which is especially useful in controlling for baseline variation in the study of immune responses. Blood RNA profiling is advantageous in screening for biomarkers as well; it can be used to study variation in immune response and develop gene signatures predictive of inflammatory and/or disease status. An example is given by the PRRS host genome consortium (PHGC) project, where blood of 200 infected animals per trial is collected at day 0 and 8 different times post-infection (Rowland et al. 2012). However, since whole blood comprises a number of cell types, gene expression differences should be handled with great caution. With the aid of complete blood counts, the transcriptional response data can be deconvoluted to help identify the unique regulatory control of specific cellular responses to pathogens (Shen-Orr et al. 2010).

Overall value of transcriptomics in important infectious swine diseases

At the end of the day, the ultimate goal is to see how the results of all these individual transcriptomic studies fit into an improved understanding of porcine immune response. Recently, such a meta-analysis was performed by combining results of several microarray-based pig immune studies to find PRRS-specific responses (Badaoui et al. 2013). This meta-analysis successfully summarized the general pathway(s) believed to be induced by PRRSv. Several interferon regulatory factors (IRFs) were highlighted in this analysis, and interferons clearly play an important role in viral infections. In agreement with the digital gene expression experiment (Xiao et al. 2010a), in several microarray and qPCR experiments, a dampened expression type I IFN response can be seen, which indicates an inadequate stimulation of the innate anti-viral immune response (Genini et al. 2008a; Xiao et al. 2010a; Ait-Ali et al. 2011; Garcia-Nicolas et al. 2014). Genini and colleagues observed a strong elevation of *IFNβ* at 9hpi, but only a slightly elevated expression of *IFNα* (Genini et al. 2008a). Ait-Ali and colleagues noted a similar low *IFNα* expression, but an, albeit late, accumulation of *IFNβ* expression. They stated that PRRSv could delay type I interferon transcriptional response in an attempt to counteract the host's early immune response (Ait-Ali et al. 2011). Van Reeth and colleagues measured the IFNα levels in bronchoalveolar fluids during PRRSv infection and saw that its presence was low, a thousand-fold lower than with an infection with swine influenza virus or porcine respiratory coronavirus (Van Reeth et al. 1999). However, different PRRSv isolates were shown to invoke different (and sometimes significantly higher) *IFNα* expression levels, but no detectable IFNα protein levels were found by ELISA (Lee et al. 2004). Zhang and colleagues found that PRRSv does not fail to induce *IFNα* or *IFNβ* mRNA expression in monocyte-derived dendritic cells, but the protein seems to be blocked post-transcriptionally (Zhang et al. 2012). Although all described data point out to a weakened IFN response, greatly responsible for a persistent viral infection, the data by these last two studies demonstrate the incomplete information achieved from looking solely at transcriptomic data.

Another overall PRRS finding is the induction of pro-inflammatory chemokines and cytokines. The differential expression of a cell surface receptor involved in cytokine regulation, *TREM1*, was found through the meta-analyses by Badaoui et al. (2013) and was also present in the list of top ten upregulated transcripts in the RNA-seq experiment of Miller et al. (2012). In the meta-analysis study, TREM1 changed, among others, the expression of chemokines such as CCL2 and CCL3, interleukins IL6, IL18, and IL1β, and toll-like receptors TLR2 and TLR4. Xiao et al. (2010a) showed an upregulation in the inflammatory response toll-like receptor genes TLR2, TLR4, cytokines (among which IL1β), and chemokines. The acute-phase protein SAA2 and inflammasome genes *RETN*, *s100A8*, *s100A9*, and *s100A12* were upregulated in the RNA-seq experiment by Miller and colleagues.

For *S. typhimurium*, as mentioned earlier, transcriptomic studies in blood have examined differences between LS and PS pigs. In the in vivo study by Knetter and colleagues, when looking at cytokine presence in serum at 2 dpi, the pro-inflammatory cytokines IL1β, TNFα, and IFNγ levels were higher in PS pigs compared to LS and control pigs, and the anti-inflammatory IL10 was upregulated in both LS and PS pigs, while only CXCL8 was elevated in the LS animals (Knetter et al. 2014). Also, Uthe and colleagues saw a correlation between IFNγ levels and shedding status. It seems that the PS animals have a much more extreme inflammatory response, as if the PS animals respond less quickly and thus extend their inflammatory response (Uthe et al. 2009). Additionally, looking at gene expression differences at 2 dpi compared to 0 dpi, PS animals showed a more extensive transcriptomic response, both in number of differentially expressed genes, as well as in level of expression compared to the LS animals. The most over-represented regulation networks in PS animals at 2dpi involve the *STAT1*, *IFNβ1*, and *IFNγ* networks, showing a complex pro-inflammatory profile (Knetter et al. 2014). The genes *CASP1*, *TNFα*, and *IL10* were also found upregulated

in these networks, and hence, a nice correlation between serum cytokines and gene expression could be noted. Other important regulators *CEBPB*, *SPI1*, and *TLR4* in the PS upregulated expression pathways as well as the *TNFα* and *IFNγ* pathways were earlier reported by Huang and colleagues in similar challenge studies (Huang et al. 2011). Not surprisingly, the microbiota that differ between LS and PS on 2dpi point to microbiota that play a role in gastrointestinal inflammation (Bearson et al. 2013). There are, however, regional differences in the inflammatory response to *S. typhimurium* expression pattern in the gut on 2dpi (Collado-Romero et al. 2010). While cytokine genes such as *TNFα, IL6*, *IL1β*, and *IFNγ* are upregulated in the jejunum and colon, they are not induced in the ileum. Collado-Romero and co-workers proposed that the ileum mucosa reacts slowly against the pathogen. Martins and colleagues and Wang and colleagues both examined transcriptomic differences due to *S. typhimurium* in mesenteric lymph nodes. Martins and colleagues describe an elevation of *IFNγ*, *IL1β*, *CXCL2*, *CXCL8*, *CASP1*, *SLC11A*, *DEFB2*, *TLR8*, and *NOD2* at 2dpi. *NFκB* was significantly down-regulated at 2 and 6dpi (Martins et al. 2013a). Wang and colleagues also note an early repression of the *NFκB* pathway from 24 to 48 hpi in mesenteric lymph nodes (Wang et al. 2007). When looking at gene expression at only 2, 4, or 8hpi in jejunal scrapings, elevated gene expression was observed of inflammatory genes such as *IL8*, *IL1β*, *PAP*, *and S100A9* (Hulst et al. 2013). They also noted an upregulation of *NFKBIA*, an NFκB inhibitor at this early time point.

A study using in vitro stimulation with endotoxin of blood of animals prior to infection was also able to find cytokine differences between LS and PS pigs, showing an attenuated response in LS animals, in contrast to a clear pro-inflammatory response in PS pigs (Knetter 2013). Interestingly, while gene expression on day 0 showed a similar magnitude of response in LS and PS pigs, response differences to LPS in blood at 2 dpi between LS and PS pigs were dramatic. Only 14 probesets were differentially expressed in LS animals after endotoxin stimulation, while 959 probesets in PS animals changed significantly, showing an apparent tolerization mechanism in LS animals. Since differences in gene expression patterns between LS and PS animals on day 0 were not significant enough to create predictor sets of genes in this and earlier studies, Kommadath and co-workers used the more sensitive weighted gene co-expression network analysis (WGCNA) technique after RNA-seq profiling of 8 LS and 8 PS animals. WGCNA creates modules of co-expressed genes that are significantly correlated with shedding levels. They include interesting genes such as cytokine genes, genes involved in TLR, NFκB, and NOD-like receptor pathways and genes linked to bacterial infections (Kommadath et al. 2014).

Future directions: a clear need for standard bioinformatic annotation!

For over a decade, microarrays have provided an enormous amount of information concerning different immune-related questions in pig transcriptomics. They have become increasingly inexpensive tools to search for gene expression differences between distinct immune phenotypes. Recently, RNA-seq, and other sequence-based methods such as miRNA-seq, BS-seq, ChIP-seq, and MeDIP-seq, experiments have become more cost-effective. The advantage of gaining information about all expressed and modified genes, different regulation of splice variants, as well as information about sequence-specific and histone code regulation, is a big plus for RNA-seq over microarrays. However, in the near future, the biggest challenge lies in comparing all existing data from many different kinds of platforms, so as to integrate such orthogonal data and better understand the physiology behind the disease phenotype and to find regulatory networks or biomarkers for disease resistance. Such meta-analyses use a set of statistical techniques to combine results from different studies (Badaoui et al. 2013), requiring only that the platform elements can be matched. It is not necessary that the exact same questions were addressed; e.g., an experiment looking at high and low responders to an infection can be compared to a study with infected versus control animals. Or an acute response study can be matched to a chronic response study. Adding to this, the possibility to integrate new (and broader) information obtained from upcoming next generation sequencing studies would really improve our transcriptomic and epigenomic insights into pig immunity.

An example of combining microarray studies for deeper insight is given by Pérez-Montarelo and co-workers. A meta-analysis was performed on 20 independent gene expression studies, using data from 480 of the same Affymetrix array. By doing so, the expression of 12,320 genes could be checked in 27 tissues and they could identify tissue-specific genes and tissue-specific regulatory networks and transcription factors (Perez-Montarelo et al. 2012). The meta-analysis study by Badaoui and colleagues described above illustrates how a meta-analysis experiment could be achieved for a PRRS-specific research question even across different platforms. Disparate microarray elements that mapped to the same IPA cDNA assembly (Couture et al. 2009) were considered to be comparable. 30,504 such elements could be compared across all 779 datasets used, of which more than a third was investigating PRRS. To facilitate these kinds of meta-analyses, lessons can be learned from the human ENCODE project by conducting experiments in a similar way and processing and archiving data using standard procedures (The ENCODE Consortium Project 2011b; Birney 2012; Landt et al.

2012). In this way, data quality is assured, data utility can be extended, and it becomes easier to compare datasets, combine computational analyses, and consequently perform meta-analysis.

Besides cross-platform meta-analyses, also cross-species meta-analyses are very promising, and R packages are freely available to conduct them (Kuhn et al. 2008; Kristiansson et al. 2013). However, until now, the main goal of these cross-species expression comparisons is often to employ model organisms for human diagnostic or therapeutic research (Yu et al. 2012; Grigoryev et al. 2013). Kapetanovic and colleagues show how the clustering software BioLayout Express 3D (BE3D) can be used to visualize inter-species expression comparisons (Kapetanovic et al. 2013). When comparing differential gene expression in mouse, human, and pig macrophages after LPS stimulation, they noted that pig macrophages act more human-like than do mouse macrophages. In a first paper, this group used BE3D to identify and visualize sets of genes responding similarly over time to LPS. They found that a subset of these genes had similar patterns of induction with human macrophage response, but not with mouse, where much lower stimulation or even repression was observed (Kapetanovic et al. 2012). They extended this work in a later paper by using the larger Snowball array. Taking only the differential genes between mouse and human, and removing those that did not have pig orthologues on the Snowball array, BE3D showed a large upregulated cluster in human compared to mouse macrophages which contained *IDO1* as the hub gene. This cluster was highly upregulated in pig macrophages as well. Other genes such as those clustering around *NOS2A* also behaved in a murine-specific manner, with an upregulated expression in mouse macrophages while no differential expression in human or pig (Kapetanovic et al. 2013).

In addition to cross-platform and cross-species comparisons, it is important to investigate simultaneously different biological levels influencing the pig's immune status. In the past, disease research very often focused only on one part of the host–pathogen interaction (Smits and Schokker 2011). One examined a portion of the host response, and looked at its ability to fight off an infection and examining its degree of disease susceptibility. Or one focused solely on the pathogen, describing level of virulence among different pathogen variants. As well, very often, only a small part of the host immune response was measured, such as a specific cell type or tissue, or only one particular timeframe was targeted. Narrow time windows or even single stages can be quite limiting, as immune response varies dramatically over time, and thus, time is a particularly crucial variable in an expression study of immune responses. With a systems biology approach, many levels of knowledge are gathered on both host and pathogen in a challenge study (genomic, transcriptomic, epigenomic, and metabolomic) and at different time points (Smits and Schokker 2011; Tuggle et al. 2011). The ultimate goal is to combine that data to fully explain host-pathogen interactions and discover emergent properties of the system that are difficult to reveal with current approaches. To disseminate public data and improve transcriptomic and epigenomic data mining, a livestock expression and epigenetic database (EpiDB) is under development. EpiDB includes data from chickens, cattle, pigs, sheep, and horses and provides a useful repository source, as well as tools to process and visualize expression and epigenetic data (Koltes et al. 2014). Examples of systems biology approaches integrating transcriptomic and epigenomic data can already be found in many miRNA-seq experiments, where one miRNA can regulate a network of several mRNAs (Giles et al. 2013; Valdmanis et al. 2013; Szeto et al. 2014; Yang et al. 2014). A porcine mRNA-seq study often precedes a miRNA-seq experiment on the same sample set to test for correlations between mRNA and miRNA in order to predict influences of specific miRNAs on components of the whole transcriptome (Bao et al. unpublished; Endale Ahanda et al. 2012).

However, the accuracy and depth of understanding stand or fall with the quality of the pig draft genome assembly and its annotation. Recently, the Immunome Response Annotation Group (IRAG) was able to improve the characterization of the pig immunome by manual annotation of almost 3,500 transcripts mapping to over 1,400 genes (Dawson et al. 2013). This was accomplished using the latest swine genome assembly version 10.2. For the genes without porcine RNA sequence evidence, RNA sequences of other species (often of human) were used to annotate more than 1,100 transcripts using the alignment tools in Otterlace (Searle et al. 2004; Loveland et al. 2012). Furthermore, gene expression clustering after infection or stimulation for many independent challenge experiments provided evidence for the involvement of over 500 genes not previously annotated for function in immune response processes (Dawson et al. 2013). Ongoing improvements of the draft assembly and additional annotation of the immunome will greatly improve the value of pig disease transcriptomic studies as well as further support the pig as model for human immune response.

Since immune networks are very complex (Gardy et al. 2009), a deeper understanding of such complexity is needed for advancements in unraveling porcine disease response mechanisms and in developing the pig as a viable model for human immunity. It is encouraging that substantial new high-throughput data have been reported in this area, and that analysis of such data is moving toward a systems biology approach by integrating different methods and combining multiple datasets. With the even higher throughput whole genome techniques coming to the forefront and performed at a relatively low cost, these

comprehensive experiments will become more commonplace in the near future.

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