

Graduate School for Cellular and Biomedical Sciences

University of Bern

# **Influences of porcine circo-, arteriviruses and cathelicidins in plasmacytoid dendritic cell-derived interferon-alpha responses**

PhD Thesis submitted by

**Baumann Arnaud**

from **Freimettigen (BE)**

**Switzerland**

Thesis advisor

Prof. Artur Summerfield

Institute of Virology and Immunology

Vetsuisse Faculty of the University of Bern

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Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

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Knowledge is power! "M.H."

Arnaud

## 2. Abbreviations

AP-1: activator protein 1  
ATP: adenosine triphosphate  
BMDC: bone marrow-derived dendritic cell  
Cap: capsid protein  
CARD: caspase activation and recruitment domain  
CARDIF: CARD adaptor inducing IFN- $\beta$   
cDC: conventional dendritic cell  
ConA: Concanavalin A  
CPE: cytopathogenic effect  
CSFV: classical swine fever virus  
CTL: cytotoxic T lymphocyte  
DAI: DNA-dependent activator of IFN-regulatory factor  
DExD/H: aspartate-glutamate-any amino acid-aspartate/histidine  
ds: double-stranded  
EAV: equine arteritis virus  
eIF2: eukaryotic initiation factor 2  
Flt3-L: Fms-related tyrosine kinase 3 ligand  
FMDV: foot and mouth disease virus  
GAG: glycosaminoglycan  
GAS: IFN- $\gamma$ -activated site  
GDP: guanosine diphosphate  
GTP: guanosine triphosphate  
HDP: host defense peptide  
IFI16: IFN-inducible protein 16  
IFN: interferon  
IPS-1: IFN- $\beta$  promoter stimulator-1  
IRF: interferon regulatory factor  
ISG: IFN-stimulated gene  
ISG15: IFN-stimulated protein of 15 kDa  
ISGF3: ISG factor 3  
ISRE: IFN-stimulated response elements  
JAK: Janus activated kinase  
LDV: lactate dehydrogenase-elevating virus  
LGP-2: laboratory of genetics and physiology-2  
LPS: lipopolysaccharide  
LRR: leucine-rich repeats  
LV: Lelystad virus  
M $\Phi$ : macrophage  
MAPK: mitogen-activated protein kinase  
MDA5: melanoma differentiation-associated protein 5  
MDM: monocyte-derived macrophage  
MoDC: monocyte-derived dendritic cell  
Mx: myxovirus resistance  
MyD88: myeloid differentiation primary-response 88  
NETs: neutrophils extracellular traps  
NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells  
NIPC: natural IFN-producing cells  
NK: natural killer  
OAS: 2', 5'-oligoadenylate synthetase  
ORF: open reading frame  
Ori: origin of replication

PAM: porcine alveolar macrophages  
 PAMP: pathogen-associated molecular patterns  
 PBMC: peripheral blood mononuclear cell  
 PCV1: porcine circovirus type 1  
 PCV2: porcine circovirus type 2  
 PCVAD: porcine circovirus-associated disease  
 PCVD: porcine circovirus disease  
 pDC: plasmacytoid dendritic cell  
 PG: protegrin  
 PI3K: phosphatidylinositol 3-kinase  
 PKR: protein kinase R  
 PMWS: postweaning multisystemic wasting syndrome  
 PMA: phorbol 12-myristate 13-acetate  
 PMAP: porcine myeloid antimicrobial peptide  
 PMNC: polymorphonuclear cells  
 PPV: porcine parvovirus  
 PR-39: proline-arginine-rich 39-amino-acid peptide  
 PRR: pattern recognition receptor  
 PRRS: porcine reproductive and respiratory syndrome  
 PRRSV: porcine reproductive and respiratory syndrome virus  
 RCR: rolling-circle replication  
 RIG-I: retinoic acid-inducible gene I  
 RLR: RIG-I-like receptor  
 RNaseL: ribonuclease L  
 SHFV: simian hemorrhagic fever virus  
 RTC: replication and transcription complex  
 ss: single-stranded  
 STAT: signal transducer and activator of transcription  
 STING: stimulator of interferon genes  
 TGEV: transmissible gastroenteritis virus  
 T<sub>h</sub>1 cell: helper 1 T cell  
 T<sub>h</sub>2 cell: helper 2 T cell  
 TIR: Toll/IL-1R  
 TLR: toll-like receptor  
 TNF: tumor necrosis factor  
 TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$   
 TYK2: tyrosine kinase 2  
 2-5A: 2', 5'- oligoadenylates

### 3. Abstract

Type I interferons (IFNs), mainly IFN- $\alpha/\beta$  play a crucial role in innate defense against viruses. In addition to their direct antiviral activity, type I IFNs have antitumoral and immunomodulatory effects. Although all cells are virtually able to induce IFN- $\alpha$ , the plasmacytoid dendritic cell (pDC) subset represents the ultimate producers of IFN- $\alpha$  as well as other proinflammatory cytokines. Due to the specific expression of TLR7 and TLR9 recognizing single-stranded (ss) RNA and unmethylated CpG motifs respectively, pDCs can secrete up to 1000 times more IFN- $\alpha$  than any cellular types. Additionally, it is well known that several cytokines including type I and II IFNs, Flt3-L, IL-4 and GM-CSF favor pDC-derived IFN- $\alpha$  responses to unmethylated CpG motifs. In a first step, we aimed to characterize and clarify the interactions of two porcine viruses with pDCs. The double-stranded DNA replicative forms of porcine circovirus type 2 (PCV2) were demonstrated to inhibit CpG-induced IFN- $\alpha$  by pDCs. Our study showed that none of the cytokines known to enhance pDC responsiveness can counter-regulate the PCV2-mediated inhibition of IFN- $\alpha$  induced by CpG, albeit IFN- $\gamma$  significantly reduced the level of inhibition. Interestingly, the presence of IFN- $\gamma$  enabled pDCs to induce IFN- $\alpha$  to low doses of PCV2. We also noted that after DNase treatment, PCV2 preparations were still able to stimulate pDCs. These data suggest that encapsulated viral ssDNA promotes the induction of IFN- $\alpha$  in pDCs treated with IFN- $\gamma$  whereas free DNA, presumably as double-stranded forms, was responsible for inhibiting pDC responses. Regarding PRRSV, it has been reported that North American isolates did not induce and even inhibited IFN- $\alpha$  response in pDCs. However, PRRSV infection was also shown to lead to an induction of IFN- $\alpha$  in the serum and in the lungs suggesting that certain cells are responsive to the virus. Contrasting to previous reports we found that numerous PRRSV isolates directly induced IFN- $\alpha$  in pDCs. This response was still observed after UV-inactivation of viruses and required TLR7 signaling. The inhibition of CpG-induced IFN- $\alpha$  was weak and strain dependent, again contrasting with a previous report. We also observed that IFN- $\gamma$  and IL-4 enhanced IFN- $\alpha$  response to two prototype strains, VR-2332 and LVP23. In summary, we demonstrated that both PCV2 and PRRSV promote IFN- $\alpha$  secretion in pDCs *in vitro* suggesting that IFN- $\alpha$  detected in PCV2- or PRRSV-infected animal might originate from pDCs. On the other hand, PRRSV replication is restricted to the macrophage (M $\Phi$ ) lineage. These innate immune cells represent a heterogeneous population which can be induced to “classical” (M1) and “alternative” (M2) activated M $\Phi$  acquiring inflammatory or “wound-healing” functional properties, respectively. Nonetheless, little is known about the effect of polarization into M1 or M2 and the susceptibility of these cells to PRRSV. Thus, we examined the impact of cytokine on M $\Phi$  polarization into M1 or M2. Infections of these cells by several PRRSV isolates enabled the discrimination of PRRSV isolate in a genotype- and virulence-dependent manner in M1 and IFN- $\beta$ -activated M $\Phi$ . In contrast, the expression of PRRSV nucleocapsid in M2 or inactivated M $\Phi$  was indistinguishable among the PRRSV isolates tested. In the last part of my Thesis, we investigated the influence of three synthetic porcine cathelicidin peptides for their ability to deliver nucleic acid to pDCs. We reported that all cathelicidins tested can complex and quickly deliver nucleic acids resulting in IFN- $\alpha$  induction. Moreover, we show that the typical  $\alpha$ -helical amphipathic conformation is required to mediate killing of bacteria but not for inducing IFN- $\alpha$  secretion by pDCs. Furthermore, we found that *E.coli* treated with one of these cathelicidins is able to induce significantly higher levels of IFN- $\alpha$  compared to a non-sense version of the peptide. These data suggest that cathelicidins could influence the immune response in a two-step process. First, these peptides target bacteria leading to cell lysis. In turn, cathelicidins form complexes and deliver extracellular microbial nucleic acids released into pDCs. These pDC-derived IFN- $\alpha$  responses could be of particular relevance in driving the adaptive immune responses against microbial infections.

## 4. Introduction

### 4.1. Overview of the IFN system

Defense mechanisms of vertebrates can be roughly divided into innate and adaptive immunity. Innate immunity represents the first line of host defense and is characterized by well-conserved mechanisms of pathogen recognition leading to a rapid inflammation and antiviral state induction. In contrast, adaptive immunity includes slow processes, which involves highly specific B and T cell-mediated immune responses. The innate immune reactions upon pathogen infection are dedicated to slow down viral and bacterial spreading in the host and prime cells from the adaptive immunity to mount an efficient immune response. Such responses are triggered by the recognition of pathogens through pattern recognition receptors (PRRs) in host cells. The repertoire of PRR is limited since they are expressed by a series of germline-encoded genes, which is in contrast to the high specificity of recognition of acquired immunity receptors, involving complex processes of gene rearrangements. The fact that PRR do not require a wide spectrum of specificity is explained by recognition of common structures and components of pathogens termed pathogen-associated molecular patterns (PAMPs) which are often primordial for pathogen survival. One important feature of the immune system is to discriminate between self and non-self in order to combat potent harmful pathogens and at the same time avoid damages to the host. The innate immune system has evolved in a way that PRRs recognize structural component of pathogens. For nucleic acids, their localization in specific cellular compartments has a major impact on their stimulatory activity. The recognition of PAMPs induces signaling cascades, which will lead to the expression of cytokines and other innate mediators. Accordingly, interferons (IFNs) are critical cytokines, which are produced by cells exposed to viruses or bacteria. In the host cell, the IFNs are essentially induced by the recognition of microbial nucleic acid, even though other PAMPs can be involved but in to a lesser extent. The IFN family is crucial for a direct antiviral activity and share also antitumoral and immunomodulatory functions. Although all cell types can induce IFNs, the plasmacytoid dendritic cells (pDCs) represents the ultimate cell subset to produce type I IFNs and other proinflammatory cytokines. The pDCs may act as sentinels of the innate immune system and alert non- and immune cells in case of viral infections.

#### 4.1.1. IFN family

After more than 50 years of the discovery of IFNs defined by their capacity to “interfere” with viral replication [1], intensive research in the field described these cytokines as a large family comprising 3 major groups of cytokines referred as type I, II and III, type I known for being the largest with many subtypes within the group [2].

##### Type I IFN:

The type I IFNs are the most well-characterized type and demonstrate antiviral and antitumoral activity as well as several immunomodulatory functions [3, 4]. They encompass several members including IFN- $\alpha$ , - $\beta$ , - $\omega$ , - $\epsilon$  and - $\kappa$ . Other IFN species such IFN- $\delta$  and - $\tau$  have been found in pig and sheep respectively [5, 6]. In mice, IFN- $\zeta$  (referred as limitin) has also been noted [7]. The most dominant type is represented by IFN- $\alpha$ , which comprises many subtypes, whereas only one has been reported for IFN- $\beta$  in human and pig [2, 8]. All these members are induced by the recognition of PAMPs through PRRs including Toll-like receptors (TLRs), cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytosolic DNA sensors. The secreted type I IFNs can act both locally and systemically and signal through a heterodimeric IFNAR1 and IFNAR2 receptor in resting

cells. This will lead to the expression of several hundred IFN-stimulated genes (ISGs), which will participate in fighting against invading pathogens especially viruses and will modulate the adaptive immune responses [9, 10].

#### Type II IFN:

Type II IFNs comprise only IFN- $\gamma$  [11]. In contrast to type I IFNs, IFN- $\gamma$  is not produced by virus-infected cells but rather by activated natural killer (NK), helper 1 T ( $T_H1$ ) and cytotoxic T cells [12]. In addition to its antiviral activity, type II IFN contributes to the induction of CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) and mount efficient responses against tumors [13]. This cytokine is also important to activate macrophages (M $\Phi$ ) and mediate intracellular killing of pathogens [14]. Like for type I IFNs, IFN- $\gamma$  is recognized by a receptor complex containing a pair of two subunits of IFNGR1 and IFNGR2 [15].

#### Type III IFN:

Recently, a type III IFNs has been described comprising IFN- $\lambda$ . This type III shows antiviral activity stimulating the expression of ISGs as the other type I IFN species do. Whilst IFN- $\lambda$  interacts with a unique heterodimeric receptor complex composed of an IFN- $\lambda$ R1 chain and IL-10R2 chain, it activates the identical signal transduction pathway than type I IFN [16].

#### 4.1.2. Induction of type I IFN by nucleic acids

The induction of type I IFNs, especially IFN- $\alpha/\beta$ , has been well investigated in response to viruses. Since the discovery of TLRs, a battery of PRRs including RLRs and cytosolic sensors have been shown to bind to different nucleic acid molecules. When a cell is infected by viruses or bacteria, the foreign nucleic acids either in a single-stranded (ss) or double-stranded (ds) form of DNA or RNA can be exposed to the PRRs for the recognition by the host cells. Here, I will focus on PRRs recognizing nucleic acids because these molecules might be the most important PAMPs during viral infections.

The family of TLRs includes several transmembrane proteins (10 in human) which share common domains including leucine-rich repeats (LRR) in the extracellular moiety and a Toll/IL-1R (TIR) domain in their cytoplasmic tail. The LRR ectodomain forms a horseshoe shape and contains two copies of 19-25 repeats of 24 to 29 amino acid sequences with typical leucine motifs [17]. Despite the fact that the LRR domains among TLRs are quite well conserved, the recognition of pathogens components is broad and diverse. Several types of nucleic acids including dsRNA (TLR3), ssRNA (TLR7 and TLR8) and DNA with unmethylated CpG motifs (TLR9) can be recognized [18-21]. These TLRs decorate endosomal compartments to avoid recognition of self nucleic acid molecules, which locate elsewhere in the host cells, such as in the nucleus or cytoplasm. Additionally, the other TLRs are expressed on the cell surface and interact with lipoproteins (heterodimers of TLR2 and TLR1 or -6), lipopolysaccharide (LPS; TLR4) and flagellin (TLR5) [22]. Upon binding to PAMPs, the TIR domain of TLRs dimerizes causing a special alteration. This conformational change of the cytoplasmic region leads to the recruitment of TIR-domain-containing adapter proteins. Except for TLR3, all TLRs seem to be dependent on the myeloid differentiation primary-response 88 (MyD88) adaptor protein. For TLR3-activation, another adapter protein referred as TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) is involved to trigger the signaling cascade. Thus, two pathways for type I IFN induction have been described based on the differential expression of TLRs and adapter proteins [23]. MyD88-dependent pathway involved a phosphorylation cascade leading to the activation and translocation of interferon regulatory factors (IRF) 7 and IRF5, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1) into the nucleus. Whilst IRFs

bind to the promoter of *IFNA* genes or ISGs, the transcription factors NF- $\kappa$ B and AP-1 mainly activate the transcription of proinflammatory cytokines such as IL-6 or tumor necrosis factor (TNF)- $\alpha$ . In some extent, NF- $\kappa$ B binds also to the promoter of *IFNB1* gene, leading to the expression of IFN- $\beta$  [24]. The TRIF-dependent pathway recruit different protein complexes than MyD88 adapter protein, however the signaling cascades converge partly to the translocation of IRF3 and NF- $\kappa$ B into the nucleus resulting in the initiation of *IFNB1* and proinflammatory cytokine transcription respectively. Additionally, TLR4 triggering can induce TRIF-dependent signaling but the resulting production of IFN- $\beta$  is induced by LPS instead of nucleic acids. The MyD88-dependent pathway is mainly associated to pDCs whereas TRIF-dependent pathway is characteristic of conventional dendritic cells (cDC) and M $\Phi$  [25-27].

While TLR3 is found to recognize poly(I:C) mimicking dsRNA molecules, transcriptional levels of IFN- $\alpha/\beta$  were not completely abolished in M $\Phi$  from TLR3<sup>-/-</sup> mice [18] indicating that other receptors must be involved. Further investigation by screening cDNA library demonstrated that RIG-I was responsible for sensing dsRNA in the cytosol in TLR3-independent manner [28]. This finding conducted to the discovery of other members of RLR family such as melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology-2 (LGP-2) showing homology in their amino acid sequence [29]. The RLR members possess an aspartate-glutamate-any amino acid-aspartate/histidine (DEXD/H)-box region common to RNA helicases and a regulatory domain in the C-terminal part. Additionally, RIG-I and MDA5 comprise two caspase activation and recruitment domains (CARD) in the N-terminal which enables the interaction with the adapter protein IFN- $\beta$  promoter stimulator-1 (IPS-1) also known as CARD adaptor inducing IFN- $\beta$  (CARDIF) important for downstream signaling [30, 31]. The final signaling events include the recruitment of IRF3 and NF- $\kappa$ B into the nucleus to activate the transcription of ISGs. While RIG-I and MDA5 have been found to recognize 5'-triphosphate replicative form of RNA viruses and long dsRNA respectively, it is not clear which motifs are essential for RLR binding to allow discrimination between self-RNA molecules [32]. Further investigation must be conducted to clarify the exact viral nucleic acid structure that is crucial for RLR recognition.

Since the past few years, new classes of receptor regrouping diverse cytosolic DNA sensors have been identified. The first member, the DNA-dependent activator of IFN-regulatory factor (DAI) was found to bind to dsDNA in the cytosol and induce a signaling cascade, which ends with the translocation of IRF3 into the nucleus to express type I IFN genes [33]. The IFN-inducible protein 16 (IFI16) was also demonstrated to bind to dsDNA and induce type I IFNs [34]. The expression of type I IFNs through DAI or IFI16 depends on the adapter protein referred as stimulator of interferon genes (STING) which is associated with reticulum endoplasmic [35]. While IFI16 signalization depends on IRF3, DAI recruits both IRF3 and NF- $\kappa$ B to induce the expression of type I IFN and proinflammatory cytokines respectively [36].

More recently, cytosolic DNA sensors belonging to the DEXD/H-box RNA helicase family have been identified in pDCs. They are referred as DHX36 and DHX9 and bind to type-A and type-B CpG respectively. Both of them interact with MyD88 but only DHX36-driven signaling promote IRF7 translocation and expression of type I IFNs. In contrast, DHX9 induces proinflammatory cytokines such as TNF- $\alpha$  by the activation of NF- $\kappa$ B [37].

It becomes clear that the type I IFN induction is finely regulated and depends on the cell types which differentially express certain PRRs. Ongoing research on network signaling improves our understanding of the type I IFN induction by nucleic acids and will clarify the exact role of individual PRR in the induction of innate immune reactions against self and non-self components [25, 26].

#### 4.1.3. Signaling pathways of type I IFN

Type I IFNs bind to a single heterodimeric receptor comprising two subunits IFNAR1 and IFNAR2 expressed by all cell lineages. Each subunit is associated with members of Janus activated kinase (JAK) family including tyrosine kinase 2 (TYK2) for IFNAR1 and JAK1 for IFNAR2. Upon binding to type I IFNs, dimerization of the receptor occurs and induces an auto-phosphorylation of the associated JAKs [38, 39].

So far, several pathways have been defined for the downstream signaling events but one referred as the “classical pathway” has been intensively characterized and involved signal transducer and activator of transcription (STAT) proteins. The activation of JAKs TYK2 and JAK1 lead to the phosphorylation and activation of STATs including STAT1, STAT2, STAT3 and STAT5. In turn, homo- or heterodimers of STATs are formed and translocate into the nucleus where they bind to the promoters of ISGs [40]. The STAT1 and STAT2 heterodimers can be also associated to IRF9 to constitute ISG factor 3 (ISGF3) complex. The complex ISGF3 acts as transcription factor and binds to specific DNA sequence of IFN-stimulated response elements (ISRE) located in some of promoters of ISGs. In addition to ISRE, other specific regions known as IFN- $\gamma$ -activated site (GAS) can be recognized by several other STAT homo- or heterodimers promoting the transcription of another class of ISGs. Stated another way, the type I IFNs signal through a single IFNRA receptor leading to phosphorylation of STAT members by tyrosine kinases resulting in the expression of ISGs.

It becomes clear that many activation combinations of STATs are induced according to the IFN subtype, leading to the specific expression of only certain ISGs. Additionally, others adapter proteins have been found to be activated by type I IFNs. These protein are part to the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling cascades [41].

#### 4.1.4. Effector functions

The effects mediated by type I IFNs are characterized by antiviral, antitumoral and immunomodulatory activities including NK and T<sub>H</sub>1 cell activation [42], antigen cross-presentation by dendritic cells [43], as well as antibody production and isotype class switching in B cell [44]. On another hand, the cells exposed to type I IFNs possess an array of ISGs. These genes encodes for proteins, which operate as direct antiviral effectors. This includes the GTPase myxovirus resistance (Mx) proteins, the 2', 5'-oligoadenylate synthetase (OAS)/ribonuclease L (RNaseL), the protein kinase R (PKR) and the IFN-stimulated protein of 15 kDa (ISG15) [9]. In this respect, many other proteins may contribute to the antiviral effects but their exact role is not clearly defined.

The Mx proteins are the most characterized family of IFN-induced GTPases out of four. In human, only two members composed this class, MxA and MxB [45]. It was demonstrated that MxA confers resistance to several viruses [46, 47]. This protein has been shown to self-assemble and might interact with nucleocapsid-like viral proteins, trapping viral component to prevent replication [48]. Importantly, the MxA protein locates in the endoplasmic reticulum suggesting that the traffic between cellular compartments might be controlled and screened for viral components [49]. Thereby, early virus replication can be affected by the Mx proteins.

The OAS proteins comprise a class of enzymes that are able synthesize 2', 5'- oligoadenylates (2-5A) from adenosine triphosphate (ATP). Originally, 2-5A has been found to inhibit protein synthesis in a cell-free system [50]. This mechanism is now confirmed by the activation of RNaseL through 2-5A. Upon binding to 2-5A, RNaseL undergoes conformational changes [51], which leads to its activation and to the degradation of ssRNA molecules [52]. Moreover, OAS needs to recognize dsRNA to further catalyze ATP to 2-5A in the cytosol. With this duality, OAS protein can be considered as a PRR as

well as an effector protein. Consequently, OAS/RNaseL pathway represents a unique antiviral late phase system enabling the degradation of viral RNA in the infected host cells.

PKR is a serine threonine kinase, which recognizes dsRNA and participates in many cellular processes. A first role was discovered in a cell-free system in which the translation was inhibited by this protein [53], a mechanism which have been so far clarified. When the dsRNA ligand binds to PKR, it autophosphorylates and following dimerization, activates the alpha subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ) changing its conformation. The protein participates in the translation initiation with 40S ribosome and depends on guanosine triphosphate (GTP). Recycling of eIF2 $\alpha$  requires eIF2B, which catalyzes eIF2 $\alpha$ -guanosine diphosphate (GDP) to eIF2 $\alpha$ -GTP. When eIF2 $\alpha$  is phosphorylated by PKR, its higher affinity for eIF2B leads to sequestration, blocking the synthesis of eIF2 $\alpha$ -GTP [54]. Consequently, PKR activation leads to the inhibition of the viral RNA translation. In addition, PKR also participates to apoptosis and activation of transcription factors such as NF- $\kappa$ B [55]. The ISG15 shares some homology with the ubiquitin protein, which is important in many cellular processes. In addition, ISG15 was found to be conjugated to several cellular proteins after exposure to type I IFNs [56]. The conjugation of ISG15 to certain proteins is termed as ISGylation and require a set of enzymes which is analogous to the ubiquitin modification system. It has been shown that 158 potential targets can be associate to ISGylation [57]. Thus, ISG15 enables to control cellular processes by a similar mechanism which is observed during the ubiquitination [58].

#### 4.1.5. Plasmacytoid dendritic cell subset

Although all cell types can theoretically produce type I IFNs, the characterization of pDC subset demonstrated that these cells, also referred as natural IFN-producing cells (NIPC) are able to secrete 200 to 1000 times more type I IFN than any other cells [59]. The remarkable capacity of pDCs to promptly respond to viruses is mainly due to the expression of TLR7 and 9 in the endosomes, which interact with ssRNA and unmethylated CpG motifs respectively [60]. Moreover, a unique pathway involving a constitutive expression of IRF7 is responsible for the rapid transcription and of type I IFNs. This pathway allows pDCs to respond efficiently and very rapidly to viruses, a mechanism which is in contrast to type I IFN induction in cDCs and M $\Phi$  where upon TLR3 activation leading to IRF3 translocation, only limited quantities of type I IFN are secreted. Another striking difference compared to the biological activity of cDC is the low phagocytosis levels and antigen presentation capacity of pDCs making them less efficient to activate T cell proliferation. Several routes of entry can be used by viruses including phagocytosis, macropinocytosis caveolae- or clathrin-dependent endocytosis [23, 61]. During the maturation of endosomes, low pH is required to expose nucleic acid to the compartments that express TLR7/9. Inhibitors of endosomal acidification such as chloroquine or bafilomycin have been found to abolish the immunostimulatory properties of CpG [62]. Investigation on pDC ontogeny showed that this subset arise from the hematopoietic lineage. The development of pDCs is mainly driven by the cytokine Fms-related tyrosine kinase 3 ligand (Flt3-L) [63]. Furthermore, due to a high expression of IL-3R, the survival of pDCs can be increased by IL-3 treatment [64]. Throughout life, pDCs originate from hematopoietic cells and migrate from the blood to lymphoid organs fulfilling their role of sentinels [61].

Porcine pDCs were identified as a population of non-adherent, non-T, non-B, CD4<sup>+</sup> and MHCII<sup>+</sup> cells and by their ability to respond to transmissible gastroenteritis virus (TGEV) secreting massive amounts of IFN- $\alpha$  [65]. Further investigations by immunohistochemistry in organs from TGEV-infected pig revealed the presence of pDCs in lymphoid tissues indicating that these cells are able to migrate in lymph nodes [66, 67]. Few years later, this subset was phenotypically characterized as CD172a<sup>low</sup>CD4<sup>high</sup>CD14<sup>+</sup>CD163<sup>+</sup> in peripheral blood mononuclear cells (PBMCs) with a frequency of

0.1-0.5% [68]. In addition to TGEV and type-A CpG [69], numerous porcine viruses including influenza virus [70], foot and mouth disease virus (FMDV) [71, 72] and classical swine fever virus (CSFV) [73] can be sensed and differentially promote IFN- $\alpha$  induction in pDCs *in vitro*. This function of viral sensing is in part influenced by the cytokine microenvironment. It has been noticed that type I and II IFNs, Flt3-L and IL-4 enhance the IFN- $\alpha$  levels produced by pDCs after CpG and FMDV, while IL-10 reduces this response [74].

#### 4.2. Brief history of porcine circovirus-associated diseases

In 1991, a first outbreak of porcine wasting disease was observed in Canada and multiple tests of diagnosis failed to enable the identification of known pathogens. In 1996, Harding and Clark described an emerging disease characterized by wasting and dyspnea referred to postweaning multisystemic wasting syndrome (PMWS) [75]. Porcine circovirus type 2 (PCV2) was isolated from pigs with PMWS in 1998 [76, 77] and referred as Stoon 1010 isolate (isolate of Saskatoon). Interestingly, a different type of non-pathogenic circovirus, namely the porcine circovirus type 1 (PCV1), was previously reported to infect the PK-15 cell line [78], which prompted the identification of PCV2 as one of the causes in the development of PMWS [79]. The two circoviruses were genetically related but less than 80% nucleotide sequence identity were found between several type 2 and the non-pathogenic type 1 of circovirus [80]. Reproduction of PMWS was achieved in gnotobiotic pigs inoculated with homogenous material from PMWS-suffering animals. Nevertheless, porcine parvovirus (PPV) was also reisolated during this study [81]. It was postulated that PCV2 was required for the development of PMWS, however, other unidentified factors such as PPV were also thought to contribute to the development of characteristic lesions and other clinical symptoms. Few years later, a report by Krakowka and colleagues demonstrated that PMWS can be caused by the inoculation of PCV2 implemented by non-infectious stimulation of the host immune system [82]. Reflecting the fact that many infected animals are asymptomatic, it is now accepted that PCV2 itself is not sufficient to induce the disease, but that concurrent pathogens or cofactors lead to the appearance of PMWS [83]. Currently, PMWS has been enclosed with many other syndromes disease termed as porcine circovirus diseases (PCVD) in Europe [84] or porcine circovirus-associated disease (PCVAD) in North America [85] and PCV2 is ubiquitously distributed in the pig population worldwide. How PCV2 evolved and its origin are not well defined but few evidences by comparing the nucleotide sequences of several ssDNA viruses indicate some similarities with plant nanoviruses and geminiviruses [86]. It was hypothesized that PCV2 was transfer to vertebrates from plant infected sap [87]. The scientific community agreed on a nomenclature defining three genotypes of PCV2, termed as PCV2a (Stoon 1010, GenBank accession number AF055392), PCV2b (48285, accession number AF055394) and PCV2c (GenBank accession number EU148503) [88]. It has been proposed that circovirus isolates related to PCV2b genotype were more associated to PCVD outbreaks than PCV2a genotype, albeit it has not been confirmed yet [89]. In Switzerland, both PCV2a and PCV2b have been isolated in swine populations [90]. Since the past 10 years, several vaccines have been developed and are available on the market for the pig industry. Although they confer protection and reduce the impact of PCVD in swine industry [91], the virus is now circulating in the vaccinated pig population.

##### 4.2.1. Impact and clinical signs of PCVD

PCVD encompasses a variety of syndromes, which include PMWS, the most characterized of circovirus-associated diseases. Actually, it is assumed that most of PCV2 infections lead to subclinical manifestation only. Nonetheless, with a worldwide distribution, PCVD morbidity and mortality varies

between 4 to 30% and 4 to 20% depending on the farms, respectively. A new nomenclature has been proposed to term the syndromes based on accurate and defined symptoms observed in PCVD-suffering pig [92]. PMWS is considered as a multifactorial disease where infections of PCV2 is necessary, but experimental infection hardly reproduces the disease described in the fields [93-95]. The main clinical features of pigs suffering from PMWS include wasting, growth retardation, dyspnea and enlargement of subcutaneous lymph nodes. Reports from PMWS-affected farms also described that other symptoms such jaundice, pallor of the skin, diarrhea can be associated [75, 96]. Further investigations using *in situ* hybridization and immunohistochemistry in PMWS-affected pig revealed macro- and microscopic lesions characterized by a loss of architecture in lymph nodes, lymphoid depletion and histiocytic infiltrations. Furthermore, PCV2 nucleic acids and antigens are strongly associated with lymphoid organs [97, 98]. Since the symptoms characterizing PMWS are not specific for PCV2 infections, the presence of PCV2 antigens and DNA as well as the typical lesions in lymphoid organs are required for an appropriate diagnosis of the disease. These findings indicate that PCV2 has a tropism for the cells of the lymphoid system and accordingly, it has been proposed that the virus causes immunosuppression by a not well understood mechanism [91, 99-101]. Many research groups attempted to reproduce with moderate success the clinical form of PMWS using several model of coinfections with bacterial pathogens [102-104] or viruses [81, 94, 105, 106]. Even though many extensive efforts have been carried out to explain the immunological basis of PCV2, it is not clearly understood why certain animals will develop PCVD and others not. Since the past few years, successful vaccinations have drastically reduced the impact of PCVD and little attention is currently paid to the immunobiology of PCV2.

#### 4.2.2. Biology of PCV2

##### Genome organization:

PCV2 belongs to the Circoviridae family. The non-encapsulated ssDNA genome of the prototype PCV2 Stoon strain is 1768 nucleotides long [80]. Therein, circoviruses represent one of the smallest viral genomes autonomously replicating in mammalian cells. The icosahedral virions form a 17-25 nm diameter size particle. PCV2 genome is composed of 3 major open reading frames (ORFs) which encode for 4 proteins, although 6 additional viral transcripts (including 3 *rep*-associated RNAs: *Rep3a*, *Rep3b* and *Rep3c* and 3 minor *NS*-associated RNAs: *NS515*, *NS672* and *NS0*) have been detected from PCV2-infected cells [107]. The ORF1 is located on the genomic strand leading to the expression of 2 viral RNA spliced transcripts, termed as *rep* and *rep'* which are translated into Rep and Rep' proteins, both involved in viral replication [108]. ORF2 and ORF3 are found on the complementary strand of PCV2 genome. ORF2 encodes the capsid protein (Cap), which represents the only structural protein. It has been shown that recombinant Cap self-assembles to form virus-like particles of similar size as PCV2 virion [109], which can be employed as vaccines [110]. ORF3 encodes a small protein, which is involved in virus-induced apoptosis in PK-15A cell line [111]. The role of the 6 viral RNA transcripts is not known and their expression into protein has never been shown suggesting that they may contribute to facilitate PCV2 replication [112]. The origin (*ori*) of viral replication has been mapped for PCV1 genome only [113]. The intergenic region between the 5'-ends of *rep* and *cap* contains a potential stem-loop structure shared by other nanoviruses and geminiviruses [86], which is one of the feature of viruses replicating through rolling-circle replication (RCR) [114].

##### Target cells:

*In vivo*, PCV2 DNA and antigens have been associated with numerous cell types of the monocytic lineage using *in situ* hybridization and immunohistochemical approaches [97, 98, 115]. Due to the

observed depletion of lymphocytes in PCVD-affected pigs, the ability to PCV2 to replicate in these cells has been examined and the data are controversial. In the study of Gilpin and colleagues, PCV2 did not infect resting lymphocytes *in vitro* according to flow cytometry analysis [116]. Conversely, Yu *et al.* detected *cap* mRNA and PCV2 DNA in both T and B lymphocytes either from PBMC or bronchial lymph nodes from PCV2-infected animals [117]. In another study, PCV2 antigens have been observed in certain subpopulations of CD4<sup>+</sup>, CD8<sup>+</sup> and IgM<sup>+</sup> cells [118]. These cells were extracted from the inguinal lymph node of infected pigs treated with Concanavalin A (ConA), which induces lymphocyte proliferation. More recently, increasing copy number of PCV2 genome has been shown in cocultures of PCV2-free monocyte-derived dendritic cells (MoDC) and ConA-stimulated PCV2-inoculated lymphocytes. In contrast, no increase in PCV2 copy numbers was found in PCV2-inoculated MoDC and ConA-stimulated lymphocytes cocultures [119]. Although these data confirmed that PCV2 can replicate *in vitro* in mitogen-activated lymphocytes, it cannot be confirmed that lymphocytes represent the main target cells for PCV2 replication. Interestingly, PCV2 quantification has also been performed in fetuses after intra-foetal inoculation of sows resulting in a high viral titer and significant high number of PCV2 positive cells in the heart and liver of infected fetuses [120]. Furthermore, MΦ, cardiomyocytes and hepatocytes were also infected in foetal stages whereas only MΦ were susceptible after post-natal infection suggesting that PCV2 changes its tropism during pig development [121]. From *in vitro* studies, it has been shown that multiple cell types can be infected by PCV2. Infection was observed in PK-15 cell line [77], porcine alveolar macrophages (PAM), fetal cardiomyocytes [122], porcine monocytic 3D4/31 cell line [123], endothelial PEDSV.15 cell line, primary porcine aortic endothelial cells, gut epithelial cells, MoDC and fibrocytes [124]. In summary, PCV2 is strongly associated with lymphoid tissues and monocytic cells but is not restricted to these subsets. The primary *in vivo* target cell type for PCV2 replication has not been identified yet.

#### Replication:

PCV2 was found to bind to heparan sulfate and chondroitin sulfate B glycosaminoglycans (GAGs) and was internalized slowly in a clathrin-dependent process in the porcine monocytic 3D4/31 cell line [123, 125]. Following internalization, PCV2 may escape the endosome during pH acidification process since chloroquine inhibits PCV2 internalization [123]. However, PCV2 internalization seems to occur in a different way in the PK-15 epithelial cell line because inhibition of pH acidification enhances PCV2 replication [126]. Once PCV2 capsid is disassembled by host proteases, PCV2 DNA must enter the nucleus to have access to host replication machinery [127]. The stem-loop structure located in the *ori* and the amino acid sequence in Rep protein form motifs which suggest that circoviruses replicate through RCR [114]. This mechanism of replication have been described for other phylogenetically related viruses such as geminiviruses [128] and nanoviruses [129]. Basically, RCR includes 4 steps: conversion of ss- to dsDNA replicative forms, initiation of replication, elongation and termination [112]. The first step of replication consists in synthesizing the complementary strand by the host enzymes. Accordingly, it was suggested that PCV2 could only replicate in dividing cells, which are in the S phase of the cellular cycle. This idea is supported by the fact that only ConA-stimulated lymphocytes could support PCV2 replication [118, 119] and not resting lymphocytes [116]. The replication of PCV2 requires the formation Rep-Rep' complex to synthesize viral DNA. Progeny of virus can be observed after 20 h post-infection [130] but this does not cause typical cytopathogenic effect (CPE) in PK-15A. The mechanism of export used by PCV2 to reach the extracellular space has not been investigated yet. Interestingly, both IFN- $\alpha$  and IFN- $\gamma$  can enhance yield of PCV2 progeny in PK-15A [131, 132]. This observation can be attributed to the presence of an ISRE-like sequence in the PCV2 genome [133].

#### 4.2.3. Type I IFN response to PCV2

The fact that PCVD is often associated with other viral or bacterial pathogen infections, or that an activation of the immune system is required to trigger the disease suggested that PCV2 could modulate the host immune response [91, 99-101]. Although PCV2 is associated with cells of the monocytic lineage, the virus does not efficiently replicate in these cells but rather accumulates insight [116]. Moreover, PCV2 amasses in MoDC or BMDC without showing any signs of replication or disturbing the capacity of these cells to present antigens [134, 135]. An early report showed that PCV2 alone can induce IFN- $\alpha$  in PAM *in vitro* [136]. In contrast, it has been shown that PCV2 did not induce IFN- $\alpha$  or even impaired CpG-induced IFN- $\alpha$  response in CD172a-enriched cells [135]. A closer examination of the interactions between PCV2 and pDCs revealed that the inhibition is mediated by PCV2 DNA. Moreover, the IFN- $\alpha$  inhibition is also affected when other ligand such as TGEV or the TLR7 agonist R837 are employed [137]. Further analysis of PCV2 DNA content in viral preparations showed that only free dsDNA forms of PCV2 have the capacity to interfere with IFN- $\alpha$  secretion [138]. This observation could reflect the fact that large quantities of PCV2 DNA replicative forms are found in lymphoid tissues of PMWS-suffering pigs [139], thus modulating the immune response in these animals. Other reports demonstrated that five CpG motifs derived from PCV2 genome have different stimulatory properties in PBMCs. Four of these sequences are stimulatory, whereas the remaining CpG motif inhibits *in vitro* secretion of IFN- $\alpha$  in PBMCs [140]. The IFN- $\alpha$  inhibition mediated by this CpG motif is dependent on the hairpin structure formed by the sequence [141], while the methylation status does not alter its inhibitory property [142]. Nonetheless, IFN- $\alpha$  has been detected in the serum of PCV2-infected pigs [143, 144], indicating that the interaction of PCV2 and pDCs might be much more complex. However, the relevance of such a response is not known in *in vivo* context yet. A type I IFN response can be thought to favor PCV2 replication [133] increasing the chance for disease development.

#### 4.3. Brief history of porcine reproductive and respiratory syndrome

The first cases of porcine reproductive and respiratory syndrome (PRRS) appeared in the United States of America and in Europe in latest 1980s. Even though several clinical manifestations have been observed, characteristic syndromes are reproductive failures and respiratory distress [145, 146]. Porcine reproductive and respiratory syndrome virus (PRRSV) was then recognized as the causative agent of PRRS in Europe [147] and the United States [148, 149] and was referred as Lelystad virus (LV) and VR-2332, respectively. While both isolates emerged simultaneously in Europe and in the US inducing similar clinical signs, it appeared that only 55-79% identity in amino acid sequences were shared by these two viruses [150]. Since the early 1990s, PRRSV has started to spread across Europe and North America especially in pig-growing areas. It becomes clear that two distinct genotypes of PRRSV evolved divergently which were assigned as to either genotype 1 (European; PRRSV-1) represented by the prototype LV, or to genotype 2 (North American; PRRSV-2) with VR-2332 as reference strain. Moreover, sequence analysis of PRRSV isolates showed a considerable genetic variation within a genotype, which can be cluster into several subtypes of PRRSV [151, 152]. So far, phylogenetic analysis of ORF5 and ORF7 from genotype 1 classified the European PRRSV into 4 subtypes including Western European subtype I, II and III and Russian subtype I. Moreover, a larger heterogeneity has been described for PRRSV-2, which comprise at least 9 lineages [153]. Currently, PRRSV is spreading throughout the world and episodically give rise to the emergence of highly virulent isolates related to PRRSV-2 in North America but also in Asia since 1997 [154, 155]. The origin of PRRSV is still unknown although the PRRSV seems to be related to the lactate

dehydrogenase-elevating virus (LDV) suggesting that both PRRSV genotypes diverged from a common precursor and that the speciation of the two current known genotypes arose latter than LDV variants [150]. Due to contaminations of semen used for inseminations, some sows in the Eastern region of Switzerland were found to be PRRSV positive by qPCR in December 2012. The infected herds were slaughtered to avoid any outbreaks and virus spreading. Thus, the worst scenario was avoided because the immune status is naïve in pigs growing in Swiss area. Due to the large heterogeneity of the virus and its weak immunogenicity, the development of successful vaccines is currently of major importance and many efforts are consumed to improve the knowledge of immunological basis of PRRSV which will help for vaccine design.

#### 4.3.1. Impact and clinical signs of PRRS

PRRS appears as one of the most important emerging disease affecting the swine industry. For instance, the cost associated to PRRS is estimated at up to 560 million US dollars per year in the USA [156]. The outcome of PRRS results of complex interactions between the virus and host factors including the age, the presence of other bacterial or viral pathogens as well as the pregnant status of sows, in which the infection can lead to late-term abortion and stillborn mummified fetuses. The symptoms in sows greatly vary from none to fever, lethargy, pneumonia and blue coloration of the ears. Infections of neonatal pigs demonstrate more profound respiratory distress and higher susceptibility to bacterial or viral co-infections where mortality can reach 100% in certain herds [157]. According to several reports, it is well accepted that PRRSV-2 causes more profound pathological lesions [158-160]. However, it seems that emergence of new isolate belonging to genotype 1 can provoke severe clinical signs [161]. Additionally, evidence from published observations showed that PRRSV can persist in infected animals and can be detected at 105 days post infection [162]. Although the lung tissues, especially PAM are the main sites of PRRSV replication [163], Rowland and colleagues were able to recover the virus from lymphoid organs or tonsils at 132 days after birth in congenital infected pigs [164]. These data suggest that PRRSV can persist for long period and may escape the immune responses. Indeed, PRRSV infection is characterized by a delayed appearance of neutralizing antibodies and a poor cell-mediated immunity [165]. Accordingly, PRRSV increases the susceptibility of pigs to secondary bacterial and viral infections in the respiratory tract [166]. The mechanisms by which PRRSV evades the host immune system have unfortunately not yet been elucidated. Moreover, due to large heterogeneity amongst PRRSV isolates and limited understanding of PRRSV immunobiology, vaccine development remains one of the biggest challenge in the future research [167].

#### 4.3.2. Biology of PRRSV

##### Genome organization:

PRRSV is an enveloped positive ssRNA virus and belongs to the Arteriviridae family which also include LDV, equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) [168]. The viral genome of approximately 15kb encodes for 7 subgenomic mRNA transcripts, each of them containing one or two ORFs for protein translation [169]. The non-structural pp1a and the larger pp1ab polyproteins are translated from full-length viral RNA. The pp1ab is expressed from a ribosomal frame shift. These two polyproteins are then cleaved by viral proteases to give rise to 14 non-structural proteins [168, 170] including proteases (NSP1 $\alpha$ , NSP1 $\beta$ , NSP2 and NSP4), an RNA-dependent RNA polymerase (NSP9), a helicase (NSP10) and an endoribonuclease (NSP11) [171]. Whilst some of the non-structural proteins of PRRSV have still unidentified structure and unknown

function, NSP2 seems to be of particular interest since the amino acid sequence represent only 32% identity between PRRSV genotypes [172]. The structural proteins are translated from ORFs 2-7. The ORFs 2-5 encode for the glycosylated envelope proteins GP2, GP3, GP4 and GP5. The non-glycosylated M and the nucleocapsid (N) proteins are expressed through ORF6 and ORF7 respectively [173]. The small E protein is translated from ORF2b, which is included in the ORF2 [174].

#### Target cells:

PRRSV has shown to have a restricted tropism for the MΦ/monocytic lineage. Antigens of PRRSV have been observed in alveolar MΦ in the lungs and in other lymphoid tissues [163, 175]. Replication of PRRSV *in vitro* has been detected in PAM [176], MoDC [177-180], bone marrow-derived dendritic cell (BMDC) [181], monocyte-derived macrophages (MDM) [182] and MARC-145 [183]. Whilst PRRSV can infect MoDC and produce virus progeny, it is not clear if replication may occur in primary DC *in vivo*. In the study of Loving and colleagues, virus quantification using an end-point titration and qPCR in the supernatants of lung DC did not show an increase of PRRSV titers or viral copy number respectively [184]. Using a GFP-tagged PRRSV it was observed that pDCs are refractory and do not support virus replication [185]. Additionally, PRRSV does not replicate *in vitro* in primary monocytes [176]. In summary, it can be concluded that cells susceptible to PRRSV are closely associated to the MΦ lineage and less to the DC subsets. However, since MΦ comprise a heterogeneous cell population [186], future studies might be devoted to assess if some of the MΦ subsets are differentially susceptible to PRRSV.

The MΦ originates from blood monocytes following cellular differentiation in specific tissues. Since PRRSV specifically targets the MΦ lineage in the lung, the understanding of MΦ biology is highly relevant. The renewing of lung MΦ mainly comes from blood precursors but also from local progenitor cells. Based on the anatomical location, the lung MΦ comprise three major subsets including PAM, interstitial and intravascular MΦ which differentially express TLRs [187]. The differentiation of MΦ is driven by the cytokine microenvironment, which plays a critical role in the activation of different functional MΦ. This functional classification is based on MΦ polarization into classically (M1) or alternatively (M2) activated MΦ. While M1 MΦ polarization is stimulated by IFN-γ, the M2 MΦ mainly require the presence IL-4 or IL-13. The M1 activation promotes inflammatory responses and increases the microbicidal activities of MΦ to destroy and eliminate intracellular pathogens [14]. Important sources of IFN-γ which results into M1 polarization include the NK and T<sub>H</sub>1 cells [188]. Alternatively, M2-driven responses by MΦ lead to anti-inflammatory cytokine secretions and tissue healing. The cytokines which activate such MΦ are secreted by polymorphonuclear cells (PMNC) as well as the CD4<sup>+</sup> lymphocytes from T<sub>H</sub>2-like responses [189]. Thus, M2 MΦ can be considered as “regulatory” MΦ by their ability to secrete IL-10 and other mediators involving in the processes of wound healing and fibrosis. Nonetheless, little information is available on the phenotype and functionality of M1- or M2-activated MΦ in the pig model. It is not known if cytokine-mediated polarization of MΦ could influence the susceptibility of these cells to PRRSV.

#### Replication:

The scavenger receptor CD163 is employed by PRRSV for binding and penetration into the cells. This receptor was isolated from screening of a PAM cDNA library. The transfection of cDNA encoding CD163 showed that it was sufficient to render non-permissive cells susceptible to PRRSV infection and replication [190]. Following entry and uncoating of PRRSV, the translation of nonstructural proteins starts at ORF1a and employs full-length genomic RNA. The ORF1a is connected to ORF1b by a ribosomal frame shift, which leads two polyproteins pp1a and pp1ab. Post-translationally processing by viral autoproteins encoded by pp1a is required to release the nonstructural proteins

and assemble into a membrane-associated viral replication and transcription complex (RTC). The NSP9 RNA-dependent RNA polymerase recognizes a signal sequence in the 3'-end of genome and initiates the synthesis of the genomic negative-stranded RNA as well as subgenomic RNAs [191]. It is thought that these minus-strands serve as template for the transcription of positive subgenomic mRNAs and facilitate PRRSV replication. The structural proteins are then translated from subgenomic mRNAs by host ribosomes [192]. Following the encapsulation of the genome by the N protein, new virions are formed by budding from the membrane of the endoplasmic reticulum to the Golgi pathway. The replication cycle of PRRSV takes around 12 h in PAM and is followed by CPE and cell lysis.

#### 4.3.3. Type I IFN responses to PRRSV

One important feature of PRRSV infection is the ability of the virus to induce any or only limited quantities of type I IFN and other proinflammatory cytokines in the lung tissues [193, 194]. Since the lack of type I IFN could play a role in PRRSV pathogenesis and explain the weak adaptive immune response and persistence of the virus [165], many studies have been performed to understand how PRRSV manipulate host immunity. Quantifications of PRRSV-induced IFN- $\alpha$  *in vitro* revealed that this cytokine is not secreted by PAM [193] and MoDC [195, 196]. In the study of Lee and colleagues, it was reported that the ability of the virus to induce IFN- $\alpha$  in PAM depends on the PRRSV isolates and even isolate-derived plaque clones [197]. These data suggest that PRRSV can strongly differ in its ability to influence type I IFN secretion, which may relate to virus isolates or even to quasispecies. Importantly, PRRSV is highly sensitive to the presence of type I and III IFN *in vitro* [184, 198, 199] and to type I IFN *in vivo* [200].

Many research groups have focused on the mechanism used by PRRSV to inhibit type I IFN secretion in cells susceptible to infection such as MARC-145. No significant upregulation of IFN- $\alpha_2$  and IFN- $\beta_1$  transcripts has been noted in PRRSV-infected MARC-145 [201]. Further investigations using IFN- $\beta$  promoter luciferase reporter system suggested that PRRSV has the ability to inhibit IRF3 activation but not NF- $\kappa$ B and AP-1. The authors propose that the inhibition of IFN- $\beta$  is attributed to the interaction of PRRSV with IPS-1, the downstream element of RIG-I signaling cascade [202]. Work by others showed that four of PRRSV nonstructural proteins including NSP1, NSP2, NSP4 and NSP11 inhibit IRF3- and NF- $\kappa$ B-dependent gene expression upon dsRNA stimulation in TLR3-expressing HEK293T cells [195]. In another study, it was also noticed that NSP1 reduced phosphorylation of IRF3 which in turn limits the induction of IFN- $\beta$  in MARC-145 [203]. In addition to its ability to suppress type I IFN induction, PRRSV NSP1 $\beta$  can alter the signaling pathway by inhibiting STAT1 phosphorylation [204]. On the other hand, PRRSV is found to prevent the translocation of ISG3F into the nucleus [205]. However, this model based on MARC-145 does not seem to fully reflect the susceptible host cells, since Gemini and colleague observed an upregulation of IFN- $\beta$  transcripts in PAM [206]. Similarly, IFN- $\alpha/\beta$  transcripts have been detected in MoDCs [196]. PRRSV must rather inhibit the type I IFN induction through its rapid cytopathogenicity in host cells because UV-inactivated viruses lose the capacity to interfere with type I IFN production by TGEV or poly(I:C) [207]. Recent reports showed that pDCs did not respond to North American PRRSV [208] and that CpG-induced IFN- $\alpha$  was inhibited in these cells [185]. However, accumulating evidence from published reports suggest that certain cell types are able to sense PRRSV infection since significant levels of IFN- $\alpha$  have been detected in the serum of infected pigs [193, 209, 210]. The report from Barranco *et al.* even demonstrated the presence of IFN- $\alpha$  secreting cells in lymphoid organs after PRRSV-1 challenge [211]. Despite many reports suggesting the suppressive activity of PRRSV regarding the type I IFN system [212, 213], this alone may not explain the delayed adaptive immune response. It seems that many additional factors may contribute to the uncommon immune response

against PRRSV. These include a particular cytokine microenvironment, downregulation of molecules related to antigen presentation and the presence of a decoy epitope in GP5 distracting the adaptive immune response for the production of neutralizing antibodies [214].

#### 4.4. Cathelicidins and the type I IFN system

Host defense peptides (HDP), also termed as antimicrobial peptides encompass a variety of small proteins, which are involved in the innate defense mechanisms. HDPs comprise two main families including the defensins and cathelicidins. Defensins are short peptides characterized by 6 cysteins with various disulfide bound configurations and have strong antimicrobial properties as well immunomodulatory functions [215]. Regarding the cathelicidins, they represent a more heterogeneous group of peptides. All of them possess an N-terminal signal sequence, a cathelin-like domain and various antimicrobial regions in the C-terminal part [216]. The great diversity of the C-terminal enables to categorize the cathelicidins in 3 classes which include the proline- and arginine-rich, cysteine-rich and the  $\alpha$ -helical peptides [217]. These peptides are temporally stored as propeptide form within neutrophil granules. The C-terminal domain of the molecule requires activation by elastases which cleave off the propeptide form to release the bioactive cathelicidin [218-220]. Additionally, the expression of cathelicidins is inducible in keratinocytes and has been detected in NK cells, B cells, monocytes/M $\Phi$  and bone marrow cells [221-223]. Interestingly, only one member referred as LL-37 has been found in humans, whereas 11 cathelicidins have been isolated in swine. The porcine cathelicidin family comprises the prophenin-1 and -2, proline-arginine-rich 39-amino-acid peptide (PR-39), disulfide-bridged cystein-rich protegrins (PG-1 to PG-5) and  $\alpha$ -helical porcine myeloid antimicrobial peptides (PMAP-23, PMAP-36 and PMAP-37). Remarkably, all cathelicidins harbour a high cationic charge for a size of 12–100 amino-acid residues [224]. The immunological properties of LL-37 have been the most studied so far. This peptide belongs to the  $\alpha$ -helical peptides and has typical amphipathic configuration. It has been demonstrated that LL-37 displays a wide antimicrobial activity against several Gram<sup>-</sup> and Gram<sup>+</sup> bacteria as well as immunomodulatory functions [225]. For instance, recent reports demonstrated that human LL-37 is able to promote rapid sensing of CpG motif-containing oligodeoxyribonucleotides (CpG-ODN) by pDCs [226]. The complexing of self DNA by LL-37 resulting in pDC activation has been proposed to play a role in the pathogenesis of some autoimmune diseases [227, 228]. Although the antimicrobial activity of porcine cathelicidins is well documented, little is known about their immunomodulatory function and their interaction with pDCs.

##### 4.4.1. Antimicrobial activity

The interface between cathelicidin and the target organisms has been quite well characterized for the human LL-37. Early publication already demonstrated that LL-37 is a potent antimicrobial agent [229, 230]. Bacterial killing is mediated by disrupting bacterial membrane integrity [231]. The cationic moieties of LL-37 enable an interaction with negatively charge residues on bacterial surface. Subsequently, the peptide accumulates in carpet like-structure due to the parallel orientation of the amphipathic helix on the bacterial surface. This mechanism induces leakage probably by bytoroidal peptide-lipid pore formation until the membrane completely collapses [217, 232]. In contrast, the biochemical properties of PR-39 suggest that the microbicidal activity is not mediated by lytic mechanism but rather by targeting cellular components [224]. The peptides PR-39 [233, 234], PMAP-23 [235, 236], PMAP-36 [237, 238], PMAP-37 [239] have been reported to kill many pathogens including Gram<sup>+</sup> and Gram<sup>-</sup> bacteria. The PGs as well as the two prophenins have also been found to

mediate antimicrobial activity summarized in a review [224]. Depending on the pathogens, the minimal inhibitory concentration ranges from 0.5 to 50  $\mu$ M but can be higher for resistant pathogens such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* [224]. Importantly, cholesterol prevents membrane damage. This explains why physiological concentrations of cathelicidins do not injury host cells which contain cholesterol in their membrane [240].

#### 4.4.2. Immunomodulatory functions

The human LL-37 have been reported to have multiple effects on the immune system. First, it has been shown that this cationic peptide can neutralize LPS toxin reducing the danger of septic shock [241]. In addition, it exhibits cell chemotaxis [222] and participates to M $\Phi$  differentiation [242] and activation [243, 244]. Investigations on MoDC differentiation indicated that the presence of LL-37 influences the expression of several cell surface markers relevant for the antigen presentation process. Moreover, LL-37-treated MoDC have higher phagocytic activity and stronger potential for inducing T<sub>h</sub>1 cell polarization compared to control MoDC [245]. Due to the implication of LL-37 in antigen presentation as well as in the process of inflammation, it can be proposed that this peptide links both innate and adaptive immunity. Following infection, tissue damage needs to be repaired. The binding polyvalence of LL-37 to multiple receptors involving the processes of wound repair and angiogenesis also suggest implication of this peptide in tissue healing [246]. Finally, another report showed that copious amounts of IFN- $\alpha$  can be induced by pDCs due to the rapid delivery of CpG-ODN complexed to LL-37 [226]. This mechanism has been proposed to be involved in the pathogenesis of psoriasis and Lupus Erythematosus autoimmune diseases where high levels of LL-37 have been detected in the lesions of patients [227, 228].

Regarding the porcine cathelicidin only PR-39 has been described to have other functions than antimicrobial effects. This includes the chemotaxis of neutrophils [247] and angiogenesis [248]. Interestingly, PR-39 has potent capacity to deliver small interfering RNA into the cells [249]. Apart from the antimicrobial activity, nothing is currently described on the influence that PMAPs could have in the modulation of some immune cells.

## 5. Goals

Due to the important and crucial role of type I IFNs during an immune response, the first objectives were to evaluate and clarify the interaction of two known immunosuppressive viruses, PCV2 and PRRSV, with pDCs. Previous report demonstrated that PCV2 inhibits IFN- $\alpha$  production in pDCs *in vitro* in response to TLR7 or 9 agonists [137]. This inhibition of IFN- $\alpha$  has been attributed to the presence of the dsDNA replicative form of PCV2. Conversely, IFN- $\alpha$  was not inhibited in pDCs in some PCV2 preparations [138]. Furthermore, detectable levels of systemic IFN- $\alpha$  were found in the serum of PCV2-infected pigs [143, 144], thereby questioning the function of PCV2 in modulating IFN- $\alpha$  in pDC responses. In parallel, a recent study on North American PRRSV genotype 2 isolates showed that the virus did not induce, or even strongly inhibited IFN- $\alpha$  in pDCs [185]. However, again many studies demonstrated that significant IFN- $\alpha$  amounts were quantified in pigs which were infected with various PRRSV strains [193, 209, 210]. Thus, the aims of the first part of my Thesis were to clarify host and viral factors, which could potentially influence the function of pDCs and type I IFN induction by PCV2 and PRRSV.

PRRSV has natural tropism for cells of monocytic lineage with a particular affinity for alveolar M $\Phi$  in the lung tissues and lymphoid organs. Therein, the understanding of M $\Phi$  biology is primordial to study PRRSV within its natural host. In the second part of this Thesis we therefore focused on the influence of IFN- $\gamma$  (M1), IL-4 (M2) and IFN- $\beta$  activation of M $\Phi$  on the susceptibility to genotype 1 and 2 PRRSV strains varying in virulence.

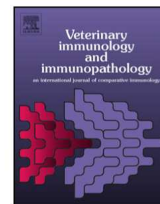
The third part of my Thesis is based on a recent report demonstrating that the human LL-37 promotes rapid sensing of CpG-ODN inducing large amounts of IFN- $\alpha$  by pDCs [226]. Except to their broad antimicrobial activity, nothing is known about the participation of porcine cathelicidins in pDC-driven innate immune responses. We considered that such functions are relevant to both PRRSV- and PCV2-mediated diseases, which are very often associated with bacterial co-infections. Consequently, we evaluated the ability of three selected porcine cathelicidins, PR-39, PMAP-23 and PMAP-36, to interact with different types of nucleic acids and mediate pDC activation.

## 6. **Results**

### 6.1. *Manuscript 1*

*Porcine circovirus type 2 stimulates plasmacytoid dendritic cells in the presence of IFN-gamma*

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## Short communication

Porcine circovirus type 2 stimulates plasmacytoid dendritic cells in the presence of IFN- $\gamma$ Arnaud Baumann<sup>a,b</sup>, Kenneth C. McCullough<sup>a</sup>, Artur Summerfield<sup>a,\*</sup><sup>a</sup> Institute of Virology and Immunology (IVI), Sensemattstrasse 293, 3147 Mithelhäusern, Switzerland<sup>b</sup> Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

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## ABSTRACT

Porcine circovirus type 2 (PCV2) is recognized as the primary cause for the development of porcine circovirus-associated disease (PCVD). A number of reports demonstrated that PCV2 double-stranded (ds) DNA inhibits interferon- $\alpha$  (IFN- $\alpha$ ) production in cultures of porcine plasmacytoid dendritic cells (pDC). In addition, a short-lived peak of systemic IFN- $\alpha$  was detectable in the serum of PCV2-infected pigs, suggesting that the interaction of PCV2 with pDC may be more complex. Culturing pDC supplemented with IFN- $\gamma$  actually rendered the cells responsive to the presence of PCV2. Accordingly, viral genomic single-stranded (ss) and replicative dsDNA forms have been examined for their ability to activate pDC. It was noted that the encapsulated viral ssDNA stimulated pDC in the presence of IFN- $\gamma$ ; free viral DNA, presumably as double-stranded forms, was responsible for inhibiting pDC responses, even in the presence of the several cytokines known to promote pDC responses. These data suggest that the equilibrium between the levels of encapsulated genomic ssDNA and free dsDNA replicative forms of PCV2 is determinant in defining the immunomodulatory characteristics of the virus infection.

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## 1. Introduction

Porcine circovirus associated-disease (PCVD) currently encompasses a variety of disease syndromes including post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) (Gillespie et al., 2009). Whilst many pathogens can be associated with PCVD and contribute to the clinical manifestations of the disease (Ellis et al., 1999, 2008; Harms et al., 2001; Opriessnig et al., 2004, 2011; Rovira et al., 2002), porcine circovirus type 2 (PCV2), a small non-enveloped single-stranded (ss) circular DNA virus of 1.7 kb, is the defining cause of PCVD pathogenesis. Even though

most PCV2 infections are asymptomatic, pigs developing PCVD symptoms are characterized by lymphoid depletions, loss of lymph node architecture and histiocytic infiltrations (Segales et al., 2004b). *In vivo*, PCV2 antigens are predominantly found in dendritic cells (DC) and macrophages (Allan and Ellis, 2000; Rosell et al., 1999). Moreover, *in vitro* studies revealed that PCV2 does infect DCs and macrophages (Gilpin et al., 2003; Vincent et al., 2003). Interestingly, the virus accumulates within DCs without showing any signs of replication or disturbing the capacity of these cells to present antigens (Vincent et al., 2003, 2005). According to the observed lymphoid depletion in PCVD-affected pigs and due to the fact that others factors are required for the expression of clinical signs of the disease, it is hypothesized that PCV2 infection causes an immunosuppression leaving the host susceptible to disease development, although the mechanism is still not well understood (Kekarainen et al., 2010; Ramamoorthy

\* Corresponding author. Tel.: +41 31 848 9377; fax: +41 31 848 9222.  
E-mail addresses: [artur.summerfield@ivi.admin.ch](mailto:artur.summerfield@ivi.admin.ch),  
[abauman2@hotmail.com](mailto:abauman2@hotmail.com) (A. Summerfield).

and Meng, 2009; Segales et al., 2004a; Segales and Mateu, 2006).

The induction of type I interferons (IFNs), especially IFN- $\alpha/\beta$ , is a common defence mechanism important in promoting innate defences and efficient adaptive immune responses, which has been adopted in higher vertebrates to prevent viral replication (Fitzgerald-Bocarsly and Feng, 2007; Sadler and Williams, 2008; Wang and Fish, 2012). Unsurprisingly, certain viruses have evolved strategies to counteract this innate defence mechanism by inhibiting the induction or pathways of the IFN signalling cascade (Haller et al., 2006). Despite their low frequency in peripheral blood mononuclear cells (PBMC), plasmacytoid DCs (pDC) represent a cell type, which most efficiently senses viruses. They are the major source of IFN- $\alpha$  in response to viral RNA and both viral and bacterial DNA containing unmethylated CpG motifs, which are recognized by Toll-like receptor (TLR) 7 and TLR9, respectively (Colonna et al., 2004; Liu, 2005). Thus, pDC represent an important target for viruses, which have evolved to suppress such “danger signals”, with the downstream effect of modulating innate immune pathways required for induction of adaptive immune responses (McCullough et al., 2009). In pigs, pDC are identified as CD14<sup>+</sup>CD172a<sup>+</sup> with high levels of CD4, CD123 and CD135 (Guzylack-Piriou et al., 2010; Summerfield and McCullough, 2009). As expected, pDC are strongly influenced by the cytokine microenvironment changing in the course of an immune response. For example, IL-4, type I and II IFNs, Flt3-L and GM-CSF enhance the ability of porcine pDC to express IFN- $\alpha$  in response to CpG-oligodeoxynucleotides (ODNs) or foot-and-mouth disease virus (FMDV) (Lannes and Summerfield, 2013).

Interestingly, published observations showed that PCV2 inhibits IFN- $\alpha$  production *in vitro* with enriched pDC in response to TLR7 or 9 agonists (Vincent et al., 2007). This PCV2-mediated inhibition of pDC-derived IFN- $\alpha$  has been linked to the amount of the double-stranded (ds) DNA replicative form of PCV2. Furthermore, it was shown that PCV2 preparations which did not inhibit IFN- $\alpha$  responses were characterized by absence or low levels of dsDNA replicative form of PCV2, implying that it is this form of DNA which is responsible for pDC suppression by PCV2 (Balmelli et al., 2011). In contrast, detectable levels of IFN- $\alpha$  were identified in the serum of PCV2-infected pigs, days before reaching the peak of viraemia (Fort et al., 2009; Stevenson et al., 2006), suggesting that the influence of PCV2 on pDC activity may be more complex and diverse. Accordingly, the aim of the present study was to determine how host and viral factors may lead to enhanced pDC type I IFN responses following encounter with PCV2.

## 2. Material and methods

### 2.1. PCV2 preparations

Viral stocks of PCV2 1010 Stoon strain (Allan et al., 1998) were prepared as previously described (Vincent et al., 2003). Briefly, PK-15A cells, free of PCV1, were infected with PCV2 in T150 flasks. Uninfected cells were used as mock control. After 24 h incubation, cells were treated

with 300 mM D-glucosamine in Hanks balanced salt solution (HBSS) for 30 min. Then, the glucosamine solution was replaced by fresh medium and the cells were cultured for a further 48 h. Finally, cells were scraped, frozen and thawed three times, clarified at 3000 g at 4 °C for 30 min and used for a super-infection of PK-15A. Following two super-infection steps, mock- or PCV2-infected cells were scraped, frozen and thawed three times, sonicated and clarified by centrifugation (3000 g at 4 °C for 30 min). Lysates were aliquoted and stored at –70 °C until use. PCV2 or mock lysates were titrated in PK-15A using immunofluorescence assay with anti-PCV2 capsid mAb (7G5-G4-A1) and titres were expressed in 50% tissue culture infectivity dose per ml (TCID<sub>50</sub>/ml). The content of dsDNA forms of PCV2 present in the preparation A, B and C was examined on agarose gel electrophoresis. Briefly, a volume corresponding to  $4 \times 10^5$  TCID<sub>50</sub> of PCV2 was used for DNA purification (NucleoSpin® Gel and PCR Clean-up kit, Macherey-Nagel, Switzerland). After DNA elution, FastDigest EcoRI (Thermo Scientific) was used to linearize PCV2 dsDNA and ethidium bromide revealed the presence of PCV2 dsDNA (1.7 kb) on 0.8% agarose gel after UV light exposure.

### 2.2. pDC enrichment

Enrichments of pDC were performed as described earlier (Guzylack-Piriou et al., 2004). Briefly, peripheral blood mononuclear cells (PBMC) from 6 weeks to 12 months old PCV2 seronegative specific pathogen-free (SPF) pigs were isolated by Ficoll-Paque differential centrifugation, followed by CD172a (mAb 74-22-15a) enrichment using MACS sorting LD columns (Miltenyi Biotec GmbH, Germany). These CD172a<sup>+</sup> fractions (purity >90%) were enriched to 2–5% for CD172<sup>low</sup>CD4<sup>high</sup> pDC, and cultured in DMEM with 10% foetal bovine serum (FBS; Biowest, France) and 20  $\mu$ M  $\beta$ -mercaptoethanol (Life Technologies, Switzerland).

### 2.3. Stimulation of pDC and IFN- $\alpha$ ELISA

Enriched pDC were incubated at  $4 \times 10^5$  cells per microwell in a total of 200  $\mu$ l with PCV2 at a multiplicity of infection (MOI) of 0.06–0.6 TCID<sub>50</sub>/cell. PCV2-mediated inhibition was evaluated by the ability to inhibit pDC responses to the type A CpG-ODN D32 (10  $\mu$ g/ml; Biosource Int., Camarillo, USA). This type A CpG was found to induce strong IFN- $\alpha$  response in porcine pDC (Guzylack-Piriou et al., 2004). The relative inhibition was calculated as follows:  $100 - ((\text{IFN-}\alpha \text{ produced by PCV2 treated cells} + \text{CpG-ODN}) / (\text{IFN-}\alpha \text{ produced by mock-treated cells} + \text{CpG-ODN}) \times 100)$ .

Cytokine treatments included IFN- $\beta$  (100 U/ml (Husser et al., 2011)), IFN- $\gamma$  (10 ng/ml, R&D Systems, UK), Flt3-L (100 U/ml (Guzylack-Piriou et al., 2010)), GM-CSF (100 U/ml (Summerfield et al., 2003b)). Secreted IFN- $\alpha$  after 20 h of incubation was quantified by ELISA as described (Díaz de Arce et al., 1992).

The contribution of free DNA was evaluated by the use of DNase I. In the experiment shown in Fig. 3, transmissible gastroenteritis virus (TGEV, strain Perdue) was employed at an MOI of 0.1 TCID<sub>50</sub>/cell as described previously

(Summerfield et al., 2003a). *E.coli* DNA (10 µg/ml; Invivo-gen, France) was also included as positive control to assess the efficiency of nuclease activity. To this end, mock lysates, PCV2 preparations (MOI of 0.06 TCID<sub>50</sub>/cell), *E.coli* DNA and TGEV were treated with DNase I (Ambion, Cambridgeshire, UK) at 400 U/ml at 37 °C for 30 min before addition to enriched pDC. This DNase concentration employed was based on the ability to digest *E. coli* DNA (100 µg/ml) making its visualization by gel electrophoresis impossible.

#### 2.4. Statistical analyses

Statistical analyses were performed using Graph-Pad Prism 6.0. Gaussian distribution was assessed with D'Agostino & Pearson normality test. Statistical significance of difference between groups was considered using one way ANOVA and Bonferroni's multiple comparison test ( $P < 0.05$ ). When the data did not follow a Gaussian distribution, Kruskal–Wallis test and Dunn's multiple comparison test were employed ( $P < 0.05$ ).

### 3. Results and discussion

Based on recent findings showing that different preparations of the same PCV2 isolate varied in their ability to inhibit CpG-induced IFN-α response by pDC (Balmelli et al., 2011), three different preparations of PCV2 using at an MOI of 0.6 TCID<sub>50</sub>/cell were tested. With respect to their inhibitory potential, these could be classified as “strong” (prep A), “moderate” (prep B) and “negative” (prep C) (Fig. 1A). The levels of IFN-α induced by CpG alone reached between 5,000 and 25,000 U/ml, depending on the experiment. Due to this variability the percentage of inhibition was used to normalized the data. For each PCV2 preparation the levels of inhibition observed in pDC related to the amount of free dsDNA form of PCV2 as shown by the intensity of the 1.7 kb band (Fig. 1B).

Since the biological activity of pDC can be influenced by stimuli present in the microenvironment (Lannes and Summerfield, 2013), the effect of a selected panel of cytokines known to enhance IFN-α responses to CpG by pDC were examined with respect to their capacity to reverse the inhibitory effect mediated by PCV2. To this end, enriched pDC were simultaneously incubated with the inhibitory PCV2 prep A at an MOI of 0.6 TCID<sub>50</sub>/cell and stimulated with CpG for 20 h. None of the cytokines tested was able to abolish the PCV2-mediated inhibition of CpG-induced IFN-α in enriched pDC (Fig. 1C). However, the level of inhibition was significantly reduced in enriched pDC cultured in presence of IFN-γ (58% inhibition on average) in comparison with pDC cultures treated with CpG and PCV2 in the absence of cytokine (81% inhibition).

Certain cytokines and haematopoietic growth factors display a known promoting effect on porcine pDC responses to FMDV (Lannes and Summerfield, 2013) and to porcine reproductive and respiratory syndrome virus (PRRSV) (Baumann et al., 2013). Accordingly, these cytokines were evaluated for their potential at promoting pDC responses to PCV2. Considering the results in Fig. 1C, prep A of PCV2 used either at an MOI 0.6 or 0.06 TCID<sub>50</sub>/cell was examined for the induction of IFN-α in enriched pDC

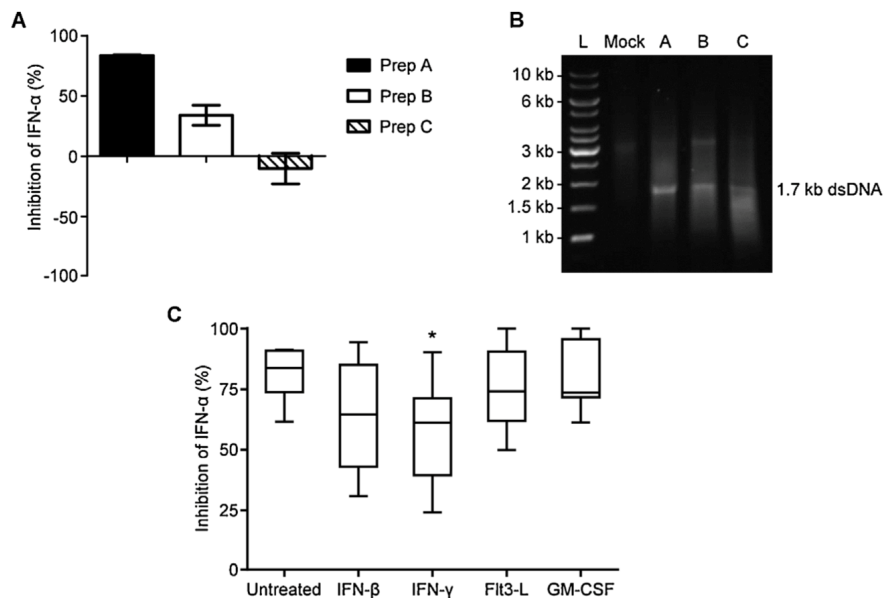
cultures supplemented with IFN-β, IFN-γ, Flt3-L and GM-CSF. As noted in Fig. 2A, these cytokines did not abolish the inhibitory effect of PCV2 at the MOI 0.6 TCID<sub>50</sub>/cell. This clearly contrasted with the results obtained at the lower MOI of 0.06 TCID<sub>50</sub>/cell, whereby the additional cytokine modulated this inhibitory PCV2 prep A to induce IFN-α secretion; the most prominent effect was in presence of IFN-γ (Fig. 2A).

At the higher MOI of PCV2 prep A, the inhibitory properties dominated as described elsewhere, which would suggest a relationship to high levels of dsDNA replicative forms of PCV2 (Balmelli et al., 2011; Vincent et al., 2007). A dose-response analyses of the PCV2 prep A in the presence of IFN-γ indicated that inhibitory effects were most prominent at higher MOIs, and stimulatory effects became apparent as the MOI was reduced (Fig. 2B); this reflects that only at high MOIs did the inhibitory effects exceed the stimulatory activity observed in presence of IFN-γ (Fig. 2C).

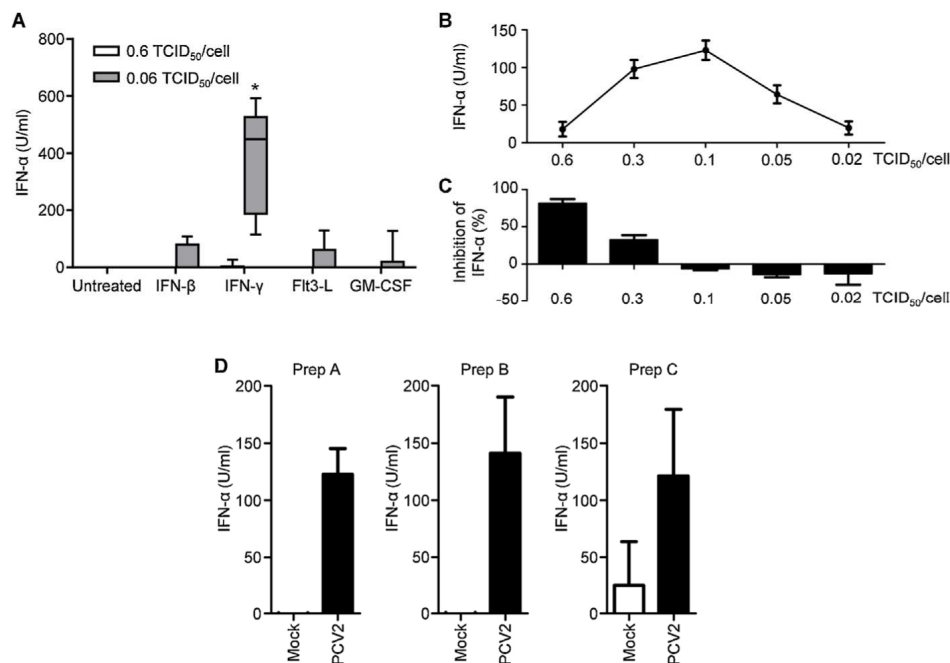
Other PCV2 preparations presented in Fig. 1A were also tested for their immunomodulatory influence on pDC in presence of IFN-γ, and found to induce similar levels of IFN-α responses (Fig. 2D). These findings demonstrate that the induction of IFN-α by PCV2 in enriched pDC is an IFN-γ-dependent process. Considering that all PCV2 preparations induced similar levels of IFN-α independent of their inhibitory activity, we postulated that pDC activation was not caused by the dsDNA replicative forms of PCV2 previously implicated in the inhibition of IFN-α induction.

In order to investigate the role of PCV2 DNA in the induction of IFN-α, free DNAs in the viral preparations were digested by an endonuclease (DNase I). The PCV2 prep A was used at an MOI which in the presence of IFN-γ induced IFN-α responses. It was treated with DNase I before incubation with enriched pDC cultured in presence of IFN-γ. Parallel controls of *E.coli* DNA and TGEV were handled in the same manner. TGEV was insensitive to DNase treatment, reflecting its capacity to induce IFN-α independently of nucleic acid (it is also an RNA virus); as expected, the capacity of the *E.coli* DNA to induce IFN-α was abrogated by the DNase I treatment (Fig. 3). In contrast to the *E.coli* DNA, the DNase treatment had no significant influence on the PCV2-induced IFN-α. This indicated that free PCV2 DNA was not required for the induction of IFN-α in enriched pDC. Although we currently cannot exclude a role for the capsid protein, we propose that the encapsulated ssDNA form of the PCV2 genome was responsible for inducing IFN-α. This is based on the observation that in most cases pDC produce IFN-α in response to nucleic acids as pathogen-associated molecular patterns, but not proteins (Kaisho, 2012).

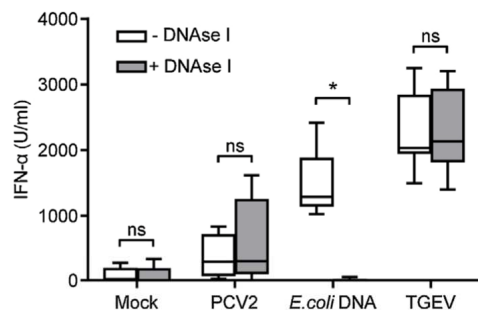
The pDC-derived IFN-α responses described in the present study relate to the *in vivo* reports in which PCV2-inoculated pigs elicited a systemic IFN-α response (Fort et al., 2009; Stevenson et al., 2006). Considering that the replication of PCV2 *in vitro* is increased by type I IFN in PK-15A (Meerts et al., 2005) and in 3D4/31 cell lines (Ramamoorthy et al., 2009), the protective value of these IFN responses in pDC may prove the contrary to protective. Nevertheless, PCV2 does not appear to replicate efficiently in pDC, rather accumulating therein. Thus, in absence of a direct antiviral activity, the IFN-α response by pDC would



**Fig. 1.** (A) Individual PCV2 preparations differentially inhibit CpG-induced IFN- $\alpha$  production in enriched pDC. PCV2 (MOI 0.6 TCID<sub>50</sub>/cell) and type-A CpG (10  $\mu$ g/ml) were simultaneously added to enriched pDC for 20 h and IFN- $\alpha$  was quantified in the supernatant by ELISA. The relative percentage of IFN- $\alpha$  inhibition was calculated to classify different PCV2 preparations as “strong” (prep A), “moderate” (prep B) or “negative” (prep C). One representative experiment performed in triplicate culture is shown. (B) Analysis of DNA content in PCV2 prep A, B and C. Volumes of prep A, B and C corresponding to  $4 \times 10^5$  TCID<sub>50</sub> were used for DNA extraction and digested with EcoRI. The digested products were loaded on 0.8% agarose gel. The 1.7 kb band represents the PCV2 dsDNA replicative form. (C) Impact of IFN- $\beta$  (100 U/ml), IFN- $\gamma$  (10 ng/ml), Flt3-L (100 U/ml), GM-CSF (100 U/ml) on the inhibition of CpG-induced IFN- $\alpha$  responses by PCV2 (prep A). Only IFN- $\gamma$  significantly reduced the inhibition compared to untreated control. The Boxplots shown were generated from a total four independent experiments performed in triplicate with cells obtained from 3 different pigs. Significant differences ( $p < 0.05$ ) between the groups are denoted by (\*).



**Fig. 2.** (A) Low doses of PCV2 (prep A) induced IFN- $\alpha$  production in pDC when cultured in presence of IFN- $\gamma$ . PCV2 (MOI 0.6 or 0.06 TCID<sub>50</sub>/cell) was added to enriched pDC and incubated with IFN- $\beta$  (100 U/ml), IFN- $\gamma$  (10 ng/ml), Flt3-L (100 U/ml), GM-CSF (100 U/ml) for 20 h. The amounts of IFN- $\alpha$  in the supernatant were quantified by ELISA. Boxplots and whiskers represent four independent experiments each performed in triplicate cultures. Significant effects of cytokine treatment ( $p < 0.05$ ) compared to PCV2 alone (untreated) is denoted by (\*). (B and C) Stimulatory activity of PCV2 in the presence of IFN- $\gamma$  is observed at different doses compared with the inhibitory activity. (B) shows the dose-dependent induction of IFN- $\alpha$  by PCV2 in the presence of IFN- $\gamma$ , and (C) the dose-dependent PCV2-mediated inhibition of CpG-induced IFN- $\alpha$  in enriched pDC. One representative experiment in triplicates out of two is shown. (D) All PCV2 preparations tested were equally stimulatory in the presence of IFN- $\gamma$ . The individual PCV2 preparations from Fig. 1A, varying in their inhibitory activity at high MOI (0.6 TCID<sub>50</sub>/cell), were used at lower MOI (0.06 TCID<sub>50</sub>/cell) to induce IFN- $\alpha$  responses whereas mock control (lysate from uninfected PK-15A) did not reproductively induce IFN- $\alpha$  (data from one representative experiment in triplicate out of two).



**Fig. 3.** Encapsulated genomic DNA of PCV2 is responsible for the induction of IFN- $\alpha$  in enriched pDC cultured with IFN- $\gamma$ . Mock (uninfected PK-15A lysate), PCV2 (prep A), *E.coli* DNA and TGEV were either treated with DNase I (+DNase I) or remained untreated (-DNase I). Enriched pDC were stimulated for 20 h and IFN- $\alpha$  was measured in supernatants by ELISA. Boxplots of a total four independent experiments performed in triplicate cultures are shown. Significance ( $p < 0.05$ ) is denoted with (\*). Ns = not significant.

remain an important alarm for the immune system; its role in modulating the effector and memory responses of the adaptive immune system is also of major consequence.

The present study provides evidence that PCV2 can show both stimulatory and inhibitory properties regarding the induction of type I IFNs by pDC. This relates to previous work showing that different motifs of synthetic CpG-ODNs derived from the PCV2 genome could differentially modulate IFN- $\alpha$  responses in porcine PBMCs – four motifs were stimulatory and one inhibitory (Hasslung et al., 2003; Wikstrom et al., 2007). This study also confirms reports on other viruses including FMDV (Lannes and Summerfield, 2013) and PRRSV (Baumann et al., 2013), in which IFN- $\gamma$  contributes in favour of promoting pDC responses. Taken together, these observations support the idea that involvement of IFN- $\gamma$  could display a critical indirect role in sensing of viruses by the innate immune system. Therein, one can propose that during innate antiviral responses an important bidirectional crosstalk exists between natural killer (NK) cells and pDC – respectively important sources of IFN- $\gamma$  and IFN- $\alpha$  (Walzer et al., 2005; Wehner et al., 2011). Under such conditions, the pDC-derived IFN- $\alpha$  would promote NK-cell-derived IFN- $\gamma$  and vice versa. With respect to PCV2 pathogenesis, we conclude that the balance between ssDNA (genomic and encapsulated) and dsDNA (replicative forms) of PCV2 works together with the host cytokine environment in the recognition of PCV2 by the innate immune system. This determines the characteristics of the virus interplay with and impact on the innate immune defences, thus influencing the development of PCVD.

### Conflict of interest

None declared.

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6.2. Manuscript 2

*Impact of genotype 1 and 2 of porcine respiratory syndrome viruses on interferon- $\alpha$  responses by plasmacytoid dendritic cells*

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# Impact of genotype 1 and 2 of porcine reproductive and respiratory syndrome viruses on interferon- $\alpha$ responses by plasmacytoid dendritic cells

Arnaud Baumann<sup>1,2</sup>, Enric Mateu<sup>3</sup>, Michael P Murtaugh<sup>4</sup> and Artur Summerfield<sup>1\*</sup>

## Abstract

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) infections are characterized by prolonged viremia and viral shedding consistent with incomplete immunity. Type I interferons (IFN) are essential for mounting efficient antiviral innate and adaptive immune responses, but in a recent study, North American PRRSV genotype 2 isolates did not induce, or even strongly inhibited, IFN- $\alpha$  in plasmacytoid dendritic cells (pDC), representing “professional IFN- $\alpha$ -producing cells”. Since inhibition of IFN- $\alpha$  expression might initiate PRRSV pathogenesis, we further characterized PRRSV effects and host modifying factors on IFN- $\alpha$  responses of pDC. Surprisingly, a variety of type 1 and type 2 PRRSV directly stimulated IFN- $\alpha$  secretion by pDC. The effect did not require live virus and was mediated through the TLR7 pathway. Furthermore, both IFN- $\gamma$  and IL-4 significantly enhanced the pDC production of IFN- $\alpha$  in response to PRRSV exposure. PRRSV inhibition of IFN- $\alpha$  responses from enriched pDC stimulated by CpG oligodeoxynucleotides was weak or absent. VR-2332, the prototype genotype 2 PRRSV, only suppressed the responses by 34%, and the highest level of suppression (51%) was induced by a Chinese highly pathogenic PRRSV isolate. Taken together, these findings demonstrate that pDC respond to PRRSV and suggest that suppressive activities on pDC, if any, are moderate and strain-dependent. Thus, pDC may be a source of systemic IFN- $\alpha$  responses reported in PRRSV-infected animals, further contributing to the puzzling immunopathogenesis of PRRS.

## Introduction

Type I interferons (IFN), mainly IFN- $\alpha/\beta$ , are essential to the innate immune system for direct antiviral activity as well as efficient induction of adaptive immune responses [1,2]. This critical role is underlined by the fact that seemingly all viral pathogens have evolved strategies to counteract this innate defense system [3].

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV), an enveloped positive-sense, single-stranded RNA virus, has been associated with a low innate and delayed adaptive immune response [4]. The virus is characterized by an enormous genetic variability with the existence of two genotypes of PRRSV referred as genotype 1 (European) and 2 (North American), and

the emergence of highly virulent isolates in Asia within genotype 2 [5]. Macrophages in lung and lymphoid tissues are the primary site of PRRSV replication [6,7], although other cell types such as monocyte-derived dendritic cells (MoDC) and monocyte-derived macrophages are susceptible to infection [8,9]. Due to the persistence of PRRSV in infected pigs, it was proposed that the virus modulates host innate and acquired immune responses [10,11]. While PRRSV is highly sensitive to IFN- $\alpha$  both in vitro [12-14] and in vivo [15], the virus promotes weakly or not at all in vitro synthesis of type I IFN in porcine alveolar macrophages (PAM) and MoDC [16-18]. However, systemic IFN- $\alpha$  was observed after infections with various PRRSV isolates [15,16,19,20], indicating that certain cell types are able to sense infection.

Plasmacytoid dendritic cells (pDC) are a major source of IFN- $\alpha$  and other inflammatory cytokines after exposure to TLR7 and TLR9 ligands, including many viruses

\* Correspondence: artur.summerfield@ivi.admin.ch

<sup>1</sup>Institute of Virology and Immunoprophylaxis (IVI), Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland

Full list of author information is available at the end of the article

and bacterial DNA [21]. Although pDC are a rare cell type, they can produce around 100 times more IFN- $\alpha$  than any other cellular type. They are often able to sense viruses in the absence of viral replication. Consequently, they represent an important candidate cell type for investigating early immune events that could influence early control of virus replication or induction of adaptive antiviral immune response [22]. In the pig, these cells have been identified as CD4<sup>+</sup>CD123<sup>+</sup>CD135<sup>+</sup>CD172a<sup>+</sup>CD14<sup>-</sup>, which can be differentiated from monocytes, macrophages and MoDC which lack CD4, CD123 and CD135 but express CD14 and in the case of macrophages and monocyte subset also CD163 [23,24]. Interestingly, stimulation of pDC with genotype 2 PRRSV was reported not to result in detectable IFN- $\alpha$  release [25]. Moreover, the IFN- $\alpha$  production induced by CpG oligodeoxynucleotides (ODN) or transmissible gastroenteritis virus (TGEV) was potently inhibited by North American PRRSV [26]. Considering the observation of in vivo IFN- $\alpha$ , important differences in the virulence of genotype 1 and genotype 2, and the possible regulation of pDC responses by cytokines induced during PRRS infection, we examined how PRRSV of different genotypes and virulence interact with pDC and how cytokines influence pDC responses.

## Material and methods

### Viruses

For the European genotype 1 PRRSV we used Lelystad virus (LV; kindly obtained from Dr Gert Wensvoort, Central Veterinary Institute, Lelystad, The Netherlands) [27] and its counterpart adapted to grow in MARC-145 (LVP23; kindly obtained from Dr Barbara Thür, IVI, Switzerland), 2982, 3267 [28] and Olot/91 (passaged several times, kindly obtained from the PoRRSCon Consortium through Dr Luis Enjuanes, Universidad Autónoma, Madrid, Spain). For the genotype 2 PRRSV we employed the prototype VR-2332 [29] (ATCC, LGC Standards, Molsheim, France), SS144, MN184, JA-1262 and SY0608 (kindly obtained from Dr Martin Beer, Friedrich-Loeffler-Institut, Riems, Germany) representing a highly pathogenic field isolate in China from 2006 [30]. The PRRSV isolate SS144 is from a severe reproductive and respiratory outbreak with high levels of mortality in a previously PRRSV-negative herd in 2010, Missouri, USA. The MN184 isolate is from a farm experiencing severe reproductive disease and sow mortality in 2001, Minnesota, USA. The JA-1262 isolate was obtained in 2009 from a midwestern USA sow herd experiencing abortions and PRRSV-infected weaning piglets. Viral stocks of LV, 2982 and 3267 Spanish field isolates, MN184 and SS144 were propagated in PAM. Strains of Olot/91, LVP23 representing LV adapted to grow in MARC-145 cells after 23 passages, SY0608, JA-1262 and prototype VR-2332 were

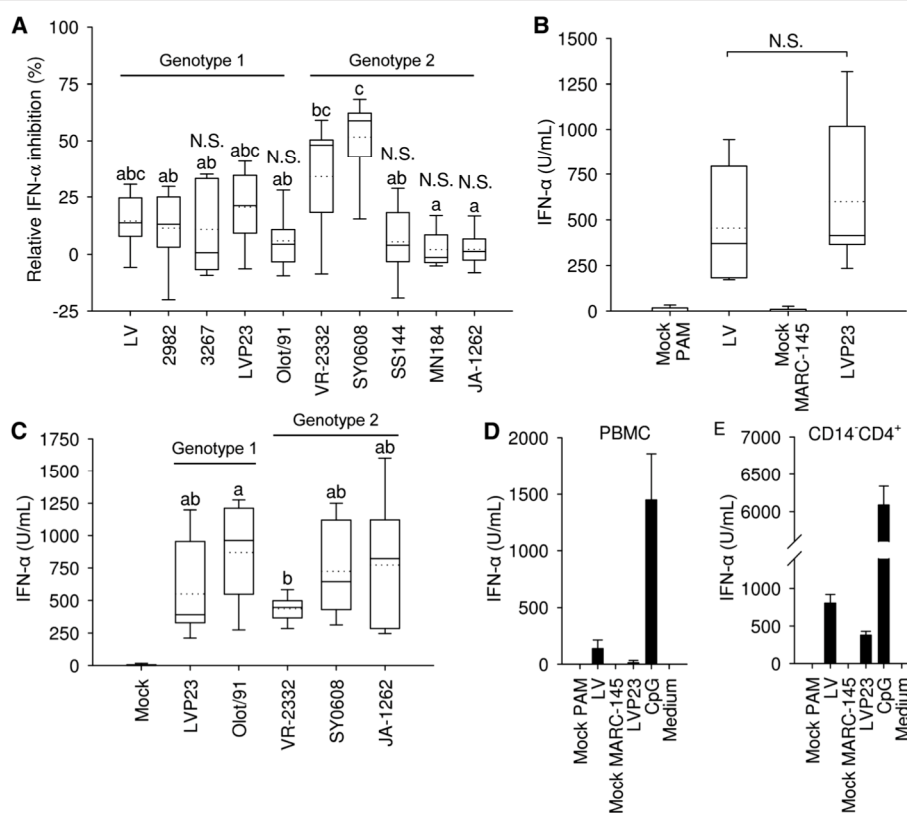
propagated in the MARC-145 cell line. Cells were lysed by freezing when 50% cytopathic effect (CPE) was reached, clarified by 2500 *g* centrifugation at 4°C for 15 min, and frozen at -70°C until use. Lysates from PAM or MARC-145 cells were used as mock-infected controls. All strains were titrated in their corresponding propagating cell type by CPE evaluation or by using the immunoperoxidase monolayer assay (IPMA) with PRRSV anti-nucleocapsid monoclonal antibodies (mAb) SDOW17-A or SR30-A (Rural Technology Inc., South Dakota, USA). Titers were calculated and expressed as 50% tissue culture infective dose per mL (TCID<sub>50</sub>/mL).

### Cells and pDC enrichment

MARC-145 cells (ATCC, LGC Standards, Molsheim, France) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Switzerland) supplemented with 10% fetal bovine serum (FBS; Biowest, France). PAM were obtained from bronchoalveolar lung lavages [31]. Specific-free pathogen (SPF) pigs from 6 week- to 12 month-old were euthanized and lungs were aseptically removed. Briefly, lungs were filled up with approximately one to two liters of PBS containing a 2× concentrated penicillin/streptomycin (Pen/Strep) solution (Gibco, Invitrogen, Switzerland). The lavage was collected and cells were recovered by centrifugation (350 *g*, 10 min, 4°C), followed by three wash steps with 2 × Pen/Strep PBS and centrifugation at 350 *g* for 10 min. PAM were maintained in RPMI 1640 medium (Gibco) supplemented with Pen/Strep and 10% FBS or frozen in liquid nitrogen until use. MARC-145 cells and PAM were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere. MoDC were prepared using interleukin-4 (IL-4)/granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described [32]. For all experiment except in Figure 1D and 1E, CD172a enrichment of pDC was performed as described earlier [33]. Briefly, peripheral blood mononuclear cells (PBMC) from 6 week- to 12 month-old pigs were isolated by Ficoll-Paque differential centrifugation [34] followed by CD172a (mAb 74-22-15a) enrichment using MACS sorting LD columns (Miltenyi Biotec GmbH, Germany) leading to > 80% of CD172a positive cells and 2-8% CD172<sup>low</sup>CD4<sup>high</sup> pDC. The cells were cultured in DMEM with 10% FBS and 20  $\mu$ M of  $\beta$ -mercaptoethanol (Invitrogen, Switzerland). For the experiment shown in Figure 1D and 1E, PBMC were depleted of monocytes by anti-CD14 (mAb CAM36A, VMRD Inc., Washington, USA) followed by CD4 (mAb PT90A, VMRD Inc.) selection with MACS sorting LS column (Miltenyi Biotec GmbH, Germany). This sorting resulted in a pDC purity of 10%.

### Stimulation of pDC and IFN- $\alpha$ ELISA

Enriched pDC were incubated at 400'000 per microwell with CpG-ODN D32 [33] (10  $\mu$ g/mL; Biosource Int.,



**Figure 1 Effects of PRRSV genotype 1 and 2 isolates on IFN-α responses of enriched pDC.** (A) PRRSV impact on CpG-induced IFN-α production by pDC. Enriched pDC were stimulated with CpG in presence of the indicated PRRSV isolates added at an MOI of 0.1 TCID<sub>50</sub>/cell. (B) Induction of IFN-α by prototype 1 LV and LVP23 (MOI of 1 TCID<sub>50</sub>/cell) in CD172a-enriched pDC (C) Comparative analysis of pDC IFN-α responses induced by various PRRSV strains (MOI of 1 TCID<sub>50</sub>/cell). (D-E) PBMC (D) and CD14<sup>+</sup>CD4<sup>+</sup> monocyte-depleted enriched pDC (E) stimulated with the prototype of genotype 1 LV, LVP23 and CpG-ODN as control. IFN-α was determined by ELISA in supernatants harvested after 20 h. Boxplots in A, B and C indicate the median (middle line), 25<sup>th</sup> and 75<sup>th</sup> percentiles (boxes), maximum and minimum (whiskers) and the mean values (dotted line) calculated from at least three independent experiments with cells from different animals each performed in culture triplicates. Bars in (D) and (E) indicate culture triplicates ± 1 standard deviation. One of two representative experiments is shown. For (A) and (C), significance between isolates are indicated by different letters based on an ANOVA on Ranks and Dunn's Method pairwise multiple comparison ( $P < 0.05$ ). In (A) not statistically significant suppression compared to mock-treated cells was determined by Mann-Whitney Rank Sum test ( $P < 0.02$ ) and noted N.S. = not significant.

Camarillo, USA) and PRRSV strains at a multiplicity of infection (MOI) of 0.1 to 2.5. Inactivation of PRRSV was performed in a UV chamber (Biorad; GS Gene Linker) at 100 mJ on ice. Virus inactivation was verified in MARC-145 cultures. Cytokine and other treatments included IFN-β (100 U/mL [35]), IFN-γ (10 ng/mL, R&D Systems, UK), Flt3-L (100 U/mL [24]), GM-CSF (100 U/mL [36]), IL-4 (100 U/mL [32]), TLR7 inhibitor IRS661 (5'-TGCTTGCAAGCTTGCAAGCA-3', Biosource Int. Camarillo, USA), and TLR7 agonist R837 (10 μg/mL; Biosource Int., Camarillo, USA, [37]). Secreted IFN-α after 20 h of incubation was measured by ELISA as described [38]. The relative proportion of stimulation or inhibition was calculated as the absolute percentage of  $100 - ((\text{IFN-}\alpha \text{ produced by PRRSV treated cells} + \text{CpG-ODN}) / (\text{IFN-}\alpha \text{ produced by mock-treated cells} + \text{CpG-ODN}) \cdot 100)$ .

#### Detection of PRRSV antigen and intracellular IFN-α by flow cytometry

To detect PRRSV replication, two million CD172a<sup>+</sup> cells were seeded in 24-well plates and infected with LV, 2982 and 3267 PRRSV strains at an MOI of 2.5 TCID<sub>50</sub>/cell. Cells were incubated for 24, 48 and 72 h and supernatants tested for IFN-α by ELISA. At each time point the cells were analyzed by three-color flow cytometry for expression of CD172a, CD4 and viral nucleocapsid protein. After staining with the cell surface marker followed by goat isotype specific anti-mouse fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE) conjugates (SouthernBiotech, Birmingham, AL, USA), the cells were fixed with 4% paraformaldehyde, washed and permeabilized with 0.3% (wt/vol) saponin in PBS. The anti-nucleocapsid mAb SDOW-17A was added during the permeabilization step for 15 min, followed by a wash

step with 0.1% (wt/vol) saponin and addition of biotinylated goat anti-mouse IgG1 conjugate (SouthernBiotech), diluted in 0.3% (wt/vol) saponin for 20 min at 4°C. After washing, Streptavidin SpectralRed® (SouthernBiotech) was added as fluorochrome for the FL3 channel. Electronic gating based on the forward/side scatter plots were applied to identify living cells and pDC were defined as CD172<sup>low</sup>CD4<sup>high</sup> and monocytes as CD172<sup>high</sup>CD4<sup>neg</sup> population [23]. For intracellular IFN-α staining, one million CD172a<sup>+</sup> cells were seeded in a 48-well plate and infected with LVP23 strain at an MOI of 1 TCID<sub>50</sub>/cell. After 12 h of culture, Brefeldin A (eBioscience, Austria) was added to the cells to block IFN-α secretion for 4 h. As positive control cells were stimulated with CpG-ODN for 2 h and incubated with Brefeldin A for further 4 h. Cells were then stained for surface CD4 and CD172a markers as mentioned above. Cell fixation and permeabilization for intracellular staining of IFN-α was performed with the Fix & Perm kit (Caltag, UK). Anti-IFN-α mAb F17 (0.3 µg/mL; R&D Systems), biotinylated goat anti-mouse IgG1 conjugate (SouthernBiotech) and Streptavidin SpectralRed® (SouthernBiotech) were added to the cells to detect intracellular IFN-α. The data were acquired using a FACScalibur and analysed using CellQuest Pro Software (BD Biosciences, Mountain View, CA, USA).

#### Statistical analysis

Data were analyzed by SigmaPlot 11.0 software. Significant differences between groups were assessed by the Kruskal-Wallis One Way Analysis of Variance (ANOVA on Ranks) and Dunn's Method pairwise multiple comparison ( $P < 0.05$  was considered significant). For significance of cytokine-enhancement experiment, the Mann-Whitney Rank Sum test was employed ( $P < 0.02$ ).

#### Results

##### No or weak suppression of IFN-α in enriched pDC by various strains of PRRSV

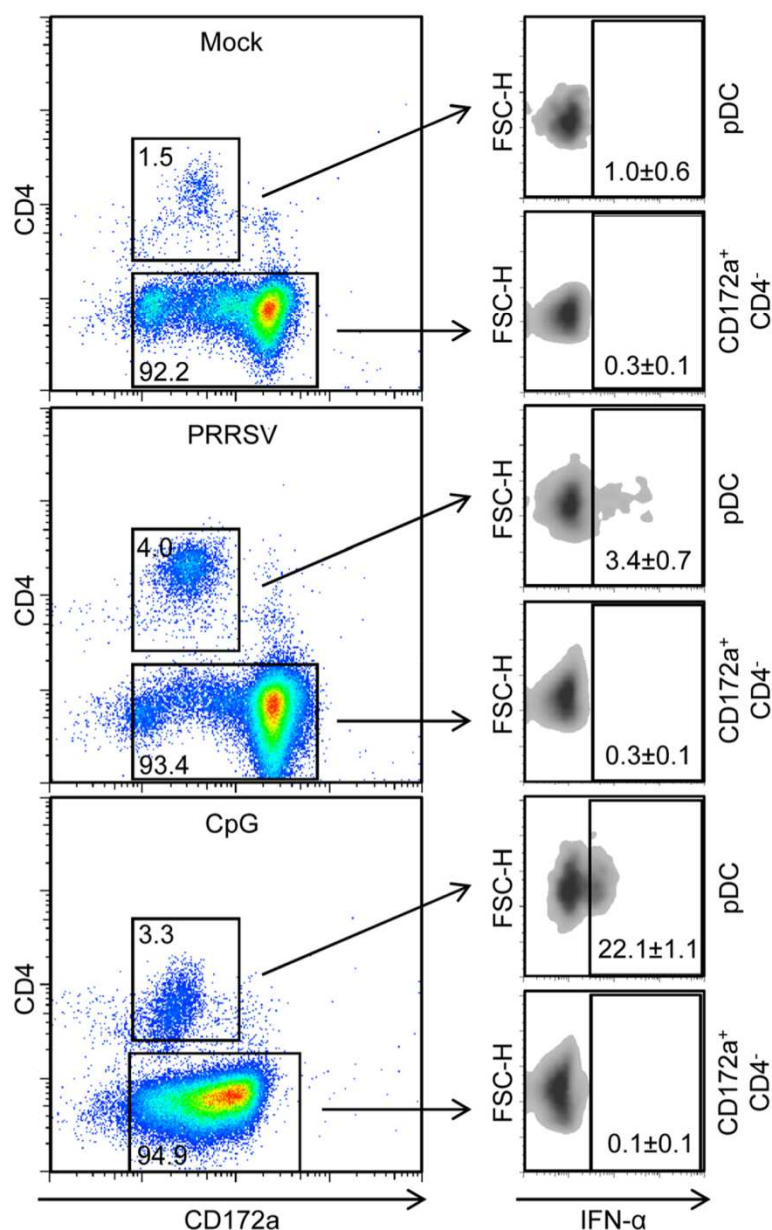
Considering the reported suppressive activity of PRRSV genotype 2 isolates on pDC activation [26], we compared the ability of virulent type 1 and type 2 PRRSV to suppress potent IFN-α induction by CpG-ODN. To this end, we simultaneously exposed enriched pDC to various PRRSV strains and CpG-ODN for 20 h and measured IFN-α in the supernatants. The highly pathogenic Chinese type 2 isolate SY0608 showed the highest inhibitory effect at 52% and was the only isolate with inhibitory activity in every replicate (Figure 1A). Type 2 isolate VR-2332 inhibited CpG-ODN induced IFN-α secretion by 34% and all other isolates, including highly virulent type 2 isolates SS144, MN184 and JA-1262, showed lower levels or no inhibitory activity (Figure 1A). Stated another way, CpG-ODN stimulated high levels of IFN-α secretion by pDC in the absence or presence of numerous PRRSV genotypes.

##### Genotype 1 and 2 PRRSV induce IFN-α in pDC

Since both type 1 and type 2 PRRSV isolates were not strongly suppressive, we analyzed their ability to directly activate pDC secretion of IFN-α. Incubation of CD172a<sup>+</sup> cells with LV or its MARC-145 cell-adapted form, LVP23, at an MOI of 0.1 did not elicit reproducible IFN-α expression (data not shown), but showed robust IFN-α production at an MOI of 1 (Figure 1B). To further investigate if pDC production of IFN-α was a universal response to PRRSV, type 2 strains VR-2332, JA-1262, a virulent recent USA field isolate, SY0608, a highly pathogenic field isolate from China, and the avirulent type 1 Olot/91 strain were incubated with enriched pDC. All isolates directly elicited IFN-α secretion by pDC, with type 2 PRRSV prototype VR-2332 displaying the lowest activity, whereas type 1 strain Olot/91 showing the highest average effect (Figure 1C). Overall, all isolates induced IFN-α secretion and the range of IFN-α production was independent of genotype or isolate virulence. To assess whether monocytes could be involved in the PRRSV-induced IFN-α secretion, we incubated unsorted PBMCs, CD14<sup>+</sup>, CD14<sup>+</sup>CD4<sup>-</sup> and CD14<sup>-</sup>CD4<sup>+</sup> sorted cells with LV, LVP23, and CpG-ODN as a positive control. Whereas no or only low levels of IFN-α were found in PBMCs after PRRSV exposure (Figure 1D), high amounts were detected in the CD14<sup>-</sup>CD4<sup>+</sup> cell fraction (Figure 1E). No IFN-α was detected in CD14<sup>+</sup> cells (data not shown) excluding the possibility that monocytes induce IFN-α in response to PRRSV. To confirm that pDC were indeed the source of IFN-α in enriched CD172a cells, CD4, CD172a and intracellular IFN-α staining was performed. It revealed that only a proportion of CD4<sup>+</sup> cells among the CD172a<sup>+</sup> fraction were IFN-α positive after PRRSV or CpG-ODN stimulation whereas no IFN-α expressing cells were observed in the CD172<sup>+</sup>CD4<sup>-</sup> cells (Figure 2). Together with the data shown in Figure 1E, these results demonstrate that CD172a<sup>+</sup>CD14<sup>-</sup>CD4<sup>+</sup> pDC [23] are the source of PRRSV-derived IFN-α responses. We also observed a higher percentage of pDC when they were stimulated by PRRSV (4%) compared to mock (1.5%) after 16 h of culture suggesting that PRRSV promotes survival of the pDC. It confirms that PRRSV interacted with pDC and promoted IFN-α secretion since the frequency of mock-treated pDC decreased in absence of stimulus.

##### PRRSV sensing by pDC does not require live virus and is mediated via TLR7

UV-inactivated type 1 LVP23 and type 2 VR-2332 PRRSV were used to evaluate if PRRSV-induced IFN-α responses in enriched pDC required live virus. As shown in Figure 3A, the intensity of IFN-α production was not altered by UV-inactivation indicating that the pDC response did not require replicating PRRSV. In the presence of IFN-γ, pDC-derived IFN-α secretion was



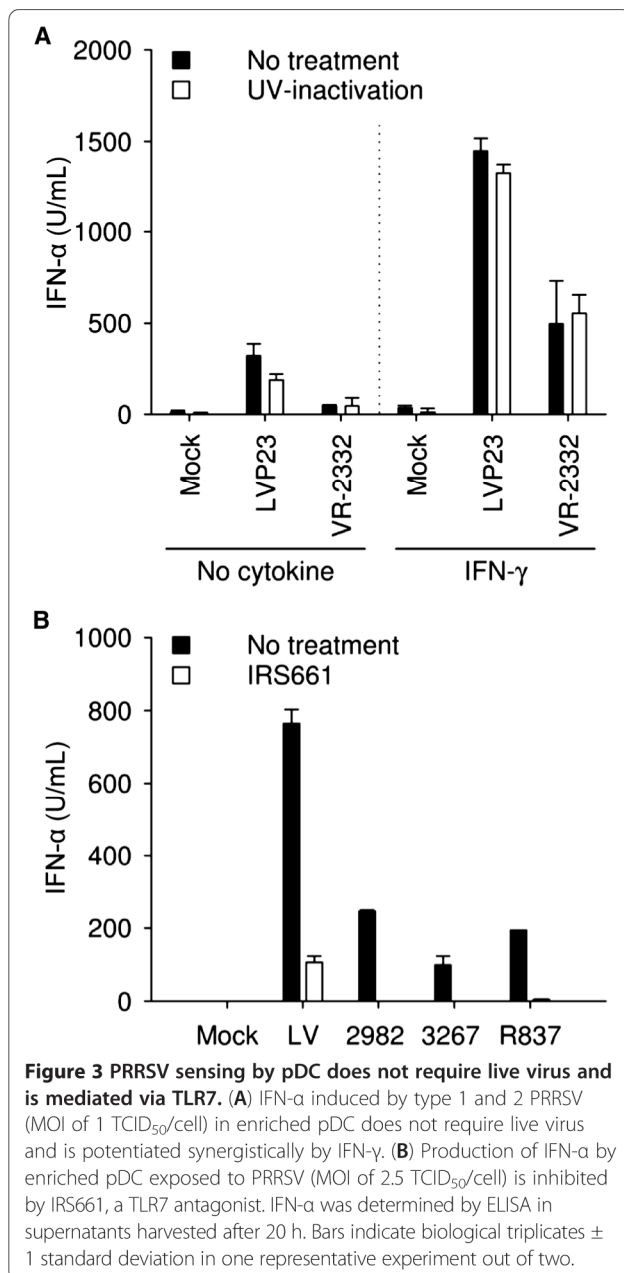
**Figure 2** IFN  $\alpha$  is induced by CD172<sup>low</sup>CD4<sup>high</sup> pDC. Intracellular staining of IFN- $\alpha$  was performed in CD172a enriched cells exposed to mock, PRRSV (MOI of 1 TCID<sub>50</sub>/cell) or CpG. Pseudo-color plot of pDC defined as CD172<sup>low</sup>CD4<sup>high</sup> and CD172<sup>+</sup>CD4<sup>-</sup> populations are gated (left panel) and density plot show that only gated pDC are positive for intracellular IFN- $\alpha$  (right panel) after PRRSV or CpG stimulation. Gate frequency is indicated as mean  $\pm$  1 standard deviation of one experiment performed in triplicate.

increased with both UV-untreated and UV-treated PRRSV (see also Figure 4). Considering the central role of TLR7 in sensing RNA viruses by pDC [39], we investigated the effects of the specific TLR7 inhibitor IRS661 [40] to inhibit PRRSV-induced IFN- $\alpha$  production. IRS661 is active on porcine cells and inhibits influenza virus-mediated pDC activation compared to a scrambled oligonucleotide [37]. We observed that pDC-derived IFN- $\alpha$  responses were drastically reduced or abrogated by TLR7 inhibition (Figure 3B), indicating that

the TLR7 pathway is intimately involved in pDC sensing of PRRSV.

#### Host factors enhancing PRRSV-induced IFN- $\alpha$ responses by pDC

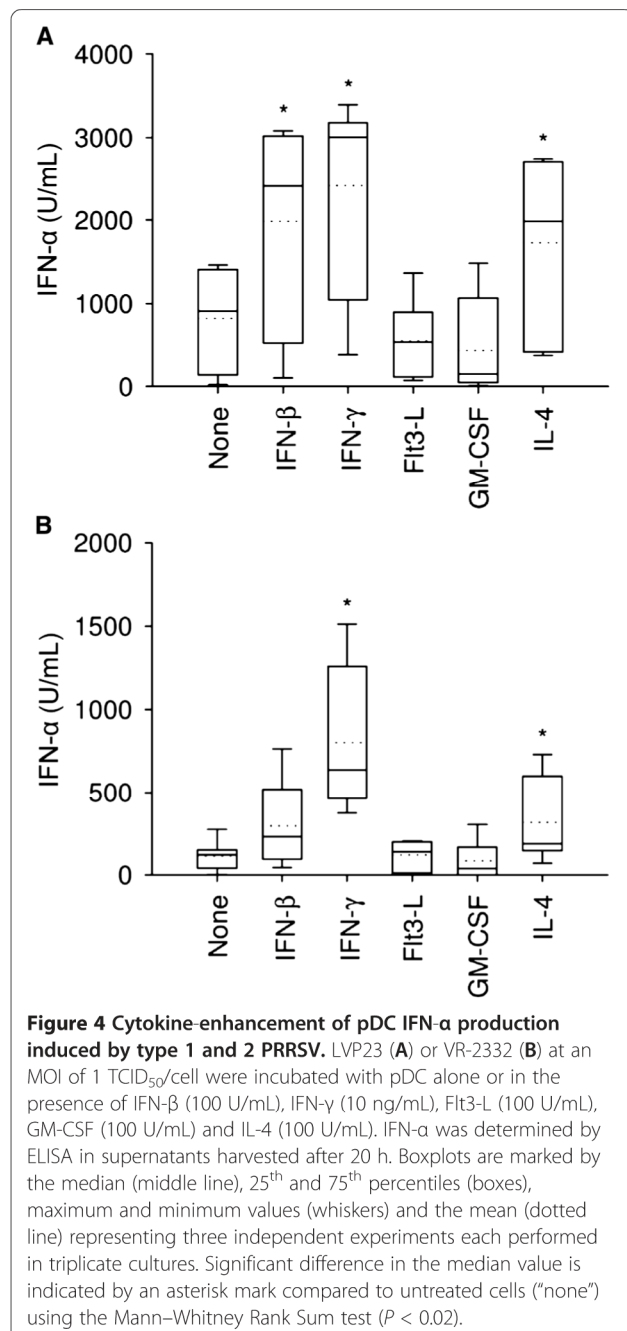
The function of pDC may be influenced by a cytokine microenvironment in vivo. Therefore, we were interested to determine the impact of cytokines on PRRSV-induced pDC activation. Type I and II IFN, Flt3-L, GM-CSF and IL-4, were evaluated for modulation of IFN- $\alpha$  secretion.



As shown in Figure 4A, LVP23-induced secretion of IFN-α was enhanced by IFN-β, IFN-γ and IL-4, whereas strain VR-2332 induced production was enhanced by IFN-γ and IL-4 (Figure 4B). These results show that cytokines known to modulate macrophage function were able to promote pDC responses to PRRSV.

#### PRRSV does not infect pDC

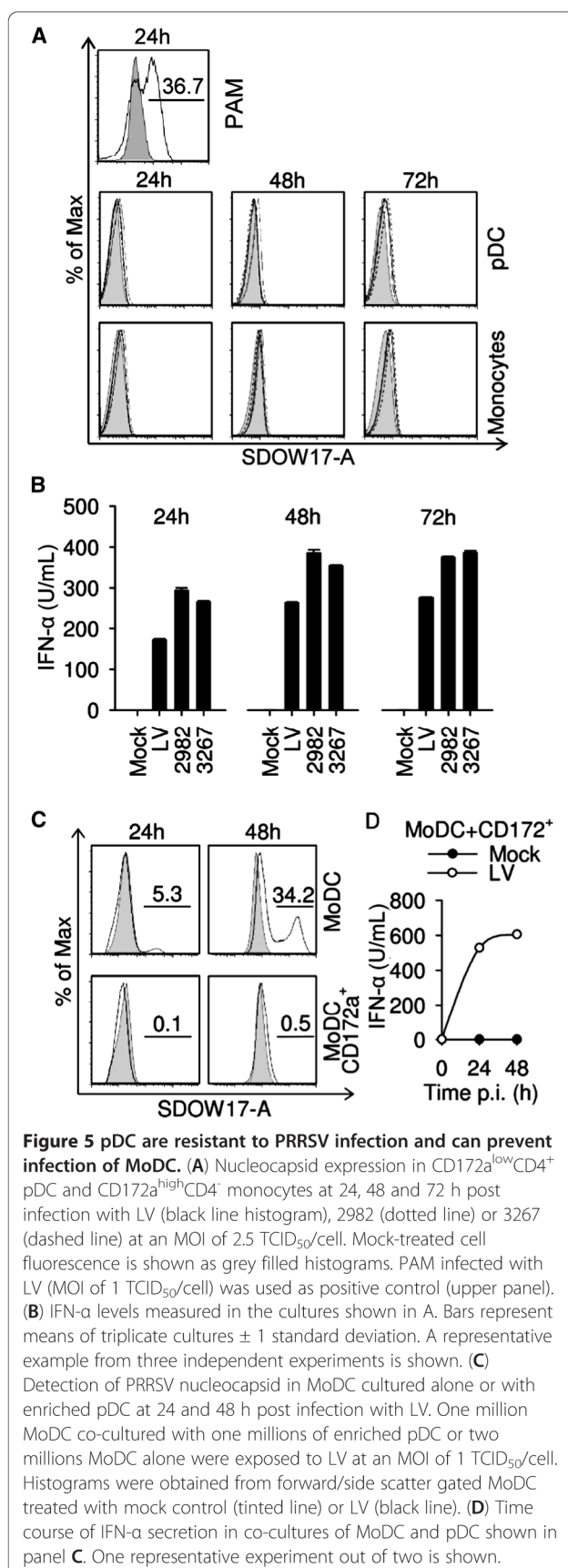
PRRSV infects macrophages, MoDC, and monocyte-derived macrophages in vitro [6,8,9]. However, sorted CD172a<sup>+</sup> cell populations of pDC were not permissive to infection. Flow cytometric three-colour analysis did not reveal the presence PRRSV nucleocapsid in gated



pDC or monocytes even after prolonged incubation with PRRSV for three days (Figure 5A). These results are consistent with a previous report employing GFP tagged PRRSV [26], indicating that pDC are not permissive to PRRSV. High levels of IFN-α were detected in these cultures, confirming that productive infection is not required for pDC sensing of PRRSV (Figure 5B).

#### pDC protect MoDCs from PRRSV infection

PRRSV appears to be highly sensitive to type I IFN [12-15]. Thus, we examined if IFN-α production by pDC



was able to protect permissive cells from PRRSV infection. MoDC were cultured with PRRSV alone or in the presence of enriched pDC. While MoDC alone were infected by PRRSV, it was prevented in the presence of enriched pDC (Figure 5C). Figure 5D shows that IFN-α was present in co-culture supernatants, but not in the supernatant of MoDC alone (data not shown). It must be noted that IFN-α is efficient to limit PRRSV replication in MoDC [12] however other important proteins and antiviral factors such as IFN-β might contribute to protection of the permissive MoDC against PRRSV.

## Discussion

In contrast to previous studies which showed that genotype 2 PRRSV are potent antagonists of type I IFN responses in pDC [26], we demonstrate that neither type 1 nor type 2 PRRSV isolates strongly inhibit CpG-ODN-induced IFN-α by enriched pDC. Furthermore, the prototype type 2 strain VR-2332, although more inhibitory than the genotype 1 viruses tested, was not as inhibitory as previously reported [26]. Indeed, porcine pDC respond positively to the presence of viable or inactivated PRRSV by secretion of IFN-α in amounts sufficient to protect permissive MoDC from PRRSV infection. Differences in animal age were not important, as we obtained similar results with 6 week-old and 1 year-old blood donors. However, the genetic of the pigs could possibly play a role since differential expression genes, especially *IFNA*, have been highlighted in phenotypic pig groups in response to PRRSV [41]. Another difference compared to the data from Calzada-Nova et al. could be the methodology and cell isolation procedures. Interestingly, the highly pathogenic Chinese isolate was the most suppressive virus tested, pointing to possible strain-dependent differences in the interaction of PRRSV with pDC which should be further investigated with similarly pathogenic viruses. The strain differences in the suppression of IFN-α might relate to certain PRRSV proteins that counteract downstream elements of the pathway [42].

By this work, we also demonstrated that both type 1 and type 2 PRRSV isolates induce pDC-derived IFN-α production which is mainly triggered by the TLR7 signaling pathway. Compared to other viruses, including TGEV [43,44], influenza virus [45], classical swine fever virus (CSFV) [46] and foot-and-mouth disease virus (FMDV) [37,47], tested in our laboratory using the same methodology, the levels of IFN-α induced by PRRSV could be classified as moderate. PRRSV is clearly a stronger inducer than CSFV or FMDV, but less potent than TGEV or influenza virus, which reach the same levels as CpG-ODN. The responses observed in vitro are relevant to in vivo conditions, in which systemic IFN-α or IFN-α secreting cells in the lung were reported in several pigs infected with various PRRSV isolates [15,16,19,20,48]. Also, Barranco et al.

reported increased numbers of non-identified IFN- $\alpha$  expressing cells by immunohistochemistry in lymph nodes of animals infected with the Spanish isolate 2982 [49]. Interestingly, the ability of PRRSV to induce IFN- $\alpha$  does not seem to be related to the suppression of TLR9 ligand-induced IFN- $\alpha$ . The highly pathogenic Chinese strain, SY0608, induced similar levels of IFN- $\alpha$  as did LVP23, but was more suppressive for CpG-induced IFN- $\alpha$  compared to all strains tested.

Host factors regulating pDC responses may be critical to anti-PRRSV responses, since the presence of different patterns of cytokines could promote an immunological micro-environment favoring or inhibiting pDC responses. For example, in mice Th1-matured pDC stimulated by TLR7 ligand respond more strongly with higher secretion of IL-6 and IFN- $\alpha$  than do Th2-pDC, but both Th1- and Th2-pDC were more strongly activated compared to unpolarized pDC [50]. As we show here in swine, both IFN- $\gamma$  and IL-4 enhance pDC responses to PRRSV. IFN- $\beta$  only enhanced pDC responsiveness to genotype 1 PRRSV; it is not yet known if this is a general feature of type 1 PRRSV or a strain-specific effect. The regulatory cytokine IL-10 was thought to strongly impact PRRSV pathogenesis and was one of the proposed mechanisms used by PRRSV to escape the innate and adaptive immune system. However, the induction of IL-10 by PRRSV is not clearly established [11,51], although its presence suppresses type I IFN induction in pDC [52,53]. It has been reported that PRRSV could induce IL-4 in the serum [20] and IFN- $\gamma$  both in the serum [54] and in the lungs [48] of infected pigs. In particular IFN- $\gamma$  could play multiple beneficial roles against PRRSV. It is known to have an antiviral activity against PRRSV [55], to generally promote natural killer and cytotoxic T-cell activity and to induce classical macrophages activation with increased anti-microbial functions, antigen-presenting activities and reduced ability to secrete regulatory cytokines such as IL-10 [56]. As IFN- $\alpha$  is known to promote IFN- $\gamma$ , our data showing that vice versa IFN- $\gamma$  also promotes IFN- $\alpha$  in pDC, it would appear that stimulating this axis will be beneficial for immunity against PRRSV. IL-4 on the other hand is known to promote the alternative pathway of macrophage activation resulting in "wound-healing" macrophages [56], which would be important to repair tissue damage in the lung during PRRSV infection. Through promoting pDC activation by PRRSV, this cytokine would also help establishing an antiviral state.

The presence of pDC prevented PRRSV infection and killing of MoDC, and thereby indirectly promoting cross-presentation of PRRSV antigens derived from apoptotic infected lung macrophages. This effect may be caused not only by IFN- $\alpha$  but also by other type I IFN's such as IFN- $\beta$ , known to be produced by pDC [57]. In addition to its antiviral activity, IFN- $\alpha$  is an efficient natural adjuvant

promoting adaptive immune responses [15,58]. For these reasons pDC responses induced by PRRSV are relevant to understanding antiviral immune responses and the pathogenesis of PRRS.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

AB and AS designed experiments. Experimental work performed by AB, EM and MM provided the viruses employed. AB, AS, EM and MM contributed to the manuscript preparation, revision and provided important intellectual input. All authors read and approved the final manuscript.

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#### Author details

<sup>1</sup>Institute of Virology and Immunoprophylaxis (IVI), Sensemettstrasse 293, 3147 Mittelhäusern, Switzerland. <sup>2</sup>Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland. <sup>3</sup>Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193, Bellaterra, Barcelona, Spain. <sup>4</sup>Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN 55108, USA.

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**CORRECTION**

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# Correction: Impact of genotype 1 and 2 of porcine reproductive and respiratory syndrome viruses on interferon- $\alpha$ responses by plasmacytoid dendritic cells

Arnaud Baumann<sup>1,2</sup>, Enric Mateu<sup>3</sup>, Michael P Murtaugh<sup>4</sup> and Artur Summerfield<sup>1\*</sup>

## Correction

After the publication of our article [1], we noted that the porcine reproductive and respiratory syndrome virus (PRRSV) strain referred to as SY0608 was not referred to correctly.

The correct reference for this PRRSV strain is RVB-581 [2,3], not SY0608.

We apologise for this oversight and any inconvenience this may have caused.

## Author details

<sup>1</sup>Institute of Virology and Immunoprophylaxis (IVI), Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland. <sup>2</sup>Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland. <sup>3</sup>Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193, Bellaterra, Barcelona, Spain. <sup>4</sup>Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, MN 55108, USA.

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\* Correspondence: [artur.summerfield@ivi.admin.ch](mailto:artur.summerfield@ivi.admin.ch)

<sup>1</sup>Institute of Virology and Immunoprophylaxis (IVI), Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland

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# Virulence and genotype-associated infectivity of interferon-treated macrophages by porcine reproductive and respiratory syndrome viruses

Obdulio García-Nicolás<sup>a,1</sup>, Arnaud Baumann<sup>b,c,1</sup>, Nathalie Jane Vielle<sup>b</sup>,  
Jaime Gómez-Laguna<sup>d</sup>, Juan José Quereda<sup>a</sup>, Francisco José Pallarés<sup>a</sup>, Guillermo Ramis<sup>a</sup>,  
Librado Carrasco<sup>d</sup>, Artur Summerfield<sup>b,\*</sup>

<sup>a</sup> Faculty of Veterinary Medicine, University of Murcia, "Mare Nostrum Excellence Campus – 37 38", Murcia, Spain

<sup>b</sup> Institute of Virology and Immunology, Mittelhäusern, Switzerland

<sup>c</sup> Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland

<sup>d</sup> Faculty of Veterinary Medicine, University of Córdoba, Campus Universitario de Rabanales, 'International Excellence Agrifood Campus, CeIA3', 14071 Córdoba, Spain

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## ABSTRACT

The polarization into M1 and M2 macrophages (MΦ) is essential to understand MΦ function. Consequently, the aim of this study was to determine the impact of IFN-γ (M1), IL-4 (M2) and IFN-β activation of MΦ on the susceptibility to genotype 1 and 2 porcine reproductive respiratory syndrome (PRRS) virus (PRRSV) strains varying in virulence. To this end, monocyte-derived MΦ were generated by culture during 72 h and polarization was induced for another 24 h by addition of IFN-γ, IL-4 or IFN-β. MΦ were infected with a collection of PRRSV isolates belonging to genotype 1 and genotype 2. Undifferentiated and M2 MΦ were highly susceptible to all PRRSV isolates. In contrast, M1 and IFN-β activated MΦ were resistant to low pathogenic genotype 1 PRRSV but not or only partially to genotype 2 PRRSV strains. Interestingly, highly virulent PRRSV isolates of both genotypes showed particularly high levels of infection compared with the prototype viruses in both M1 and IFN-β-treated MΦ ( $P < 0.05$ ). This was seen at the level of nucleocapsid expression, viral titres and virus-induced cell death. In conclusion, by using IFN-γ and IFN-β stimulated MΦ it is possible to discriminate between PRRSV varying in genotype and virulence. Genotype 2 PRRSV strains are more efficient at escaping the intrinsic antiviral effects induced by type I and II IFNs. Our *in vitro* model will help to identify viral genetic elements responsible for virulence, an information important not only to understand PRRS pathogenesis but also for a rational vaccine design. Our results also suggest that monocyte-derived MΦ can be used as a PRRSV infection model instead of alveolar MΦ, avoiding the killing of pigs.

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## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) has a high prevalence in intensive swine farming and causes reproductive failure in sows and respiratory disease in piglets and growing pigs. It is considered as one of the most economically significant swine diseases worldwide. PRRSV is classified in type 1 or PRRSV-1 (European genotype) and type 2 or PRRSV-2 (North American genotype) (Nelsen et al., 1999). Although these genotypes cause similar systemic clinical manifestations, there are

differences in the severity of respiratory signs (Martinez-Lobo et al., 2011), and a high inter- and intra-genotype genetic variability, with an average homology of only 60%. This high variability is seen for both non-structural (55–63%) and structural proteins (61–81%) (Murtaugh et al., 2010; Shi et al., 2010). In consequence, different PRRSV isolates present a high antigenic and pathogenic variability. The European PRRSV-1 genotype has been divided into a Western European Subtype I, a distinct Russian Subtype I and East European Subtypes II and III, whereas PRRSV-2 has been divided into at least nine lineages, including the highly pathogenic (HP)-PRRSV which emerged in 2006 in China (Shi et al., 2010).

PRRSV shows a restricted cell tropism for cells from the monocyte/macrophage lineage, the main *in vivo* target cells for replication being alveolar lung macrophages (MΦ), and other tissue MΦ (Van Breedam et al., 2010). Consequently, the interaction of the virus with this cell type is of major importance to understand

\* Corresponding author at: Institute of Virology and Immunology, Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland. Tel.: +41 31 8489377.

E-mail address: [artur.summerfield@ivi.admin.ch](mailto:artur.summerfield@ivi.admin.ch) (A. Summerfield).

<sup>1</sup> Equal contributors.

the pathogenesis. PRRSV has numerous pathways to prevent antiviral responses in MΦ as well as in cell lines used for experimental *in vitro* studies (Chand et al., 2012), but is sensitive to the action of many type I interferon (IFN)'s (Sang et al., 2010) as well as IFN-γ (Rowland et al., 2001). Despite the ability to suppress antiviral responses in MΦ *in vivo*, IFN-α is found both locally and systemically at early time points post infection (Albina et al., 1998; Dwivedi et al., 2012; Liu et al., 2010; van Reeth et al., 2002). This response is weak with low pathogenic PRRSV isolates but can reach high levels with HP-PRRSV isolates (Guo et al., 2013). *In vitro* studies indicate that plasmacytoid dendritic cells are likely to represent a source of IFN-α since they can be activated by PRRSV (Baumann et al., 2013). In addition, IFN-γ is found locally and systemically during PRRSV infection of pigs, although this is found at later time points, most likely reflecting adaptive T cell responses (Darwich et al., 2010).

MΦ, although all of monocyte origin, represent a heterogenic family of cells as a result of cellular differentiation in specific tissues and local microenvironments including cytokine milieu (Gordon, 2003). One functionally important classification of MΦ is based on their polarization into classically (M1) or alternatively (M2) activated MΦ. M1 differentiation is typically induced by IFN-γ and results in an inflammatory phenotype. These MΦ secrete high levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α in response to toll-like receptor (TLR) ligands. Furthermore, they have a reduced phagocytic activity but enhanced antigen-presenting functions. IL-4 and IL-13 induce the alternative MΦ activation (M2), which have more anti-inflammatory and tissue re-modelling functions. Typically, M2 MΦ produce more IL-10, are highly phagocytic, but inefficient at antigen presentation (Mosser, 2003).

Considering the central importance of MΦ for PRRS pathogenesis and the role of IFNs and cytokines in regulating MΦ, we hypothesized that PRRSV of different virulence should differ in their interaction with cytokine-modulated MΦ. To test this, IFN-γ-(M1), IL-4-(M2) and IFN-β-activated MΦ were evaluated for their susceptibility to different PRRSV-1 and PRRSV-2 strains as well their ability to secrete cytokines after infection.

## 2. Materials and methods

### 2.1. Monocyte-derived MΦ

Monocyte-derived MΦ were generated as previously described (Carrasco et al., 2001). Briefly, peripheral blood mononuclear cells were isolated from 3 to 12 months old specific pathogen-free (SPF) pigs using ficoll-paque density centrifugation (1.077 g/l; Amersham Pharmacia Biotech). Monocytes were then enriched by CD172a (clone 74-22-15A, hybridomas kindly given by Dr. A. Saalmüller, Veterinary University of Vienna, Austria) positive selection using the magnetic cell sorting system (MACS) with LS columns (Miltenyi Biotec GmbH, Germany), and seeded in 24-well culture plates at a density of  $5 \times 10^5$  cells per well in Dulbecco's modified Eagle's medium with Glutamax (Life Technologies, Switzerland) and 10% heat-inactivated porcine serum (Sigma Chemicals, Switzerland). The cells were cultured at 39 °C with 5% CO<sub>2</sub> for 72 h and then stimulated by using either IFN-γ (10 ng/ml, R&D Systems, UK), IL-4 (100 U/ml, own production, (Carrasco et al., 2001), IFN-β (100 U/ml, own production, (Husser et al., 2012) or were left untreated for another 24 h. These MΦ were termed "IFN-γ MΦ", "IL-4 MΦ", "IFN-β MΦ" or MΦ, respectively.

### 2.2. Viruses

As a genotype 1 PRRSV we used Lelystad virus adapted to grow in MARC-145 (LVP23; kindly obtained from Dr. Barbara

Thür, IVI, Switzerland), two Spanish field isolates 2982, 3267 (kindly provided by Dr. Enric Mateu, Centre de Recerca en Sanitat Animal-CReSA, Barcelona, Spain) (Gimeno et al., 2011) and Olot/91 (passaged several times, kindly obtained from the PoRRSCon Consortium through Dr. Luis Enjuanes, Universidad Autónoma, Madrid, Spain). The HP-PRRSV Lena strain (kindly provided by Prof. Hans Nauwynck, Ghent University, Belgium) which belongs to Eastern European subtype III (Karniychuk et al., 2010) was also included. As a genotype 2 PRRSV we used VR-2332 (Collins et al., 1992) (ATCC, LGC Standards, Molsheim, France), JA-1262, MN184, SS144 (kindly obtained from Dr. Michael Murtaugh, University of Minnesota, St. Paul, MN, USA) and RVB-581 (kindly obtained from Dr. Martin Beer, Friedrich-Loeffler-Institut, Riems, Germany) (Wernike et al., 2012) representing a highly pathogenic field isolated in China. The PRRSV isolate SS144 was isolated in 2010 from a severe PRRS outbreak with high mortality rates in Missouri, USA. The MN184 isolate was obtained in 2001 from a swine farm experiencing severe reproductive disorder and sow mortality in Minnesota, USA. JA-1262 represents a 2009 Midwestern US isolate from a sow herd enduring abortions and holding infected piglets. Lena, 2982, 3267, MN184 and SS144 were propagated in alveolar MΦ isolated and cultured as described (Basta et al., 2000). Strains of Olot/91, LVP23, RVB-581, JA-1262 and VR-2332 were propagated in the MARC-145 cell line (ATCC, LGC Standards, Molsheim, France) cultured in DMEM GlutaMax supplemented with 10% of foetal bovine serum (Biowest, France). Viral stock was obtained from cells lysed by freezing when 50% cytopathic effect (CPE) was reached, clarified by  $2500 \times g$  centrifugation at 4 °C for 15 min, and stored at -70 °C. Lysates from uninfected MΦ or MARC-145 cells were used as mock-infected controls. All strains were titrated in their corresponding propagating cell type by CPE evaluation or by using the immunoperoxidase monolayer assay (IPMA) with PRRSV anti-nucleocapsid monoclonal antibody (mAb) SDOW17-A (Rural Technology Inc., South Dakota, USA). Titres were calculated and expressed as 50% tissue culture infective dose per ml (TCID<sub>50</sub>/ml). All inocula were tested for PCV2 by qPCR as described by Olvera et al. and found to be negative (Olvera et al., 2004).

### 2.3. PRRSV infection of MΦ

MΦ were infected at a multiplicity of infection (MOI) of 0.1 TCID<sub>50</sub>/cell in medium containing 10% SPF pig serum, free of antibodies against PRRSV. Mock-treated MΦ were included as controls. Virus adsorption was allowed for 1 h before the cells were washed three times with PBS, new cell culture medium was added and the cells were cultured at 39 °C. Culture supernatants were collected at 0, 6, 12, 16, 20 and 24 h post-infection (hpi) for virus titrations, PRRSV nucleocapsid (N) expression or cytokine measurements. All experiments were performed in triplicates and repeated at least two times with different blood donors.

### 2.4. Cytokines measurements

Supernatants of MΦ were harvested at 20 hpi. IFN-α production was determined by in-house ELISA (Guzylack-Piriou et al., 2004). TNF-α and IL-10 expression was measured using commercial kits (R&D Systems, UK). The detection limits are given at 30 and 60 pg/ml, respectively.

### 2.5. Monoclonal antibodies and flow cytometry

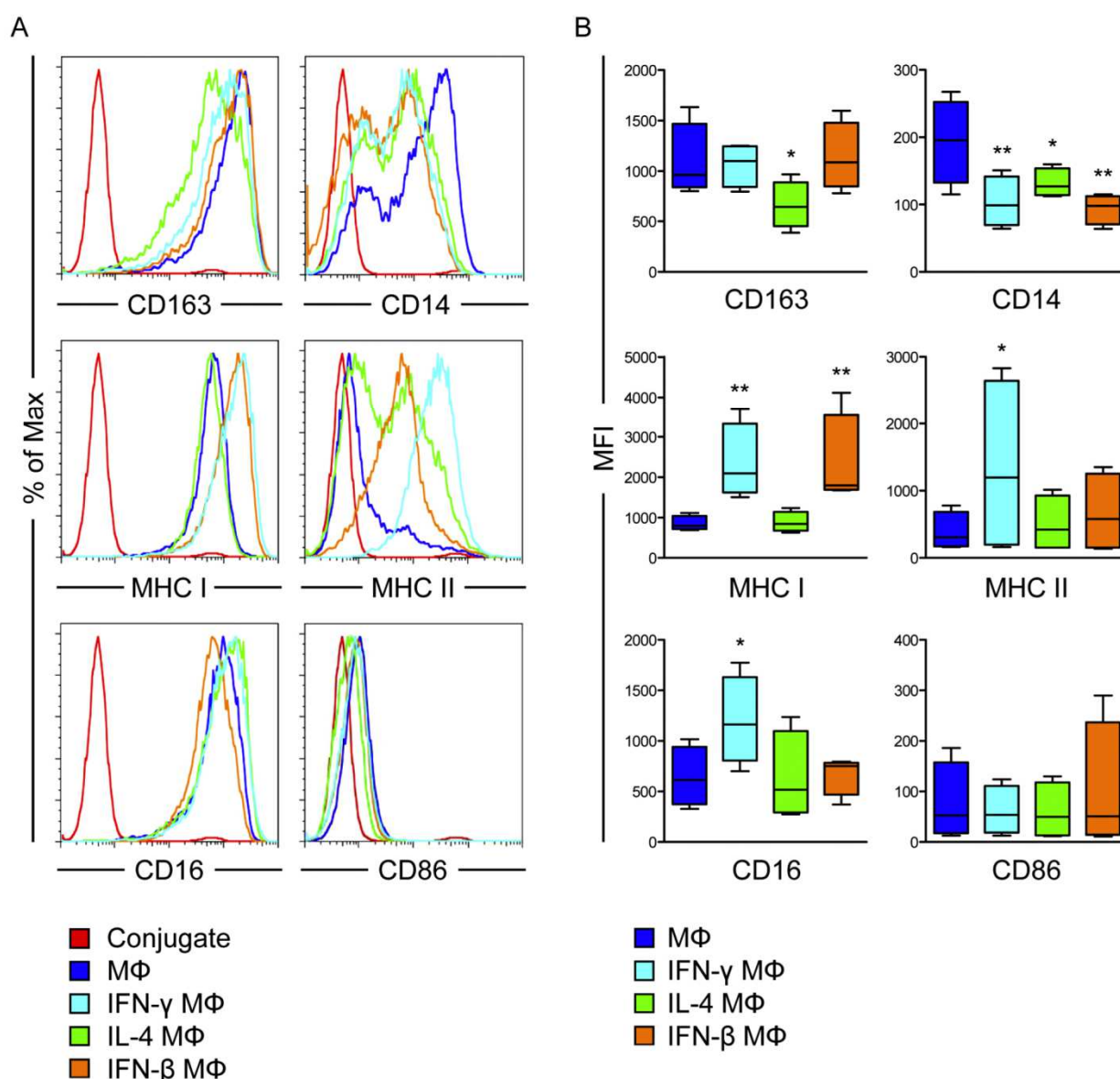
The following mouse anti-pig cell surface antibodies were used: anti-CD172a (clone 74-22-15A), anti-CD163 (clone 2A10/11; AbD Serotec, Puchheim, Germany), anti-α-MHC I and anti-MHC II (clone 74-22-15A, 74-11-10 and MSA3, respectively, hybridomas kindly

given by Dr. A. Saalmüller, Veterinary University of Vienna, Austria), anti-CD86 (clone HA5.2B7, Beckman Coulter, Nyon Switzerland), anti-CD16 (clone G7; AbD Serotec) and anti-CD14 (clone CAM36A; VMRD, Inc., Pullman, WA, USA). Specific goat anti-mouse IgG conjugated with R-phycoerythrin (RPE) (Dako, Zug, Switzerland) was used as secondary antibody. At each time point post infection the cells were seeded and fixed with 4% paraformaldehyde, washed and permeabilized with 0.3% (wt/vol) saponin in PBS. For detection of the PRRSV N protein the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed and then permeabilized with 0.3% (wt/vol) saponin, during which staining with the monoclonal antibody SDOW17-A (Rural Technologies Inc., South Dakota, USA) was performed for 15 min on ice. Acquisition was done on a FACSCalibur (Becton Dickinson, Mountain View,

CA, USA). Electronic gating based on the forward/side scatter plots was applied to identify living cells using the FlowJo V.7.2.5 software (Tree stars Inc., Ashland, OR, USA).

## 2.6. Statistical analysis

Figures and statistical analyses were done with GraphPad Prism V.5 software (GraphPad Software, San Diego, California, USA). Significance of PRRSV N expression was indicated by different letters based on Kruskal–Wallis tests. Significant effects of cytokines compared to unpolarized MΦ (Fig. 1b, Table 1 and data not shown) were assessed by repeated measures ANOVA and Dunnett's Multiple Comparison tests.  $P < 0.05$  was at least considered statistically significant.



**Fig. 1.** Differential expression of cellular markers after MΦ polarization. (A) MΦ were incubated with IFN-γ (IFN-γ MΦ), IL-4 (IL-4 MΦ), IFN-β (IFN-β MΦ) or with medium only (MΦ) for 24 h, and CD163, CD14, MHC I, MHC II, CD16 and CD86 were measured by flow cytometry. The data are representative of four independent experiments performed in culture triplicates. (B) Mean fluorescence intensity (MFI) of unpolarized MΦ, IFN-γ MΦ, IL-4 MΦ and IFN-β MΦ shown in A. Boxplots represent four independent experiments performed in culture triplicates. Significant differences compared to unpolarized MΦ were denoted by \* and \*\* where  $P < 0.05$  and  $P < 0.01$  respectively.

**Table 1**

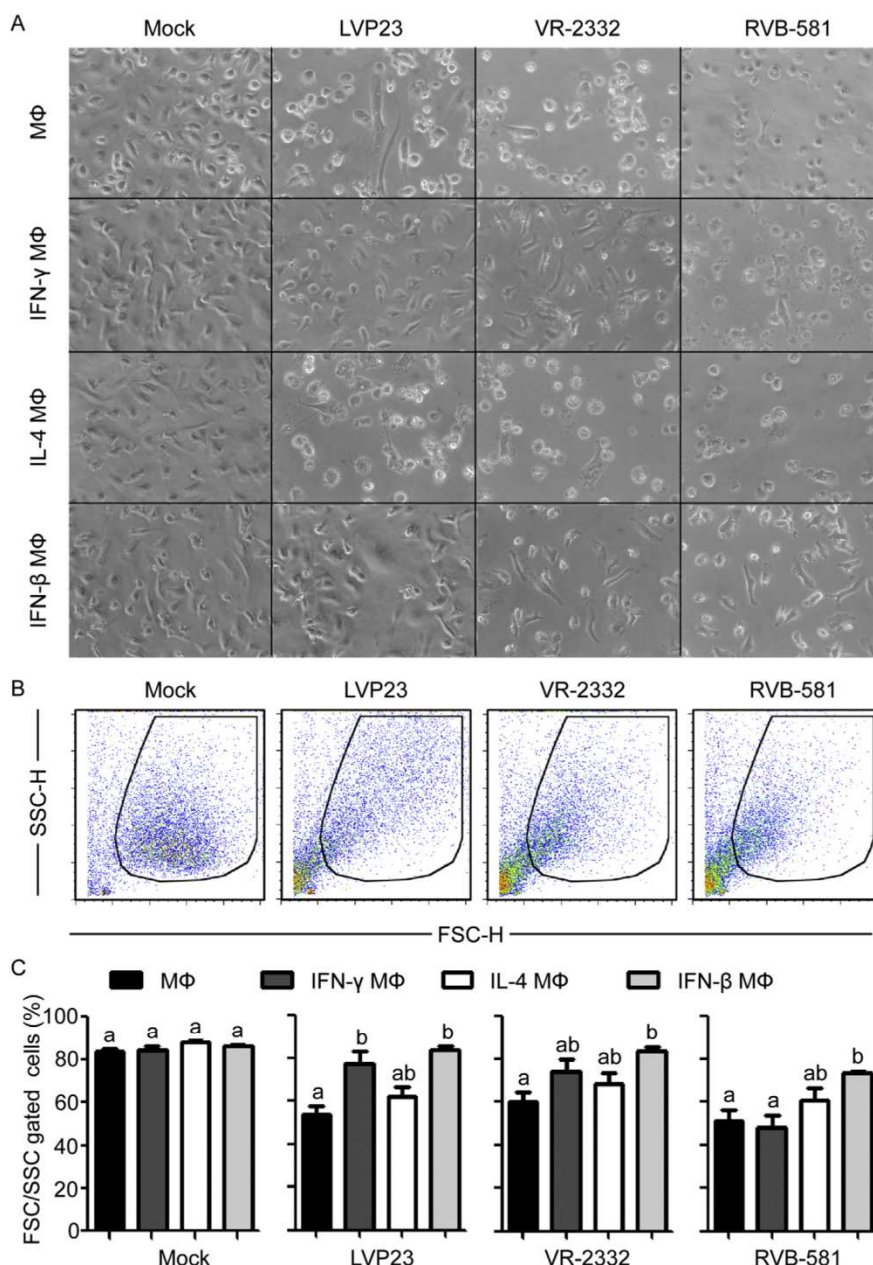
MΦ polarization with IFN-β and IFN-γ impacts on PRRSV infection and viral titre.

		MΦ	IFN-γ MΦ	IL-4 MΦ	IFN-β MΦ
PRRSV N <sup>+</sup> cells (%) <sup>a</sup>	LVP23	55.73 ± 9.07	7.67 ± 7.59 <sup>*</sup>	43.1 ± 4.42	17.81 ± 15.95 <sup>*</sup>
	VR-2332	65.68 ± 4.89	18.3 ± 7.48 <sup>*</sup>	57.23 ± 1.24	32.83 ± 24.44 <sup>*</sup>
	RVB-581	74.7 ± 10.49	52.48 ± 15.03	75.45 ± 9.29	59.98 ± 24.13
Titre log <sub>10</sub> (TCID <sub>50</sub> /ml) <sup>b</sup>	LVP23	5.31 ± 0.12	3.81 ± 0.24 <sup>*</sup>	6 ± 0.35	4 ± 0.58 <sup>*</sup>
	VR-2332	6.12 ± 0.14	4.94 ± 0.37 <sup>*</sup>	5.87 ± 0.25	4.81 ± 0.37 <sup>*</sup>
	RVB-581	5 ± 0.41	4.25 ± 0.46	5.62 ± 0.32	4.56 ± 0.51

<sup>a</sup> Percentage of PRRSV N<sup>+</sup> cells at 16 hpi (MOI of 0.1 TCID<sub>50</sub>/cell).

<sup>b</sup> Viral titre at 16 hpi ± standard deviation of two independent experiments performed in culture duplicates are shown.

<sup>\*</sup> Significant difference compared to unpolarized MΦ using ANOVA and Dunnett's multiple comparison test ( $P < 0.05$ ). The data are from the kinetic experiment (Fig. 5).



**Fig. 2.** Cell morphology and FSC/SSC of MΦ after PRRSV exposure. (A) Photography (400×) of MΦ, IFN-γ MΦ, IL-4 MΦ and IFN-β MΦ infected at an MOI of 0.1 TCID<sub>50</sub>/cell with LVP23, VR-2332, RVB-581 or treated with mock. Phase-contrast microscopy analysis was performed at 20 hpi. (B and C) FSC/SSC data of PRRSV infected MΦ. B. FSC/SSC plot of unpolarized MΦ. FSC/SSC plot with gating to exclude shrunken (dead) cells and debris at 20 hpi with LVP23, VR-2332, RVB-581 (MOI of 0.1 TCID<sub>50</sub>/cell) or mock treatment. (C) Percentage of FSC/SSC-gated MΦ defined in B with bars show the mean ± standard deviations of three independent experiments performed in culture triplicates. Different letters indicate statistically significant differences ( $P < 0.05$ ).

### 3. Results

#### 3.1. MΦ phenotype

In order to determine the impact of the selected cytokines on MΦ differentiation, CD163, CD14, MHC I, MHC II, CD16 and CD86 were measured by flow cytometry (Fig. 1a). The PRRSV receptor CD163 was significantly down-regulated by IL-4 as shown in Fig. 1b. The expression of CD14 was down-regulated by each cytokine. As expected, MHC I was up-regulated by IFN-β and IFN-γ but not by IL-4. MHC II was induced by IFN-γ whereas IFN-β and IL-4 did not significantly influence MHC II expression. Similarly, CD16 was upregulated by IFN-γ only. Finally, CD80/86 remained at low level even after cytokine stimulation. In conclusion, while these results demonstrate that the cytokines used modulate MΦ, the main effect of the IFN's was found to be an upregulation of MHC I and MHC II, whereas the main effect of IL-4 was found to be a downregulation of CD163.

#### 3.2. MΦ viability

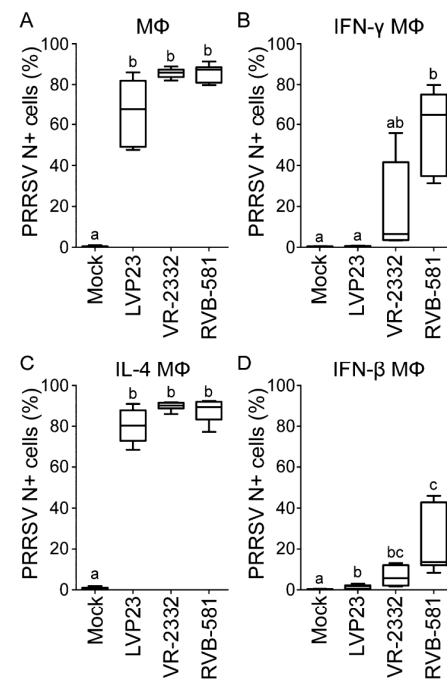
To measure MΦ, we evaluated the cultures by microscopy but did not employ a cell viability stain such as propidium iodide, as MΦ are highly phagocytic and rapidly remove necrotic and apoptotic cells. The viability of MΦ varied depending on the MΦ activation and the PRRSV strain used (Fig. 2A). As determined by the disappearance of adherent MΦ and presence of detached shrunken cells with highly granular cytoplasm vesicles and cell debris, undifferentiated MΦ and “IL-4 MΦ” were sensitive to all PRRSV strains. Infection with VR-2332 and RVB-581 strain particularly destroyed the MΦ. In contrast, IFN-γ protected the MΦ against cytopathogenic effects induced by LVP23 and VR-2332 but not against RVB-581. “IFN-β MΦ” were the most resistant against all 3 viruses, although the density of MΦ was reduced as well after infection with VR-2332 and RVB-581.

To quantify MΦ viability we employed the FSC/SSC profiles obtained by flow cytometry. To this end, an electronic gate was defined in the mock-treated MΦ. After infection, many MΦ had strongly reduced FSC/SSC and remained outside of this gate implying a shrinking process or cellular disintegration (Fig. 2B). As shown in Fig. 2C, the cytokine treatments did not influence the percentage of MΦ in the FSC/SSC gate. This analysis confirmed that all viruses reduced MΦ viability of undifferentiated MΦ. IFN-γ induced good protection against LVP23 and VR-2332 but not RVB-581. IFN-β induced complete protection against LVP23 and VR-2332 but only partial protection against RVB-581. IL-4 appeared to induce a partial resistance against all viruses.

#### 3.3. MΦ susceptibility to PRRSV infection

Considering these results, we tested MΦ infection by quantification of the nucleocapsid (N) expression by flow cytometry. Undifferentiated MΦ and “IL-4 MΦ” were highly susceptible to all PRRSV tested with higher infection rates obtained with the PRRSV-2 isolates when compared to LVP23, although this was not statistically significant (Fig. 3A and C). In contrast, IFN-γ and IFN-β almost completely prevented MΦ infection by LVP23 but only partially against VR-2332 and RVB-581 (Fig. 3B and D). RVB-581 was significantly more resistant to the antiviral effects of both IFN's when compared to LVP23.

Taking this into account, we performed a kinetic analysis of PRRSV infection and replication in MΦ. As for LVP23 infection, the results confirmed that both IFN prevented the expression of N protein in MΦ, but did not completely stop virus replication (Fig. 4A–D, left panels). Nevertheless, the highest titres found at 16 hpi were reduced by 1.5 logs by IFN-γ and 1.3 logs by IFN-β

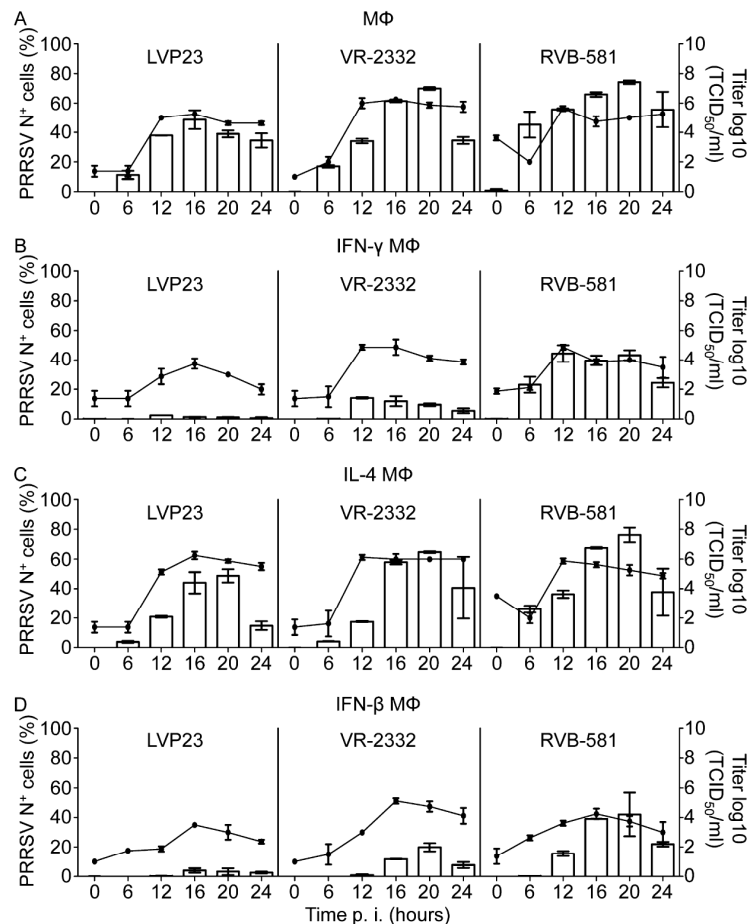


**Fig. 3.** Effect of cytokine polarization on MΦ susceptibility to infection with LVP23, VR-2332 and RVB-581. Unpolarized MΦ (A), IFN-γ MΦ (B), IL-4 MΦ (C) and IFN-β MΦ (D) were infected with LVP23, VR-2332, RVB-581 or mock control for 20 h. Percentage of PRRSV nucleocapsid (N) expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N<sup>+</sup>) cells of three independent experiments performed in culture triplicates. The different letters indicate significant differences between viruses ( $P < 0.05$ ).

(Table 1). As for VR-2332, again a clear antiviral effect was found with the IFN-γ and IFN-β both at the level of N expression and viral titres (Fig. 4A–D, middle panels, Table 1). Nevertheless, the reduction in N<sup>+</sup> MΦ and viral titres was lower when compared to LVP23 infected MΦ (Table 1). As for RVB-581, the IFN's only had a minor effect on the peak of N<sup>+</sup> MΦ and on viral titres (Fig. 4A–D, right panels and Table 1). These findings confirm that PRRSV isolates differ in their IFN sensitivity, with LVP23 being the most sensitive isolate and the HP isolate RVB-581 the most resistant.

We consequently tested more PRRSV-1 and PRRSV-2 isolates of different virulence to confirm these findings. Statistical analysis of at least two independent experiments demonstrated that all PRRSV-2 isolates had a significantly higher resistance to IFN-γ when compared to LVP23, while this discrimination was not possible in undifferentiated MΦ (Fig. 5 A and B). Furthermore, the Chinese HP PRRSV isolate RVB-581 was significantly more resistant than all PRRSV-1 isolates. In undifferentiated MΦ, the infection rates by the Olot/91 and the Spanish field isolate 2982 were lower than with the other viruses tested but this was not observed in the IFN-γ- and IFN-β-treated MΦ. Differences in infection rates were observed in the “IL-4 MΦ” as well but these did neither relate to genotype nor virulence (Fig. 5C). It appeared also that differences relating to virulence and genotypes could be found using “IFN-β MΦ” (Fig. 5D). PRRSV-2 isolates were more resistant to the effect of IFN-β but the PRRSV-1 isolate 2982 did not differ from this group. A statistical analysis demonstrated that both IFN-γ and IFN-β significantly reduced the N expression for all isolates compared to MΦ treated with medium only.

These findings pointed on a genotype-dependent resistance in IFN-treated MΦ. Considering that PRRSV-2 is generally more virulent than PRRSV-1, an alternative interpretation would be an



**Fig. 4.** Kinetic of N expression and virus titre in polarized MΦ infected with LVP23, VR-2332 and RVB-581. Unpolarized MΦ (A), IFN-γ MΦ (B), IL-4 MΦ (C) and IFN-β MΦ (D) were infected with LVP23, VR-2332 and RVB-581 (MOI of 0.1 TCID<sub>50</sub>/cell) for the indicated time on the x-axis. Bars indicate the mean ± standard deviation of PRRSV nucleocapsid expression (%N<sup>+</sup> cells, left y-axis) measured by flow cytometry. The dotted shows viral titre (TCID<sub>50</sub>/ml, right y-axis) measured in the supernatants. One out of two representative experiments in duplicate is shown.

inverse relationship of resistance to virulence independent of the genotype. We therefore tested HP-PRRSV isolate Lena, a PRRSV-1 belonging to subtype III in MΦ polarized by IFN-γ and IFN-β. The levels of N expression in MΦ infected with Lena were clearly and significantly higher than LVP23, and at least as high as those reached by RVB-581 (Fig. 6A and B). From these data, we propose that IFN-treated MΦ may allow to discriminate PRRSV isolate also relating to their virulence.

#### 3.4. Production of cytokines and IFN-α in infected MΦ

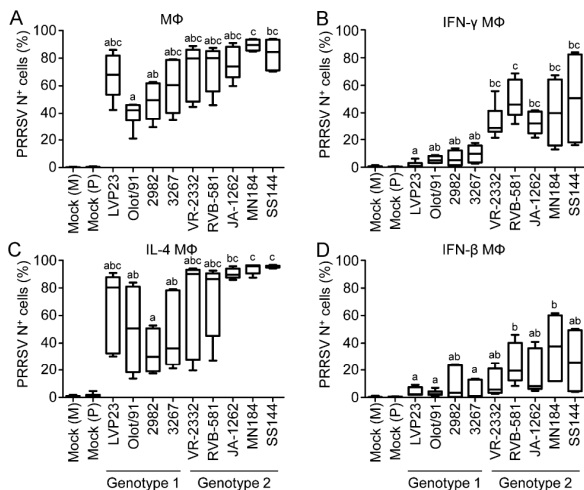
We did not find TNF-α or IL-10 production in any of the supernatants harvested from the infected MΦ. Whereas TNF-α was induced by LPS (10 μg/ml) in undifferentiated MΦ (2.3 ng/ml), IFN-γ MΦ (4.3 ng/ml), IL-4 MΦ (4.1 ng/ml) and IFN-β MΦ (3.3 ng/ml), no detectable amounts of IL-10 were found in any types of LPS-stimulated MΦ. Furthermore, IFN-α was not detected with one exception. LVP23 induced IFN-α in “IFN-γ MΦ” at 20 hpi (82.65 U/ml,  $P < 0.05$ ; data not shown).

#### 4. Discussion

Differentiated MΦ exhibit marked phenotypic and functional heterogeneity due to polarization depending on the imprinting by

the environment and the phase of the immune response (Gordon and Taylor, 2005; Mosser and Edwards, 2008). At least three distinct functional states have been proposed, classically activated M1, alternatively activated M2 and regulatory MΦ, also called M2b or M2-like. In mouse and man, the differentiation of these states is typically but not exclusively promoted by IFN-γ, IL-4/IL-13 and IL-10, respectively. The hypothesis of the present study was that the early interaction of PRRSV with MΦ plays an important role in the outcome of infection with PRRSV varying in virulence, and that MΦ modulation could highlight differences relating to the virulence of PRRSV.

The functional and phenotypic characteristics of M1 and M2 macrophages have not yet been described in pigs. As this was not the objective of the present study, we cannot give details on phenotypic and functional markers of this system in the pig. Nevertheless, we can state that as expected M1 MΦ have a higher MHC I and MHC II expression than unpolarized and M2 MΦ. Compared to unpolarized MΦ, both M1 and M2 have reduced CD14, CD16 is upregulated in M1, while CD163 is downregulated in M2. Considering that CD163 represents one of the PRRSV receptors also involved in virus internalization (Welch and Calvert, 2010), a reduced infection rate could be expected in M2 MΦ. Indeed, the levels of N expressions of LVP23, VR-2332 and RVB-581 in IL-4 MΦ were significantly reduced compared to those observed in unpolarized MΦ



**Fig. 5.** Differential infection of IFN- $\gamma$  M $\Phi$  by PRRSV-1 and PRRSV-2 strains. Unpolarized M $\Phi$  (A), IFN- $\gamma$  M $\Phi$  (B), IL-4 M $\Phi$  (C) and IFN- $\beta$  M $\Phi$  (D) were infected with various genotype 1 PRRSV (LVP23, Olot/91, 2982 and 3267) or various genotype 2 PRRSV (VR-2332, RVB-581, JA-1262, SS144 and MN184) at an MOI of 0.1 TCID<sub>50</sub>/cell or treated with mock for 20 h. Mock (M) and mock (P) stands for MARC-145 cells and PAM lysates. The percentage of PRRSV N expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N<sup>+</sup>) cells of a pooled data from triplicate culture of at least two independent experiments. The different letters indicate significance between viruses ( $P < 0.05$ ).

at 6 and 12 hpi ( $P < 0.05$ ), but not at later time points (20 and 20 hpi). Furthermore, the end point titres reached in the “IL-4 M $\Phi$ ” were not statistically different from those in unstimulated M $\Phi$ , indicating that M2 polarization does not interfere with virus replication. In contrast to IL-4, IFN- $\beta$  and IFN- $\gamma$  treatment of M $\Phi$  induced a clear antiviral state in M $\Phi$  with reductions in virus infection and replication. Importantly, these effects were significantly stronger against low virulent PRRSV-1 strains when compared to the PRRSV-2 and HP-PRRSV-1 Lena isolates. This was particularly evident for IFN- $\gamma$ . Furthermore, it appeared that the HP-PRRSV isolate from China and Eastern Europe were particularly resistant to the effects of IFN- $\gamma$ . Consequently, we propose that such M $\Phi$  cultures could help to better understand the immunological and genetic basis of PRRSV

virulence. It could be speculated that most of the PRRSV-2 and the HP isolates possess enhanced abilities to interfere with antiviral genes induced by both IFN- $\beta$  and IFN- $\gamma$ , and this could be one of the mechanisms of their increased virulence. In the same idea, the reduced replication yield of low virulent PRRSV-1 isolates in IFN- $\gamma$  and IFN- $\beta$  M $\Phi$  should be more deeply investigated to identify which cellular elements could be of particular relevance for M $\Phi$  resistance to PRRSV. Another important aspect which might reflect the fact that HP-PRRSV isolates differentially replicate in IFN- $\gamma$  M $\Phi$ , could be the use of alternative receptors than CD163 for cell binding and entry. In this study, CD16 upregulation was observed in M1 M $\Phi$ . Accordingly, it can be suggested that other potential receptors for PRRSV binding would be upregulated. It would lead to a more efficient viral uptake, increasing the antigen expression per cell. Such phenomena would explain why HP-PRRSV have higher N expression compare to low virulent isolates.

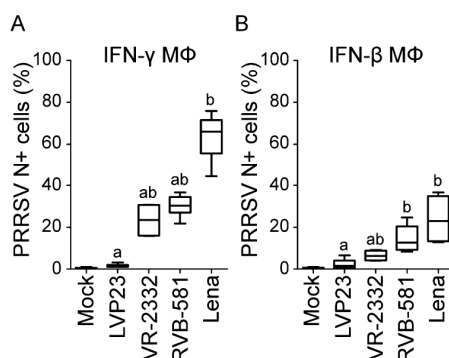
Interestingly, none of the PRRSV isolates tested induced pro-inflammatory TNF- $\alpha$  or anti-inflammatory IL-10, at least at the protein level detectable by ELISA. This challenges a direct role of M $\Phi$ -derived cytokines during the pathogenesis of PRRSV. Nevertheless, both pro- and anti-inflammatory cytokines are found in the lung and lymphoid organs of PRRSV infected pigs and have been associated to M $\Phi$  and other accessory cells (Barranco et al., 2012a,b; Gomez-Laguna et al., 2010, 2013). We therefore conclude that the currently available cell culture models cannot fully reflect the complex events induced in the cytokine network of a multitude of cell types found *in vivo*. On the other hand, PRRSV infection of M $\Phi$  and cell lines inhibits IFN- $\alpha$  and IFN- $\beta$  production both at the mRNA and protein levels through interactions of several viral non-structural proteins with various cellular products including IRF-3, IRF-7, NF- $\kappa$ B and STAT1/2/IRF-9 (Sang et al., 2011). These pathways are likely to also affect cytokine responses in M $\Phi$ , which would fit our *in vitro* data.

Considering that lung M $\Phi$  are the main PRRSV target cells *in vivo*, they represent an appropriate model for *in vitro* studies to identify viral and cellular factors of importance for virus replication and host responses at the cellular level. Nevertheless, it is necessary to sacrifice pigs to collect sufficient M $\Phi$  from lung lavages. Our data support an alternative model using monocyte-derived M $\Phi$  which can be obtained without slaughtering animals. In addition, these M $\Phi$  can be modulated and polarized by cytokines to mimic certain functions of tissue M $\Phi$  during steady state conditions or during the course of immune responses. In fact, it can be expected that during early phases of PRRSV infection the virus would encounter unprimed as well as IFN type I-primed M $\Phi$ , followed by M1 “IFN- $\gamma$  M $\Phi$ ”, M2 “IL-4 M $\Phi$ ” and finally M2b “IL-10 M $\Phi$ ”. M2 and M2b functional states would be important in re-storing tissue integrity and counteracting damaging inflammatory responses, and thereby protect lung functions. Future studies are still required to address the interaction of PRRSV with such regulatory M $\Phi$ .

In conclusion, the present study has identified important strain differences using cytokine-primed M $\Phi$  cultures, which will be very valuable to understand the virological and cellular elements important for PRRSV-cell interactions and relating to virulence. This knowledge is crucial for the rational design and development of more effective vaccines against PRRSV.

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**Fig. 6.** Genotype-independent IFN-resistance of HP-PRRSV in M $\Phi$ . IFN- $\gamma$  (A) and IFN- $\beta$  M $\Phi$  were infected with LVP23, VR-2332, RVB-581 and Lena (MOI of 0.1 TCID<sub>50</sub>/cell) or mock control. Percentage of PRRSV nucleocapsid (N) expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N<sup>+</sup> cells of two independent experiments performed in culture triplicates. The different letters indicate significant differences between viruses ( $P < 0.05$ ).

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6.4. Manuscript 4

*Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytoid dendritic cells and can thereby mediate bacteria-induced interferon-alpha responses.*

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**1 Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytoid**  
**2 dendritic cells and can thereby mediate bacteria-induced interferon-alpha responses**

**3** Arnaud Baumann\*†, Thomas Démoulin\*, Sylvie Python\* and Artur Summerfield\*

**4** \*Institute of Virology and Immunology (IVI), Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland.

**5** †Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland.

**6** Running title: PMAP-36 influences plasmacytoid dendritic cell responses.

**7** Corresponding author: Artur Summerfield

**8** Institute of Virology and Immunology, Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland

**9** Phone: 0041 31 848 9377

**10** Fax: 0041 31 848 9222

**11** E-mail: [artur.summerfield@ivi.admin.ch](mailto:artur.summerfield@ivi.admin.ch)

**12**

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**14**

## Abstract

Cathelicidins constitute potent antimicrobial peptides characterized by a high cationic charge that enable strong interactions with nucleic acids. In fact, the only human cathelicidin LL-37 triggers rapid sensing of nucleic acids by plasmacytoid dendritic cells (pDC). Amongst the porcine cathelicidins, phylogenetic analysis of the C-terminal mature peptide showed that the porcine myeloid antimicrobial peptide (PMAP)-36 was the most closely related of the 11 porcine cathelicidins to human LL-37. Despite several investigations evaluating potent antimicrobial functions of the porcine cathelicidins, nothing is known about their ability to promote pDC activation. We therefore investigated the capacity of the proline-arginine-rich 39-amino-acid peptide (PR-39), PMAP-23 and PMAP-36 to complex with bacterial DNA or synthetic RNA molecules and facilitate pDC activation. We demonstrate that these peptides mediate a rapid and efficient uptake of nucleic acids within minutes followed by robust IFN- $\alpha$  responses. PMAP-36 which harbors the highest cationic net charge was found to be the most potent peptide tested for this effect. The peptide-DNA complexes were internalized and also found to associate with the cell membranes of pDC. The amphipathic conformation typical of PMAP-36 was not required for IFN- $\alpha$  induction in pDC. We also demonstrate that this cathelicidin can mediate IFN- $\alpha$  induction in pDC stimulated by *E. coli*, which alone fail to activate pDC. This response was weaker with a non-sense PMAP-36, relating to its lower antimicrobial activity. Collectively, our data suggest that the antimicrobial and nucleic acid complexing properties of cathelicidins can mediate pDC activation promoting adaptive immune responses against microbial infections.

## 34 Introduction

35 The cathelicidin family together with defensins encompass a wide spectrum of host defense peptides  
36 (HDP) widely referred to as antimicrobial peptides. Cathelicidins are predominantly expressed by  
37 neutrophils in pre-propeptide forms which share an N-terminal signal sequence, a cathelin-like domain  
38 and a highly diverse structural C-terminal part (1). The inactive propeptides are temporally stored  
39 within neutrophil granules, and require activation by elastases which cleave off the C-terminal  
40 bioactive domain of the molecule (2). Interestingly, swine possesses all classes of cathelicidins  
41 observed in higher vertebrates, whereas in humans LL-37 alone has so far been described. The porcine  
42 cathelicidin family includes the prophenin-1 and -2, proline-arginine-rich 39-amino-acid peptide (PR-  
43 39), disulfide-bridged cysteine-rich protegrins (PG-1 to PG-5) and  $\alpha$ -helical porcine myeloid  
44 antimicrobial peptides (PMAP-23, PMAP-36 and PMAP-37) (3). Antimicrobial activity has been well  
45 characterized for PR-39 (4, 5), PMAP-23 (6, 7) and PMAP-36 (8). It was also shown that PR-39  
46 displays chemotactic activity in neutrophils (9). Despite several investigations evaluating the  
47 antimicrobial activity of cathelicidins, nothing is known about their participation in pDC-driven innate  
48 immune responses.

49 It is widely accepted that type I IFNs, mainly IFN- $\alpha/\beta$ , are essential to the innate immune system for  
50 direct antiviral defenses as well as robust adaptive immune responses (10, 11). Although plasmacytoid  
51 dendritic cells (pDC) are rare, they act as the ultimate producer of IFN- $\alpha$ , secreting up to 1000-fold  
52 higher levels of type I IFN than any other cellular types (12). They represent the major source of type I  
53 IFNs and other inflammatory cytokines after exposure to TLR7 and TLR9 ligands, including many  
54 viruses and bacterial DNA (13). Consequently, they represent an important target for investigating  
55 early immune events that could influence the induction of adaptive immune responses (14). In pig,  
56 these cells are identified as CD4<sup>+</sup>CD123<sup>+</sup>CD135<sup>+</sup>CD172a<sup>+</sup>CD14<sup>-</sup> and can be differentiated from  
57 conventional blood dendritic cells and monocytes that lack CD4 expression (15).

58 Recent reports demonstrated that human LL-37 was able to promote rapid sensing of unmethylated  
59 CpG motifs inducing large amounts of IFN- $\alpha$  by pDC (16). This mechanism has been proposed for the

60 pathogenesis of some autoimmune diseases such as psoriasis and Lupus Erythematosus (17, 18).  
61 Similar to what has been observed with LL-37, human  $\beta$ -defensin 2 and 3 have been shown to enhance  
62 IFN- $\alpha$  response in pDC (19). However, nothing has been described regarding the effect of porcine  
63 cathelicidin in the activation of pDC by microbial nucleic acids. Consequently, the ability of PR-39,  
64 PMAP-23 and PMAP-36 to interact with different types of nucleic acids and their contribution to  
65 activate pDC have been investigated.

## 66 **Materials and Methods**

### 67 *Reagents*

68 The PR-39 (RRRPRPPYLPRPRPPFFPPRLPPRIPPGFPPRFPPRFP), PMAP-23  
69 (RIIDLLWRVRRPQKPKFVTWVR), PMAP-36  
70 (GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG), a non-sense version (scrambled) of  
71 PMAP-36 (GKIRGKRKKVRPGTIFRLRVLKWIPPIGCKRLSKLG) was generated using solid-phase  
72 peptide synthesis by ChinaPeptides (Shanghai, China). All peptides showed >98% purity by HPLC  
73 analysis and mass spectrometry. Peptides were dissolved in deionized sterile water and stored at -20°C  
74 until further use. Polyuridylic acid (poly(U)) potassium salt and chloroquine diphosphate were  
75 provided by Sigma-Aldrich (Switzerland). Endotoxin-free genomic DNA from *E.coli* K12 was  
76 purchased from InvivoGen (San Diego, CA, USA). The type-A guanine cytosine  
77 oligodeoxynucleotide (CpG) D32 known to induce IFN- $\alpha$  in porcine pDC (20) was obtained from  
78 Microsynth AG (Switzerland). Another type-A CpG-2216-FITC was purchased from Invivogen and  
79 used for confocal microscopy. Plasmid pEAK8 was propagated in chemocompetent bacteria XL1-Blue  
80 *E.coli* and transformed bacteria were selected with ampicillin on agar plate. After overnight growth in  
81 lysogeny broth (LB; AppliChem, Switzerland) medium supplemented with ampicillin, plasmid was  
82 extracted from bacteria using the extraction NucleoBond® Xtra Midi kit (Macherey-Nagel GmbH,  
83 Switzerland). The genomic *E.coli* DNA was labeled with FITC using Label IT® Nucleic Acid  
84 Labeling kit following the manufacture's instructions (Mirus, Madison, WI, USA). Alternatively, the  
85 Ulysis™ Alexa Fluor® 488 Nucleic Acid Labeling kit was employed for confocal microscopy  
86 following manufacture's protocol (Life Technologies, Switzerland). The transfection reagent N-[1-  
87 (2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) was purchased from  
88 Roche (Switzerland) and used following the manufacturer's instructions. The following mouse mAb  
89 were used: anti-CD172a (mAb 74-22-15a, kindly obtained from Dr. Armin Saalmüller, Veterinary  
90 University of Vienna, Austria), anti-CD4 (mAb PT90A, VMRD Inc., Washington, USA) and anti-  
91 IFN- $\alpha$  (mAb F17; R&D Systems). Isotype specific anti-mouse conjugated with FITC, RPE, biotin and

Streptavidin SpectralRed® (SouthernBiotech, Birmingham, AL, USA), as well as isotype specific Alexa 546 and Alexa 647 conjugates from Life Technologies (Basel, Switzerland) were employed as secondary reagents.

#### *Isolation of pDC*

Enrichment of pDC was performed as described earlier (20). Briefly, peripheral blood mononuclear cells (PBMC) from 6 week- to 12 month-old specific pathogen free (SPF) pigs were isolated by Ficoll-Paque differential centrifugation (21) followed by CD172a enrichment using MACS sorting (Miltenyi Biotec GmbH, Germany). Each enrichment lead to > 80% of CD172a positive cells and 2-5% pDC. Herein, these CD172a-sorted cells are referred to as “enriched pDC”. To examine the cellular localization of *E.coli* DNA in pDC, CD172a-enriched cells were purify by electrostatic sorting based on a gate on CD172lowCD4high population using a FACSAria Cell Sorter (BD biosciences, Switzerland).

#### *Stimulation of pDC and quantification of IFN-α by ELISA*

Stimulation of pDC was performed in 96-microwell plates at  $4 \times 10^6$  cells/ml. The following amounts of nucleic acid molecules and cathelicidins were employed to form complexes at room temperature (RT) for 10 min: 1 µg of *E.coli* DNA (final concentration of 10 µg/ml), 1 µg of pEAK8 plasmid (final concentration of 10 µg/ml), 0.25 µg of type-A CpG (final concentration of 2.5 µg/ml) or 1 µg of poly(U) (final concentration of 10 µg/ml) were incubated with  $4 \times 10^{-9}$  mol and with 4-fold dilutions of each cathelicidins (final concentration of 40 µM, 10 µM, 2.5 µM and 0.6 µM) in 60 µl of DMEM containing 10% FBS. A volume of 40 µl containing  $4 \times 10^5$  enriched pDC were finally added to reach the concentrations listed above. Cells were incubated for 10 min, washed three times with 200 µl cold PBS (350g at 4°C for 10 min), and then cultured in 200 µl of fresh DMEM supplemented of 10% FBS and 20 µM of β-mercaptoethanol (Invitrogen, Switzerland). After 20 h, cultures were centrifuged and cell-free supernatants were harvested. The levels of secreted IFN-α was quantified in collected supernatants by ELISA as described in (22).

Chloroquine (1.25-10  $\mu$ M) was employed to examine the role of pH endosomal acidification, which is required for TLR7/9 sensing of nucleic acids (23). A similar protocol was employed except that enriched pDC were pretreated with increasing amount of chloroquine before being added to *E.coli* DNA (10  $\mu$ g/ml) complexed to PR-39 (40 $\mu$ M), PMAP-23 (40  $\mu$ M) and PMAP-36 (2.5  $\mu$ M). Exposure of the cells to CpG alone was included as control. The cultures were incubated at 39°C for 20 h and IFN- $\alpha$  was quantified in the culture supernatant by ELISA.

When enriched pDC were stimulated with XL1-blue *E.coli*,  $8 \times 10^{-9}$  mol of PMAP-36 or scrambled peptide followed by 4-fold dilutions (final concentration of 40  $\mu$ M, 10  $\mu$ M, 2.5  $\mu$ M and 0.6  $\mu$ M) were incubated with  $2 \times 10^6$  bacteria in a volume of 100  $\mu$ l of FBS free DMEM for 1 h. Finally,  $4 \times 10^5$  enriched pDC suspended in 100  $\mu$ l of supplemented DMEM were added to reach 10% FBS, 20  $\mu$ M of  $\beta$ -mercaptoethanol, 1X Pen/Strep (Life Technologies, Switzerland) and the peptide concentrations mentioned above. Cultures were incubated for 20 h and IFN- $\alpha$  was quantified by ELISA.

#### *Flow cytometry and confocal microscopy*

The uptake of *E.coli* DNA complexed to cathelicidin peptides was performed in 48-well plate. Briefly, 5  $\mu$ g of FITC-labeled DNA from *E.coli* K12 (final concentration of 10  $\mu$ g/ml) was mixed with  $1.25 \times 10^{-9}$  mol of PMAP-36 (final concentration of 2.5  $\mu$ M) or  $2 \times 10^{-8}$  mol of PR-39 or PMAP-23 (final concentration of 40  $\mu$ M) in 400  $\mu$ l of DMEM at RT for 10 min. Then, 100  $\mu$ l containing  $1 \times 10^6$  CD172a<sup>+</sup> cells was added to reach the final concentrations mentioned above. The cultures were incubated at 39°C for 1, 5, 10 or 30 minutes. Cells were then washed with cold PBS and stained with a monoclonal antibody directed against a CD172a or CD4 epitope in order to identify pDC characterized as CD172<sup>low</sup>CD4<sup>high</sup> cells. To characterize the intracellular levels of IFN- $\alpha$ ,  $1 \times 10^6$  CD172a<sup>+</sup> cells were stimulated in a 48-well plate in a final volume 500 $\mu$ l. Similarly to the uptake experiment, complexes were formed at RT for 10 min before the addition of enriched pDC. Cultures were incubated at 39°C with either CpG (2.5  $\mu$ g/ml), *E.coli* DNA (10  $\mu$ g/ml) or *E.coli* DNA + PMAP-36 (10  $\mu$ g/ml + 2.5  $\mu$ M) for 10 min. Cells were then washed three times with cold PBS and cultured in 500 $\mu$ l of fresh medium for several time points. Brefeldin A (eBioscience, Austria) was added to the

cells to block IFN- $\alpha$  secretion for 4 h before stopping the cultures. Culture supernatants were collected and used to quantify the levels of IFN- $\alpha$  by ELISA. Cells were subsequently stained for surface CD4 and CD172a expression as mentioned above. Cell fixation and permeabilization for intracellular staining of IFN- $\alpha$  was performed using a Fix & Perm kit (Caltag, UK). Anti-IFN- $\alpha$ , biotinylated goat anti-mouse IgG1 conjugate and Streptavidin SpectralRed® were added to the cells to detect intracellular IFN- $\alpha$ . The data were acquired using a FACScalibur (BD Biosciences, Mountain View, CA) and data analysis was performed using FlowJo software.

For confocal images, pure FACS-sorted pDC were stimulated following a similar protocol used for the IFN- $\alpha$  quantification. In 96-well plate, 1  $\mu$ g of *E.coli* DNA labeled with Alexa 488 (final concentration of 10  $\mu$ g/ml) was complexed to  $4 \times 10^{-9}$  mol of PR-39 or PMAP-23 (final concentration of 40  $\mu$ M) or  $2.5 \times 10^{-10}$  mol of PMAP-36 (final concentration of 2.5  $\mu$ M) in a volume of 75  $\mu$ l at RT for 10 min. The control *E.coli* DNA labeled with Alexa 488 was employed alone. A volume of 25  $\mu$ l containing  $1 \times 10^5$  pure pDC was added in each well and incubated at 39°C for 10 min. Cells were then washed three times with cold PBS and cultured in 200  $\mu$ l of fresh medium. The type-A CpG-2216-FITC (1  $\mu$ g/ml) was also included as positive control. After 5 h incubation pDC were stained against CD4 and isotype specific Alexa 546 conjugate. Finally, the cells were fixed with the Fix & Perm kit and transferred in 8-well Lab-Tek® chambers (Nunc, Wiesbaden, Germany). The slides were mounted with Moviol and analyzed by confocal microscopy (Leica TCS-SL confocal microscope and software; Leica Microsystems AG, Switzerland). All images were acquired using a 63 $\times$  oil-immersion objective, with settings for high-resolution images acquired at optimum voxel size and automatic threshold.

### *Bacterial culture and cytotoxic assay*

The ability of killing or inhibiting the growth of XL1-Blue *E.coli* was investigated for the scrambled peptide and PMAP-36 using adapted protocols (8, 24). All inoculums were harvested in mid-log phase growth of XL1-Blue *E.coli* and the CFU/ml was calculated with the OD600 value (0.3-0.6) from a previous known standard curve. To determine the killing activity of the peptides,  $4 \times 10^{-9}$  mol followed by 4-fold dilutions of scrambled peptide and PMAP-36 (final concentration of 40  $\mu$ M, 10  $\mu$ M, 2.5  $\mu$ M

169 and 0.6  $\mu$ M) were added in 50  $\mu$ l of PBS in 96-microwell plate. A volume of 50  $\mu$ l of PBS containing  
170  $2 \times 10^6$  CFU was added to each peptide dilution to reach the above concentrations. Following 1 h  
171 incubation at 37°C, 10  $\mu$ l from serial 10-fold dilutions were plated on agar to count the CFU. The  
172 experiments were performed in triplicates.

173 The minimal inhibitory concentration of the scrambled peptide and PMAP-36 was determined as  
174 being the lowest peptide concentration where no bacterial growth was monitored by reading the  
175 OD600. Briefly, serial 2-fold dilutions starting by 90  $\mu$ l of 10  $\mu$ M of scrambled peptide or PMAP-36  
176 were inoculated with 10  $\mu$ l of LB containing  $10^6$  CFU. Cultures were incubated at 37°C under 220  
177 rpm and absorbance was quantified by photospectrometry reading the OD600 at several time points.  
178 The experiments were performed in quadruplicates.

#### 179 *Phylogenetic analysis*

180 Published sequences of the C-terminal mature form of cathelicidin were retrieved from Zanetti (1).  
181 The unrooted phylogenetic tree was constructed using the neighbour-joining method (p distance  
182 model) on the MEGA5 program.

#### 183 *Software and statistical analysis*

184 Peptide helical projection and net charge were obtained on HeliQuest (<http://heliquet.ipmc.cnrs.fr/>).  
185 Confocal images were analyzed on IMARIS 7.6 software (Bitplane AG) to subtract threshold and  
186 gamma-correction compared to negative control such that no false-positive emissions could occur.  
187 Conformation of the scrambled peptide was predicted on PsiPred (<http://bioinf.cs.ucl.ac.uk/psipred/>)  
188 and Jpred (<http://www.compbio.dundee.ac.uk/www-jpred/>). Data were analyzed using GraphPad  
189 Prism 6.0 and multiple comparisons were considered using a One-way or Two-way ANOVA.  
190 Dunnett's multiple comparison post-hoc test was employed to compare data with a control group  
191 whilst Bonferroni was employed to compare all treatment groups. Significant differences are noted \*  
192 ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) or \*\*\* ( $p < 0.001$ ).

## 193 Results

### 194 *Porcine cathelicidins complex DNA with an efficacy relating to their cationic charge.*

195 Cathelicidins are usually subcategorized in different classes according to the highly diverse peptide  
196 conformations that can be observed in certain vertebrates. Phylogenetic analysis of the C-terminal part  
197 of several cathelicidin members showed at least two clusters including  $\alpha$ -helical and prolin/arginine or  
198 cystein-rich peptides (Fig. 1A; (1, 3)). Interestingly, each species expresses at least one cathelicidin  
199 peptide from the  $\alpha$ -helical class. On the other hand, the closest porcine cathelicidin related to the  
200 human LL-37 was found to be PMAP-36. The pig possesses a wide range of structurally diverse  
201 cationic peptides, which could in theory interact with negatively charged molecules such as nucleic  
202 acids. However, except to their antimicrobial activity, nothing is known about their role and their  
203 interaction with innate immune cells. Therefore, we investigated the influence of three synthetic  
204 porcine cathelicidin members including two  $\alpha$ -helical peptides, PMAP-36 and PMAP-23, and PR-39  
205 in delivering nucleic acid complexes to porcine pDC. The helical wheel projection of these synthetic  
206 peptides showed that both PMAP-23 and PMAP-36 share an amphipathic conformation but differ in  
207 their net charge, PMAP-36 being the most cationic (Fig. 1B). The cationic charge of PR-39 was found  
208 to be an intermediate between PMAP-23 and PMAP-36. A shift assay revealed that the synthetic  
209 peptides could complex a plasmid DNA through electrostatic interactions (Fig. 1C). As expected, a  
210 lower concentration of PMAP-36 was required to inhibit the migration of DNA compared to PR-39  
211 and PMAP-23. These results indicate that the three synthetic peptides interact and complex DNA with  
212 different efficacy, which closely relates to the cationic charge of the peptide.

### 213 *Cathelicidins promote nucleic acid-mediated secretion of IFN- $\alpha$ after short term incubation in pDC.*

214 We next examined whether complexes of nucleic acids with porcine cathelicidins could trigger a  
215 potent activation of porcine pDC. The synthetic peptides PR-39, PMAP-23 and PMAP-36 were  
216 incubated at RT with several types of nucleic acids including *E.coli* DNA, plasmid DNA, type-A CpG  
217 and poly(U). After 10 min incubation at RT, these complexes were exposed to enriched pDC for 10

min and then extensively washed and cultured for a further 20 h at 39°C. All three peptides complexed to *E.coli* DNA induced high levels of IFN- $\alpha$  whilst *E.coli* DNA alone following removal after 10 min, was unable to trigger IFN- $\alpha$  (Fig. 2A). The highest levels of IFN- $\alpha$  were observed with 40  $\mu$ M for PR-39 and PMAP-23 while 2.5  $\mu$ M PMAP-36 induced the maximal response. These responses were even higher than those induced by *E. coli* DNA left in the cultures during the 20 h stimulation period (ctrl). When complexes of plasmid DNA with the three peptides were employed, it was again the PMAP-36, which was the most potent (Fig. 2B). Complexes of CpG with PR-39 and PMAP-36 also revealed a similar dose-response pattern (Fig. 2C). Complexes of poly(U), a synthetic RNA-like molecule, with PMAP-36 induced the highest levels of IFN- $\alpha$ , even higher than when complexed with DOTAP, a transfection reagent. Exposure of pDC to poly(U) alone did not induce IFN- $\alpha$  secretion (Fig. 2D). None of the cathelicidins tested alone induced IFN- $\alpha$  in enriched pDC (data not shown). Altogether, these data indicate that complexes of porcine cathelicidins with different types of nucleic acids were potent factors to induce IFN- $\alpha$  responses in enriched pDC. Such immune responses were the most efficient with PMAP-36 independently of the size and type of nucleic acids employed.

To test if complexes of nucleic acids and cathelicidins enables a faster delivery into the cells, the association of *E.coli* DNA complexed to PMAP-36 with pDC was examined over time. As shown in Fig. 3A, the percentage of *E.coli* DNA<sup>+</sup> pDC reached almost 80% after only 10 min of exposure and did not increase after 30 min incubation. Similar results were obtained with PMAP-23 and PR-39 (Supplemental Fig. 1). These findings demonstrate that porcine cathelicidin enable a rapid interaction of nucleic acid molecules with pDC.

We next investigated if the production of IFN- $\alpha$  was mainly mediated by pDC using intracellular cytokine staining. Enriched pDC were incubated with *E.coli* DNA complexed to PMAP-36 for 10 min and then were washed extensively. After an 8 h incubation, flow cytometry analysis revealed that only CD172<sup>low</sup>CD4<sup>high</sup> pDC stimulated with CpG or *E.coli* DNA complexed to PMAP-36 showed IFN- $\alpha$  present inside the cells (Fig. 3B). No fluorescence signal was detected in the CD4<sup>-</sup> populations indicating that neither conventional blood dendritic cells nor monocytes were involved in this IFN- $\alpha$

response. A kinetic assay of intracellular and secreted IFN- $\alpha$  was performed to characterize pDC responsiveness over time (Fig. 3C). Detectable amounts of IFN- $\alpha$  in the supernatants were already observed at 8 h post-stimulation when *E.coli* DNA was complexed to PMAP-36, whereas no IFN- $\alpha$  was observed in CpG-treated cells. Only minimal amounts of IFN- $\alpha$  were noted with *E.coli* DNA alone.

To confirm that nucleic acids were internalized by pDC, the latter subset was purified by FACS sorting (Supplemental Fig. 2) and loaded with peptide DNA complexes. Examination by confocal microscopy revealed that *E.coli* DNA when added alone was undetectable in pDC, similarly to untreated cells (Fig. 4A and 4B). In pDC exposed to CpG-FITC or to *E.coli* DNA complexed to PMAP-23, a comparable punctual distribution of internalized DNA was observed (Fig. 4C and 4D). In contrast, PR-39 and PMAP-36 formed large complexes with the *E.coli* DNA localized at the surface and inside pDC (Fig. 4E and 4F).

Since recognition of DNA delivered by LL-37 is recognized by TLR9 in human, depending on pH endosomal acidification (17, 23), the effect of chloroquine regarding its ability to inhibit IFN- $\alpha$  responses triggered by *E.coli* DNA complexed to cathelicidins was investigated. At all chloroquine concentrations, a complete inhibition of IFN- $\alpha$  was observed in enriched pDC stimulated with CpG or *E.coli* DNA complexed to PMAP-23, whereas the responses induced by *E.coli* DNA coupled with PR-39 or PMAP-36 was only inhibited at high doses of chloroquine (Fig. 5). These data indicate that the induction of IFN- $\alpha$  in response to nucleic acid delivered by PR-39 and PMAP-36 is less sensitive to pH perturbation compared to the CpG-induced IFN- $\alpha$  responses in pDC.

*PMAP-36-mediated-killing is dependent on its amphipathic conformation enhancing the levels of IFN- $\alpha$  responses to E.coli in enriched pDC.*

Considering the reported antimicrobial activity of porcine  $\alpha$ -helical cathelicidins against several pathogens including Gram<sup>+</sup> and Gram<sup>-</sup> bacteria (3), we investigated whether cathelicidins can mediate pDC activation by bacteria employing both, their bacteriolytic activity and their capacity to deliver

released bacterial DNA to pDC. To this end, we employed a scrambled version of PMAP-36 predicted to be incapable of forming an  $\alpha$ -helix. Moreover, bioinformatical helical wheel projection demonstrated that the hydrophobic residues were no more allocated in a transversely amphipathic arrangement than the original peptide (Fig. 6A). As expected, the antimicrobial activity was reduced when comparing the scrambled peptide to PMAP-36, both in terms of bacterial killing (Fig. 6B) and bacterial growth inhibition (Fig. 6C). At all concentrations employed to inhibit the growth, the scrambled peptide only delayed *E.coli* propagation, whilst 2.5  $\mu$ M of PMAP-36 was already enough to completely abolish the bacterial growth. Nonetheless, the capacity of these peptides to form complexes with DNA and induce potent IFN- $\alpha$  responses in enriched pDC was similar, even more efficient at 10  $\mu$ M for the scrambled counterpart (Fig. 6C). Altogether, these data indicate that the  $\alpha$ -helical amphipathic conformation of PMAP-36 displays a critical role in killing bacteria but is not essential to promote IFN- $\alpha$  responses in pDC. We next tested the effect of PMAP-36 on pDC stimulation by *E. coli*. To this end, the bacteria were incubated alone, with the scrambled peptide or PAMP-36 peptide for 1 h, afterwards enriched pDC were added. At 40  $\mu$ M, PMAP-36 significantly enhanced IFN- $\alpha$  compared to its non-sense version (Fig. 7). The bacteria *E. coli* alone were unable to activate pDC. These data suggest that through its combined antimicrobial and DNA complexing activity, PMAP-36 can mediate pDC activation in response to bacteria.

## 286 Discussion

287 Porcine cathelicidins represent a large group of structurally diverse HDP, including  $\alpha$ -helical,  
288 prolin/arginin-rich and cystein-rich peptides. In contrast, LL-37 representing an  $\alpha$ -helical peptide is the  
289 only known human cathelicidin. Instead, much more members of the defensin family are found in  
290 human (3). Interestingly, phylogenic analyses of the C-terminal bioactive form of cathelicidins from  
291 several vertebrates revealed that the PMAP-36 clusters closely with the human LL-37, suggesting a  
292 certain homology in the functionality of these peptides. In addition to its bactericidal activity (25, 26),  
293 LL-37 contributes to many other immune functions including neutralization of LPS (27), chemotaxis  
294 of cells (28), regulation of macrophage differentiation (29) and activation (30, 31). In the study of  
295 Hurtado and Peh, it was shown that this cationic peptide contributes to a rapid sensing of CpG in B  
296 cells and pDC (16). With the results obtained from the phylogenic analysis and the stimulation of  
297 porcine pDC, it is possible to consider PMAP-36 as the functional homolog of the human LL-37. In  
298 accordance with the observations in human (16), the sensing of nucleic acids delivered by PMAP-36  
299 to pDC is also independent on its amphipathic configuration or its microbicidal properties. One can  
300 propose that the range of action of PMAP-36 and some of other porcine cathelicidin members might  
301 certainly be broader than only mediating killing of bacteria and sensing of nucleic acids by pDC.  
302 Observations such as these invite further investigations to decipher the interactions of porcine  
303 cathelicidins with other immune cells.

304 The ability of pDC to sense CpG motifs, has mainly been attributed to the expression of TLR9 that  
305 decorates their endosomal compartments (32). In humans, it was demonstrated that induction of IFN- $\alpha$   
306 by self-DNA complexed with LL-37, is dependent on TLR9 recognition, a feature of certain  
307 autoimmune diseases (17, 18). Similarly, self-RNA complexed to LL-37 also triggered IFN- $\alpha$  in pDC  
308 through TLR7-mediated pathway (33). Although TLR9 was shown to be critical for the recognition of  
309 microbial DNA, a recent report demonstrated the presence of cytosolic aspartate-glutamate-any amino  
310 acid-aspartate/histidine (DEXD/H)-box helicases, DHX36 and DHX9, which were able to bind to  
311 CpG. These receptors signal through MyD88 (34). The fact that CpG-induced IFN- $\alpha$  is abolished by

the presence of chloroquine is attributed to perturbations of endosomal and lysosomal pH (23). The recognition of type-A CpG is well characterized and mainly signals through TLR9 in the endosome (35). Therefore, the weak chloroquine-mediated inhibition of IFN- $\alpha$  responses to *E.coli* DNA complexed to PR-39 and PMAP-36 could indicate that receptors other than TLR9 are involved in such responses. Unfortunately, porcine TLR9 antagonist is not yet available to assess the contribution of this receptor.

The enhancement of IFN- $\alpha$  observed in response to whole bacteria might be explained by the rapid sensing of newly released DNA and RNA molecules from PMAP-36-mediated lysis of bacteria, which in turn are complexed to the cathelicidin. The observation that high concentrations of PMAP-36 were required to kill bacteria and form stimulatory nucleic acids complexes, may be explained by the association of the peptides bacterial membranes, and the possible presence of inhibitory factors for pDC activation. Nevertheless, we propose a model in which cathelicidins secreted by neutrophils would act in tandem with pDC to promote IFN- $\alpha$  induction, favoring a microenvironment suitable for an efficient adaptive immune response. In addition to their antiviral effect, type I IFNs have been shown to promote Th1 response in human (36), and antigen cross-presentation by dendritic cells (37), as well as stimulating antibody production and isotype class switching in B cells (38). Moreover, the signaling of type I IFNs was associated to host resistance against group B streptococci, pneumococci, and *E. coli* (39) underlining the important role of type I IFN in bacterial infections. Another important aspect regarding cathelicidin might be their use in vaccine formulation to deliver nucleic acids to PRR (40). However, exposure to excessive levels of cathelicidin can have deleterious effects during the development of an immune response (41). In this respect, we found an inhibition of IFN- $\alpha$  production at the highest PMAP-36 concentration employed, indicating that cathelicidins can also impair the recognition of nucleic acids by PRR. In vivo studies questioning the efficiency of cathelicidin-formulated vaccine are required to determine their potential in improving vaccine formulations.

In conclusion, this study demonstrates that three porcine cathelicidins efficiently complex nucleic acids for delivery to pDC resulting in potent IFN- $\alpha$  release. In contrast to the antimicrobial activity of

338 these peptides, this was only related to the net charge of the peptide. With this dual function, we have  
339 demonstrated that the peptide PMAP-36 can promote pDC activation in response to *E.coli*.  
340 Collectively, the present data confirm the remarkable link between cathelicidins and IFN- $\alpha$  release by  
341 pDC also for the porcine immune system, which could be of significance during viral-bacterial co-  
342 infections.

### 343 **Disclosures**

344 The authors have no financial conflicts of interest.

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469 **Footnote to the title page**

470 \*Institute of Virology and Immunology (IVI), Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland.

471 †Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland.

472 Corresponding author: Artur Summerfield

473 Institute of Virology and Immunology, Sensemattstrasse 293, 3147 Mittelhäusern, Switzerland

474 Phone: 0041 31 848 9377

475 Fax: 0041 31 848 9222

476 E-mail: [artur.summerfield@ivi.admin.ch](mailto:artur.summerfield@ivi.admin.ch)

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480 Abbreviations used in this article: CpG, guanine cytosine oligodeoxynucleotide; DOTAP, N-[1-(2,3-  
481 dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; HDP, host defense peptide; LB,  
482 lysogeny broth; pDC, plasmacytoid dendritic cell; PG, protegrin; PMAP, porcine myeloid  
483 antimicrobial peptide; poly(U), Polyuridylic acid; PR-39, proline-arginine-rich 39-amino-acid peptide;  
484 RT, room temperature.

## Figure legends

**Figure 1. Porcine cathelicidins complex nucleic acids with different efficacy relating to their cationic charge.** (A) Unrooted phylogenetic tree of the C-terminal active form of cathelicidin in mammals including human (LL-37), rhesus monkey (RL-37), rabbit (CAP18), mouse (CRAMP), Rat (rCRAMP), dog (canine cath), horse (eCATH-1, eCATH-2 and eCATH-3), cow (Bac5, Bac7, BMAP-27, BMAP-34, Indolicidin and Dodecapeptide), sheep (OADode, SMAP-29, SMAP-34, OaBac5, OaBac6 OaBac7.5 and OaBac11) and pig (PR-39, Prophenin-1, Prophenin-2, PMAP-23, PMAP-36, PMAP-37, PG-1, PG-2, PG-3, PG-4 and PG-5) is shown. The tree was constructed using the neighbor-joining method (p distance model). The putative clade of  $\alpha$ -helical peptides is encircled. (B) Helical wheel projection of PR-39, PMAP-23 and PMAP-36 are presented. The hydrophobic residues are displayed in yellow, the N- and C-terminal parts of the peptide are noted N and C in red, respectively. Analyses of the net charge and the helical projections were determined with HeliQuest (<http://heliquest.ipmc.cnrs.fr/>). (C) PR-39, PMAP-23 and PMAP-36 complex nucleic acid with different efficacy. Serial dilutions of PR-39, PMAP-23 and PMAP-36 were mixed with 250 ng of plasmid DNA in PBS for 10 min. The mixtures were run on 0.8% gel agarose gel and DNA was visualized with ethidium bromide under UV radiation.

**Figure 2. Cathelicidins enable nucleic acids-mediated pDC activation after short term incubation.** *E.coli* DNA (10  $\mu$ g/ml) (A), plasmid DNA (10  $\mu$ g/ml) (B), CpG (2.5  $\mu$ g/ml) (C) or poly(U) (10  $\mu$ g/ml) (D) were complexed with PR-39, PMAP-23 or PMAP-36 for 10 min before being added to enriched pDC. Cells were then extensively washed with cold PBS and cultured for 20 h. An incubation of 20 h without washing the cells was used as positive control (ctrl). Since poly(U) poorly induced IFN- $\alpha$  even after 20 h incubation, DOTAP incubated for 10 min (DOTAP) or 20 h (DOTAP ctrl) was used to confirm that cells were responsive (D). Secreted IFN- $\alpha$  was quantified by ELISA. Bars show the mean  $\pm$  SD of triplicate. Significance compared to the absence of peptide was denoted \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) or \*\*\* ( $p < 0.001$ ) following Dunnett's multiple comparison test. All data have been reproduced at least two times in independent experiments.

**Figure 3. PMAP-36 enables a rapid association of *E.coli* DNA to trigger IFN- $\alpha$  response in pDC.**

(A) Flow cytometry demonstrated a rapid association of *E.coli* DNA with pDC. *E.coli* DNA labeled with FITC (at 10 $\mu$ g/ml) was complexed to PMAP-36 (final concentration 2.5  $\mu$ M) for 10 min at RT. Enriched pDC were added and incubated at 39°C for 1, 5, 10 and 30 min. Cells were washed and stained against CD172a and CD4 to identify pDC. Representative figures are shown (B) Intracellular IFN- $\alpha$  was only associated with CD172<sup>low</sup>CD4<sup>high</sup> pDC. Enriched pDC were incubated either with *E.coli* DNA, CpG or *E.coli* DNA complexed to PMAP-36 for 10 min or left untreated. Cells were washed extensively and cultured for a further 8 h. Pseudo-color plot of pDC defined as CD172<sup>low</sup>CD4<sup>high</sup> and CD172<sup>+</sup>CD4<sup>-</sup> populations are gated (left panel) and contour plots demonstrate that only the subsets of pDC are positive for intracellular IFN- $\alpha$  (right panel) after *E.coli* DNA complexed to PMAP-36 or CpG stimulation. Gate frequency is indicated as mean  $\pm$  SD of duplicate. One out of two independent experiments is shown. (C) Kinetics of intracellular and secreted IFN- $\alpha$  in pDC revealed a peak of production after 8-10 h post-stimulation. The Amount of IFN- $\alpha$  detected in the supernatants (left axis; dotted lines) and percentage of IFN- $\alpha$ <sup>+</sup> pDC (right axis; open bars) is shown. One representative out of two independent experiments is shown.

**Figure 4. Complexes of *E.coli* DNA with cathelicidins and their association with pDC.** (A, B, D-F) *E.coli* DNA labeled with Alexa 488 (10  $\mu$ g/ml) was complexed to PR-39, PMAP-23 (40  $\mu$ M) and PMAP-36 (2.5  $\mu$ M) for 10 min at RT or left untreated. Afterwards, 1x10<sup>5</sup> pure pDC were added for 10 min. Cells were then extensively washed and incubated in fresh medium at 39°C for 5h. (C) CpG 2216-FITC (1  $\mu$ g/ml) was employed as positive control. Cells were then stained against membrane CD4 (in red) and were finally fixed. The presence of *E.coli* DNA or CpG is shown in green. Bars represent 10  $\mu$ m. Representative pictures, observed in three independent experiments, are shown.

**Figure 5. IFN- $\alpha$  responses to *E.coli* DNA complexed to PMAP-36 and PR-39 are relatively resistant to inhibition endosomal acidification.** Complexes of *E.coli* DNA (10  $\mu$ g/ml) with PR-39, PMAP-23 (40  $\mu$ M) or PMAP-36 (2.5 $\mu$ M) for 10 min were added to enriched pDC pretreated with increasing amount of chloroquine. Cells were then extensively washed with cold PBS and cultured for

20 h. Cells treated with CpG were employed as positive control. The relative inhibition compared to untreated cells was calculated as following:  $100 - (\text{stimulated cells}) / (\text{stimulated cells} + \text{chloroquine}) \times 100$ . Data are representative of two independent experiment. Significance compared to the chloroquine-mediated inhibition of CpG-induced IFN- $\alpha$  is denoted by \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ) following a Two-way ANOVA and Bonferroni post-tests.

**Figure 6. The amphipathic conformation of PMAP-36 plays a crucial role in antimicrobial activity but not in delivering nucleic acids to pDC.** (A) The random sequence of PMAP-36 (scrambled) is shown by an helical wheel projection. Hydrophobic residues are displayed in yellow, the N- and C-terminus parts of the peptide are noted N and C in red, respectively. (B-C) PMAP-36 is more efficient at killing or inhibiting the growth of *E.coli* than its scrambled counterpart. (B) Different concentrations of scrambled peptide and PMAP-36 peptides were incubated with  $2 \times 10^6$  CFU for 1 h in PBS. Serial 10-fold dilution were then plated on agar to count colonies and data were expressed in CFU/ml. Data show the mean  $\pm$  SD of culture triplicates and are representative of three independent experiments. Significance is indicated by \*\*\* ( $p < 0.001$ ) following a Two-way ANOVA and Bonferroni post-hoc tests. (C) Several 2-fold dilutions of scrambled peptide and PMAP-36 were incubated with  $1 \times 10^6$  CFU in LB medium at 37°C for 1 to 8 and 24 h. The growth of *E.coli* was determined by reading the absorbance (OD600). Data show the mean  $\pm$  SD of culture quadruplicates and are representative of three independent experiments. (D) A similar dose response of *E.coli* DNA-induced IFN- $\alpha$  is observed for the scrambled peptide and PMAP-36 in enriched pDC. *E.coli* DNA (10 $\mu$ g/ml) was complexed to scrambled peptide or PMAP-36 for 10 min before addition to enriched pDC. Cells were then extensively washed with cold PBS and cultured for 20 h. Secreted IFN- $\alpha$  was quantified by ELISA. Bars show the mean  $\pm$  SD of triplicate. Data are representative of three independent experiments. Significance is denoted by \*\*\* ( $p < 0.001$ ) following a Two-way ANOVA and Bonferroni post-tests.

**Figure 7. PMAP-36 enhances the levels of IFN- $\alpha$  responses to *E.coli* in enriched pDC.** A total of  $2 \times 10^6$  *E.coli* were incubated with 40  $\mu$ M of scrambled peptide or PMAP-36 in FBS free DMEM. After

563 1 h, 4x10<sup>5</sup> enriched pDC were added and incubated in medium containing 1X Pen/strep and 10% FBS  
564 for 20 h. The levels of IFN- $\alpha$  were quantified by ELISA in cell-free supernatants. Each color represent  
565 an independent experiment performed in triplicate. The lines show the mean. One-way repeated  
566 measures ANOVA and Bonferroni post-tests were employed and significance between groups is  
567 denoted by \*\* (p<0.01) or \*\*\* (p<0.001).

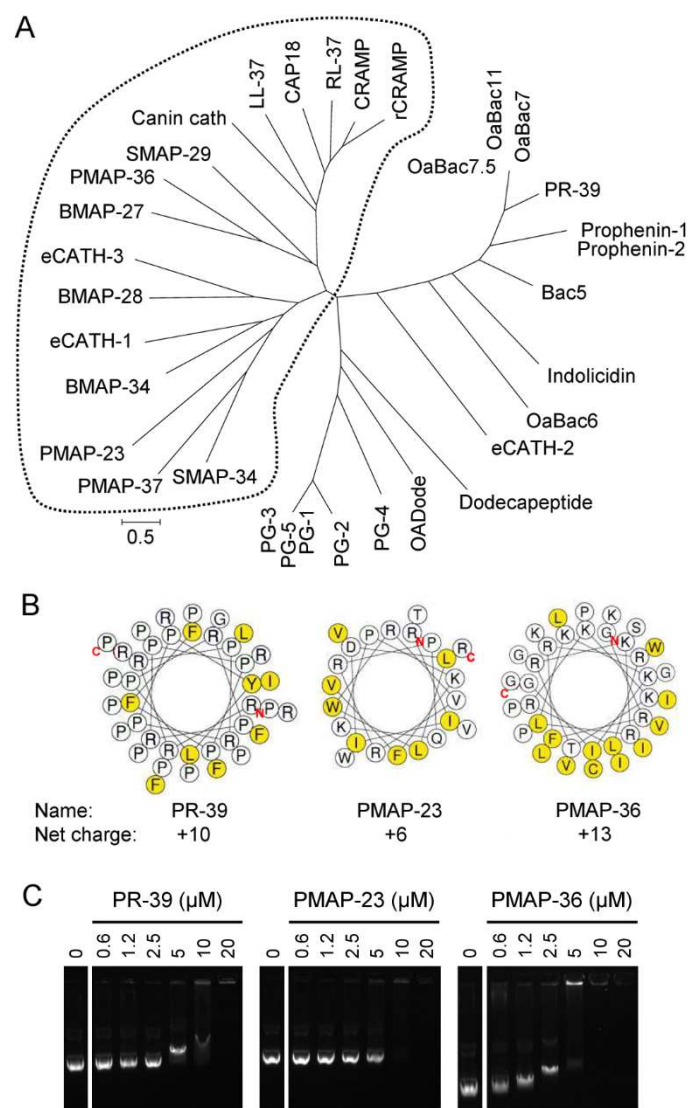


Figure 1

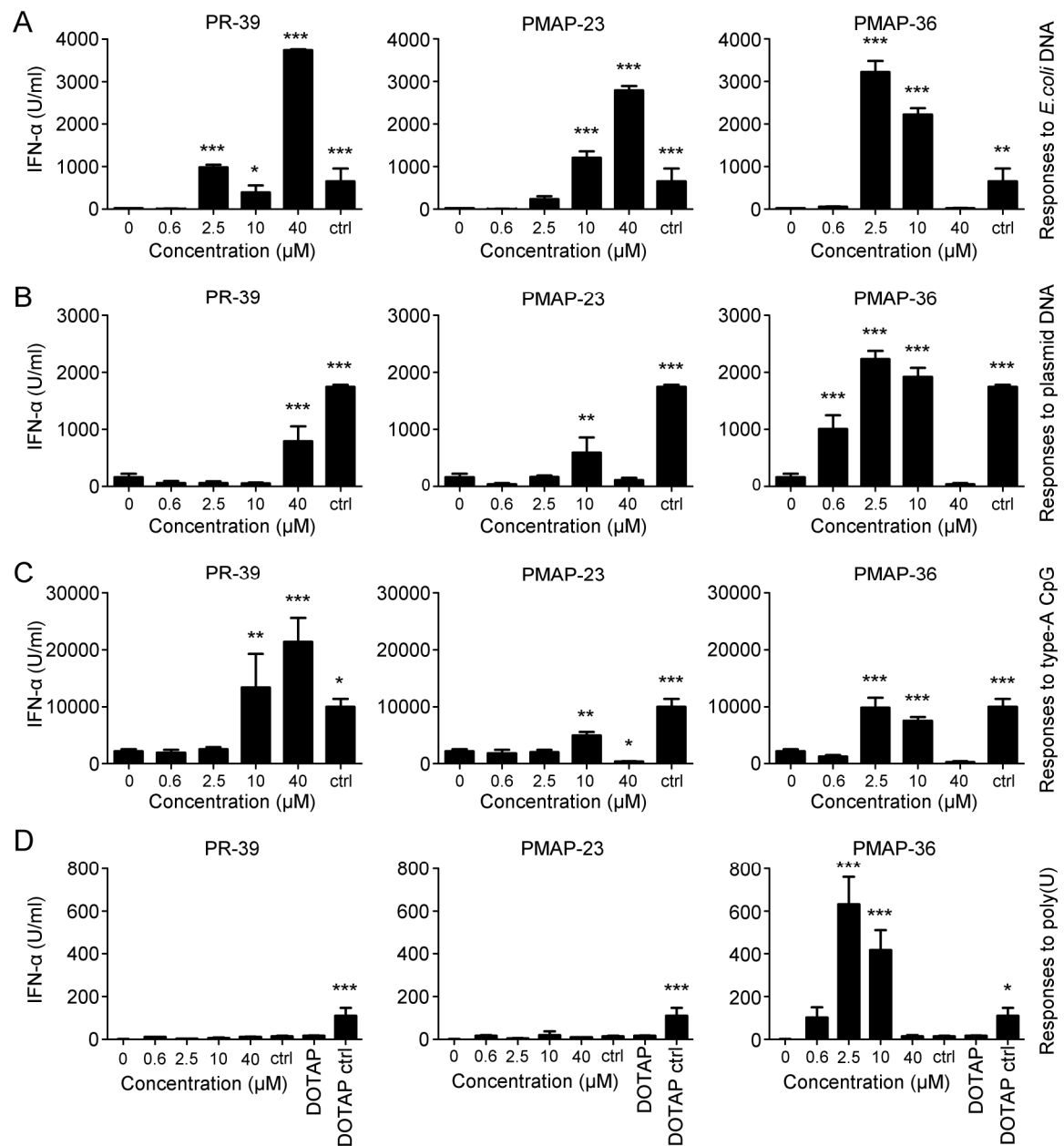


Figure 2

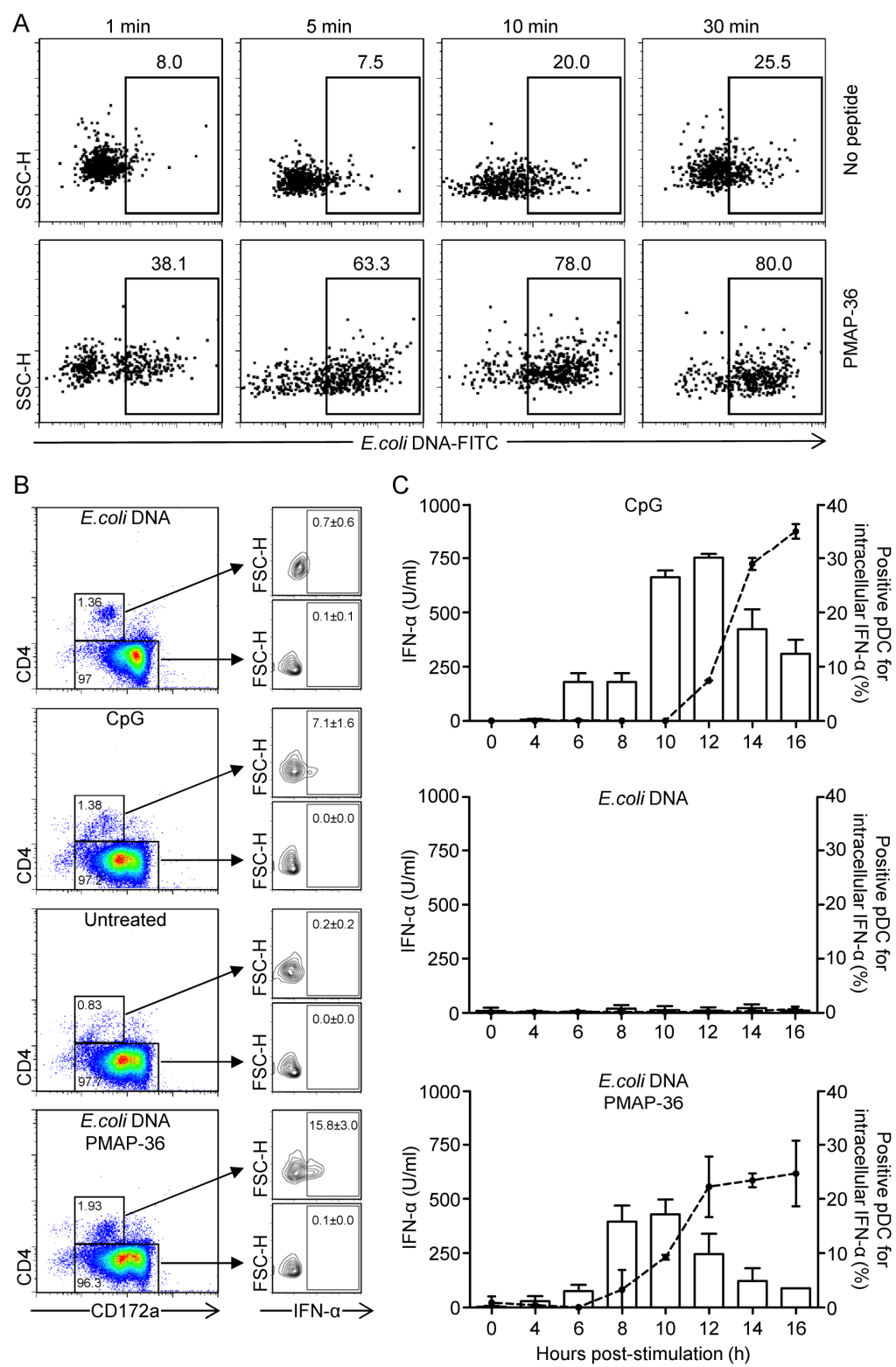


Figure 3

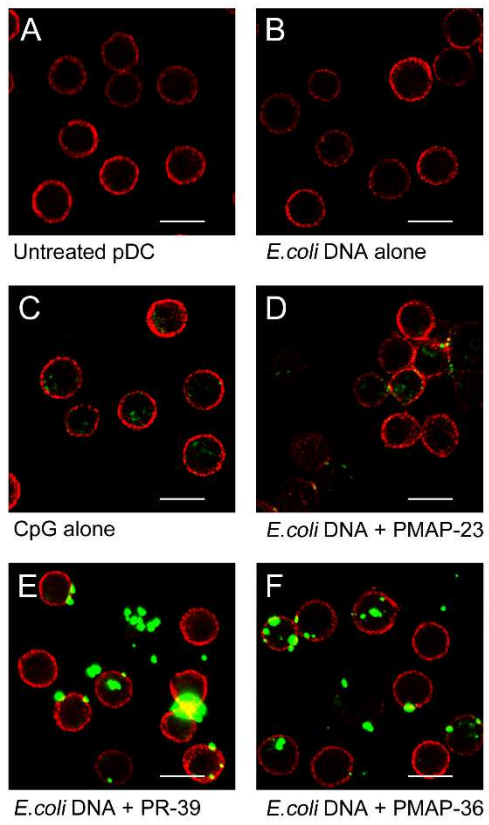


Figure 4

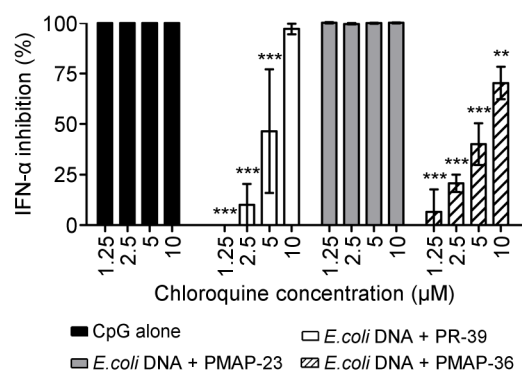


Figure 5

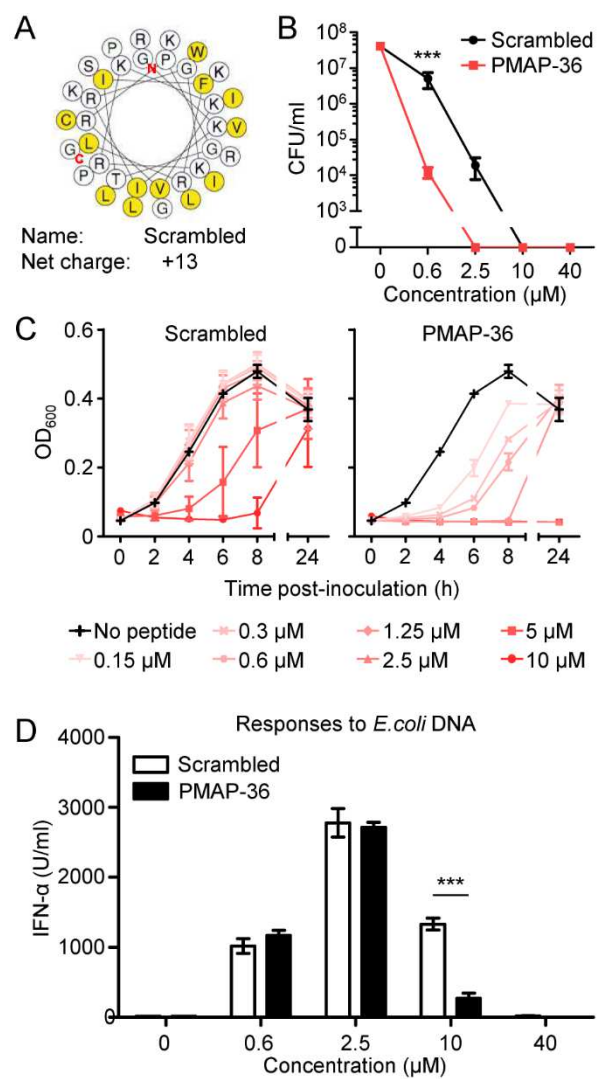


Figure 6

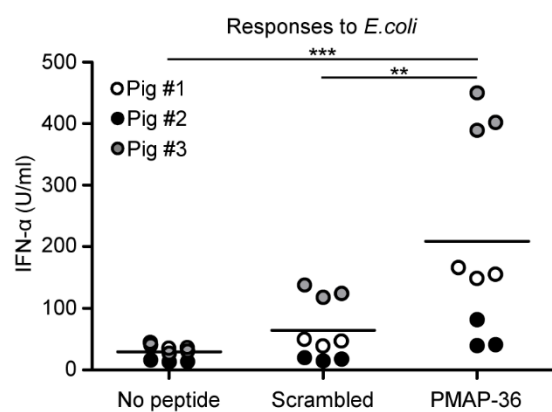
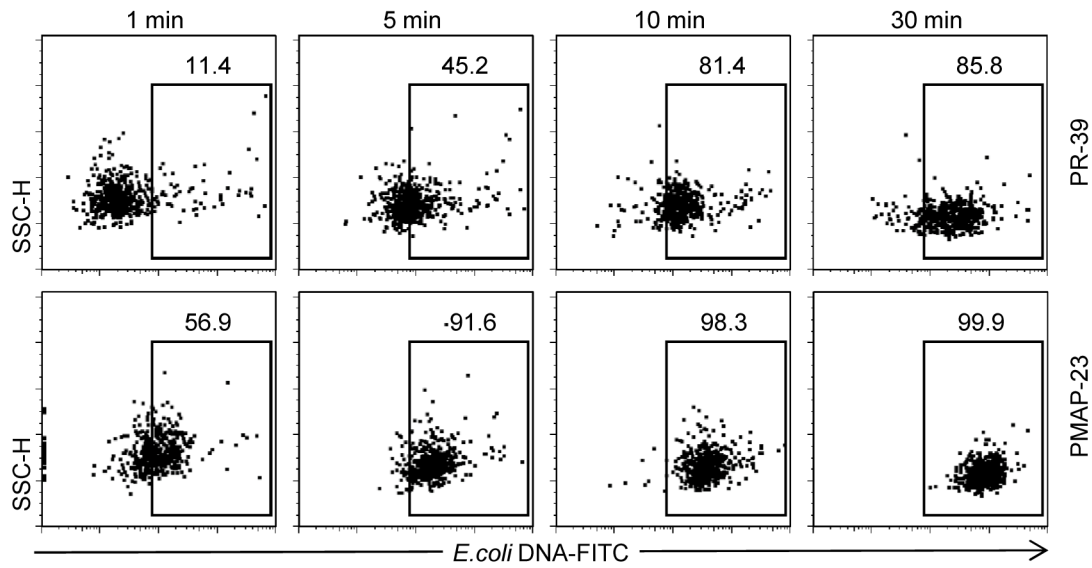
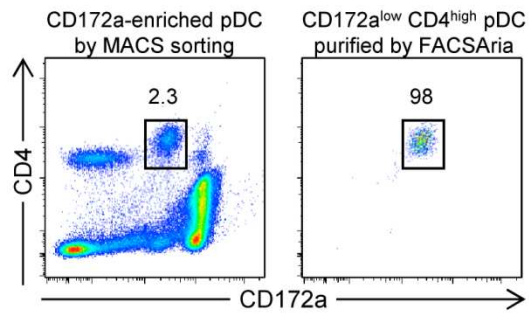


Figure 7



**Supplementary figure 1. Rapid association of *E.coli* DNA coupled to PMAP-23 and PR-39 with pDC.** Flow cytometry demonstrated a rapid association of *E.coli* DNA with pDC. *E.coli* DNA labeled with FITC (final concentration 10 $\mu$ g/ml) was complexed to PR-39 or PMAP-23 (final concentration 40  $\mu$ M) for 10 min at room temperature. Enriched pDC were added and incubated at 39°C for 1, 5, 10 and 30 min. Cells were washed and stained against CD172 and CD4 to identify pDC. One out of three independent experiment is shown.



**Supplementary figure 2. Purity of pDC was examined after FACS sorting procedure.**

CD172a-enriched cells were purified by electrostatic sorting based on a gate on CD172<sup>low</sup>CD4<sup>high</sup> population using a FACS Aria Cell Sorter. Left panel shows the purity of CD172a-enriched cell whereas right panel represent pure pDC population after the sorting procedure.

## 7. Discussion and Perspectives

PCV2 is associated with immunosuppression resulting in increased host susceptibility to disease progression and secondary infections [91, 100, 101]. Although PCV2 DNA has been described to inhibit pDC function, our work provides evidence that PCV2 can have both stimulatory and inhibitory properties regarding the induction of type I IFNs by pDCs. These findings confirm earlier studies performed by others showing that different motifs of synthetic CpG-ODN sequences derived from the PCV2 genome could differentially modulate IFN- $\alpha$  responses in porcine PBMCs [140-142]. We demonstrated that PCV2 is able to induce IFN- $\alpha$  in an IFN- $\gamma$ -dependent process in pDCs. We also found that using DNase treatment, the secretion of IFN- $\alpha$  was not altered indicating that the stimulation properties of PCV2 can be attributed to the encapsulated genomic ssDNA. However, we cannot exclude the participation of the capsid itself in the release of IFN- $\alpha$  by pDCs. We proposed that pDCs might be the early original source of type I IFNs in pigs infected by PCV2 since few reports detected IFN- $\alpha$  in the serum of animals after PCV2 challenge [143, 144].

In contrast to a previous publication where PRRSV-2 was assigned as potent antagonist of type I IFN responses in pDCs, we demonstrate that neither genotype 1 nor 2 PRRSV isolates strongly inhibit CpG-ODN induced IFN- $\alpha$  by pDCs. The highest level of inhibition (51%) was reached by a Chinese isolate but was not as suppressive as PCV2 in our parallel study (82% inhibition). Additionally, we observed that all PRRSV isolates tested could induce IFN- $\alpha$  secretion by pDCs, a response which was triggered through TLR7 signaling pathway. Similarly to PCV2 infection, we hypothesize that systemic IFN- $\alpha$  detected in PRRSV infected pigs might originate from pDCs [193, 209, 210]. Furthermore, we found that PRRSV-induced IFN- $\alpha$  secretion by pDCs protected permissive MoDC from PRRSV infection. These data confirm previous reports indicating that PRRSV is sensitive to the effect of type I IFNs [184, 198, 199] and might be potent factor to limit viral replication during early steps of an immune response.

Interestingly, the levels of IFN- $\alpha$  induced by both PCV2 and PRRSV can be significantly increased by the presence of IFN- $\gamma$ . Moreover, similar effects were observed in pDCs stimulated with FMDV [74]. These data suggest that IFN- $\gamma$  could play an indirect role in viral sensing by the innate immune system. It is well known that type I IFNs promote NK cell activation which is required for the secretion of IFN- $\gamma$  and cytotoxic activity. Altogether, it seems that a cytokine-mediated bidirectional crosstalk exists between NK cells and pDCs [250], which could be of importance in early control of virus infections.

In pig, the functional and phenotypic characteristics of M1 and M2 M $\Phi$  have not been clarified yet. We demonstrated that the polarization of MDM into M1-like-M $\Phi$  by IFN- $\gamma$  or IFN- $\beta$ -activated M $\Phi$  enables the distinction of PRRSV isolates based on the genotype and more importantly on their virulence. Such an *in vitro* model will be helpful for investigating important viral elements relating to virulence, or alternately, for the identification of efficient defense mechanisms against PRRSV. For instance, transcriptomic tools will help to identify genes involved in the inhibition of PRRSV replication in relevant host cells.

In our last study, we showed that porcine cathelicidins were potent factors to mediate a rapid uptake of nucleic acids resulting in a consequent IFN- $\alpha$  secretion by pDCs. Furthermore, we hypothesized that a certain homologies in the functions are shared between PMAP-36 and the human LL-37 [226]. Consequently, PMAP-36 as well as other porcine cathelicidins could potentially impact on anti-microbial immune responses by modulating cellular functions. We also proposed that cathelicidin-mediated killing of bacteria followed by the induction of pDC-derived IFN- $\alpha$  is relevant *in vivo* to mount an efficient adaptive immune response upon bacterial infection. Nonetheless, we attempted to compare the efficacy between *ex vivo*-derived and synthetic cathelicidins in order to confirm this

mechanism previously observed *in vitro*. Neutrophils are important players of innate immunity promoting neutrophils extracellular traps (NETs) [251]. It has been shown that neutrophil-mediated killing of bacteria happens at both intracellular and extracellular interfaces [252, 253]. Scapinello and colleagues reported that porcine neutrophil secretions have strong bactericidal activity. Mass spectrometry analysis revealed that among many secreted proteins, PMAP-36 and lactotransferrin are upregulated by phorbol 12-myristate 13-acetate (PMA) in neutrophils. Thus, the antimicrobial activity of neutrophil secretions could be in part attributed to these two identified proteins, which are known to have antibacterial effects *in vitro* [238, 254]. In our hand, whilst preparations of PMA-stimulated neutrophil supernatants inhibited DNA migration in a gel-shift assay, they did not increase pDC-derived IFN- $\alpha$  in response to *E.coli* DNA compared to supernatants of unactivated neutrophils. However, both PMA- and unstimulated neutrophil supernatants tended to promote higher levels of IFN- $\alpha$  than a stimulation with *E.coli* DNA alone but were not as potent as the synthetic peptides. These observations might be explained by the presence of inhibitory factors for pDC responsiveness. Another aspect, which interferes with the secretion of IFN- $\alpha$  by pDCs, would be the PMA, since it was found to abrogate CpG-induced IFN- $\alpha$  responses in our model. Even though neutrophil secretions partly increased IFN- $\alpha$  responses by pDCs, we were not able to confirm that such responses are attributed to *ex vivo*-derived cathelicidins. In this respect, a close examination implying purification steps of these secretion content might corroborate the results observed with the synthetic cathelicidins. Certain DNA motifs of PCV2 genome have been described to modulate the cytokine production in PBMCs [140-142]. Our reports strongly suggest that the free dsDNA replicative intermediates of PCV2 are responsible for the suppression of pDC responsiveness *in vitro*, while encapsulated genomic ssDNA is stimulatory. Interestingly, dsDNA replicative forms have been detected in lymphoid tissues of PMWS-suffering pigs [97, 139]. On the other hand, it seems that the activation of the immune system represent a crucial event to induce PMWS development [82]. During the course of an immune response, it might be correct to think that the cathelicidins might be upregulated and released by several innate immune cells [255]. Thus, cathelicidin would play an important role in delivering the inhibitory dsDNA forms of PCV2 into immune cells leaving the host susceptible to disease development. This idea is supported by the fact that most of the pigs infected by PCV2 alone are asymptomatic and the reproduction of PMWS has only been achieved in coinfection models with other pathogens [81, 94, 102-106].

Another important aspect which could be further investigated, is the influence of cathelicidins in cell development, especially M $\Phi$ . In the study of van der Does *et al.*, it has been demonstrated that anti-inflammatory M2 M $\Phi$  cultured with LL-37 demonstrate a pro-inflammatory signature characterized by a low expression of CD163 and poor secretion of IL-10 after LPS stimulation [242]. These findings could be translated to the porcine model suggesting that the expression of important receptors or cellular factors implicated in PRRSV immunobiology could be altered by the presence of cathelicidins. Accordingly, the levels of two cathelicidins, PR-39 and prophenine-2, as well as the number of PMNC in the bronchoalveolar lavages were shown to increase in the lungs of pigs following challenge with *Actinobacillus pleuropneumoniae* [256]. Due to the increased susceptibility to other pathogens in the lungs of PRRSV-infected pigs and persistence of the virus [166], it could be considered that some important factors must impair the clearance of the virus by immune cells. In this respect, cathelicidins could be involved in the resistance of the virus by modulating the differentiation of monocytic cells and M $\Phi$  activation. It therefore could be attractive to evaluate the impact of porcine cathelicidins on M $\Phi$  differentiation and their susceptibility to PRRSV. In our hand, M2-activated M $\Phi$  show a down-regulation of CD163 but were still highly inclined to infection. A combination of certain

activation stages of MΦ as well as immunomodulating factors such cathelicidins might contribute to the puzzling PRRS pathogenesis.

In conclusion, we demonstrated that pDCs can induce IFN- $\alpha$  in response to two known immunosuppressive viruses, PCV2 and PRRSV. These data suggest that pDCs might be the source of IFN- $\alpha$  *in vivo*. Additionally, we showed that porcine cathelicidins represent important factors involved the type I IFN induction by pDCs at least *in vitro*. These recent findings indicating that cathelicidin peptides display multiple functions should be implicated in concepts explaining the immunobiology of viral and bacterial co-infections.

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## 9. **Annexes**

### 9.1. *Declaration of originality*

## **Declaration of Originality**

**Last name, first name:** Baumann Arnaud

**Matriculation number:** 05-501-945

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, January 10th 2013

Signature

A handwritten signature in black ink, appearing to read 'Baumann', followed by a long horizontal line.