3. Immune responses against African swine fever virus infection

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Abstract

Infection with African swine fever virus (ASFV) leads to a short haemorrhagic course of disease that, depending on the virus isolate, results in up to 100% lethality in domestic and Eurasian wild pigs. Consequently, ASFV infection in swine is of considerable economic significance. This chapter explains the basics of antiviral immunity in swine, focusing on the 'knowns' and 'unknowns' of the response against ASFV. In particular, monocytes and macrophages play an essential role as the main targets of infection and are crucial in viral persistence and dissemination. Furthermore, ASFV has developed several mechanisms to influence the antiviral and cell biological activity of infected monocytes, including down-regulation of cell surface receptors (e.g. CD14 and MHC-I) and modulation of interferon and cytokine/chemokine responses. ASFV infected pigs also develop virus-specific antibodies that can be used diagnostically, and while the neutralising effect of these antibodies has led to their involvement in protective immunity being controversially discussed, they may still exhibit protective functions through complement-mediated lysis and/ or antibody dependent cell-mediated cytotoxicity. Indeed, T cells (presumably CD8+) also play a central role in the elimination of the virus, as can be seen in experiments where, after depletion of these cells, pigs previously primed with an avirulent ASFV become ill, while non-depleted animals are protected from highly virulent challenge. Nonetheless, despite these advances in our knowledge, much remains unknown about antiviral immunity generated during the course of

a natural ASFV infection, or in response to attenuated virus strains or immunisation. Although such studies would undoubtedly be technically challenging, a deeper understanding of the immunity developed by the natural hosts (i.e. bushpigs and warthogs) against ASFV infection would teach us a lot about an effective protection from ASFV infection, and the involvement of both the innate and adaptive immune systems in this process.

Keywords: innate immunity, adaptive immunity, pig, anti-viral immune responses

3.1 Introduction to anti-viral immune responses

The main aim of the immune system is to detect and subsequently to eliminate harmful pathogens whilst maintaining a homeostatic relationship with beneficial microbes. Pathogens can be very different – parasites, fungi, bacteria, viruses – that must be recognised by the immune system as harmful and foreign. This chapter focuses on the arms of the immune system that are required for anti-viral immune responses in general and particularly the 'knowns' and 'unknowns' of such responses against African swine fever virus (ASFV).

When any pathogen comes into contact with a possible host, the first line of defence is provided by the innate immune system, which comprises soluble factors like type I interferons (IFNs) and cells, like macrophages or dendritic cells that detect pathogens through distinct pathogen-associated molecular patterns (PAMPs). PAMPs are found only in microbes but not in the host and are recognised by innate microbial sensors, collectively known as pattern recognition receptors (PRRs). PRRs consist of several families, each of which recognises unique PAMPs associated with infections. Noteworthy, most if not all components of replicating viruses are synthesised by the host cell using host cell machinery. Thus, viruses can interact with PAMPs either extracellularly or intracellularly. The result of stimulating PRRs is a transcriptional activation of genes involved in innate defence as well as those that activate antigen-presenting cells for successful priming of adaptive responses.

Unlike the innate immune system, which is activated only based on the identification of general threats, adaptive immunity is activated by exposure to specific pathogens, in this case viruses, and uses an immunological memory to learn about the threat to enhance the immune response accordingly. The adaptive immune response is much slower to respond to pathogens than innate immune response, but it is more specific. They respond to pathogens by recognising parts of them as antigens, which are considered harmful or foreign substances entering the body. Soluble factors such as cytokines or chemokines and effector cells such as T or B cells are the main players in adaptive immune responses. Figure 3.1 shows the main stages of an antiviral immune response. Section 2 in this chapter comprises the elements of the innate immune systems that have been characterised during ASFV infection whereas section 3 and 4 will focus on adaptive humoral and cellular responses respectively in the context of ASFV infection.
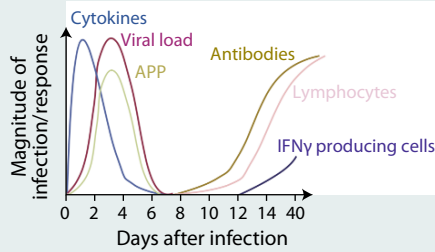
Figure 3.1. General kinetics of lung viral load, acute phase proteins (APPs), cytokines and different immune system components (lymphocytes, interferon (IFN)-γ producing cells and antibodies) during African swine fever viral infection in pigs. The magnitude of responses is related to the days after infection (0-14).

3.2 Innate immunity: the first line of defence

As mentioned, innate immune responses provide a first line of defence against pathogens and promote the development of acquired immunity (Medzhitov and Janeway, 2000). Virulent ASFV isolates have developed mechanisms to outpace these defences and to evade the innate immune system (Dixon *et al.*, 2019; Franzoni *et al.*, 2018). In this subchapter, an overview of how ASFV infection impacts two key cell types of the innate immune system, macrophages and dendritic cells, is provided. In addition, the main findings on ASFV modulation of type I IFN, a crucial element of the innate response to viral infection, are summarised.

PAMPs are recognised by PRRs involving (1) membrane PPRs, associated to plasmatic membrane such as toll-like receptors (TLR)7, TLR8 and TLR13, or TLR3 and TLR9, associated to endoplasmic reticulum membranes (Takeuchi and Akira *et al.*, 2010), and (2) cytoplasmic sensors, mainly the retinoic acid-inducible gene I/melanoma differentiation-associated gene 5 (RIG-I/MDA5) and cyclic GMP-AMP synthase (cGAS), sensing respectively exogenous RNA or DNA (Wu and Chen, 2014). All of them are able to activate signal transduction pathways to induce innate immune responses, such as type I IFN. In fact, the antiviral innate immunity is directed to avoid the viral entry at the fusion step through IFN-induced membrane proteins named interferon-induced proteins with tetratricopeptide repeats (IFITs), which are abundant at the late endosomal membrane and have the ability to impair fusion (Munoz-Moreno *et al.*, 2016).

Cytoplasmic dsDNA acts like a potent PAMP, sensed by cGAS. cGAS detects cytoplasmic dsDNA and catalyses the synthesis of GMP-AMP cyclic dinucleotide (cGAMP) (Sun *et al.*, 2013). cGAMP acts as a second messenger and binds to the stimulator of interferon genes protein (STING), which traffics from the endoplasmic reticulum (ER) to the trans-Golgi network (TGN), where tumour necrosis factor (TNF) receptor-associated factors (TRAF)-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) is recruited and phosphorylated. This event allows the recruitment of interferon regulatory factor (IRF)3, subsequently phosphorylated by TBK1 and translocated to the nucleus, acting as a transcription factor for IFN-β gene expression (Granja *et al.*, 2008; Perez-Nunez *et al.*, 2015; Wu and Chen *et al.*, 2014). Recently, Garcia-Belmonte and co-workers demonstrated that whereas virulent Arm/07 inhibits the synthesis of IFN-β, and consequently

IFIT-1 and C-X-C motif chemokine ligand 10 (CXCL10), by modulating IRF3 activation and STING phosphorylation, the naturally attenuated strain NH/P68 does not (Garcia-Belmonte *et al.*, 2019). Indeed, the infection by the ASFV NH/P68 strain results in: (1) the induction of significant levels of IFN-β compared to those found in Arm/07-infected macrophages; (2) phosphorylation of STING; (3) traffic of STING through the Golgi to perinuclear punctuated structures; and (4) localisation of IRF3 to the nuclear soluble fraction and binding to chromatin. Furthermore, inhibition of cGAS by the specific inhibitor Ru521, impairs NH/P68-induced STING phosphorylation, suggesting that cGAS is likely the main DNA sensor activating the STING cascade by the attenuated ASFV.

In contrast, cGAMP-induced phosphorylation of STING was prevented by infection of cells with Arm/07. STING trafficking to perinuclear punctuated structures was also severely impaired in Arm/07-infected cells, indicating that the virulent ASFV strain prevents the activation and trafficking of STING. These results are in agreement with works describing the inhibition of IFN-β production during virulent ASFV strain infection, despite that the molecular mechanisms involved were not approached by these authors (Gil *et al.*, 2008; Reis *et al.*, 2016). In contrast, Portugal *et al.* (2018) reported recently that IFN-β inhibition was exerted by both virulent and attenuated ASFV strains.

3.2.1 Macrophages

Macrophages (Mφ) are phagocytic cells which are at the frontline of pathogen defence. They detect foreign molecules as they are equipped with an array of sensing molecules named PRRs (Hume, 2015). These cells contribute to the initiation of acquired immune responses, by processing and presenting antigens to naïve T lymphocytes (Hume, 2015). Both monocytes and Mφ are the main targets of ASFV and are thought to be crucial for viral persistence and dissemination (Sánchez-Cordón *et al.*, 2008). Virulent ASFV isolates can inhibit Mφ defences, in order to survive and efficiently replicate in these cells.

Considering the central role of Mφ in ASFV immunopathogenesis, several studies analysed the impact of this virus on the phenotype and functionality of these cells. Susceptibility of myeloid cells to ASFV seems to be linked to maturity: *in vitro* differentiation of porcine blood monocytes into Mφ, with accompanying CD163, major histocompatibility complex (MHC) class II DR, and CD203a up-regulation, increased susceptibility to ASFV infection (Basta *et al.*, 1999; McCullough *et al.*, 1999; Sánchez-Torres *et al.*, 2003). A role for CD163 as an ASFV receptor was postulated in the past (Sánchez-Torres *et al.*, 2003), but subsequent studies showed that CD163 expression was not necessary for ASFV infection of Mφ (Popescu *et al.*, 2017) whereas it was demonstrated that both virulent and attenuated ASFV down-regulated expression of CD14 and CD16 on infected Mφ (Franzoni *et al.*, 2017). Down-regulation of CD14 (lipopolysaccharide (LPS) receptor) and CD16 (a low-affinity receptor for the immunoglobulin (Ig)G Fc involved in antibody opsonisation) might impair Mφ's antimicrobial and antiviral activities (Franzoni *et al.*, 2018). Overall, ASFV infection seems to compromise Mφ ability to respond to different external stimuli: it was recently described that both attenuated NH/P68 and virulent 22653/14-infected Mφ released lower amounts of interleukin (IL)-6, IL-12 and TNF-α compared to mock-infected Mφ in response to stimulation with IFN-γ and LPS or a TLR2 agonist (Franzoni *et al.*, 2020).

ASFV strains of diverse virulence can differently modulate MHC class I expression on Mφ. It was observed that attenuated (BA71V, NH/P68) but not virulent (22653/14) ASFV strains down-regulated MHC I expression on infected Mφ (Franzoni *et al.,* 2017, 2020), and this might promote natural killer (NK) cell activation *in vivo* (Lanier*.,* 2005). In fact, an *in vivo* study reported a correlation between NK activation and protection in pigs inoculated with NH/P68 and then challenged with the homologous virulent L60 strain (Leitão *et al.,* 2001). Through this mechanism, virulent ASFV isolates might elude the early recognition of the innate immune system; however, differences between ASFV genotypes have been observed: Mφ infected with virulent isolates belonging to genotype II (Arm07), IX (Ken06.Bus) or VIII (Malawi Lil20) resulted in MHC I expression similar to those infected with attenuated NH/P68 and lower than those infected with virulent genotype I isolates (Benin 97/1, Sardinian 22653/14) (Franzoni *et al.*, 2020; Arav, personal communication). In addition, deletion of the gene coding for CD2v, whose ORF is interrupted in NH/P68 (Portugal *et al.,* 2015), from genotype I Benin 97/1 resulted in lower MHC I expression on bone marrow derived Mφ comparable to that of NH/P68 infected Mφ (Arav, personal communication), suggesting that CD2v might represent a relevant virulence factor for genotype I ASFV isolates.

Several studies have analysed the chemokine/cytokine responses of Mφ to ASFV infection *in vitro.* It was speculated that cytokine release by infected Mφ might contribute to ASFV pathogenesis, and it was recently reported that Mφ's infection with virulent Georgia 2007 down-regulated expression of anti-inflammatory IL-10, whereas it induced up-regulation of pro-inflammatory IL-17 and cytokines of TNF superfamily, including (Fas-ligand) FASL, leukotriene (LT)A, LTB, TNF, TNFSF4, TNFSF10, TNFSF13B and TNFSF18. *In vivo,* these cytokines might promote death of bystander cells and tissue inflammation (Zhu *et al.*, 2019). Differences between strains were observed, with attenuated ASFV strains inducing enhanced expression of key cytokines (IFN-α, IFN-β, IL-1β, IL-12p40, TNF-α) and chemokines (CCL4, CXCL8, CXCL10) compared to highly virulent strains (Fishbourne *et al.*, 2013; Franzoni *et al.*, 2020; Gil *et al.*, 2003, 2008; Reis *et al.*, 2016). Virulent ASFV isolates might have evolved mechanisms to circumvent Mφ cytokine/chemokine responses, and these mechanisms might be at least partially lost in attenuated strains, with consequent enhancement of immune surveillance and thus induction of effective adaptive immune responses against the virus. Another hypothesis is that virulent ASFV isolates are so aggressive that the Mφ ability to mount cytokine responses is affected from the early steps of infection.

Despite this central role for Mφ in ASFV infection biology, little is known about responses of polarised Mφ to ASFV. Mφ are a heterogeneous population and present extraordinary plasticity, changing *in vivo* their phenotype and functions in response to the subtle and continuous changes of environmental signals (Italiani and Boraschi, 2014; Mosser, 2003). 'Classical' and 'alternative' activated Mφ, called M1 and M2 respectively, represent the two extremes of diverse functional status, with M1 Mφ providing antimicrobial and pro-inflammatory functions, and M2 being associated with mechanisms of immunosuppression and wound healing (Mosser, 2003). Nevertheless, M1 and M2 are not ontogenetically defined and the M1/M2 paradigm is just a limited attempt to define the complexity and plasticity of the mononuclear phagocyte system (Italiani and Boraschi*,* 2014). In pigs, as in other species, M1 polarisation can be achieved *in vitro* through IFN-γ and LPS, with consequent up-regulation of MHC and co-stimulatory molecules
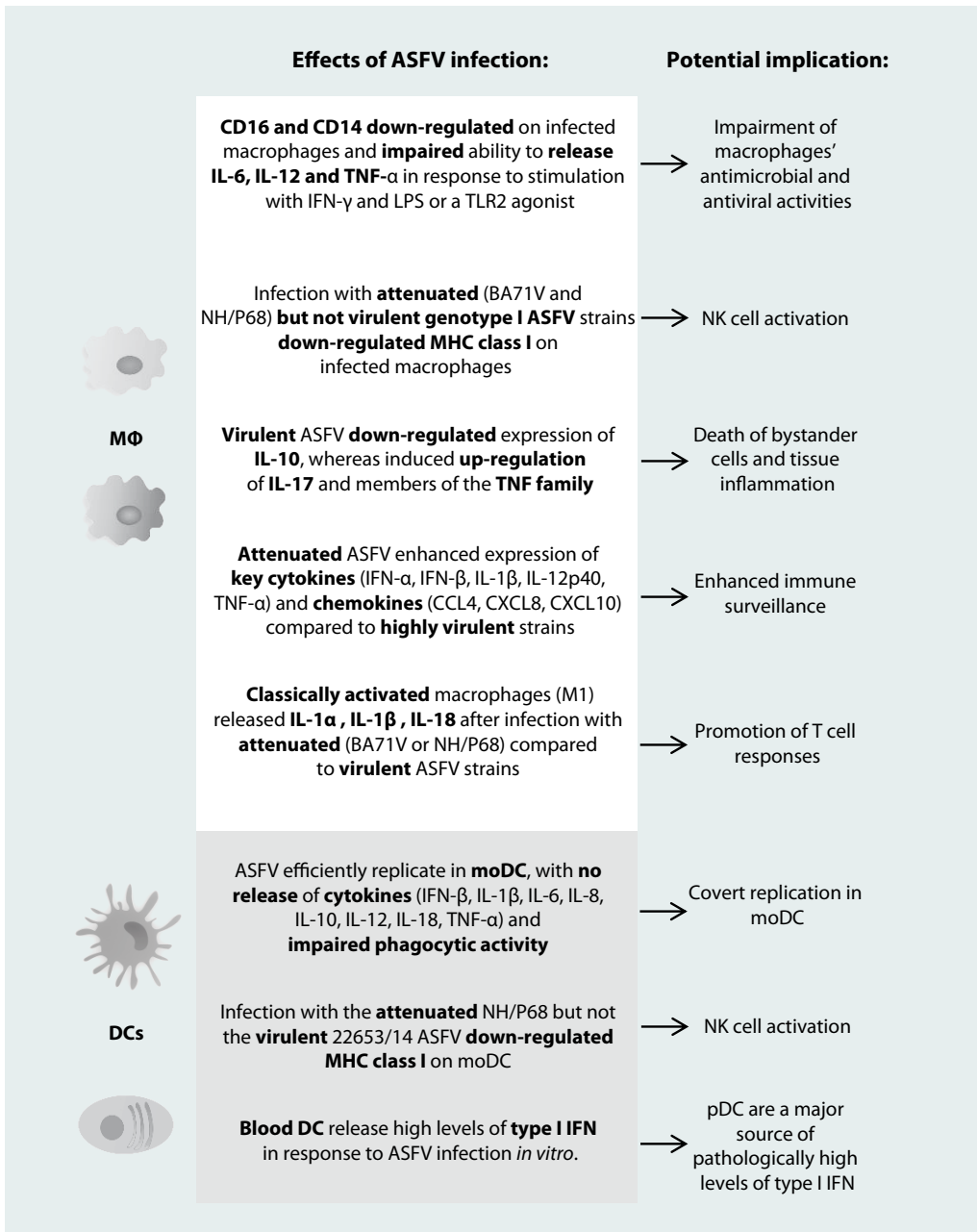
and release of pro-inflammatory cytokines, whereas M2 polarisation can be achieved through IL-4 (Mosser, 2003; Singleton *et al.*, 2016).

It was recently described that virulent isolates present a greater ability to infect Mφ activated with type I or type II IFNs compared to attenuated strains (Franzoni *et al.*, 2017, 2020; Golding *et al.*, 2016), whereas alternative activation did not affect susceptibility to ASFV infection. Both attenuated (NH/P68) and virulent (22653/14) ASFV strains efficiently replicated in different monocyte-derived macrophage subsets (moMφ, M1, M2, Mφ activated with IFN-α), but with delayed kinetics in moM1. The data suggested that the ability of ASFV to infect moM1 is initially impaired, but then the virus overcame cellular defence mechanisms and efficiently replicated in this subset (Franzoni *et al.*, 2020). MoM1 can mount a robust cytokine response after infection with attenuated ASFV strains (BA71V or NH/P68), with release of IL-1α, IL-1β and IL-18 (Franzoni *et al.*, 2017, 2020), whereas negligible release of all the tested cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α) was detected from moMφ, moM2, IFN-α-activated moMφ (Franzoni *et al.*, 2020). As previously reviewed, release of IL-18 might enhance the development of T cell responses, whereas IL-1β release might promote apoptosis of bystander uninfected cells, reducing ASFV replication and spread *in vivo* (Franzoni *et al.*, 2018). The virulent 22653/14 ASFV seems to have developed mechanisms to counteract cytokine responses by moM1, and further studies should be performed, aimed at understanding which viral factors, lost in attenuated strains, inhibit release of these key cytokines (Franzoni *et al.*, 2017, 2020). A summary of ASFV impact on Mφ phenotype and functionality is shown in Figure 3.2.

3.2.2 Dendritic cells

Dendritic cells (DCs) are regarded as sentinels of the immune system and play a central role in activating and shaping adaptive immune responses against pathogens. They express a variety of PRRs and can detect, take up and process antigen, and then migrate into secondary lymphoid tissues to present the processed antigens to T lymphocytes (Banchereau and Steinman, 1998). These cells can orchestrate host immune responses, being able to prime naïve T lymphocytes and to modulate the type and the characteristics of T and B cell responses (Banchereau and Steinman, 1998). DCs are a heterogeneous population and can be broadly divided into three subsets: conventional DC1 (cDC1), conventional DC2 (cDC2) and plasmacytoid DC (pDC) (Collin and Bigley, 2018). pDC are specialised in secreting large amounts of type I interferon following viral stimulation (Collin and Bigley, 2018), whereas cDC1 are specialised in priming a Th1 response through IL-12 secretion and antigen presentation via MHC class I to CD8^{+} T cells, and cDC2 can stimulate Th2 and Th17 responses (Collin and Bigley, 2018). Each species has its own peculiarities and DC phenotypes are also organ specific, as recently reviewed (Franzoni *et al.*, 2019).

To date, few studies have analysed DC responses to ASFV infection, as recently reviewed (Franzoni *et al.*, 2019). In the 1990s, researchers described that ASFV was able to infect skin-derived DCs *in vitro*, with subsequent impairment of DCs infection by foot-and-mouth disease virus (Gregg *et al.*, 1995). *In vivo*, it was reported that virulent ASFV L60 infected interdigitating DC (iDC): ASFV antigens were identified in iDC in mandibular lymph nodes at 3 days post infection and this was followed by a reduction in the number of iDC. These results suggest that early DC depletion in lymph nodes during infection with virulent ASFV isolate might contribute

	Effects of ASFV infection:	**Potential implication:**
MΦ	**CD16 and CD14 down-regulated** on infected macrophages and **impaired** ability to **release IL-6, IL-12 and TNF-α** in response to stimulation with IFN-γ and LPS or a TLR2 agonist	Impairment of macrophages' antimicrobial and antiviral activities
	Infection with **attenuated** (BA71V and NH/P68) **but not virulent genotype I ASFV** strains **down-regulated MHC class I** on infected macrophages	NK cell activation
	Virulent ASFV **down-regulated** expression of **IL-10**, whereas induced **up-regulation** of **IL-17** and members of the **TNF family**	Death of bystander cells and tissue inflammation
	Attenuated ASFV enhanced expression of **key cytokines** (IFN-α, IFN-β, IL-1β, IL-12p40, TNF-α) and **chemokines** (CCL4, CXCL8, CXCL10) compared to **highly virulent** strains	Enhanced immune surveillance
	Classically activated macrophages (M1) released **IL-1α , IL-1β , IL-18** after infection with **attenuated** (BA71V or NH/P68) compared to **virulent** ASFV strains	Promotion of T cell responses
DCs	ASFV efficiently replicate in **moDC**, with **no release** of **cytokines** (IFN-β, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α) and **impaired phagocytic activity**	Covert replication in moDC
	Infection with the **attenuated** NH/P68 but not the **virulent** 22653/14 ASFV **down-regulated MHC class I** on moDC	NK cell activation
	Blood DC release high levels of **type I IFN** in response to ASFV infection *in vitro*.	pDC are a major source of pathologically high levels of type I IFN

Figure 3.2. African swine fever virus modulation of macrophages and dendritic cell phenotype and functionality and possible implications. CXCL = C-X-C motif chemokine ligand; DC = dendritic cell; IFN = interferon; IL = interleukin; LPS = lipopolysaccharides; MHC = major histocompatibility complex; Mφ = macrophages; TNF = tumour necrosis factor; TRL = toll like receptor.

to unsuccessful development of a protective immune response (Gregg *et al.,* 1995). More recently, Golding *et al.* (2016) observed that *in vitro* porcine peripheral blood mononuclear cells enriched for DC (by depletion of CD3^{+} T cells, CD14^{+} monocytes and CD21^{+} B cells) released high levels of type I interferon in response to ASFV infection, suggesting that pDC might be a potential source of the pathologically high levels of type I interferon in pigs during acute ASFV infection. It was recently observed that both attenuated NH/P68 and virulent 22653/14 ASFV efficiently infected and replicated in monocyte derived DC (moDC), with infection with the virulent 22653/14 ASFV not modulating any surface marker expression and not inducing release of any of the tested cytokines (IFN-β, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α). These results suggest that, similar to Mφ, virulent 22653/14 strain covertly replicates in moDC, whereas NH/P68 infection resulted in MHC I down-regulation, thus potentially affecting NK cell activation *in vivo* (Franzoni *et al.,* 2018).

More studies focused on the interaction of ASFV with different DC subsets are needed. As recently reviewed, it would be interesting to assess ASFV interaction with different porcine blood DC subsets, i.e. cDC1, cDC2, pDC and to characterise DC interaction with other cell types, like NK cells, γδ-T cells, and Mφs. More studies focused on the infection of ASFV not only with blood derived DC, but also with DC from tissues are required, taking advantage of the great increase of the overall toolbox for the analysis of the pig immune system which occurred in the last decades (Franzoni *et al.,* 2019). Current knowledge of ASFV modulation of DC phenotype and functionality are summarised in Figure 3.2.

3.2.3 Type I interferon

Type I IFN is a crucial element of the innate immune response to viral infection and thus most viruses have developed mechanisms to counteract its induction or effects (Summerfield, 2012). Attenuated ASFV strains induce enhanced expression of type I IFNs (both IFN-β and several IFN-α subtypes) compared to highly virulent strains (Gil *et al.,* 2008; Razzuoli *et al.,* 2020; Reis *et al.*, 2016) and genes within ASFV multigene families (MGF) 360 and 530/505 may play a central role in inhibiting type I induction in ASFV-infected Mφ (Afonso *et al.,* 2004; Reis *et al.,* 2016). It was described that deletion or interruption of several genes within MGF360 and 530/505 in the genome of the virulent Benin 97/1 resulted in enhanced induction of type I IFN (Reis *et al.,* 2016). In addition, it was demonstrated that some members of these MGF suppress type I IFN induction through inhibition of transcription factors: A276R (MGF360-15R) inhibits IRF 3, whereas A528R (MGF505-7R) impaired both IRF3 and NK-kβ (Correia *et al.,* 2013). Other ASFV genes inhibited type I IFN production: the protein coded by I329L is a TLR3 homologue and targets TLR4/toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF), impairing activation of both IRF3 and nuclear factor (NF)-κB (Correia *et al.,* 2013; de Oliveira *et al.,* 2011).

ASFV can subvert not only type I IFN induction, but also that of interferon stimulated genes (ISGs). It was described that infection with attenuated NH/P68 but not virulent Armenia/07 resulted in enhanced CCLX10 and IFIT-1 expression in porcine alveolar Mφ (Garcia-Belmonte *et al.,* 2019). Portugal and colleagues (2018) reported similar findings, with virulent L60 but not attenuated NH/P68 inhibiting induction of several ISGs in Mφ following IFN-α stimulation (Portugal *et al.,* 2018). NH/P68 lacks several genes within MGF360/530 (Portugal *et al.,* 2018) and these genes seem to play a crucial role in modulating ISG responses: deletion of six MGF360 and

two MGF530 genes from the virulent Pr4 strain resulted in enhanced expression of several ISGs in infected Mφ, including cytokine IP-10, IFIT-1, and ISG43 (Afonso *et al.,* 2004).

Despite ASFV possessing strategies to inhibit type I IFN production/responses by Mφ or other cell types, the virus likely promotes release of these cytokines by pDC, as described in other viruses (Summerfield, 2012). High levels of type I IFN have been observed in pigs infected with virulent ASFV (Golding *et al.,* 2016) and pDC are likely to be the source of this cytokine (Golding *et al.,* 2016).

Viruses have evolved mechanisms to become 'tolerant' to the action of type I IFN (Summerfield, 2012) and several studies suggest that ASFV has employed this strategy. It was described that high doses of type I IFN (2,000 U/ml) impaired ability of attenuated OUR T88/3 but not virulent ASFV isolates to infect porcine alveolar Mφ (Golding *et al.,* 2016). Similar findings were obtained in moDC infected with ASFV strains of diverse virulence (attenuated BA71V and NH/P68 and virulent 22653/14) (Franzoni *et al.,* 2018). It was recently reported that IFN-α inhibited ASFV ability to infect moMφ in a dose dependent manner, and only the virulent 22653/14 was tolerant to low levels of IFN-α (100 U/ml) (Franzoni *et al.,* 2020).

Ability of virulent ASFV isolates to overcome the type I IFN induced antiviral state seems to be linked to MGF360 and MGF505 genes: attenuated OUR T88/3 lacks genes within MGF360 and 505, deletion of five genes within MGF360 and two within MGF505 from virulent Pr4 ASFV resulted in enhanced sensitivity to IFN-α (Golding *et al.,* 2016). Tolerance to type I IFNs seems to be linked to virulence *in vivo*: deletion of genes within MGF360 and 505 from the virulent genotype II Georgia 2007 or genotype I Benin 91/7 resulted in attenuation *in vivo*, and deleted mutants were also able to induce protection to homologous challenge (O'Donnell *et al.,* 2015; Reis *et al.,* 2016).

Not only type I IFN, but also some ISGs were able to inhibit ASFV infection. Two interferon-induced transmembrane proteins (IFITM2 and IFITM3) affected BA71V infectivity in Vero cells, impairing viral entry and uncoating (Munoz-Moreno *et al.,* 2016), whereas MxA protein affected BA71V entry in Vero cells due to its recruitment to perinuclear viral factories (Netherton *et al.,* 2009). It would be interesting to assess whether differences exist between strains of diverse virulence, and which viral factors are involved in resistance to type I IFN/ISG mediated antiviral activities.

3.3 Humoral responses against African swine fever virus upon infection

The way viral antigens are recognised by B cells are through inmunoglobulins (Ig). These proteins are produced by B cells in a vast range of specificities, each B cell is producing Ig of a single specificity. Membrane-bound Ig on the B cell surface serves as receptor for antigen specificity and it is known as the B cell receptor (BCR). Ig of the same antigen specificity is secreted as antibody (Ab) by terminally differentiated B cells or plasma cells (Janeway *et al.,* 2017). Even though research on B cell development has received some attention in pigs, there are still several gaps into B cell development and characterisation of B cell subpopulations. On the other hand, Ab repertoires in pigs have been extensively studied (Butler *et al.*, 2017). Their studies showed

that pigs resemble most mammals in using both kappa and lambda light chains of Ab. Similar to in humans, but unlike in many other mammals, the kappa-lambda expression ratio is essentially equal in adult swine, but not during development (reviewed in Butler *et al.,* 2017).

Antibodies are secreted in response to viral infections and they have been extensively used in diagnosis of infections such ASFV. This topic will be later explained in Chapter 5 in this book. Here, we will focus on the knowledge of humoral responses during ASFV infection.

Surprisingly, the role of Ab within ASFV infection remains controversial nowadays. It has been described that ASFV infection induces neutralising Ab but the existence of non-neutralising Ab, which binds to virus without diminishing infectivity, has also long been recognised (reviewed in Escribano *et al.,* 2013). Early experiments showed that antibodies obtained from pigs that had recovered from ASFV infection with one of the most used strains in experimental protection experiments, E75CV1-4 virus, a Spanish strain (E75) which was adapted to grow in CV1 cells and propagated in pig macrophages, appeared to confer protection against ASFV infection. Sera from E75CV1-4 infected convalescent pigs neutralised the infectivity of virulent ASFV isolates E75, E70, Lisbon 60, Malawi Lil 20/1 and a low-passage tissue culture-adapted variant of E75, namely E75CV/V3, by 86-97% in Vero and macrophage cell cultures (Zsak *et al.,* 1993). Anti-viral antibodies' role in homologous protective immunity to E75 virulent ASFV strain was examined by passive transfer experiments in swine. In those experiments, 85% of the animals receiving anti-ASFV immunoglobulin survived challenge infection. In contrast, 100% mortality was observed in the experimental group that received control immunoglobulin sera fractions or phosphate-buffered saline. With the exception of a significantly delayed and transient fever response, the animals who received anti-ASFV antibodies remained clinically normal following challenge, whereas the control group presented clinical ASF on day 4 post-challenge. In addition, a significant 3-day delay in the onset of viraemia and a 10,000-fold reduction in both mean and maximum virus titres were observed for animals given anti-ASFV Ig. These data strongly suggest that anti-ASFV antibodies alone protect swine from lethal infection with virulent ASFV (Onisk *et al.,* 1994). Moreover, they support the view that the antibody-mediated protective effect is an early event that effectively delays disease onset (Onisk *et al.,* 1994). Following this line of thought, other reports showed that when ASFV antibodies are transferred through colostrum, they also confer a degree of protection against viral challenge in suckling piglets (Schlafer *et al.,* 1984a,b) showing a representation of *in vivo* antibody-mediated protection against ASFV. Several ASFV proteins are able to induce neutralising antibodies in immunised pigs. Among these proteins, p54 and p30 (Gómez-Puertas and Escribano, 1997; Gómez-Puertas *et al.,* 1996) were shown to be involved in various steps of virus attachment and internalisation. Nevertheless, immunisation of pigs with recombinant p54 and p30 proteins expressed in baculovirus did not protect against virulent ASFV challenge. Similarly, a vaccine based on ASFV proteins p30, p54, p22, and p72 produced in baculovirus failed to protect pigs against a virulent ASFV challenge, despite producing neutralising antibodies (Neilan *et al.,* 2004); the above results added to the controversy about the role of antibody-mediated neutralisation of ASFV in protection against ASF infection (Escribano *et al.,* 2013).

Several *in vivo* and *in vitro* studies indicate a potential protective role of Ab by mechanisms including complement mediated cell lysis or antibody dependent cell-mediated cytotoxicity (ADCC) (Rock*,* 2017; Takamatsu *et al.,* 2013). A remarkable correlation has been established

between the presence of haemadsorption (HAD) inhibitory antibodies in serum with its capacity to inhibit the infection of ASFV *in vitro* and to partially protect against ASFV challenge *in vivo* (Burmakina *et al.,* 2019; Ruiz-Gonzalvo *et al.,* 1996).

In summary, ASFV Ab and their neutralisation ability have received little attention recently, and most of the publications only use antibodies to monitor infection without further analysis on their activity, mechanisms, localisation and isotypes. For example, virus antigens important for protection or cross-protection have not been fully characterised although antibodies against the virus CD2-like protein have been shown to be involved in cross-protection (Burmakina *et al.,* 2019). Further studies on Ab generated after infection with different isolates of ASFV are required to elucidate their role in ASFV infection.

3.4 Cellular response against African swine fever virus upon infection

T cells are defined in pigs, as in other species, by the expression of the CD3 T cell co-receptor complex on their surface. Additionally, they express either αβ or γδ T cell receptors (TCR) as antigen receptors. T cells, like all antigen-specific lymphocytes, are able to recognise antigens specifically and react differently depending on their function (helper function, cytotoxicity, cytokine production, and sometimes even antigen presentation). A special characteristic of the adaptive immune system is that after antigen contact and the immune response that follows, antigen-specific memory cells are produced, so that a subsequent reaction to an antigen can be faster and/or stronger. However, both the antigen presentation, followed by the antigen-specific reaction and even more so the generation of memory cells take time. Therefore, the adaptive immune system reacts with very high antigen-specificity but with a significant time delay compared to the innate immune system. This time factor is problematic in the case of an acute and usually lethal virus infection such as that caused by infection with ASFV. Therefore, failure to detect a specific cellular immune response after infection with virulent ASFV strains is commonly reported. However, mechanisms of both specific and non-specific cellular immune responses to infections with moderately virulent ASF viruses and after immunisations with ASFV are described.

3.4.1 αβ TCR+ T cells

When T cells contact their specific antigen using their TCR, they differentiate into effector cells. Apart from a small population of regulatory T cells, the main distinction is made between cytotoxic T cells and helper T cells.

3.4.1.1 CD8+ cytotoxic T cells

Cytotoxic T cells play an important role in protection against intracellular antigens. These cells recognise short sections of viral antigen displayed on the surface of infected cells via MHC class I (referred to as swine leukocyte antigen I (SLA I) in pigs), and then kill such cells usually by releasing perforin and granzyme. Cytotoxic T cells can also kill cells by engaging Fas/FasL, but this is only marginally described in pigs. In pigs, classical MHC I restricted T cells with lytic activity are described as CD2+CD3+CD4-CD5^{high}CD6+CD8α^{high}CD8β+ (Denyer *et al.,* 2006).

Depending on their function as effector or memory cells, they can also express CCR7 and CD27. As CD8 T cells also produce IFN-γ and TNF-α they play an important role in antimicrobial immunity. Not least because of this cytokine secretion and the production of effector molecules, such as perforin, cytotoxic T cells can also be responsible for severe immunopathology after viral infection.

T cells, especially CD8α+ T cells, play an important role in protective immunity against ASFV (Oura *et al.,* 2005). After exposure to the low virulent ASFV strain OUR T88/3, pigs were depleted of CD8α+ lymphocytes with monoclonal anti-CD8 antibodies (clones 76-2-11 and 11/295/33). A subsequent challenge with homologous, but virulent, OUR T88/1 revealed that depleted animals suffered from severe acute ASF and died, whereas non-depleted animals showed only mild clinical signs and survived. Since CD8α is expressed on different T cell populations like cytotoxic T cells, γδ T cells, NK cells, invariant T cells or memory helper T cells, it remains unclear which of these subpopulations might mediate the described protection. As cytotoxic T cells in the pig typically express a heterodimer of CD8α and CD8β, in order to define the protective CD8 expressing cell type the authors tried to specifically deplete CD8β T cells in a follow-on experiment. However, depletion with the anti CD8β antibody (clone PPT22) was not successful in all animals. Only one animal out of seven showed a significant depletion of CD8β+ cells. Interestingly, this pig did not survive the challenge infection. Overall, these depletion experiments proved the crucial role of CD8α+ T cells for protection. It remains to be clarified which CD8+ T cell type is responsible for the protective effect. Furthermore, although this experiment proves the protective effect of CD8+ ASFV-specific memory cells, it does not explain the role of cytotoxic T cells in acute infections.

An early reference to ASFV specific CD8+ T effector cells is found in Norley and Wardley (1984): the authors infected pigs with a virulent Uganda isolate and examined peripheral blood mononuclear cells (PBMCs) of these animals in a killing assay with virus-infected autologous testicular cells as target. The effector cells were able to lyse the ASFV-infected cells virus-specifically. Since unseparated PBMCs were used, it can only be assumed that CD8+ cells were involved in cytotoxicity. Furthermore, the statement is limited to early effector cells, since the animals in this experiment did not survive long enough to investigate the cytotoxic properties of ASFV-specific memory cells. Such ASFV specific CD8+ memory cells could be described in a study by Martins *et al.* (1993): the authors used SLA inbred mini-pigs that had survived infection with the non-lethal ASFV strain NH/P68. Their PBMCs were re-stimulated *in vitro* and their cytotoxic properties were tested in a killing assay with homologous infected macrophages. A specific lysis of ASFV infected macrophages against non-infected or classical swine fever virus (also called hog cholera virus) infected controls could be shown. The authors demonstrated that the observed killing is SLA I dependent, by showing that both anti-SLA I antibodies and anti-CD8 antibodies strongly reduce cytotoxicity. In addition, they showed a preferential lysis of infected macrophages with matching SLA I.

Which viral epitopes are recognised by ASFV specific CD8 cells is still largely unclear. Knowledge of CD8 epitopes is important in any viral infection, not only for the production of a possible vaccine, but also for the elucidation of CD8-dependent protective effects and immunopathogenesis. Two studies used inbred mini-pigs immunised with two different attenuated isolates that were subsequently challenged with homologous virulent strains (Alonso *et al.,* 1997; Leitão *et al.,* 1998). In the study by Alonso *et al.* (1997), these pigs served as donors for the effector cells

tested in killing assays against infected alveolar macrophages. Specific lysis was achieved with isolated CD8+ cells but not with CD4+ cells. In a further experiment, the target cells were not infected with complete ASFV, but with a recombinant vaccinia virus expressing the early ASFV protein encoded by the CP204L gene (also referred to as p30 or p32). These target cells were also specifically lysed, albeit less strongly than those infected with the full virus. The result indicates that specific CD8 epitopes are present in CP204L (Alonso *et al.*, 1997). Leitão *et al.* (1998) showed that effector cells isolated from ASF recovered pigs were capable of lysing macrophages loaded with a 25 amino acid peptide derived from the B646L gene that encodes the major capsid protein p72. This lysis could be efficiently blocked with antibodies against SLA-I, again suggesting a role for CD8+ cells (Leitão *et al.*, 1998). More recent work using an attenuated strain of ASFV has shown that both B646L and CP204L, along with other 16 antigens, are capable of inducing secretion of IFNγ from cells from recovered inbred pigs (Netherton *et al.*, 2019). Individual epitopes have also been mapped within the EP153R and EP402R proteins (Argilaguet *et al.*, 2012; Burmakina *et al.*, 2019) genes; however, the relative importance of a given antigen or epitope in the protection mediated by these attenuated viruses is unclear.

An unusual facet of the swine immune system is the significant number of CD4+CD8+ double positive (DP) T cells (Saalmüller *et al.*, 1987). DP T cells play a distinct role in porcine immune responses (Okutani *et al.*, 2018) and are often described as memory or effector T cells (Saalmüller *et al.*, 2002). *In vitro* stimulation studies demonstrated CD8 upregulation on porcine CD4 T cells (Reutner *et al.*, 2013) or showed proliferation and cytokine production in DP T cells e.g. (Lefevre *et al.*, 2012). DP T cells are capable of expressing perforin and granzyme, also indicating a role as cytotoxic effector cells. Alonso *et al.* (1997) discussed in the abovementioned study the involvement of DP T cells, which account for a considerable proportion of T cells after re-stimulation, in the measurable specific cytotoxicity (Schäfer *et al.*, 2019). Antigen stimulation of PBMCs from ASF recovered pigs induced both proliferation of DP T cells that are also perforin positive (Takamatsu *et al.*, 2013), as well as secretion of IFN-γ (Netherton *et al.*, 2019). After infection with the virulent Armenia strain, an increase of DP T cell frequency with a corresponding decrease of CD4+ T cell frequency in peripheral blood and lymphoid organs of domestic pigs was detectable. In wild boar, no direct correlation between CD4+ T helper cells and DP T cells was detectable. DP T cells of wild boar showed a high proliferative activity. However, data on the role of DP T cells during acute primary infections are not available. Nevertheless, these studies indicate an activation-dependent expression of CD8 on CD4+ T cells and underline the effector functions of DP T cells (Hühr *et al.*, 2020).

A rare cell population of αβTCR+ T cells are the invariant NK T cells (iNKT). Their frequency in swine is typically between 0.01 and 1% among CD3+ T cells and decreases with age. These cells bridge and orchestrate both untargeted innate and specific adaptive responses, which are crucial for pathogen clearance and survival. In contrast to the vast heterogeneity of TCRs among conventional CD3+ T cells (cTC), iNKT cells possess a semi-invariant TCR. This TCR is restricted to the non-classical MHC class I-related CD1d, presenting lipid or glycolipid antigens. iNKT cells can be activated antigen-dependently with glycolipids derived from microbes or the host by TCR-CD1d interactions or antigen-independently via cytokines, mainly IL-12 and IL-18 or type I IFN. Effector cytokines are present as immediately available preformed mRNA transcripts in iNKT cells. Therefore, iNKT cells rapidly proliferate and secrete effector molecules like IFN-γ, IL-17 or granulocyte-macrophage colony-stimulating factor after activation. Moreover, they are able to

lyse infected cells by perforin and Fas/FasL interaction. Pig iNKT cells can be stained by a cross reacting murine CD1d tetramer, loaded with PBS57. After infection with the virulent Armenia strain, an average 5-fold increase in the frequency of iNKT cells can be observed in blood. In the examined gastrohepatic lymph nodes, liver and lung a less strong increase in frequency is measurable. This first study does not yet make any statements about the activation and effector mechanisms of iNKT cells after ASFV infection, but it does provide indications of the possible involvement of this rare T cell population in the pathogenesis of ASFV infection (Schäfer *et al.*, 2019). In the same line are *in vitro* experiments by Denyer *et al.* (2006), who investigated perforin-producing lymphocytes in a co-culture experiment, in which they co-cultivated PBMCs from naïve pigs with ASFV-infected MHC I haplotype-matching cells and observed a stronger increase in perforin positive cells in culture with ASFV compared to the control. Furthermore, they also described that after stimulation with ASFV infected cells, the majority of perforin positive cells can be assigned to NK cells and MHC I independent NKT cells (CD3+CD6-CD8+CD16+), which indicates a specific proliferation of these cells (Deyner *et al.*, 2006).

3.4.1.2 CD4+ T helper cells

In contrast to the CD8+ T cells described above, CD4+ cells recognise antigens presented by MHC class II. While almost every cell can present an antigen via MHC I, only classical antigen presenting cells (APC) present phagocytosed extracellular antigen in MHC II. However, as will be discussed later, pigs also have T cell subtypes and NK cells that express MHC II on their surface and are therefore able to present antigen for CD4+ T cells. Depending on the cytokine environment, CD4+ T helper cells develop into a number of functionally different subgroups, which can be differentiated by means of different transcription factors, also in pigs. The existence of Th1, Th2, Th17 and regulatory T (Tregs) in pigs is undisputed. The effector molecules produced by the cells correspond to those of comparable helper cells in humans and mice. Of interest in connection with ASFV, is the group of perforin-producing cytolytic T-helper cells characterised by the transcription factor EOMES. These cells belong to the CD4+CD8+ phenotype and were discussed in the previous paragraph because of their cytolytic activity.

The activation of CD4+ cells after infection is measured by their proliferation and/or IL-2 and IFN-γ production. Some early studies report T cell proliferation and cytokine production in assays using T cells from pigs that had survived low virulent ASFV infection (Revilla *et al.*, 1992). However, there is usually no consistent differentiation of proliferating T cell subtypes. Regarding a specific response of CD4+ cells, Canals *et al.* (1992) presented a more detailed study: they re-stimulated PBMCs of ASFV E75 immune pigs with active virus and with ultraviolet (UV) inactivated virus. The measured proliferation after stimulation with inactivated virus (and thus extracellular antigen) could be completely inhibited with anti-CD4 antibodies, whereas anti-CD8, anti-SLA I and anti-SLA II antibodies only partially inhibited this proliferation (Canals *et al.*, 1992). ASFV seems to influence the ability of leukocytes to proliferate in general. CD4+ and CD8+ splenocytes from mini-pigs infected with the Malawi ASFV strain showed a constantly decreased proliferation performance after mitogenic stimulation with concanavalin A or pokeweed mitogen over the duration of the infection. The effect could not be reversed by rIL-2, but there was no evidence of apoptosis in T cells (Childerstone *et al.*, 1998). *In vitro* studies have also shown an effect of ASFV infection on proliferation of naïve cells and this may depend

in part on the EP402R gene that is responsible for the haemadosorption phenomenon (Borca *et al.,* 1998).

A major task of the CD4+ T cells is to assist in the so-called T cell dependent B cell proliferation and maturation. In an *in vitro* antibody synthesis assay, this T cell-help could be investigated. PBMCs of BA71 immune pigs were re-stimulated *in vitro* with ASFV and their antibody production was measured *in vitro*: if the T cells are removed by rosetting or the CD4+ cells are specifically removed with anti-CD4 antibodies, ASFV specific antibody production is significantly reduced (Casal *et al.,* 1987). This result would have to be tested for other ASFV strains, but indicates a T cell dependent antibody production and thus, probably also a T cell dependent class switch.

3.4.2 γδ T cells

Unlike many other mammals, pigs have a large number of γδ T cells in the periphery and in the lymphoid organs. In young pigs up to 50% of all PBMCs are γδ T cells, the percentage decreases with age. They are defined as CD3+ γδ TCR+ CD4-. Depending on the activation status, three cell populations can be distinguished in this population: CD2-CD8- (naïve); CD2+CD8- (proliferation and cytokine secretion) and CD2+CD8+ (cytotoxicity, proliferation and cytokine secretion) (Yang and Parkhouse, 1996).

Due to their activation via TCR, γδ T cells are, by definition, assigned to the adaptive immune system. Thus, they can be activated directly or by contact with APCs and then fulfil functions of cytotoxicity and cytokine production (mainly IFN-γ, TNF-α and IL-17A) (Sedlak *et al.,* 2014). In parallel, however, they also fulfil tasks of the innate immune system, since activation is also possible via other receptors, such as TLR. The extent of this reaction is regulated by an interaction of activating and inhibiting receptors. A third function of γδ T cells in pigs is their ability to present antigen. For this purpose, the cells express MHC II on the surface. This function may be particularly relevant after ASFV infections: it is generally agreed that ASFV replicates predominantly in monocytes/macrophages and thus influences their function. Can antigen-presenting γδ T cells fill this infection-related gap in antigen presentation?

Despite the large number of γδ T cells in pigs and their ability to present antigens, their involvement in the anti ASFV response has been studied only on few occasions. After infection with the moderately virulent strain Netherlands 86, an association between circulating γδ T cells and survival of the animals was described (Post *et al.,* 2017). Takamatsu *et al.* (2006) showed that γδ T cells, incubated with ASFV, are able to stimulate ASFV specific T cells. However, the mechanism of this cell interaction has yet to be clarified (Takamatsu *et al.,* 2006). After lethal infection with the virulent Armenia strain, the frequency of γδ T cells tends to decrease. In domestic pigs, the composition of γδ T cell-subpopulation remains unchanged over the time of infection. In infected wild boar, however, an activation and thus a percentage shift towards CD2+ CD8- or CD2+ CD8+ γδ T cells is observed in blood and spleen (Hühr *et al.,* 2020).

3.4.3 Natural killer cells

NK cells, which by definition belong to the innate immune system, play a crucial role in fighting virus-infected cells. They produce a relevant amount of IFN-γ and TNF-α in an antiviral immune

response. Their activation status is regulated by activating and inhibiting ligands that can bind ligands to different target cells. NK cells interact with APCs, especially DCs, and thus can induce their maturation and increase their functionality. In pigs, NK cells are detected as CD3-CD2+CD8+CD16+perforin+ cells. Based on the expression of NKp46, NK subtypes with different functionality can be described. While cytolytic activity could be measured independently from NKp46 expression, NKp46+ NK cells produce significantly higher levels of IFNγ after stimulation (Mair *et al.,* 2012).

Similarly, as described above for γδ T cells, NK cells in pigs also appear to be able to act as APCs. After cytokine stimulation (IL-2, IL-12, IL-18, IFN-α) they express MHC II on their surface (Franzoni *et al.,* 2014). Additionally, after stimulation (IL-2, IL-12, IL-18), co-stimulatory molecules CD80/CD86 are also found on the surface of NK cells (De Pelsmaeker *et al.,* 2019). The same authors also report that NK cells internalise antigen of the cells they lyse. Thus, NK cells, like γδ T cells, would theoretically be able to take over APC function in ASFV infected animals instead of the infected classical APCs. This mechanism has not yet been described after ASFV infection. However, it has been shown that the increase in cytotoxic activity of NK cells correlates with the absence of clinical signs after infection with the non-haemabsorbing NH/P68 strain. Conversely, a lower cytotoxicity of NK cells could be measured in pigs with clinical signs of chronic ASF (Lanier*,* 2005). Since in the described experiment the animals with NK cell activity were also protected from challenge with the virulent strain Lisbon 60, participation of NK cells in an antigen-specific memory response can be considered. After infection with the moderately virulent strain Malta 78, a decrease in NK activity was measured in 4 of 6 animals (Norley and Wardley, 1983). However, an interesting temperature phenomenon was also discussed: the NK activity measured *in vitro* disappears at 40 °C. Therefore, reduced or missing protective NK activity in ASFV infected pigs with high fever and severe courses of disease is possible. However, some *in vitro* experiments show a decrease of NK activity after infection with virulent virus strains, independent of temperature (Martins and Leitão*,* 1994; Mendoza *et al.,* 1991).

3.4.4 Regulatory T cells

Regulatory T (Tregs) cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. Generally, they have multiple mechanisms to mediate their suppressive effects: suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC maturation or function.

In pigs, Tregs defined as CD3+CD4α+CD25+FoxP3+ cells have not received much attention in the context of viral infections until recently. A study by Sanchez-Cordon *et al.* (2020) has recently shown that Tregs exhibited a progressive increase in two groups of immunised animals with OURT88/3 or BeninΔMGF ASFV strains. In fact, they peak at day 130 pi, showing significant statistically differences as compared with pre-immunisation values. They also showed that those Tregs were able to inhibit proliferative responses *in vitro*, suggesting that higher percentage of Tregs circulating in blood might represent an inhibition of specific immune responses. Therefore, Tregs might represent a viral strategy to prevent immune responses against ASFV.

3.5 Immunity unknowns

Given the relevance of ASFV infection in swine, it is surprising that immune responses against ASFV are still poorly characterised. Deeper understanding of the immunity developed by the natural hosts, including bushpig (*Potamochoerus larvatus*) and warthog (*Phacochoerus africanus*), by ASFV infection is an area that should be addressed. However, this approach is very difficult since few reagents are available for wild African pigs with little cross reactivity among them. Unfortunately, many gaps still exist regarding immune responses in the natural infection vs experimental infection. Besides, identification of the correlates of immune protection would allow applying the three Rs (Replace, Reduce, Refine) guiding principles of animal science and reducing unnecessary painful challenges with ASFV (Arias *et al.,* 2017). Despite these advances, very little is known about how immune responses are generated during the course of ASFV natural infection.

Acknowledgements

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