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**INTERACTION OF MONOCYTES AND DERIVED  
MACROPHAGE SUBSETS WITH AFRICAN SWINE FEVER  
VIRUSES OF DIVERSE VIRULENCE**

by

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## ABSTRACT

African swine fever (ASF) is a devastating disease for which there is no vaccine available. The ASF virus (ASFV) primarily infects cells of the myeloid lineage and this tropism is thought to be crucial for disease pathogenesis. A detailed *in vitro* characterization of the interactions of a virulent (22653/14) and a tissue culture adapted (BA71V) strains of ASFV with porcine monocytes, un-activated (moM $\Phi$ ), classically (moM1) and alternatively (moM2) activated monocyte-derived macrophages was conducted to better understand this relationship. Low concentration of hM-CSF was selected as the method of choice to generate moM $\Phi$ . Using a multiplicity-of-infection (MOI) of 1, both viruses were able to infect monocytes and macrophage subsets, but BA71V presented a reduced ability to infect moM1 compared to 22653/14, with higher expression of early compared to late proteins. Using an MOI of 0.01, only 22653/14 was able to replicate in all the macrophage subsets, with initially lowest in moM1 and moM2. ASFV down-regulated CD16 expression and BA71V-infected but not 22653/14-infected moM $\Phi$  and moM2 presented with a reduced expression of MHC class I. Higher levels of IL-18, IL1- $\beta$  and IL-1 $\alpha$  were released from moM1 after infection with BA71V. These results revealed differences between these strains, suggesting that virulent isolates have evolved mechanisms to counteract activated macrophages responses, promoting their survival, dissemination in the host and so ASF pathogenesis.

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## CHAPTER 1. INTRODUCTION

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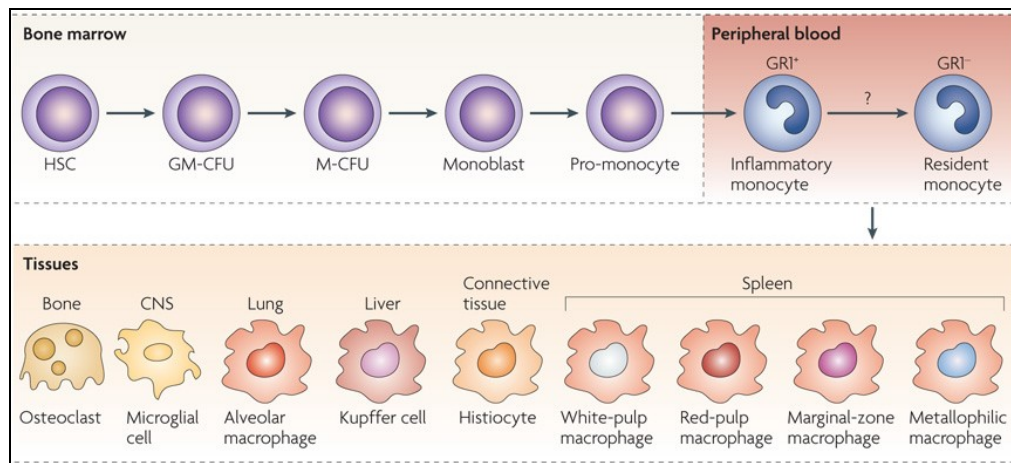
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## 1.1 Monocytes and macrophages

Monocytes and macrophages are the main target of the African Swine Fever Virus (ASFV) (Sánchez-Cordón *et al.*, 2008).

Monocytes are primary immune cells, which are derived from a common myeloid progenitor cells in the bone marrow (haematopoietic stem cell) and there undergo differentiation steps, in response to macrophage colony-stimulating factor, before enter the bloodstream (Volkman and Gowans, 1965, Mosser and Edwards, 2008). In mice, monocytes can also be differentiated into 2 populations: ‘inflammatory’ monocytes, with a  $GR1^{+}CX3CR1^{low}$  phenotype, which rapidly exit the blood, and ‘resident’ monocytes, which do not express  $GR1^{-}$  (Geissman *et al.*, 2003). As shown in figure 1.1, monocytes circulate in the peripheral blood before migrating to different tissues and replenish the tissue macrophage populations (Volkman and Gowans, 1965; Mosser and Edwards, 2008). Their half life is very short, about one day in mice (Van Furth and Cohn, 1968) and 3 days in humans (Whitelaw, 1972).



**Figure 1.1. Monocytes origin and differentiation.** Monocytes originate in the bone marrow from a common haematopoietic stem cell (HSC) and there they undergo differentiation steps during which they commit to the myeloid and then to a monocyte lineage. They differentiate into monoblasts and then pro-monocytes before becoming monocytes, which enter the bloodstream. In mice these cells can be differentiated into two populations: inflammatory and resident monocytes. It is unclear if inflammatory monocytes mature into resident monocytes or if they represent distinct populations. Monocytes migrate to different tissues, where they replenish tissue-specific macrophages. CNS, central nervous system; GM-CFU, granulocyte-macrophage colony-forming unit; M-CFU, macrophage colony-forming unit. Figure taken from Mosser and Edwards, 2008.

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Monocytes are a heterogeneous population and in human they can be divided into two subsets: CD14<sup>+</sup>CD16<sup>-</sup> (90% of circulating monocytes), which are often called classical monocytes, and CD14<sup>+</sup>CD16<sup>+</sup>, which exhibit features of tissue macrophages (Ziegler-Heitbrock, 1996). CD14<sup>+</sup>CD16<sup>+</sup> express CD32 and higher amount of MHC II molecules than classical monocytes and they have distinct chemokine–receptor expression profile (Weber *et al.*, 2000). Human monocytes can also be characterised also by their expression of CD64, also known as FcγRI (Grage-Gribenow *et al.*, 2001). CD14<sup>+</sup>CD16<sup>+</sup>CD64<sup>+</sup> monocytes present a dendritic cells (DC) phenotype, expressing higher levels of MHC II and CD80/86 than classic monocytes and having an enhanced T-cell-stimulatory activity (Grage-Gribenow *et al.*, 2001). It was speculated that CD14<sup>+</sup>CD16<sup>+</sup>CD64<sup>+</sup> cells could represent an immunoregulatory monocyte phenotype or an intermediate phenotype between monocytes and DC (Grage-Gribenow *et al.*, 2001). As previously stated, in mice monocytes can be divided according to their expression of CCR2, CX3C-chemokine receptor 1 (CX3CR1) and GR1 into two subpopulations: inflammatory CCR2<sup>+</sup>GR1<sup>+</sup>CX3CR1<sup>low</sup> and resident CCR2<sup>-</sup>GR1<sup>-</sup>CX3CR1<sup>high</sup> (Palframan, 2001, Geissman *et al.*, 2003). It is unclear if they represent distinct populations or if inflammatory monocytes are initially released into the circulation and, in the absence of inflammation, they mature into resident macrophages, altering their functional and phenotypic characteristic, passing through an intermediate phenotype (CCR2<sup>+</sup>GR1<sup>low</sup>) (Gordon and Taylor, 2005). Porcine monocytes can instead be characterised based on expression of CD163, which is scavenger receptor of haemoglobin/haptoglobin complexes (Sachez *et al.*, 1999, Kristiansen *et al.*, 2001). CD163<sup>+</sup> monocytes represent a more mature subset, in fact they produce higher amount of TNF-α, present higher levels of CD80/CD86 co-stimulatory molecules and SLA DR antigens and have better antigen-presenting capacity to primed T CD4<sup>+</sup> lymphocytes (Chamorro *et al.*, 2004).

As shown in figure 1.1, circulating monocytes migrate into tissue in the steady state or in response to inflammation, where they differentiate into macrophages or into specialised cells such as DC and osteoclasts (Volkman and Gowans 1965, Mosser and Edwards, 2008). Beyond this precursor role, monocytes can carry out specific effector functions during infection (Serbina *et al.*, 2008). In fact in mice

inflammatory monocytes are implicated in defence against bacterial, protozoal, and fungal pathogens. They respond rapidly to microbial stimuli by secreting cytokines and antimicrobial factors (Serbina, *et al.*, 2008). In addition, a recent study reported that in mice monocytes can participate in steady-state surveillance of the lung, in a complementary way to resident macrophages and DC, without differentiating into macrophages (Rodero *et al.*, 2015).

Macrophages as recognised by Metchnikoff are first and foremost professional phagocytes, which express all the genes required to internalize particles and to degrade those particles in lysosomes (Hume, 2015). Tissue macrophages maintain tissue homeostasis through the clearance of senescent cells and the repair/remodelling of tissues after inflammation (Gordon, 1988). They clear the interstitial environment of extraneous cellular materials, independently of immune-cell signalling and in the absence of other immune cells. Macrophages are involved in removal of cellular debris that are generated during tissue remodelling and rapidly clear cells that have undergone apoptosis. In human they also clear approximately  $2 \times 10^{11}$  erythrocytes each day, recovering iron and haemoglobin (Mosser and Edwards, 2008). These cells can rapidly respond to endogenous danger signals generated following injury or infection. They recognize pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs), such as toll like receptors (TLRs), NOD-like receptors (NAPLs) and C-type lectin receptors. Through complement receptors these cells interact with the complement inflammatory cascade, crucial to ward off infection (Martinez *et al.*, 2009). Macrophages are a heterogeneous population and this heterogeneity reflects the specialization of function adopted by macrophages in different anatomical locations. For example osteoclasts are specialised in bone remodelling and instead alveolar macrophages present high expression of pathogen recognition receptors (PRRs), enabling them to clear microorganisms, virus and environmental particles in the lung (Gordon and Taylor, 2005).

*In vitro* human monocytes can be differentiated into macrophages (MoM $\Phi$ ) with the addition of macrophage colony stimulator factor (M-CSF) to culture media (Gordon and Taylor, 2005). M-CSF, also known as colony-stimulating factor-1 (CSF-1), controls the survival, proliferation, and differentiation of mononuclear phagocytes

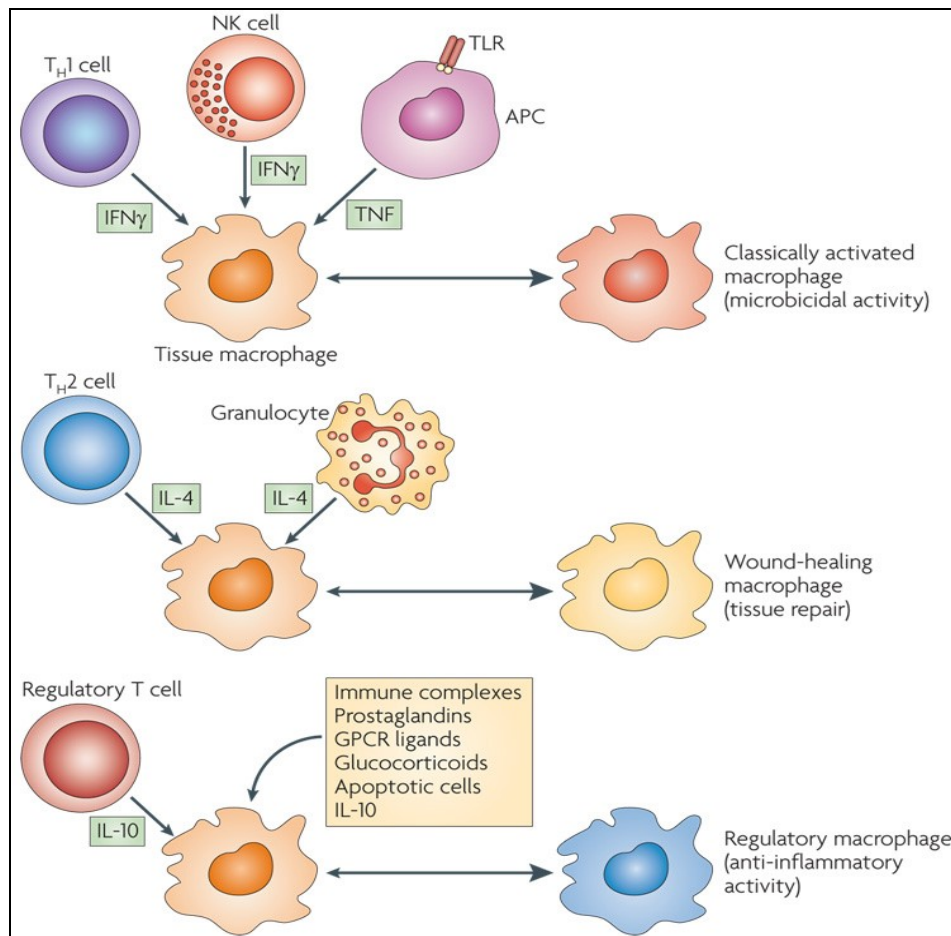
(Stanley *et al.*, 1997). It drives the differentiation of monocytes into macrophages acting via a cell surface tyrosine kinase receptor, known as colony stimulating factor receptor 1 (CSF-1R), expressed on cells of the mononuclear phagocyte lineage (Stanley *et al.*, 1997). CSF-1 bind to the CSF receptor (CSF-1R) and it has been reported that treatment of mice with monoclonal antibodies against this receptor depleted most tissue macrophage populations (McDonald *et al.*, 2010).

In pigs monocytes can be differentiated into macrophages through culture with M-CSF for 4-7 days (Singleton *et al.*, 2016, Kyrova *et al.*, 2014) or with different concentration of porcine serum or plasma for 3-4 days (10%: Garcia-Nicolas *et al.*, 2014, Kyrova *et al.*, 2014; 20% Chamorro *et al.*, 2000, Sanchez-Torres *et al.*, 2003; 30%: McCullough *et al.*, 1997, McCullough *et al.*, 1999, Basta *et al.*, 1999, Basta *et al.*, 2001, Tsai *et al.*, 2010). In addition, some authors used the media from the murine fibroblast L929 cells line as a source of M-CSF (Genovesi *et al.*, 1990, Zsak *et al.*, 1998, Wang *et al.*, 2011). In few studies researchers differentiated monocytes into macrophages using media supplemented with just 10% fetal bovine serum (FBS) (10%: Gil *et al.*, 2003, Gil *et al.*, 2008), but the work of McCullough and colleagues (1999) reported that monocytes cultured in media supplemented with FSB remained morphologically closer to monocytes than those cultured in porcine plasma (McCullough *et al.*, 1999). As previously stated, in humans M-CSF is commonly used to *in vitro* differentiate monocytes into macrophages (Gordon and Taylor, 2005) and treatment of porcine monocytes with M-CSF increases cell viability (Genovesi *et al.*, 1990). Fairbairn *et al.* (2014) observed that porcine PBMC cultured with hM-CSF ( $10^4$  U/ml) increased in size and granularity compared with freshly isolated PBMC and that the CD14<sup>+</sup>CD172a<sup>+</sup> population was selectively expanded, suggesting that as in humans M-CSF act as a mitogen for pig monocytes (Fairbairn *et al.*, 2014). To date there is not a standardize protocol to *in vitro* differentiate porcine monocytes into macrophages and in the first part of this thesis we focus on comparing different methods described in the literature, assessing their effect on macrophages morphology, expression of surface markers, cytokine release and susceptibility to monocytotropic ASFV (Chapter 3).

Several studies analyzed the interaction of ASFV with monocytes and macrophages, and there are better described in the section 1.7.1. To date, no studies investigated the interaction of ASFV with activated macrophages.

Macrophages present remarkable plasticity, that allow them to respond to environmental signals, produced also by antigen-specific immune cells. Both the innate and the adaptive immune responses affect their phenotype and physiology (Mosser and Edwards, 2008). Macrophage activation results in their polarisation into different functional subsets: classical activated macrophages (M1), alternative activated macrophages (M2) and type II-activated or regulatory macrophages (Mosser, 2003, Mosser and Edwards, 2008), as represented in figure 1.2.

M1 polarization can be reached *in vitro* by exposure to IFN- $\gamma$  and lipopolysaccharide (LPS), which induces TNF production. Classical activation results in secretion of high levels of pro-inflammatory cytokines and increased microbicidal or tumoricidal capacity (Mosser, 2003). Stimulation with IFN- $\gamma$  results in increased production of superoxide anions, oxygen and nitrogen radicals by macrophages. In fact the main role of M1 macrophages is in host defence to intracellular pathogens and in driving Th1 cellular immune responses (Mosser and Edwards, 2008). It was reported that mice lacking IFN- $\gamma$  expression are more susceptible to various bacterial, protozoal and viral infections, as are humans with genetic mutations in these signalling pathways (Filipe-Santos *et al.*, 2006). Moreover some pathogens have developed mechanisms to interfere with IFN- $\gamma$  signalling and to prevent efficient macrophage activation (Mosser and Edwards, 2008). M1 are vital component of the host defence, but their activation might be tightly controlled, in fact they are key mediators of the immunopathology that occurs in several autoimmune diseases (Mosser and Edwards, 2008). Very few studies analysed macrophage classical activation in pigs. It was reported that in pig classical activation led to MHC II up-regulation, suggesting that also in this specie M1 have and enhanced antigen presenting functions (Garcia-Nicolas *et al.*, 2014, Singleton *et al.*, 2016).



**Figure 1.2. Cytokines produced by immune cells can give rise to macrophages with distinct physiology.** Classical activated macrophages arise in response to IFN- $\gamma$ , which can be produced by T cells or NK cells, and TNF, produced by antigen presenting cells (APCs). Alternative activated macrophages arise in response to IL-4, which can be produced by TH2 cells or by granulocytes. Regulatory macrophages are generated in response to various stimuli, such as immune complexes, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells or IL-10. Figure taken from Mosser and Edwards, 2008.

Alternative activation of macrophages was discovered later than classical activation. It was found that alternative activation occurred via the stimulation of macrophages with IL-4, which was found to up-regulate expression of the mannose receptor (CD206) (Stein *et al.*, 1992). Activation with this cytokine was clearly distinct from the classic activated macrophage, in fact M2 do not produce NO and have a poor ability to kill intracellular pathogens (Modolell *et al.*, 1995). It was later discovered that alternative activation results also from exposure to IL-13, which shares the same IL-4 receptor alpha (IL-4R $\alpha$ ) chain as IL-4 (Gordon, 2003). M2 macrophages are primarily associated with mechanisms of immunosuppression and wound repair

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(Gordon, 2003), in fact they are also called ‘wound-healing’ macrophages (Mosser and Edwards, 2008). They are not efficient at antigen presentation and instead promote cell growth, collagen formation and tissue repair; M2 secrete several fibrogenic factors and insulin-like growth factor 1 and platelet-derived growth factor C, which provide signals for tissue proliferation and repair (Mosser, 2003, Martinez *et al.*, 2009). M2 produce high levels of the pro-inflammatory cytokine IL-10 and mediate repression of pro-inflammatory factors, so are compromised in their ability to kill intracellular pathogens and down-regulate a pro-TH1 response (Mosser, 2003, Martinez *et al.*, 2009). Macrophage alternative activation is implicated in the success of TH2 responses, mainly directed toward extracellular parasites such as helminths and some protozoa. Intracellular pathogens have, as a result, created mechanism to promote macrophage alternative activation and to avoid TH1 immune control (Martinez *et al.*, 2009). Very few studies analysed macrophage alternative activation in pigs. In this specie alternative activation leads to CD163 down-regulation, but has no influence on MHC class I or II expression (Garcia-Nicolas *et al.*, 2014).

Another macrophage category is the type II-activated macrophage, which were recently classified as one M2 subtype. In fact M2 can be subdivided in three subtypes: M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1beta or LPS) and M2c (IL-10, TGF-beta or glucocorticoids) (Martinez *et al.*, 2008). Macrophage activation with immune complexes and LPS results in inhibition of IL-12 and promotion of IL-10 secretion, up-regulation of antigen presentation and promotion of a TH2 response (Martinez *et al.*, 2008). Glucocorticoids affect monocyte adherence, spreading, phagocytosis and apoptosis, whereas IL-10 is a potent inhibitor of TH1 cells (Martinez *et al.*, 2008).

Nevertheless, macrophage taxonomy and the M1/M2 paradigm is a limited attempt to define the complexity and plasticity of mononuclear phagocytes. Individual macrophage cells differ markedly from each other, and change their functions in response to the subtle and continuous changes in the surrounding environmental signals, so M1/M2 subsets represent the two extreme of diverse functional macrophage activation states (Sica and Matovani, 2012, Italiani and Boraschi, 2014, Hume, 2015).

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## 1.2 African Swine Fever

African swine fever (ASF) is caused by a highly infectious virus that affects domestic and wild pigs and it is considered a notifiable disease by the OIE World Organisation for Animal Health because of its potential for rapid dissemination and significant socio-economical consequences (Sanchez-Vizcaino, 2006).

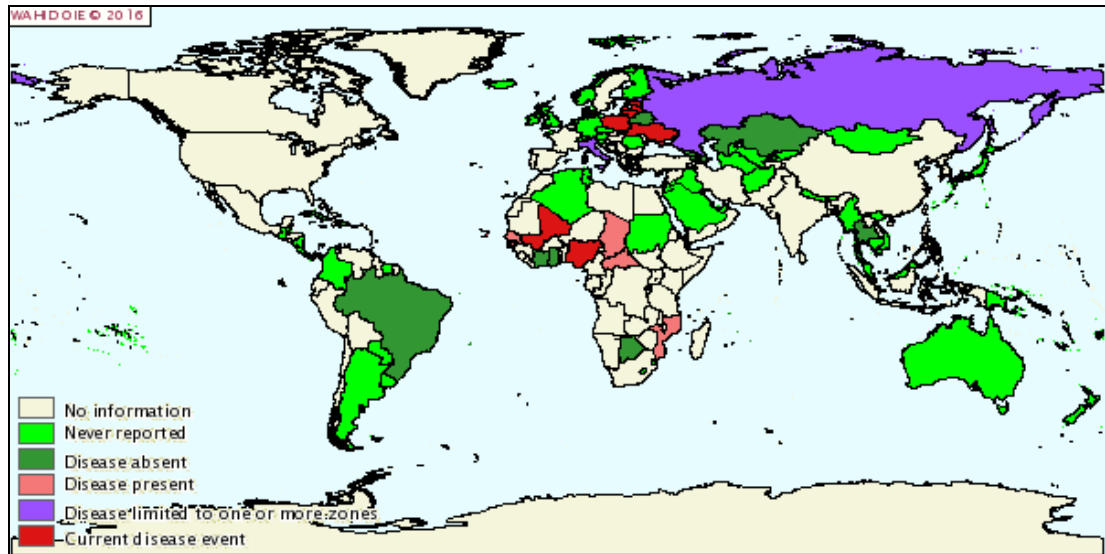
The aetiological agent is the African swine fever virus (ASFV), the only member of the *Asfarviridae* family (Dixton *et al.*, 2005).

ASF is currently endemic in many sub-Saharan countries of Africa and Sardinia (Italy). The spread of this disease to the Caucasus region in 2007, and from there to Russia and Ukraine in 2012, Belarus in 2013, and European Union countries (Lithuania, Estonia, Latvia and Poland) in 2014-2016, now endangers the pig industry worldwide (Alonso, 2013, OIE WAHIS interface).

In Russia ASFV progressively spread and 18 federal districts reported outbreaks (OIE, WAHIS interface). In this area ASFV has been isolated in wild boars and the persistence of the virus in the sylvatic cycle makes eradication more difficult (Beltran-Alcrudo *et al.*, 2008). In addition, a recent study revealed the existence of long-term infected animals in this area, which could become carriers and persist in the population, leading to the maintenance and future reappearance of the disease (Mur *et al.*, 2016a).

In Poland ASFV was first detected in early 2014 and between 2014 and 2015 65 cases of ASFV infection in wild boar have been recognised, located near the border with Belarus in Sokółka and Białystok counties (Wozniakowski *et al.*, 2016). Recently, in that country cases of ASFV have been described also in domestic pigs (OIE WAHIS interface).

The distribution of ASFV from January 2016 is illustrated in Figure 1.3.



**Figure 1.3 Distribution of ASFV from January 2016.** Figure taken from <http://web.oie.int> (disease and distribution maps).

Using retrospective analysis it was established that the first ASFV outbreak occurred in Africa in 1907, but the disease was firstly described in Kenia in 1921 by Montgomery. The first spread of ASF outside Africa was to Lisbon (Portugal) in 1957 and a further outbreak occurred in 1960 in Lisbon. ASF become endemic in all the Iberian peninsula and outbreaks were reported subsequently in a number of other European countries, including Malta, Italy, France, Belgium and The Netherlands (Costard *et al.*, 2013). In Spain and Portugal complete eradication took more than 30 years (Sanchez-Vizcaino, 2006).

In Sardinia the disease first occurred in 1978 and now is endemic, as a result of extensive pig farming that has been practised for centuries and of the presence of endemically infected wild boar (Firinù *et al.*, 1988; Costard *et al.*, 2009).

There is no vaccine or treatment available and apart from stamping out and movement control, there are no control measures, thereby potentially resulting in extreme losses for producers (Costard *et al.*, 2013).

As previously written, ASF affects both domestic and wild pigs (Sanchez-Vizcaino, 2006).

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African wild pigs are resistant to the disease and ASF persists in Africa via a natural cycle of transmission between the warthog (*Phacochoerus aethiopicus*), bushpig (*Potamochoerus porcus*) and the soft tick (*Ornithodoros moubata*). Warthogs are born free from infection and may be infected early in life following the bite of an infected tick. Virus replicates in the warthog and produces a low degree of viraemia for a few weeks, which is sufficient to infect a proportion of ticks that feed on the viraemic young warthogs (Thomson, 1985). Domestic pigs in Africa acquire infection from wildlife reservoirs of the virus primarily by the bite of an infected tick (Plowright, 1977).

In other areas, such as Sardinia, infection can occur either directly (excretions, secretions, dead animals) or indirectly (fomites, such as clothing, equipment and vehicles), in fact ASFV can persist in the environment for several days. The virus can persist in tissues for several months and feeding domestic pigs with uncooked swill can result in infection (Costard *et al.*, 2009). Soft ticks *Ornithodoros* are absent in Sardinia, so they are not involved in the persistence of the virus in the island (Costard *et al.*, 2013). In a recent study 1767 porcine serum samples collected from all around the island (1261 from domestic and 506 from wild boar) were analysed for antibodies to salivary antigens of *Ornithodoros erraticus* and only one sample resulted positive. In addition, ticks were directly searched in a number of pig premises with no success, confirming the absence of *Ornithodoros Erraticus* tick role in ASF cycle in Sardinia (Mur *et al.*, 2016b).

Recovered pigs can remain persistently infected for periods of 6 months or more, representing an obstacle for eradication of the disease from endemic areas (Wilkinson 1984, Oura *et al.* 2005).

The clinical signs associated with ASF are very varied as they depend on how virulent the viral isolate is and on the breed and physical condition of the pig. African ASFV isolates generally induce peracute or acute disease. European domestic pigs and boars are very susceptible and exhibit a wide range of clinical signs from subacute to chronic. As above stated, wild African pigs are very resistant to infection and do not generally present any lesions (Sánchez-Vizcaíno, 2006).

In Europe, it has been observed that wild boars are susceptible to the disease as domestic pigs (Jori and Bastos, 2009, McVicar *et al.*, 1981). Outbreaks of ASF in wild boars fade out, so the contact with infected domestic pigs or other source of infection is essential for the persistence of the virus in the sylvatic cycle (Laddomada *et al.*, 1994).

In the acute form, the animals show high temperatures (40-42°C), recumbency and lack of appetite, and suffer respiratory disorders. In some cases there may be nasal haemorrhaging, constipation and vomiting. Exanthemas are very evident (pinkish almost purple skin due to intense hyperaemia), and/or cyanotic foci, which appear as irregular purple-coloured marks on the skin of the extremities, ears, chest, abdomen and perineum. Abortion frequently occurs in gestating females. In acute cases, the disease causes death in 90 to 100% of affected animals. In acute and subacute courses death occurs between 7 and 20 day post infection, the signs develop more slowly. The chronic form is characterised by a large variety of clinical signs which are mainly the result of secondary bacterial complications. Mortality is low, affecting between 2 and 10% of all the sick animals (Sánchez-Vizcaíno, 2006).

Some characteristic ASF lesions are: purplish and megalic spleen, haemorrhaging in the tonsils and lymphatic ganglia, particularly in the gastrohepatic and renal ganglia, and petechial haemorrhaging in the kidneys, bladder mucosa, pharynx and larynx, pleura and heart, endocardium and pericardium, hydropericardium, ascitis, and hydrothorax and hepatic congestion (Figures 1.4, 1.5, 1.6) (Sánchez-Vizcaíno, 2006).

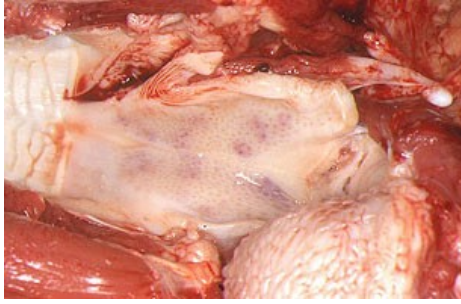
The disease is characterised by severe lymphopenia, immunodepression and thrombocytopenia (Gómez-Villamandos *et al.*, 2013).



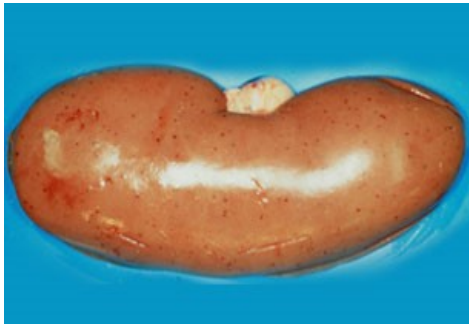
**Figure 1.4 Spleen: purplish and megalic** (Sánchez-Vizcaíno, 2002).

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**Figure 1.5 Tonsils with subepithelial haemorrhages** (Sánchez-Vizcaíno, 2002).



**Figure 1.6 Kidney: petechiae in the entire cortex area** (Sánchez-Vizcaíno, 2002).

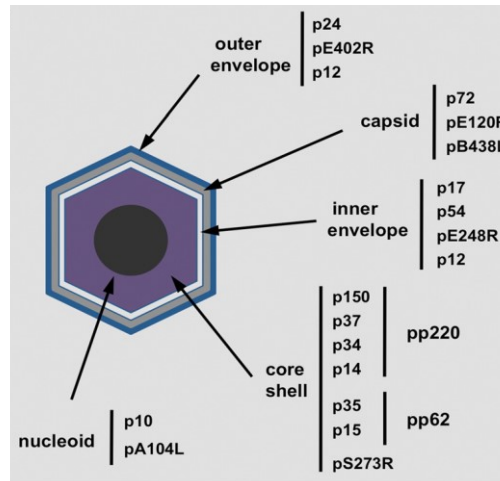
### 1.3 African Swine Fever Virus

As above stated, the aetiological agent is the African swine fever virus (ASFV), the only member of the *Asfarviridae* family (Dixon *et al.*, 2005). It is a large, enveloped virus with double-stranded DNA genome of 170 to 190 kbp. ASFV encodes for between 151 and 167 open reading frames (ORFs) (Dixon *et al.*, 2013a).

The ASFV particle has an icosahedral morphology with an average diameter of 200 nm and is composed by several concentric domains, as illustrated in Figure 1.7: 1) an internal core, 2) the core shell, a thick protein layer, 3) an inner lipid envelope, 4) the icosahedral capsid (Carrascosa *et al.*, 1984; Andres *et al.*, 1997). The extracellular virions possess also an external envelope (Breese and DeBoer, 1966).

Thanks to this structure, the virus is highly resistant to PH and temperature variations, in fact it can remain infectious for 18 months at room temperature (Sanchez-Vizcaino, 2006).

ASFV is highly resistant to PH and temperature variations, in fact it remains stable at PH 4-10 and it is inactivated only after cooking for 20 minutes at 60°C. Smoked sausages and matured ham required smoking at 32-49°C for 12 hours and maturing for 25-39 days to inactivate the virus (Plowright *et al.*, 1994).



**Figure 1.7 Localization of different ASFV structural proteins inside ASFV** (Figure taken from Salas and Andres, 2013).

ASFV entry involves dynamin-dependent and clathrin-mediated endocytosis (Hernaiz and Alonso, 2010) or through macropinocytosis (Sanchez *et al.*, 2012).

During endocytosis, ASFV viral particles undergo disassembly in various compartments, thanks to the acid pH of endosomes. In fact, the inhibition of the endosomal acidification impedes ASFV desencapsidation and so successful infection (Cuesta-Geijo *et al.*, 2012). Then ASFV requires cholesterol to exit the endosome to gain access to the cytoplasm, in order to establish productive replication. In fact a recent study reported that accumulation of cholesterol in endosomes impairs fusion, resulting in retention of ASF virions inside endosomes (Cuesta-Geijo *et al.*, 2015).

After exit from the endosome-lysosome, ASFV virions develop a strong association with the microtubular network (Netherton and Wileman, 2013). One of the major structural proteins of ASFV, p54, interacts directly with the 8-kDa light chain of the microtubule motor protein dynein (Alonso *et al.*, 2001) and it has been shown that alteration in the p54-dynein interaction in infected cells results in a marked decrease

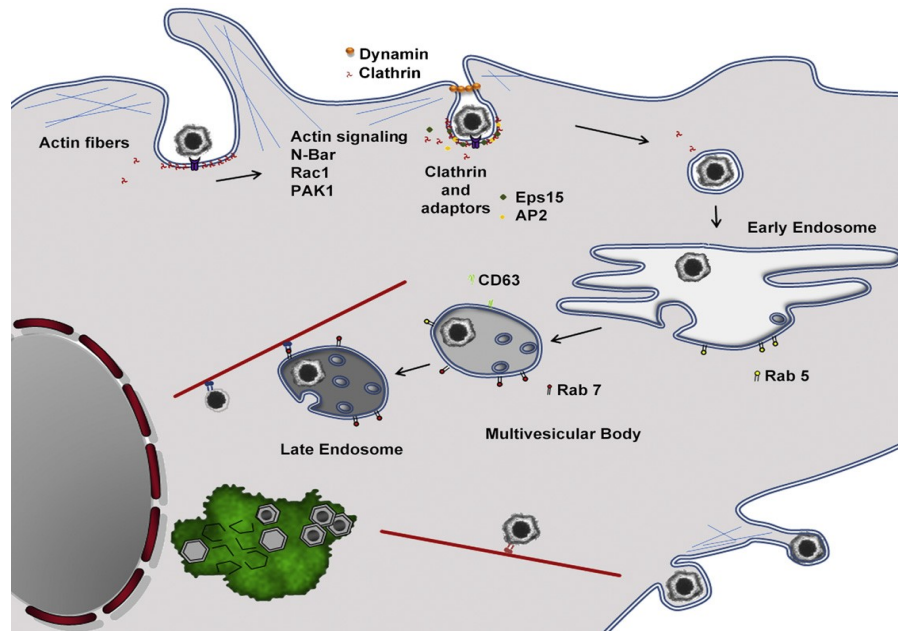
in virus infectivity, viral replication and finally virus production (Hernaez *et al.*, 2010).

ASFV DNA replication occurs mainly in perinuclear cytoplasmic viral assembly sites, close to the microtubular organizing centre (MTOC), known as virus factories (Netherton and Wileman, 2013). ASFV factories recruit different cellular components, including cellular chaperones, such as hsp70, and mitochondria (Castelló *et al.*, 2009; Heath *et al.*, 2001; Rojo *et al.*, 1998).

During ASFV infection, all the components of the translation machinery examined (eIF4G, eIF4E, eIF2, eIF3b and the eukaryotic elongation factor 2 [eEF2]) and ribosomes are relocated from a diffused distribution throughout the cytoplasm of infected cells to the viral factories. Also the mitochondria network are mobilized together with ribosomes to the viral factories. The increase in the availability of host sources in the viral factories and simultaneously its depletion in the cytoplasm may results in the shut off of the host mRNA translation (Castello *et al.*, 2009).

ASFV mRNAs are structurally similar to the cellular mRNAs and possess a cap structure in its 5'-UTR and a poly (A) tail of 33 nucleotides in average (Salas *et al.*, 1981). The fact that ASFV mRNAs are capped indicates that they drive translation by a canonical cap-dependent mechanism, as happens with most of cellular mRNAs. Also some studies reported that a part of the genome replication is initiated within the nucleus (Netherton and Wileman, 2013).

ASFV utilises the cytoskeletal network to facilitate its egress from the virus factory to the plasma membrane and out of the infected cell (Netherton and Wileman, 2013). Finally progeny virions are bud out or are propelled away along actin projections to infect new cells (Netherton and Wileman, 2013). In fact, ASFV infection induces long unbranched actin projections, similar to those detected in filopodia, that originate from the plasma membrane (Jouvenet *et al.*, 2006). Interestingly, ASFV infection evolves toward cell lysis at very late times of infection (Breese and DeBoer, 1966), which might represent an alternative mechanism of viral egress.



**Figure 1.8 Replication cycle of ASFV.** Replication cycle of ASFV, from virus entry to formation of the early endosome, its maturation, virion desencapsidation in the acidic late endosomal compartments and finally to virions exit along actin projections (Figure taken from Alonso *et al.*, 2013).

The first morphological indication of viral assembly is the accumulation within the factory of viral membranes, which are the precursors of the inner envelope of the viral particle (Salas and Andres, 2013). Then, the capsid is preassembled on the convex face of the viral membranes, which thus become polyhedral forms (Garcia-Escudero *et al.*, 1998). P72 is the major capsid protein, but capsid assembly depends also on protein pB602L, a non-structural protein that acts as a chaperone for the folding of p72 (Epifano *et al.*, 2006a), and on protein pB438L, a minor capsid component probably involved in the formation of the capsid vertices (Epifano *et al.*, 2006b).

Simultaneously to the capsid assembly, the core shell is formed underneath the concave face of the viral envelope (Andres *et al.*, 1997) and its main constituents domain are the proteolytic products of the two virus polyproteins pp220 and pp62 (Andres *et al.*, 2002).

The formation of the nucleoid is likely to be the last step in morphogenesis. Probably the viral DNA is first encapsidated, possibly together with nucleoproteins, and then condensed inside the assembling virus particles to produce the “full” mature virions (Salas and Andres, 2013).

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African swine fever virus (ASFV), like other complex DNA viruses, sets up a number of strategies to evade the host's defense systems, such as apoptosis, inflammation and immune responses (Sanchez *et al.*, 2013). Some viral proteins involved in evading host's defence are described below.

ASFV proteins involved in modulating host defence, regulating host gene transcription, are:

- A238L protein, which inhibits activation of the transcriptional factors NF $\kappa$ B and NFAT. It interacts with the p65 subunits of NF $\kappa$ B during infection, creating a complex thus inhibiting this transcriptional factor (Revilla *et al.*, 1998). In addition, A238L alters the activity of NFAT, through inhibition of the host calcineurin dependent pathways (Dixon *et al.*, 2004). This protein down-regulates the production of pro-inflammatory cytokines, such as TNF- $\alpha$ , by ASFV infected macrophages (Powell *et al.*, 1996, Granja *et al.*, 2006a). In fact it has been reported that infection with the mutant ASFV E70, deleted on A238L, is characterized by an increased synthesis of TNF- $\alpha$  and other cytokines (Salguero *et al.*, 2008). A238L has effects also on the nitric oxide synthesis. By using a recombinant ASFV lacking of the A238L gene, it has been demonstrated that A238L strongly down regulates inducible nitric oxide synthase (iNOS) activation (Granja *et al.*, 2006b).

- A224L protein, which is an inhibitor of apoptosis (Nogal *et al.*, 2001) and is also involved in the NK-kB activation (Rodriguez *et al.*, 2002).

- ASFVj4R protein, which binds the host  $\alpha$ -NAC protein (Goatley *et al.*, 2002) and influences its transcription. It has been speculated that the interaction between ASFVj4R and  $\alpha$ -NAC affects the ability of this cellular factor to act as a transcription co-activator (Sanchez *et al.*, 2013).

- The ASFV ubiquitin conjugating (UBCv) enzyme, which might play a role in regulating host gene transcription. In fact it was described that this viral protein binds the NH<sub>2</sub>-terminal end of a host nuclear protein SMCy, which is involved in gene transcription (Bulimo *et al.*, 2000).

In addition ASFV developed mechanisms to regulate host cell cycle. ASFV strongly induces Myc activation from early times after infection, which promotes cell growing and protein synthesis (Castello *et al.*, 2009). It regulates the expression of the components of eIF4F complex at transcriptional level, in order to guarantee the viral protein synthesis (Castello *et al.*, 2009). Moreover it has been reported that the viral protein A224L inhibits caspase activation, promoting cell survival (Nogal *et al.*, 2001).

This virus monopolizes all the components of the host translation machinery, relocating them to the 'viral factories', in order to guarantee viral proteins synthesis. The phosphorylation of the  $\alpha$  subunit of the eIF2 inhibits its activity and so inhibits translation and protein synthesis, so eIF2 $\alpha$  phosphorylation is one of the most important host defense mechanisms against viral infections and ASFV has developed mechanisms to avoid the phosphorylation of this factor (Sanchez *et al.*, 2013). Phosphorylated-eIF2 $\alpha$  levels decrease at early times post infection, and remain undetectable throughout the infection (Castello *et al.*, 2009), suggesting a viral mechanism to ensure the availability of this factor for viral protein synthesis.

It has been shown that DP71L induces a decrease of phosphorylated eIF2 $\alpha$  and enhances the expression of co-transfected reporters, suggesting that DP71L plays a role in keeping the translation machinery active to allow viral protein synthesis (Zhang *et al.*, 2010). However, ASFV possess multiple mechanisms to avoid eIF2 $\alpha$  phosphorylation (Zhang *et al.*, 2010). It has been observed that this virus induces phosphorylation of eIF4E, which takes place after 8 hours post infection (pi) and is associated to an enhancement of the viral replication and protein synthesis (Castello *et al.*, 2009).

Moreover, ASFV encodes several proteins involved in inhibiting apoptosis of infected cells. In fact, ASFV-infected macrophages undergo apoptosis at late time post-infection (24-48 hours pi), suggesting that there are some viral genes that negatively regulate apoptosis (Ramiro-Ibanez *et al.*, 1996), such as:

- The protein encoded by A179L, which represents the viral homolog of the anti-apoptotic protein Bcl2 (vBcl2) and inhibits the action of several pro-



apoptotic protein (Bid, BimL, BimS, BimEL, Bad, Bmf, Bik, Puma and DP5) (Galindo *et al.*, 2008).

- A224L protein, a homolog of inhibitor of apoptosis proteina (IAP), which inhibits caspase 3 (Nogal *et al.*, 2001) and activates the transcription factor NFkB (Rodriguez *et al.*, 2002).
- E153 protein, which acts on the 53 pathway (Hurtado *et al.*, 2004).
- DP71L protein, which induces dephosphorylation of the initiator factor eIF2 $\alpha$ , playing an important role in keeping the translation machinery active to synthesize new viral proteins (Rivera *et al.*, 2007, Zhang *et al.*, 2010).

Phylogenetic analysis of ASFV is based on the partial sequence of the B646L gene, which encodes for the structural protein p72 (Bastos *et al.*, 2003). This analysis showed that there is a strong correlation between isolates from West Africa, South America and Europe collected before 2007, all belonging to genotype I. In contrast, isolates from South and East Africa present more differences and are grouped in other 22 genotypes (Bastos *et al.*, 2003, Lubisi *et al.*, 2005, Boshoff *et al.*, 2007, Achenbach *et al.*, 2016). Genotype XXIII is present in Ethiopia and was only recently discovered (Achenbach *et al.*, 2016).

As previously stated, in June 2007 ASF was confirmed in Georgia, and it has since spread to neighboring countries. Sequence analysis indicated that the Georgia 2007 isolate is closely related to isolates belonging to genotype II, which is circulating in Mozambique, Madagascar, and Zambia (Rowlands *et al.*, 2008). ASFV was first detected in Russia 2007 and all the isolates collected in the Russian Federation from 2007 to 2011 revealed 100% nucleotide identity of B646L gene sequence and formed one genetic cluster within genotype II (Malogolovkin *et al.*, 2012).

Other genes have been used to discriminate between isolates on a regional level: B602L (coding for the J9L protein), CP204 (coding for the P32 protein) and E183L (coding for the envelope protein P54) (Gallardo *et al.*, 2009, Nix *et al.*, 2006, Rowlands *et al.*, 2008). The analysis of these genes is useful to study the epidemiology and evolution of different isolates and can be used to identify the source of contamination in case of new ASFV outbreaks (Nix *et al.*, 2006).

Recently the analysis of the TRS in the intergenic region between the I73R and I329L genes, at the right end of the genome, has been used to investigate the genetic variability among ASFV isolates circulating in eastern Europe. Viruses from Poland and Lithuania presented a TRS insertion identical to that present in ASFV isolates from Belarus and Ukraine, but not in the remaining viruses from eastern Europe, including those obtained in Russia in 2012 and in Georgia in 2007. These molecular data suggested that the ASFV isolates detected in Poland and Lithuania most probably originated from Belarus (Gallardo *et al.*, 2014).

The phylogenetic analysis, based on the sequence of the gene coding for the protein p72, showed that all the Sardinian isolates belong to the genotype I, along with the isolated from South America, Caribbean, West Africa and Europe (Giammaroli *et al.*, 2011). Between Sardinian isolates there are no differences in the genomic region p54, but there are differences in the B602L gene, which is involved in viral morphogenesis. Sardinian isolates can be divided in two subgroups (X and III) on the basis of the presence/absence of a deletion of 12-13 tetramers in this region. This deletion (subgroup X) is present in almost all the isolates from 1990 and there are no differences between viruses isolated from domestic pigs and wild boar (Giammaroli *et al.*, 2011). In a recent study 44 sardinian isolates collected during 1978-2014 were further characterised, through the analysis of p30, CD2V and I73R/I329L variable regions (Sanna *et al.*, 2016). Researchers showed that Sardinian isolates can be divided into two sub-groups also by the sequences comparison of the CD2v gene: oldest Sardinian isolates (1978-1990) displayed 8 PPPKPC identical hexamers interrupted in the midst by a SPPKPC and a RPPKCP motifs and followed by an hexamer with an aminoacidic substitution (PPSKPC rather than PPPKPC), in contrast viruses isolated in recent years (from 1990 to 2014) show 7 identical repeats with the same central interruption where the last hexamer was not modified (Sanna *et al.*, 2016). These studies suggest that since 1990 the ASF outbreaks in Sardinia are caused by a mutated form of the virus.

## 1.4 Control of ASF in Sardinia

As above stated, in Sardinia the disease first occurred in 1978 and now is endemic. The disease originated in the south part of Sardinia and it was probably introduced from Spain through food products containing uncooked pork, which were used to feed sardinian pigs (Mannelli *et al.*, 1997). As previously stated, *Ornithodoros* ticks are not involved in the epizootic cycle of ASF in Sardinia and the control of other risk factors present in the island is necessary for effectively eradicate the disease (Mur *et al.*, 2016b). The persistence of ASFV is probably due to extensive pig farming that has been practised for centuries and of the presence of endemically infected wild boar (Firinu *et al.*, 1988, Costard *et al.*, 2009). In a recent study a Bayesian multivariable logistic regression mixed model was used to assess the factors associated to the ASFV occurrence in Sardinia. Researchers found that ASFV persistence was associated to particular socio-cultural, productive and economical factors found in the region, particularly to large number of confined farms (most of them backyard), high road density, high mean altitude, large number of open fattening farms, and large number of pigs per commune (Martinez-Lopez *et al.*, 2015).

In the last decades several legislative measures were developed in order to eradicate this disease.

The 14<sup>th</sup> February 1968 ministerial decree ('ordinanza ministeriale') disposed that the mayor, after notification of ASF presence or suspect, ordered sequestration ('sequestro di rigore') of infected, suspected infected and suspected contaminated animals. The 'provincial veterinarian' ('veterinario provinciale'), after mayor's provisions, issued with urgency decrees of 'infectious zone' ('zona infetta') and 'protection zone' ('zona di protezione') and ordered immediate killing of infected, suspected infected and suspected contaminated animals. The decrees of 'infectious zone' ('zona infetta') and 'protection zone' ('zona di protezione') were revoked respectively at least 60 and 30 days after the last outbreak (OM 14-2-1968).

In the following years several modifications were made to this legislative measure, to more efficiently fight against ASF. The Member States of the European Union

moved from a diversity of national control policies towards a common, community wide approach based on notification of outbreaks, harmonized control measures, uniform diagnostic procedures and contingency plans. In 2002 an EU directive was issued to determine actions to be taken within the EU to fight against ASF, including measures to be applied in case of an outbreak or a suspect or presence of ASF in wild boar (direttiva 2002/60/CE). In 2005 an EU decision was issued focussing on the presence of ASF in Sardinia. This decision established rules about movements, shipments, stampings ('bollatura') of sardinian pigs and pig products, to avoid dissemination of the disease in other areas of the EU (decisione 2005/363/CE). Moreover, the decision 2005/362/CE was issued with the aim to eradicate ASF from wild boar in Sardinia (decisione 2005/362/CE). The decision 2005/363/CE has been recently abrogated and it is currently in force the decision of the European Commission of the 27<sup>th</sup> of March 2014, which will be effective till the 31<sup>st</sup> of December 2017. According to this decision, there is a prohibition to export live pigs, porcine sperm, ovules and embryos, pork and any product containing pig meat from Sardinia (and other areas within the EU where ASF cases has been recently reported). There are dispensations to export pork and products containing pig meat from Sardinia if they are made from pigs born and bred outside Sardinia and other areas with ASF. Moreover there are dispensation to export products containing pig meat if they are processed with a treatment that guarantee that there will be no risk related to ASF (decisione di esecuzione 2014/178/UE).

According to the extraordinary ASF eradication plan, since 2014 Sardinian local authorities adopted further measures to eradicate ASFV from Sardinia (Deliberazione 50/17 del 16.12.2014 Regione Autonoma della Sardegna, Legge Regionale n.34 del 22.12.2014, Determinazione 87 del 11.2.2015 Regione Autonoma della Sardegna).

According to the current legislation, in case of an ASF outbreak the official veterinarian must contact the 'Unità di Crisi Locale', a group of experts which coordinate the execution of the legislative measures.

Further measures were adopted to contrast the contact between domestic pigs and wild boars. In fact in Sardinia wild boar are likely to play an important role in facilitating virus persistence in areas where they lives in continuous contact with free

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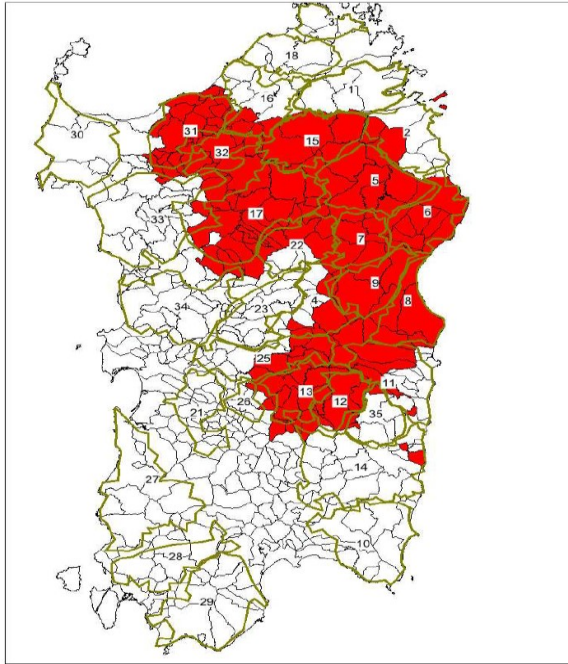
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ranging domestic pigs (Costard *et al.*, 2009). Every pig farm should adopt measure to avoid pigs-wild animals contacts and every pig should be kept on holdings, under human control. If feral pigs are detected, they are going to be killed by official veterinarians, even if they don't display ASF clinical signs (Legge Regionale n.34 del 22.12.2014, Determinazione 87 del 11.2.2015 Regione Autonoma della Sardegna).

Every pig owner should register animals at the 'Banca dati nazionale' and every farm is subjected to official controls. Pigs are examined by official veterinarians, who also assess if in the farm there are effective measures to avoid contact between domestic and feral pigs/wild boars. Official veterinarians collect pig blood, which is sent to the Istituto Zooprofilattico Sperimentale (IZS) of Sardinia, where serological assays to detect antibodies against ASFV are performed. A farm became 'Azienda certificata per PSA' if it was inspected at least once in the previous 12 months by an official veterinarian, every pig did not display ASF clinical signs and did not present antibody against ASFV (Legge Regionale n.34 del 22.12.2014, Determinazione 87 del 11.2.2015 Regione Autonoma della Sardegna).

Further measures were taken also to control the spread of the disease in the wild boar population. Hunters must not leave organs or pieces of the carcass in the field, must collect wild boar blood sample for analysis, must inform an official veterinarian in case of finding of dead wild boar Legge Regionale n.34 del 22.12.2014, (Deliberazione 50/17 del 16.12.2014 Regione Autonoma della Sardegna). In some area ('macroareali'), displayed in Figure 1.9, hunt is forbidden unless special requirements are fulfilled (Determinazione n. 7 del 15.10.2015, Regione Autonoma della Sardegna, Determinazione n. 25 del 20.11.2015, Regione Autonoma della Sardegna).



**Figura 1.9.** Area ('macroareali infetti nel selvatico') of Sardinia where wild boar hunt is forbidden (Determinazione n. 25 del 20.11.2015, Regione Autonoma della Sardegna).

### 1.5 ASF diagnosis in Sardinia

Samples of domestic pigs and wild boars, collected by official ASL veterinarians, are sent to the IZS of Sardinia. That institute is responsible for the ASF diagnosis in Sardinia (Legge Regionale n.34 del 22.12.2014, Determinazione 87 del 11.2.2015 Regione Autonoma della Sardegna).

According to the current legislation, ASF should be suspected every time that animals display: fever (more than 40°C), lack of appetite, cutaneous and subcutaneous haemorrhaging, abortion, death of pigs of any ages without identification of other causes. ASF should be suspected every time there is death, infertility or abortion without an identified cause. Samples should be collected in case of outbreaks, epidemiological link with an outbreak, serological positivity, death animal or abortion. Sample suitable for virus isolation are: heparin blood, spleen, kidney, lymph nodes, tonsils, long bone (for bone marrow collection) and foetus (Decisione della Commissione del 26 maggio 2003 recante approvazione di un

manuale di diagnostica della peste suina, OIE Manual of Diagnostic Tests for Aquatic Animals, 2012).

Techniques used to identify ASFV are:

- Direct immunofluorescence, to detect the virus in tissue samples;
- Haemadsorbing test (Malmquist test);
- PCR and RT-PCR, to detect ASFV in any kind of sample, as heparin blood, bone marrow, serum, tissue and organs not well conserved;
- Sequencing and molecular epidemiological analysis, to analyse all the isolates positive at the Malmquist test.

In case of a positive result in one of the previous assays, an ASFV outbreak is opened. Positive samples should be re-tested with a different assay, even on a different animal in the same farm. In case of an ambiguous result or in case of a positive result in the absence of clinical symptoms or pathological lesions of ASF, a test to isolate the virus should be performed. On these samples, one of the following test is performed: PCR, Real Time PCR, Malmquist Test.

Serological assays to detect antibodies against ASFV are performed on blood samples. The first serological assay to be used is the ELISA and in case of a positive result an immunoblotting assay is also performed. Results of the serological assay should be evaluated considering the clinical symptoms and the epidemiological situation, in case of a suspected or an opened ASF outbreak (Decisione della Commissione del 26 maggio 2003 recante approvazione di un manuale di diagnostica della peste suina, OIE Manual of Diagnostic Tests for Aquatic Animals, 2012).

During the hunting season, according to the extraordinary ASF eradication plan (Deliberazione 50/17 del 16.12.2014 Regione Autonoma della Sardegna) official veterinarian collect blood samples from dead wild boar, which are sent to the IZS of Sardinia to detect antibodies against ASFV, by ELISA and immunoblotting. In the area displayed in Figure 1.9, also spleens are collected by official veterinarians and are sent to the IZS of Sardinia to detect ASFV, by direct immunofluorescence, haemadsorbing test (Malmquist test), PCR and RT-PCR.

## 1.6 ASF vaccines and antiviral agents

Currently there are no vaccine against ASFV. Pigs that survive inoculation with ASFV isolates with reduced virulence can be protected from challenge with the homologous or closely related virulent viruses (Leitão *et al.*, 2001, Oura *et al.*, 2005, King *et al.*, 2011), indicating that anti-ASFV immune response(s), including protective immune response(s), developed in these pigs prior to challenge with the virulent virus (Takamatsu *et al.*, 2013).

Efforts has been done to develop attenuated ASFV strains, by deletion of specific genes from virulent ASFV isolates. Independent deletions of the UK (DP69R) or NL (DP71L) genes from the ASFV E75 (Zsak *et al.*, 1998, Zsak *et al.*, 1996) and deletion of the 9GL (B119) gene from the virulent Malawi Lil-20/1 strains (Lewis *et al.*, 2000) resulted in creation of recombinant deletion viruses with reduced virulence in swine, able to confer protection to challenge with the homologous parental virus (Zsak *et al.*, 1998, Zsak *et al.*, 1996, Lewis *et al.*, 2000). Multigene family (MGF) 360 and 530/550 have been implicated in the modulation of type I interferon (IFN) response, so additional copies of these genes were deleted or interrupted from a virulent genotype I isolate, Benin 97/1. The deleted mutant (Benin $\Delta$ MGF) was attenuated in pigs and immunisation and boost with this virus protected against challenge with a lethal dose of Benin 97/1, suggesting that deletion of IFN modulators is a promising route for rational attenuation of virulent ASFV isolates to construct candidate vaccine strains (Reis *et al.*, 2016). Recent studies focused on the creation of a deletion vaccine strain based on the attenuation of the virulent and epidemiologically relevant Georgia2007 isolate. Deletion of 9GL (B119L) resulted in a mutant (ASFV-G- $\Delta$ 9GL) virus with limited replication in swine macrophages, but able to confer protection to homologous challenge when administered at a lower dose ( $10^2$ - $10^3$  HAD<sub>50</sub>) 21-28 days before infection with the parental strain (O'Donnell *et al.*, 2015). Deletion of six genes belonging to MGF 360 and 505 lead to the creation of a vaccine strain (ASFV-G- $\Delta$ MGF) able to replicate efficiently in primary macrophage cell cultures, completely attenuated in swine, and vaccination with  $10^2$ - $10^4$  HAD<sub>50</sub> protected pigs to challenge with the virulent parental strain (O'Donnell *et al.*, 2015). Despite both vaccine strains (ASFV-G- $\Delta$ 9GL and ASFV-G- $\Delta$ MGF) were efficient in inducing protection, they presented concerns regarding their safety. So a

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new mutant, ASFV-G- $\Delta$ 9GL/ $\Delta$ MGF, harboring deletions of both 9GL and MGF360/505 genes, was constructed. This virus was highly attenuated in swine, but did not induce protection against challenge with the virulent parental ASFV-G isolate (O'Donnel *et al.*, 2016). In addition, deletion of the thymidine kinase gene was performed in a strain of Georgia adapted to replicate in Vero cells (ASFV-G/V). This mutant (ASFV-G/V- $\Delta$ TK) was completely safe in pigs, but unable to provide protection to challenge with the virulent parental strain (Sanford *et al.*, 2016). These results suggest caution towards approaches involving genomic manipulations when developing rationally designed ASFV vaccine strains isolate. In addition, even if attenuated viruses could be used to protect pigs against challenge with virulent strains, the use of this kind of vaccines has some risks, due to the fact that the attenuated virus could revert to virulence *in vivo*.

The use of vaccine strains which undergo a single cycle of infection but do not produce infectious progeny would avoid this risk. However higher virus load would most likely be required to induce protection (Dixon *et al.*, 2013). In the past it has been shown that inactivated vaccines are not useful to protect pigs against ASFV (Stone *et al.*, 1967; Mebus, 1988) and recently it has been reported that modern adjuvants, like Polygen<sup>TM</sup> or Emulsigen<sup>®</sup>-D, do not increase the efficacy of these kind of vaccines (Blome *et al.*, 2014). The construction of vaccines using techniques of molecular biology, not including the whole virus but immunological proteins/peptides, would be useful for the eradication of the disease. Nevertheless, knowledge of protective antigens is essential to create these kind of vaccines (Dixon *et al.*, 2013).

Antibody response against ASFV can be detected even 7 days pi (Parker and Plowright, 1968), but there are controversies about the ability of these antibodies to neutralise the virus (Escribano *et al.*, 2013). Nevertheless, it is not possible to exclude that antibody response does not contribute to protection. Some studies showed that administration of sera of immune animals to infected pigs delay clinical symptoms, reduce viremia and increase the survival probability (Onisk *et al.*, 1994). Instead, several studies reported the importance of the cellular immune response against ASF, especially of the CD8<sup>+</sup> T cells (Argilaguet *et al.*, 2012, Argilaguet *et al.*, 2013, Oura *et al.*, 2005).

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Recombinant viruses represent an attempt to design a safe and efficient ASFV vaccine. A recombinant Newcastle disease virus (rNDV) expressing the ASFV protein 72 (p72) was recently constructed. The recombinant virus was safe in mice and animals immunized with rNDV/p72 developed high titers of ASFV p72 specific IgG antibody and T cell response (Chen *et al.*, 2016). Further studies in pigs will evaluate the efficacy of this potential vaccine candidate for preventing ASF. Lokhandwala *et al.* (2016) recently tested the safety and immunogenicity of an adenovirus-vectored ASFV multi-antigen cocktail. Codon-optimized synthetic genes encoding p32, p54, pp62, and p72 ASFV antigens were used to generate a recombinant adenovirus. Immunization with the cocktail rapidly induced ASFV antigen-specific antibody and cellular immune responses against all the antigens. Importantly, significant antigen-specific IFN- $\gamma$  responses were detected post-priming and post-boosting (Lokhandwala *et al.*, 2016). A challenge study need to be performed in order to evaluate the relevance of the induced immune responses in regards to protection.

DNA vaccines, where one or more genes encoding immunological proteins are inserted, can be used to induce a stronger cellular response. To date several ASFV proteins have been identified as target of the immunological response, such as p30 and p73 (Leitao *et al.*, 1998, Alonso *et al.*, 1997). Moreover hemagglutinin (HA) showed the ability to confer protection in the absence of neutralising antibodies (Ruiz-Gonzalvo *et al.*, 1996). Recent studies showed that ASFV vaccines based on the proteins p30, p54 and HA were able to induce a T cell response and partially protected pigs against challenge with a virulent ASFV isolate (Argilaguuet *et al.*, 2012, Argilaguuet *et al.*, 2013). In fact a DNA vaccine containing p30, p54 and HA fused to ubiquitin was able to protect more than 50% of the vaccinated pigs against challenge with a virulent ASFV strain, in the absence of antibody response (Argilaguuet *et al.*, 2012). Protected pigs showed a peak in the number of CD8 T cells 3 days post vaccination and leukopenia was delayed and had lower intensity. In the same study two 9mers target of the T cell response were identified, both located on HA, and immunodominance hierarchy in the T cell response and SLA I restriction were observed (Argilaguuet *et al.*, 2012). In addition, in another study the ASFV proteins p54, p30 and HA were inserted in a baculovirus vector (BacMam-sHAPQ),

under control of the human cytomegalovirus promoter (CMV<sub>ie</sub>). The use of this vaccine induced a strong T cell response, positively correlated with protection in survived pigs (4 out of 6), in the absence of antibody response (Argilaguet *et al.*, 2013). The use of peptide vaccine against ASFV has also been tested. A vaccine based on 17 ASFV immunological peptides failed to protect pigs against an ASFV lethal challenge, nevertheless it was able to statistically significantly delay mortality (Ivanov *et al.*, 2011).

Recently, it has been reported that other two ASFV proteins, CD2v (EP402R) and C-type lectin (EP153R), represent significant protective antigens for ASFV, so they should be targeted in future vaccine design and development (Burmakina *et al.*, 2016).

Further studies should be performed on the interaction of ASFV with the porcine immune system, to understand how attenuated virus induce protection and to identify targets of the protective immune response, generating information useful to underpin vaccine development efforts.

Several studies focused on the development of antiviral agents against ASFV. Those treatments might be beneficial in areas located close to the affected farms, in order to isolate the epidemic area (Zakaryan and Revilla, 2016). Potential anti-ASFV compounds can be divided into two groups: 1) agents that affect ASFV replication cycle (from virus attachment to release of virus progeny); 2) agents that target host cell factors involved in virus replication. Antiviral agents belonging to the first group are: sulphated polysaccharides which affect ASFV attachment; cholesterol removing agents that inhibit viral entry; drugs blocking endosomal acidification (ammonium chloride, amantadine, chloroquine, and methylamine) required for ASFV replication (Zakaryan and Revilla, 2016). Agents belonging to the second group are: fluoroquinolones, a family of drugs which interfere ASFV replication targeting the type II topoisomerase (Mottola *et al.*, 2013); lauryl-galattate, which inhibits both cellular and viral DNA synthesis (Hurtado *et al.*, 2008); polyphenolic phytoalexins, which interfere with viral DNA replication, late viral protein synthesis and viral factory formation (Galindo *et al.*, 2011). A recent study tested the antiviral effect of five flavonoids on the replication of ASFV in Vero cells. Researchers observed a

dose-dependent anti-ASFV effect of apigenin *in vitro*. Apigenin was highly effective at the early stages of infection; it inhibited ASFV-specific protein synthesis and viral factory formation (Astghik *et al.*, 2016). Nevertheless the antiviral activities of the compounds mentioned were only tested *in vitro* and evidence of their *in vivo* efficacy is still lacking (Zakaryan and Revilla, 2016).

## **1.7 Immunology against ASFV**

As above stated, pigs that survive inoculation with ASFV isolates with reduced virulence can be protected from challenge with the same or closely related virulent viruses (Leitão *et al.*, 2001, Oura *et al.*, 2005, King *et al.*, 2011). This indicates that anti-ASFV immune response(s), including protective immune response(s), developed in these pigs prior to challenge with the virulent virus (Takamatsu *et al.*, 2013). The analysis of the immune mechanisms underlying the development of this immunity will generate information useful for the development of an efficient vaccine against this disease.

### **1.7.1 ASFV and innate immunity**

ASFV mainly targets myeloid lineage cells, especially monocytes and macrophages, which are thought to be crucial for viral persistence and dissemination (Sierra *et al.*, 1991, Sánchez-Cordón *et al.*, 2008). ASFV encode different genes to avoid apoptosis (such as A224L, A179L, EP153R), so infected macrophage can survive and disseminate the virus into the whole organism (Nogal *et al.*, 2001, Galindo *et al.*, 2008, Hurtado *et al.*, 2004, 2011).

Infected monocytes-macrophages containing virus replication sites may synthesize and release monokines (TNF- $\alpha$ , IL-1 $\alpha$ ) which would trigger lymphocytes apoptosis (Fernandez de Marco *et al.*, 2007). In fact it has been speculated that the level of apoptosis depends on the amount of cytokines released, and this in turn depends on the number of ASFV infected macrophages, and this may explain the greater presence of lymphocyte apoptosis of highly virulent isolates such as Malawi compared with the less virulent isolates such as Malta (Oura *et al.*, 1998).

To date several studies analysed the cytokine response of monocyte/macrophages to ASFV (Gomez del Moral, 1999, Gil *et al.*, 2003, Gil *et al.*, 2008, Zhang *et al.*, 2006) and more recent study reported the expression of chemokines after ASFV infection (Fishbourne *et al.*, 2013). It has been shown that the low-virulent ASFV/NH/P68 induces enhanced expression and production of relevant regulatory cytokines (IFN- $\alpha$ , TNF- $\alpha$  and IL12) in comparison to the highly virulent ASFV/L60 (Gil *et al.*, 2008) and it has been reported that the low virulence strain OURT88/3 induces higher expression of key inflammatory chemokines (CCL4, CXCL8, CXCL10) compared to infection with high virulence strain Benin 97/1 (Fishbourne *et al.*, 2013). A different response of antigen presenting cells (APCs) to ASFV isolates of reduced virulence could lead to the acquisition of a protective immunity rather than disease.

A recent study assessed the effect of ASFV on the expression of surface markers on bone marrow-derived macrophages, obtained from femur bones. Flow cytometry was used to analyse the expression of several surface markers in ASFV infected cells: MHC II, CD163, CD203a, CD45, CD16 (Lithgow *et al.*, 2014). They observed that MHC II, CD203a, CD45 are not up-regulated after ASFV infection and CD16 is instead down-regulated in macrophages after ASFV infection. This study produced results contrasting to a previous work, where the importance of CD163 in ASFV infection was described (Sanchez-Torres *et al.*, 2003). In fact Lithgow *et al.* (2014) observed that this marker is not up-regulated after ASFV infection and the virus is able to infect both CD163<sup>+</sup> and CD163<sup>-</sup> bone marrow-derived macrophages (Lithgow *et al.*, 2014). The effect of the virus on the expression of this marker on monocytes/macrophages should be better defined.

DC are one of the target of ASFV. DC are thought to be the most potent antigen presenting cells (APCs) that play an important role in induction of adaptive immune responses, by processing antigens, expressing lymphocyte co-stimulatory molecules, migrating to lymphoid organs and secreting cytokines (Banchereau and Steinman., 1988). These cells express a variety of specialised pattern recognition receptors (PRRS), including Toll-like receptors (TLRs) for the recognition of pathogen associated molecule patterns (PAMPs) (Gijzen *et al.*, 2006). DC can broadly be

divided into two subpopulations: conventional or myeloid DC (mDC), with main function in antigen presentation, and plasmacytoid DC (pDC), specialized in rapidly secreting large amounts of type I interferon (McCullough and Summerfield, 2009).

Despite the important role that DC play in innate and adaptive immunity against pathogens (Banchereau and Steinman., 1988), little is known about their interaction with ASFV. ASFV is able to infect skin-derived DC and this interferes with a subsequent infection with FMDV, in fact ASFV inoculation 3 hours before FMDV inoculation blocked FMDV infection (Gregg *et al.*, 1995a). *In vivo* it has been observed that ASFV is able to infect interdigitating DC (iDC) and in pigs infected with a virulent ASFV isolate there is reduction in the number of iDC in mandibular lymph nodes from 3 days pi (Gregg *et al.*, 1995b). A recent study reported that infection *in vitro* of porcine leucocytes enriched for DC with ASFV induced high levels of type I interferon, suggesting that ASFV-infected pDC could be a potential source of interferon in animals undergoing acute ASF (Golding *et al.*, 2016).

In the same study it was reported an important role of type I IFN in preventing replication of attenuated ASFV strains in macrophages. Researchers observed that replication of attenuated strains, containing MGF 360/530 deletions, but not virulent ASFV isolates in porcine alveolar macrophages was inhibited by recombinant porcine IFN- $\alpha$  (Goldwing *et al.*, 2016). Also a former study described that IFN- $\alpha$  has an important role in protection against ASFV: it can reduce ASFV replication in both porcine monocytes and alveolar macrophages, inhibiting synthesis of late ASFV proteins (Esparza *et al.*, 1988). Virulent isolates have developed mechanism to overcome IFN antiviral activity, in fact MFG 360/530 genes either directly or indirectly suppress a type I IFN response (Afonso *et al.*, 2004). Deletion of MGF 360/530 probably results in absence of interference with antiviral genes induced by IFN- $\alpha$ . Type I interferon can induce interferon-induced transmembrane (IFITM) protein expression, a group of antiviral restriction factors that restrict viral progression at entry. A recent study reported that infection with cell-adapted ASFV isolate BA71V, which is IFN sensitive, was able to induce IFITMs expression. High levels of this proteins caused a collapse of the endosomal pathway to the perinuclear area. Expression of IFITM1, 2 and 3 reduced virus infectivity in Vero cells, with

IFITM2 and IFITM3 having an impact on viral entry/uncoating (Munoz-Moreno *et al.*, 2016).

Little is known about the effect of ASFV on natural killer (NK) and  $\gamma\delta$ -T cells, which are two major lymphocyte populations of the innate immune system. Their activation/inhibition could be crucial, given that swine possess only a small number of cytotoxic T-cells, but large numbers of lymphocytes with innate cytotoxic activity, especially  $\gamma\delta$ -T cells (Denyer *et al.*, 2006). In young pigs,  $\gamma\delta$ -T cells and NK cells represent 50% and 10% of the total peripheral blood lymphocyte population, respectively, although their frequencies decrease with age (Denyer *et al.*, 2006, Gerner *et al.*, 2009). It is well known that NK cells possess the ability to attack pathogen-infected and malignant cells and to produce immunostimulatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$  (Gerner *et al.*, 2009). Specifically, these cells are triggered to kill or ignore cells (cancer cells and virus-infected cells) depending on a balance of inhibitory and activating signals received through ligands on potential targets. Activation of these cells by most pathogens is led by accessory cells, especially mature DC, which release cytokines and provide contact-dependent signals to NK cells (Newman *et al.*, 2007, Trinchieri, 2003, Fehniger and Caligiuri, 2001). Several studies suggest the importance of NK cells in response to ASFV (Leitao *et al.*, 2001, Norley *et al.*, 1983, Martins *et al.*, 1994). Leitao *et al.* (2001) showed that NK activity was correlated to survival after ASFV infection. In that study 31 pigs were inoculated with ASFV/NH/P68 and some pigs developed ASF chronic type lesions and others instead remained asymptomatic. In animals developing lesions and viraemia, NK activity levels were close to that of control animals, instead pigs remaining asymptomatic after infection had elevated NK cell activity (Leitao *et al.*, 2001). Instead another study reported that pigs infected with ASFV exhibited a suppressed NK activity, but that was probably due to the sensitivity of NK cells to increased temperatures. In fact NK cell activity was lost when cells were incubated at 40°C *in vitro* and the authors suggested that pyrexia might be the cause of the depressed NK cell activity (Norley *et al.*, 1983). Martins *et al.* (1994) showed that NK cell activity of PBMC derived from naïve pigs was stimulated *in vitro* by non-virulent NH/P68 virus, but depressed by virulent Lisbon 60 isolate.

$\gamma\delta$ -T cells represent another significant lymphocyte population originally defined as effector lymphocytes of the innate immune system but now being regarded, like NK-cells, as also playing an important role in the activation and regulation of immune responses.  $\gamma\delta$ -T cells express a subtype of T cell receptor (TcR) different from the conventional  $\alpha\beta$  TcR and display characteristics of both T cells and cells of the innate immune system. They recognize conserved non-peptide antigens that are up-regulated by stressed cells, by both TcR and non-TcR molecules, such as TLRs and NK receptors (NKR). Like NK cells, the balance between activating and inhibitory signals regulates the  $\gamma\delta$ -T cell response (Bonneville *et al.*, 2010).  $\gamma\delta$ -T cells can produce cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , involved in the protection against viruses and other intracellular pathogens, or cytokines that contribute to the protection against extracellular parasites (IL-4, IL-5 and IL-13), extracellular bacteria (IL-17) and also immunosuppressive cytokines (Transforming Growth Factor (TGF)- $\beta$  and IL-10) (Bonneville *et al.*, 2010). Activation of  $\gamma\delta$ -T cell responses may therefore be direct or indirect, mediated by the interaction with APCs, such as DC, through release of pro-inflammatory cytokines and expression of ligands for  $\gamma\delta$  TcR and NKRs (Devilder *et al.*, 2009). In swine,  $\gamma\delta$ -T cells act both as cytotoxic cells and professional antigen presenting cells (APCs). They can express CD8 $\alpha$  and MHC II molecules, and the latter seems to be correlated with T cell activation in swine (Gerner *et al.*, 2009).

Little is known about the interaction of ASFV with  $\gamma\delta$ -T cells. In a study researchers used lymphocytes from ASFV immune pigs and observed that these cells are able to present viral antigen (Takamatsu *et al.*, 2006), however the interaction of ASFV with  $\gamma\delta$ -T cells should be better studied in the future.

## **1.7.2 ASFV and adaptive immunity**

### **1.7.2a Humoral Immunity**

Almost all virus can be neutralised by antibodies. However, there is some controversy about antibody-mediated neutralization of ASFV with sera from



convalescent pigs and about the protective relevance of antibodies in experimentally vaccinated pigs (Escribano *et al.*, 2013).

In the past some it was indicated the lack of neutralizing activity of sera from animals infected with ASFV (Hess, 1981), nevertheless evidence of neutralising antibodies against this virus has been provided by numerous groups in the last 15 years (Escribano *et al.*, 2013).

Onisk *et al.* (1994) examined the role of anti-viral antibodies in homologous protective immunity to a virulent ASFV strain E75 by passive transfer experiments in swine. 85% of animals that received anti-ASFV immunoglobulin survived challenge infection and remained clinically normal following challenge, while 100% mortality was observed in control group animals. These data indicate that anti-ASFV antibodies alone protect swine from lethal infection with virulent ASFV (Onisk *et al.*, 1994). Also Zsak *et al.* (1993) showed that sera from convalescent swine infected with an attenuated AFV isolate (E75CV1-4, derived from the Spanish strain E75 and adapted to grow on CV1 cells and propagated in pig macrophages) neutralized the infectivity of virulent ASFV isolates E75, E70, Lisbon 60, Malawi Lil 20/1 and a low passage tissue culture adapted variant of E75 (E75CV/V3) by 86-97% in Vero and macrophage cell cultures. Unexpectedly, these immune sera failed to neutralize high passage tissue culture adapted ASFV variants including Lisbon 60, Haiti, Dominican Republic I, Dominican Republic II, and Brazil II. These results suggest that tissue culture adaptation of ASFV isolates may be associated with loss of specific determinants associated with virus neutralization (Zsak *et al.*, 1993).

In fact few years later, Gomez-Puertas *et al.* (1997) showed that highly passaged ASF viruses were resistant to neutralization by antisera from convalescent pigs or antibodies generated against individual viral proteins which neutralized low-passage viruses. They showed that the absence of neutralization of high-passage viruses is not due to antigenic variability of critical epitopes, but is linked to the relative amount of phosphatidylinositol in viral membranes, which is higher in low-passage viruses. The data suggest that phosphatidylinositol is essential for a correct epitope presentation to neutralizing antibodies. Additionally, the removal of phosphatidylinositol from a

low-passage virus by a specific lipase transformed this virus from neutralizable to non neutralizable (Gomez-Puertas *et al.*, 1997).

Anti-ASFV antibodies neutralize the virus before and after binding to susceptible cells. Gomez-Puertas *et al.* (1996) observed that neutralising antibodies inhibited about 80% of virus attachment and more than 90% of virus internalization and the combination of both mechanisms neutralized more than 95% of virus infectivity. In addition, researchers reported that antibodies to proteins p72 and p54 are involved in the inhibition of a first step of the replication cycle related to virus attachment, while antibodies to protein p30 are implicated in the inhibition of virus internalization (Gomez-Puertas *et al.*, 1996). Moreover, proteins p54 and p30 mediate specific interactions between ASF virus and cellular receptors and that simultaneous interference with these two interactions has a complementary effect in antibody-mediated protection (Gomez-Puertas *et al.*, 1998).

Even if the existence of neutralising anti-ASFV antibodies has been demonstrated, a persistent non-neutralized ASFV fraction of about 10% is found with most convalescent swine sera *in vitro* neutralization assay. Probably, the main cause for the persistent surviving virus fraction observed in neutralization assay is the induction of blocking antibodies, and this could also explain the persistent infections observed in some convalescent pigs (Gomez-Puertas and Escribano, 1997).

Although antibody-mediated immune mechanisms have been shown to be important in immunity to ASF, a study showed that neutralizing antibodies to some ASFV proteins (p30, p54, and p72) are not sufficient for antibody-mediated protection. In that study pigs immunized with baculovirus-expressed p30, p54, p72 from the pathogenic ASFV isolate Pr4 died 7-10 days post challenge with  $10^4$  TCID<sub>50</sub> of Pr4 virus. Nevertheless, test group animals exhibited a 2-day delay to onset of clinical disease and reduced viremia levels at 2 days post infection in comparison to the control group (Neilan *et al.*, 2004). Further studies are needed to clarify the role of ASFV antibodies in protection.

### 1.7.2b Cellular Immunity

Despite the controversy on the role of neutralising antibodies in protection against ASFV, several studies demonstrated the importance of cellular immune response against this virus.

IFN- $\gamma$  has an important role in protection against ASFV, in fact it reduces ASFV replication in both porcine monocytes and alveolar macrophages. Instead, TNF- $\alpha$  does not show antiviral activity in either type of cells (Esparza *et al.*, 1988).

Several study analysed the role of T cells in response to this virus. The first demonstration of ASFV specific lymphocyte proliferation was described by Wardley and Wilkinson (1980) 10 days pi with a non-virulent virus, however they did not detect ASFV specific lymphocyte proliferation after infection with a virulent virus, presumably because the pigs died too quickly. One year later Sanchez-Vizcaino *et al.* (1981) showed that infection with moderate or non-virulent strains of ASFV induced ASFV specific memory T cell proliferation against homologous virus. Also Revilla *et al.* (1992) observed that a non-virulent ASFV isolate (BA71V) induced memory T cell response against homologous virus. In fact PBMC from inbred pigs that were immunized with autologous macrophages infected with BA71V produced IFN- $\gamma$  when challenged *in vitro* with homologous or attenuated isolates of the ASF virus, but not with heterologous or virulent isolates (Revilla *et al.*, 1992).

Later experiments (Martins *et al.*, 1993, Scholl *et al.*, 1989) examined ASFV specific T cells activities in swine that had recovered from the non-haemabsorbing ASFV isolate NH/P68. Scholl *et al.* (1989) showed that blood mononuclear cells (BMC) from swine surviving experimental infection with NHV exhibited a strong virus-induced, antigen-specific blastogenic response, absent in control pigs. This ASFV-induced blastogenesis was dependent on virus dose and on the presence of adherent cells (Scholl *et al.*, 1989). Few years later Martins *et al.* (1993) show that ASFV specific CTL activity was triggered in swine infected with the NHV isolate. PBMC from such infected swine showed significant activity in CTL assays, using cultured ASFV-infected porcine blood derived macrophages as target cells. This CTL activity appeared to be SLA class I restricted because it was higher in the infected

macrophages of the same haplotype, and was blocked by anti-CD8 monoclonal antibodies (mAbs) but not by anti-CD4 mAbs. Experiments with macrophages infected with different ASFV isolates revealed that there was marked lysis of macrophages infected with the virulent L60 isolate but less lysis of macrophages infected with the DR-II and Tengani isolates (Martins *et al.*, 1993). King *et al.* (2011) observed that the ability of lymphocytes from immune pigs to release IFN- $\gamma$  *in vitro* in response to different isolates was correlated with protection against ASF. Immunisation of pigs with the non-virulent OURT88/3 isolate followed by the closely related virulent OURT88/1 isolate could confer protection against challenge with virulent isolates from Africa, including the genotype I Benin 97/1 isolate and genotype X Uganda 1965 isolate. Cross-protection was correlated with the ability of different ASFV isolates to stimulate production of IFN- $\gamma$  by lymphocytes from OURT88/3 and OURT88/1 immunised pigs, assessed *in vitro* by ELISPOT (King *et al.*, 2011).

Few recent studies underlined the importance of T cell response in ASFV-vaccine induced protection. As above stated, ASFV vaccines based on the proteins p30, p54 and HA were able to induce a T cell response and partially protected pigs against challenge with a virulent ASFV isolate (Argilaguet *et al.*, 2012, Argilaguet *et al.*, 2013). A DNA vaccine containing p30, p54 and HA fused to ubiquitin was able to protect more than 50% of the vaccinated pigs against challenge with a virulent ASFV strain, in the absence of antibody response (Argilaguet *et al.*, 2012). In another study the ASFV proteins p54, p30 and HA were inserted in a baculovirus vector (BacMam-sHAPQ), and the use of this vaccine induced a strong T cell response, positively correlated with protection in survived pigs (4 out of 6), in the absence of antibody response (Argilaguet *et al.*, 2013).

The critical importance of CD8<sup>+</sup> lymphocytes on protective immunity to ASF was demonstrated directly by depleting CD8<sup>+</sup> lymphocytes from ASF immune pigs *in vivo*. Researchers showed that depletion of CD8 T cells from pigs vaccinated with an avirulent ASFV isolate OURT88/3 resulted in a loss of protection when challenged (Oura *et al.*, 2005).

A study on the phenotype of ASFV specific CD8<sup>+</sup>T cells revealed that there are two distinctive populations: a conventional (perforin<sup>+</sup>CD2<sup>+</sup>CD3<sup>+</sup>CD4<sup>-</sup>CD5<sup>+</sup>CD6<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>) and a CD4<sup>+</sup> phenotype (perforin<sup>+</sup>CD2<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD5<sup>+</sup>CD6<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>), both able to lyse ASFV infected syngeneic target cells (Denyer *et al.*, 2006). In a recent study the phenotype of ASFV-specific double positive CTLs was characterised. Pigs were immunized by inoculating non-virulent ASFV isolate OURT88/3 and then challenged 4 weeks later with the related virulent isolate OURT88/1. Only one pig exhibited transient pyrexia and viraemia. Before challenge with OURT88/1, PBMC from immune pigs were stimulated *in vitro* for 5 days with OURT88/1 and the phenotypes of proliferated lymphocytes were analyzed. More than half (55.7%) of the proliferating CD4<sup>+</sup> cells in the protected pigs expressed perforin, in contrast to only 13.8% of proliferating CD4<sup>+</sup> cells from the diseased pig (Takamatsu *et al.*, 2013). Unpublished observations from Denyer, Stirling & Takamatsu revealed that IFN- $\gamma$  positive lymphocyte population from ASFV stimulated immune PBMC was dominated by the CD4<sup>+</sup>CD8<sup>+</sup> T cell phenotype, but only a third of these cells presented a typical memory helper T cell (CD4<sup>+</sup>CD81<sup>ow</sup>) phenotype and the rest were CD8<sup>hi</sup>CTL (Takamatsu *et al.*, 2013). Further investigation is needed to explore the contribution of CD4<sup>+</sup>CD8<sup>+</sup> T cells in protection to ASF.

Several studies were made in order to identify antigens recognized by ASF immune porcine T cells. In a study SLA inbred minipigs were experimentally infected with an attenuated isolate of the virus and CTL assays were performed using alveolar macrophages as target cells. It was observed that ASFV-specific cytotoxic T lymphocytes recognize and lysed p32, an immediate early ASFV protein (Alonso *et al.*, 1997). Also the structural ASFV protein p72 was identified as target of CTLs. Researchers used expression vectors based on the Pseudomonas outer membrane lipoprotein I gene (*oprI*) to study ASFV-specific CTL activity. They observed that the aminoacid sequence HKPHQSKPILTDENDTQRTCSHTNP from the major structural ASFV protein (VP72) is presented by macrophages, which are lysed under restriction of SLA class I antigens (Leitão *et al.*, 1998). Recently two 9-mer peptides within the ASFV haemagglutinin protein (CD2v, EP402R) were identified as CTL epitopes. Researchers tested a panel of 53 9mer peptides selected *in silico* from

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within p54, p30 and sHA protein sequences. *In silico* prediction of CTL epitopes was based on their binding affinity to TAP, due to the fact that MHC I restricted epitopes can often be transported by TAP as N-terminal elongated peptide precursors. Immunodominance hierarchy in the responses and the SLA I restriction were also observed (Argilaguet *et al.*, 2012).

## **1.8 Aim, hypothesis and objectives**

There is a need to develop alternative strategies to improve the control of ASF outbreaks and minimize the need for mass culling. As previously stated, main target of the virus are cells of the myeloid lineage and this tropism is thought to be crucial for disease pathogenesis. A detailed characterization of the interaction of ASFV isolates of differing virulence with monocytes and macrophages subsets would aid the understanding of the immunological mechanisms underlying the disease, generating information to aid the development of marker vaccines.

In this thesis, responses of monocytes and derived macrophage subsets to ASFV were investigated through the following two objectives:

- To compare different methods to differentiate porcine monocytes into macrophages, assessing their phenotype, release of cytokines and susceptibility to ASFV infection (Chaper 3).
- To determine the interaction of monocytes and macrophage subsets to the avirulent BA71V and the virulent 22653/14 ASFV strains, characterising their susceptibility to infection, expression of surface markers and release of cytokines (Chapter 4).

## CHAPTER 2. MATERIALS AND METHODS

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## 2.1 Animals and blood sampling

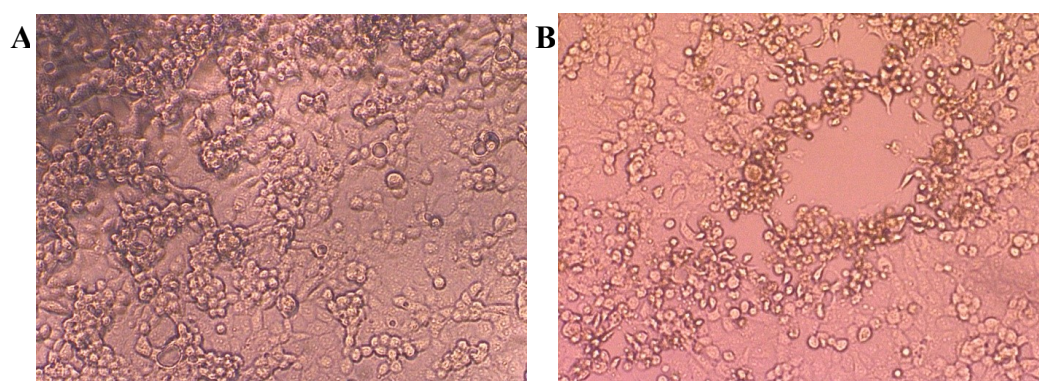
Blood samples were obtained from 6 to 18 months old healthy crossbred pigs (*Sus scrofa*) housed at the experimental facility of the IZS of Sardinia (Sassari, Italy), authorized for animal research by Italian Ministry of Health. The ASFV negative status of the animals was confirmed by a commercial ELISA test (Ingenasa, Madrid, Spain) and by an immunoblotting test (OIE, 2012). Heparinized blood was collected by cranial vena cava puncture using a 50 mL syringe containing 5000 IU of sodium heparin (Thermo Fisher, Germany), connected to a mm 14-gauge needle (Delta Med, Mantova, Italy). Animal housing, handling and sampling procedures were performed in accordance with the local ethics committee, according to the guide of use of laboratory animals of the Italian Ministry of Health.

## 2.2 Viruses

The attenuated ASFV BA71V strain (kindly provided by EU Reference laboratory CISA-INIA, Madrid, Spain) is non pathogenic in swine and was obtained by adaptation to growth in Vero cells of the BA71 isolate (a highly virulent virus isolated in Badajoz, Spain in 1971) (Enjuanes *et al.*, 1976). It belongs to the p72 genotype I and cluster within sub-group V of the B602L gene (Bastos *et al.*, 2003, Nix *et al.*, 2006). BA71V was propagated *in vitro* by inoculation of sub-confluent monolayers of Vero cells, maintained in Dulbecco's Modified Eagle Medium (DMEM) (Euroclone, Milan, Italy) supplemented with 50 µg/ml gentamicin (Gibco, Thermo Fisher Scientific, Monza, Italy) and 10% foetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific). Images of un-infected and infected Vero cells are displayed in Figure 2.1. After 1-2 days at 37°C, 5% CO<sub>2</sub>, a clear cytopathic effect



was observed and the supernatant was collected and pooled with a freeze-thawed cell lysate. The resultant pool was clarified by centrifugation at 3000g for 15 minutes in a rotating bucket centrifuge, aliquoted and stored at -80°C. Virus titres were obtained by serial dilution of the virus suspension on Vero cells followed by observation for cytopathic effect and crystal violet staining to identify infection rates as previously described (Carrascosa *et al.*, 2011).



**Figure 2.1. Infection of Vero cells with BA71V.** Vero cells were mock-infected (A) or infected for 24 hours with BA71V (B). In Figure B a clear cytopathic effect is visible. Images taken with an inverted microscope (Olympus, Tokyo, Japan), using a 10X magnification.

The highly virulent ASFV Sardinian field strain 22653/14 is placed in the p72 genotype I and was isolated from the spleen of a naturally infected pig collected from a 2014 outbreak in the province of Cagliari (Exotic Disease Laboratory ASFV Archive, IZS of Sardinia, Sassari, Italy). Genotype of 22653/14 was determined by partial p72 gene characterization, as previously described (Bastos *et al.*, 2003). ASFV 22653/14 was propagated *in vitro* by inoculation of sub-confluent monolayers of porcine monocytes/macrophages for no more than six passages (Malmquist and Hay, 1960). In brief, leukocytes were cultured in RPMI-1640 medium supplemented with 20% (v/v) autologous plasma, 100 U/ml penicillin and 100 µg/ml streptomycin.

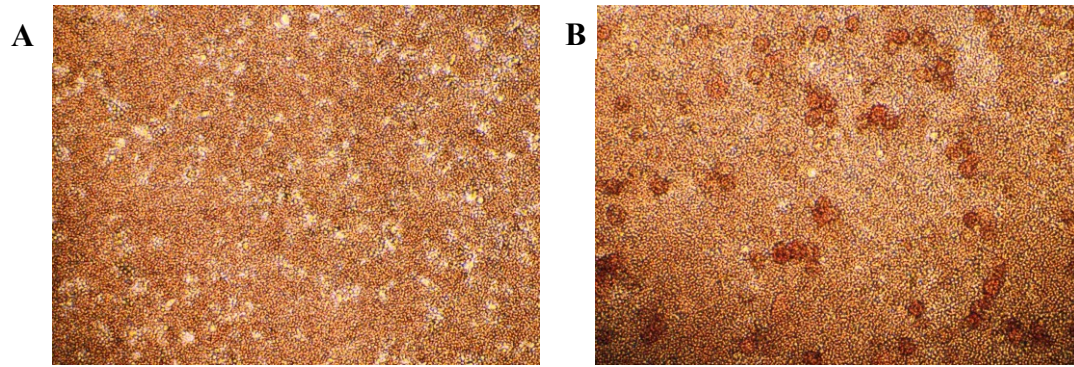
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After 2 days, non-adherent cells were removed and virus suspension was added to the adherent monocytes/macrophage monolayer. After 2 hours incubation, RPMI-1640 medium supplemented with 1% (v/v) autologous plasma, 0.1% autologous erythrocytes, 100 U/ml penicillin and 100 µg/ml streptomycin was added. After 3 days at 37°C in 5% CO<sub>2</sub>, the supernatant was collected and pooled with a freeze-thawed cell lysate. The resultant pool was clarified by centrifugation at 3000g for 15 minutes, aliquoted and stored at -80°C. Images of un-infected and infected macrophages are displayed in Figure 2.2. Viral titre of ASFV 22653/14 was obtained by serial dilution of the virus suspension on monocyte/macrophages in 96 well plates and cell cultures were observed for hemadsorption (Malmquist and Hay, 1960) or immunofluorescence (OIE, 2012). For detection of infected cells by immunofluorescence a FITC-conjugated polyclonal antibody against ASFV (kindly provided by the EU ASF Reference Laboratory CISA- INIA, Madrid, Spain) was used. In brief, medium was removed from each well, monolayer of macrophages were washed with PBS and then cells were fixed by addition of methanol for 15 minutes. After two washes with PBS, cells were incubated 1 hour at 37°C with a polyclonal antibody anti-ASFV diluted 1:200 in PBS and then washed twice with PBS. Plates were viewed under an inverted fluorescent microscope (Axiovert 200, Zeiss, Germany). There was correspondence between viral titres obtained using immunofluorescence and hemadsorption.

All the virus titres were determined using the Spearman–Kärber formula.

Mock-virus supernatants were prepared in identical manner from uninfected Vero cell ('mock Vero') or monocyte/macrophage ('mock macrophages') cultures.



**Figure 2.2 Infection of macrophages with 22653/14.** Porcine blood derived macrophages were mock-infected (A) or infected for 24 hours with 22653/14 (B). In Figure B clear haemadsorbing effects ('rosetta') are visible. Images taken with an inverted microscope (Olympus), using a 10X magnification.

### 2.3 Enrichment of monocytes

Peripheral blood mononuclear cells (PBMC) were prepared by layering 30 ml of heparinized blood diluted 2:1 in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS) over 20 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) and centrifuged at 600g for 20 minutes at 4°C without breaking (Berg *et al.*, 2013). PBMC were collected from the plasma-Histopaque interface, washed thrice in PBS at 4°C, pelleted and re-suspended in RPMI-1640 medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Thermo Fisher Scientific, Rockford, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Pen Strep, Thermo Fisher Scientific, Rockford, USA) (monocyte medium).

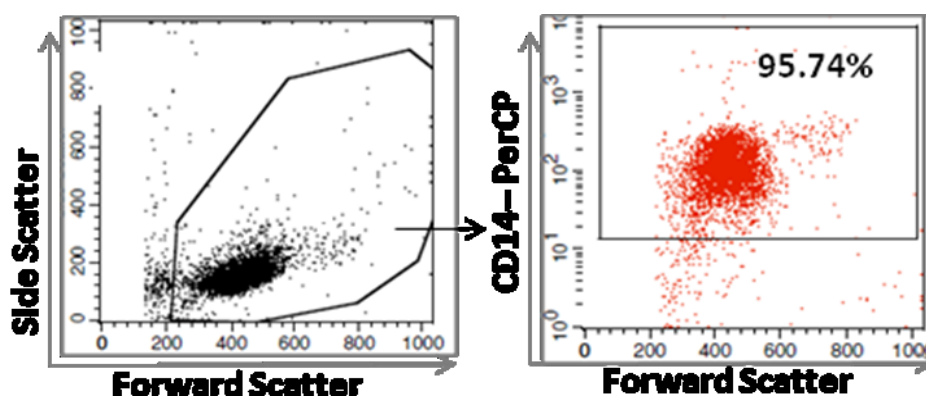
Porcine monocytes were isolated by plastic adhesion using flasks (Corning, NY, USA) pre-incubated with autologous porcine plasma, according to previous methods with slight modifications (Berg *et al.*, 2013). In brief, autologous plasma was collected from heparinized blood by centrifugation at 700g for 30 minutes at 4°C without breaking. Flasks were incubated for 1 hour at 37°C with 5% CO<sub>2</sub> with autologous plasma before removal of plasma and addition of PBMC. Flasks were

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incubated for 1 hour and non-adherent cells were then removed by 4 washes with unsupplemented RPMI-1640 and adherent cells were incubated overnight at 37°C with 5% CO<sub>2</sub> in monocyte medium. The following morning adherent cells were detached by placing the flasks on ice for 1 hour, pelleted by centrifugation at 200g for 8 minutes and re-suspended in monocyte medium; an aliquot was used to count and to assess cells viability using a Countess Automated Cell Counter (Thermo Fisher Scientific). 8-10x10<sup>5</sup> live cells/well were seeded in a 12 well plates (Greiner CELLSTAR, Sigma). In selected experiments a second aliquot was used to assess cell purity: cells were stained with CD14-PerCP (TUK4, Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at room temperature (RT), washed with PBS supplemented with 2% FBS and resuspended in PBS. Cells were analysed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) and an average of 90% CD14+ monocytes was observed, as displayed in Figure 2.3.



**Figure 2.3 Assessment of monocyte purity using flow cytometry.** Porcine blood derived monocytes were stained with CD14-PerCP for 10 minutes at RT, washed and analysed using a FACSCalibur flow cytometer. Cells with FSC and SSC typical for monocytes were analysed for their expression of surface marker CD14.

## **2.4 *In vitro* differentiation of monocyte into moMΦ and activation**

In Chapter 3, six different culture conditions were tested for moMΦ differentiation. Monocytes were cultured for 5 days at 37°C with 5% CO<sub>2</sub> in medium supplemented with different concentrations of autologous porcine plasma or human M-CSF (hM-CSF). Plasma supplemented media were composed of RPMI-1640, penicillin/streptomycin (100 U/ml/100 µg/ml) and 10%, 20% or 30% (v/v) autologous porcine plasma, whereas hM-CSF supplemented media were prepared with monocyte medium and 50 ng/ml, 100 ng/ml or 200 ng/ml of recombinant human M-CSF (eBioscience, San Diego, USA). Porcine plasma and FBS were heat-treated at 56°C for 30 minutes to inactivate complement before addition to culture media. In Chapter 4, moMΦ differentiation was achieved by culturing monocytes for 5 days in monocyte medium supplemented with and 50 ng/ml hM-CSF.

To assess macrophage activation, moMΦ obtained using 30% autologous porcine plasma or 50 ng/ml hM-CSF were both classically and alternatively activated as described in humans and pigs (Gordon and Taylor, 2005, Garcia-Nicolas *et al.*, 2014, Singleton *et al.*, 2016). In Chapter 4, only moMΦ cultured in 50 ng/ml hMCSF were activated. For classical activation, moMΦ were cultured in monocyte medium supplemented with 100 ng/ml of recombinant porcine IFN-γ (Raybiotech Inc, Norcross, GA, USA) and 100 ng/ml of LPS (Lipopolysaccharide from *Escherichia coli* 0111:B4; Sigma-Aldrich) for 24 hours at 37°C in 5% CO<sub>2</sub>, while for alternative activation, cells were stimulated with 20 ng/ml of recombinant porcine IL-4 (R&D Systems, Minneapolis, MN, USA) under the same culture conditions.

## **2.5 *In vitro* ASFV infection of monocytes/macrophages subsets and growth curves**

Monocytes, moM $\Phi$ , moM1 and moM2 were infected, following the replacement of culture media with fresh monocyte medium free of growth factors and/or cytokines, with the virulent 22653/14 or the attenuated BA71V ASFV strains. Monocytes and macrophages were infected using an MOI of 1 and mock-infected controls were included in every experiment. Cells were incubated at 37°C, harvested after 24 hours (Chapter 3) or 18 hours (Chapter 4) and infection was assessed by intracytoplasmic p72 expression using flow cytometry. In defined experiments, culture supernatants were collected to evaluate cytokine release in response to infection. To evaluate BA71V and 22653/14 growths in moM $\Phi$ , moM1 and moM2, cells were infected with an MOI of 0.01 and after 90 minutes the inoculum was removed, cells were washed with RPMI and fresh monocyte medium added to the wells. Culture supernatants were collected 0, 24, 48, 72 hours pi, cleared by centrifugation at 2000g for 3 minutes and stored at -80°C until analysed. Viral titres in culture supernatants were determined by RT-PCR, as described below. In parallel macrophages were harvested and infection was confirmed by flow cytometry, as described below.

## **2.6 Light microscopy**

Infection of Vero cells with BA71V and of monocyte/macrophages with the Sardinian field isolate 22653/14 were assessed by light microscopy, as displayed in figure 2.1 and 2.2. Differentiation of monocytes into moM $\Phi$  was observed by light microscopy. Monocytes were photographed immediately or were differentiated in

macrophages with different methods. Light microscopy was performed using an inverted microscope (Olympus, Tokyo, Japan), with 10X magnification.

## **2.7 Confocal microscopy**

Monocyte and moMΦ morphology was analysed by confocal microscopy, as previously described (Kyrova *et al*, 2014). Confocal microscopy was kindly performed by Dr Antonio Anfossi. Monocytes were cultured on two-well chamber slides (Thermo Fisher Scientific) at a concentration of  $5 \times 10^5$  live cells/well and were observed immediately or following differentiation under the different culture conditions. In defined experiment, macrophages differentiated using 50 ng/ml of hM-CSF were activated for 24 hours before observation. Cells were fixed with 4% paraformaldehyde and labelled with Hoechst 33342 (Molecular Probes, Thermo Fisher Scientific) for nuclear staining and Alexa Fluor 488 conjugated phalloidin (Molecular Probes, Thermo Fisher Scientific) to visualize actin cytoskeleton. Microscopy was performed using a Leica SP5 Confocal Microscope (Leica Microsystem, Wetzlar, Germany) equipped with a 40X Plan-Apo 1.25 NA oil immersion objective. Images were acquired on a format of 1024x1024 pixel, with a line average of 2 and scan speed of 100 Hz. Images were processed with LAS AF Lite software (Leica Microsystem) for contrast and brightness adjustments. Manipulations did not change the data content.

## **2.8 Assessment of metabolically active cells**

A colorimetric assay based on the reduction of the MTS tetrazolium salt was used to assess metabolically active cells (CellTiter 96® Aqueous Non-Radioactive Cell

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Proliferation Assay; Promega, Madison, USA). Monocytes were seeded into a 96 well plates at a concentration of  $1-2 \times 10^5$  live cells/well and differentiated into moM $\Phi$  with the six different methods as mentioned above. A control sample prepared with monocytes incubated in monocyte medium was also included. After 3 days, medium was discarded, cells were washed twice with RPMI-1640 and 100  $\mu$ l/well of fresh monocyte medium were added. The assay was performed, according to manufacturer's instruction, and absorbance was read with an Epoch microplate reader (BioTek, Winoosky, VT, USA).

## 2.9 Flow cytometry

Expression of surface markers on porcine monocytes and moM $\Phi$  were analysed by multi-parameter flow cytometry. Cells were harvested with ice-cold PBS with 10mM EDTA, washed in PBS and transferred to a round-bottom 96 well plate (1-2 x 10<sup>5</sup>/well) for staining. To assess viability, cells were stained with LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific) for 30 minutes at 4°C and washed twice with PBS supplemented with 2% FBS. Direct and indirect staining methods based on both unconjugated and fluorochrome-conjugated mAbs specific for cell surface markers were performed. The mAbs used were: MHC class II DR (clone 2E9/13, Bio-Rad, Oxford, UK), CD163-RPE (clone 2A10/11, Bio-Rad), CD203a (clone PM18-7, Bio-Rad), CD14-PerCP (clone Tük4, Miltenyi Biotec, Bergisch Gladbach, Germany), MHC class I (JM1E3; AbD Serotec), CD16-RPE (G7, Thermo Scientific Pierce, Rockford, IL, USA). Primary mAbs were added to each well and incubated for 10 min at room temperature (RT). For indirect staining, secondary antibodies (Rat anti-mouse IgG2b-RPE-R&D Systems, Goat anti-mouse



IgG Fc cross adsorbed RPE-conjugated, Thermo Fisher Scientific) specific for the corresponding unconjugated primary antibody were used and allowed to incubate at RT for additional 10 minutes after a washing step. To complete either direct or indirect immunofluorescence procedures, excess of primary or secondary antibodies were washed away with PBS. For control staining, the following irrelevant isotype-matched control antibodies were employed: mouse IgG1 isotype control PE conjugated (clone ZX3, Thermo Fisher Scientific), mouse IgG1 negative control purified, mouse IgG2b negative control purified (Bio-Rad). To assess ASFV infection, intracytoplasmic staining was performed. Cells were fixed and permeabilized using Leucoperm (Bio-Rad) according to manufacturer's suggestion. Afterwards cells were incubated with mAbs at RT for 30 minutes in the dark. mAbs used for intracellular staining were: anti-p72-FITC (18BG3, Ingenasa) and p30-FITC (kindly provided by Dr Gian Mario De Mia, IZSUM, Italy). Cells were washed twice in PBS, re-suspended in PBS and transferred to FACS tubes prior to flow cytometric analysis. All the washes used centrifugation at 836g for 3 minutes. All antibodies used for flow cytometric analysis are listed in Table 2.1. At least 5000 live monocytes/moMΦ were acquired on a FACS Calibur (FACS Calibur, BD, Franklin Lakes, USA) flow cytometer and analysed using Cell Quest Pro software (BD). Analysis of data was performed by gating on viable cells (Live/Dead Fixable Dead Cell Stain negative) in the monocyte/macrophage population, and their expression of surface and intracytoplasmic markers was assessed. Gates for surface markers were set using the corresponding isotype controls, whereas gates for ASFV proteins were set using the mock-infected controls.

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## **2.10 TNF- $\alpha$ release in response to LPS stimulation**

Analysis of TNF- $\alpha$  secretion from monocytes and moM $\Phi$  in response to LPS stimulation was also performed. Briefly, culture media were discarded and replaced with fresh monocyte medium, then cells were stimulated with 100 ng/ml of LPS. Unstimulated cells were used as a negative control. After 6 hours of culture, supernatants were collected, cleared by centrifugation at 2000g for 3 minutes and stored at -80°C until analysed. The measurement of TNF- $\alpha$  levels in culture supernatants was performed using a Porcine TNF- $\alpha$  DuoSet ELISA (R&D System), according to the manufacturer's instructions and absorbance was read with an Epoch microplate reader (BioTek).

## **2.11 Patterns of cytokine secretion**

Analysis of basal cytokine release from moM $\Phi$  differentiated in different media or in response to classical and alternative activation or after ASFV infection was performed using a multiplex immunoassay. Monocytes were seeded in 12 well plates and differentiated into moM $\Phi$  under the different conditions as described above. After 5 days, supernatants were collected, cleared by centrifugation at 2000g for 3 minutes and stored at -80°C until analysed. MoM $\Phi$  cultured in 30% autologous plasma or 50 ng/ml hM-CSF were left untreated or classically and alternatively activated. After 24 hours activation, supernatants were collected, cleared by centrifugation at 2000g for 3 minutes and stored at -80°C until analysed.

Analysis of cytokine release in response to ASFV infection was also assessed.

Monocytes and macrophage subsets were infected with the avirulent BA71V or the virulent 22653/14 strains, alongside with mock-infected controls. 18 hours pi,

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supernatants were collected, cleared by centrifugation at 2000g for 3 minutes and stored at -80°C until analysed.

The simultaneous measurement of GM-CSF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF- $\alpha$  was performed using the Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine kit (Merck Millipore, Darmstadt, Germany) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. In defined experiments, only the levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-6, IL-10, IL-12 and TNF- $\alpha$  were assessed. These experiments were kindly performed by Dr Silvia Dei Giudici, Dr Piero Bonelli and Susanna Zinellu. The intra-assay and inter-assay CV were 10% and 20%, respectively.

## **2.12 DNA extraction and real-time PCR**

Viral DNA was extracted from cell culture supernatants using High Pure PCR Template Preparation Kit according to the manufacturer's protocols (Roche, Mannheim, Germany). ASFV viral copy numbers were assessed by real-time PCR (King *et al.*, 2003), using the TaqMan Fast Advanced Master Mix (Applied Biosystems), 0.8  $\mu$ M of sense and anti-sense primers (5'-CTG CTC ATG GTA TCA ATC TTA TCG A-3' and 5'-GAT ACC ACA AGA TCR GCC GT-3'), 0.2  $\mu$ M of TaqMan probe 5'-[6-carboxy-fluorescein (FAM)]-CCA CGG GAG GAA TAC CAA CCCAGT G-3'-[6-carboxy-tetramethyl-rhodamine (TAMRA)] in a total volume of 25  $\mu$ l containing 5  $\mu$ l of extracted DNA. The incubation profile was established as follows: 40 cycles of denaturation at 95°C for 15'', annealing at 58°C for 60'', after a initial denaturation step at 95°C for 10'. The plasmid pEX-K4-ASFV-E70p72

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(Eurofins Genomics, USA) was used as the template to prepare the standard curve for the real-time PCR assay. This plasmid contains a full length p72 sequence and the copy number was calculated based on the plasmid and insert molecular weight. For each experiment, a standard curve was prepared by serial dilution ( $10^{-8}$ - $10^{-1}$ ) of pEX-K4-ASFV-E70p72 template DNA. These experiments were kindly performed by Dr Silvia Dei Giudici and Martina Sale.

### **2.13 Data analysis and statistics**

All experiments were performed in duplicates (for metabolic activity assay and multiplex cytokine immunoassay) or triplicates (all other assays), and repeated at least three times with different blood donor pigs. Graphical and statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software Inc, La Jolla, USA). Data were presented as boxplots, indicating the median (middle line), 25<sup>th</sup> and 75<sup>th</sup> percentiles (boxes), maximum and minimum (whiskers), or as means with standard deviations (SD) quoted to indicate the uncertainty around the estimate of the group mean. A Mann-Whitney test or a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis test was used; a p value <0.05 was considered statistically significant.

**Table 2.1 Antibodies used for flow cytometric analysis of surface and intracellular markers of porcine myeloid cells**

mAb	Source	Clone	Host species	Target species	Isotype	Conc (mg/ml)	Label	Working Dilution (for staining)
CD14	Miltenyi Biotec	TUK4	Mouse	Human	IgG2a	ND	PerCP-Cy5.5	Neat
CD163	AbD Serotec	2A10/11	Mouse	Pig	IgG1	ND	RPE	1/5
CD203a	AbD Serotec	PM18-7	Mouse	Pig	IgG1	1	None	1/50
MHC II	AbD Serotec	2E9/13	Mouse	Pig	IgG2b	1	None	1/20
CD16	Thermo Scientific Pierce	G7	Mouse	Pig	IgG1	ND	RPE	1/5
MHC I	AbD Serotec	JM1E3	Mouse	Pig	IgG1	1	None	1/50
IgG1 isotype control	Thermo Scientific Pierce	ZX3	Mouse	Pig	IgG1	ND	PE	1/5
IgG1 isotype control	AbD Serotec	BB238E6	Mouse	Pig	IgG1	0.1	None	1/80
IgG2b isotype control	AbD Serotec	76-2-11	Mouse	Pig	IgG2b	0.1	None	1/40
p30	IZSUM	ND	Mouse	ASFV	ND	ND	FITC	1/15
p72	Ingenasa	18BG3	Mouse	ASFV	IgG2a	1	FITC	1/25
Rat anti-mouse IgG2b PE-conjugated secondary antibody	R&D Systems	332723	Rat	Mouse	IgG1	ND	PE	1/10
Goat anti-mouse IgG Fc cross adsorbed RPE-conjugated secondary antibody	Thermo Scientific Pierce	Polyclonal	Goat	Mouse	Polyclonal	0.5	RPE	1/80

ND: not determined.

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**CHAPTER 3. RESULTS: *IN VITRO* DIFFERENTIATION OF PORCINE  
MONOCYTES INTO MACROPHAGES**

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### 3.1 Introduction

moM $\Phi$  have been employed as a model cell in numerous studies of the porcine immune system. However, the lack of a standardized method for moM $\Phi$  differentiation hampers the comparison of results coming from the use of different laboratory protocols. In the first phase of the work, we sought to determine the optimal method for porcine moM $\Phi$  generation by directly comparing the phenotype and function following differentiation induced by different protocols. The use of varying concentrations of autologous plasma (10, 20 and 30% v/v) or recombinant human macrophage-colony stimulating factor (hM-CSF; 50, 100, and 200 ng/ml) to differentiate porcine monocytes into macrophages were compared. Changes in cell morphology and surface marker expression were assessed by confocal microscopy and flow cytometry. Macrophage differentiation was evaluated by analysing TNF- $\alpha$  response to LPS stimulation and determining cytokine secretion patterns under both basal conditions and after classical and alternative activation. The effects of the differentiation methods on metabolic activity and susceptibility to infection with the myelotropic ASFV were also evaluated.

### 3.2 Phenotype characterization of moM $\Phi$

First, changes in cell morphology were evaluated by flow cytometry and confocal microscopy. As displayed in Figure 3.1, moM $\Phi$  differentiated by both autologous plasma (AP-moM $\Phi$ ) and hM-CSF (M-CSF-moM $\Phi$ ) increased significantly in dimension and granularity compared to monocytes, as respectively shown by their forward (FSC) and side angle (SSC) light scattering properties measured by flow cytometry. No significant differences were observed between moM $\Phi$  populations in

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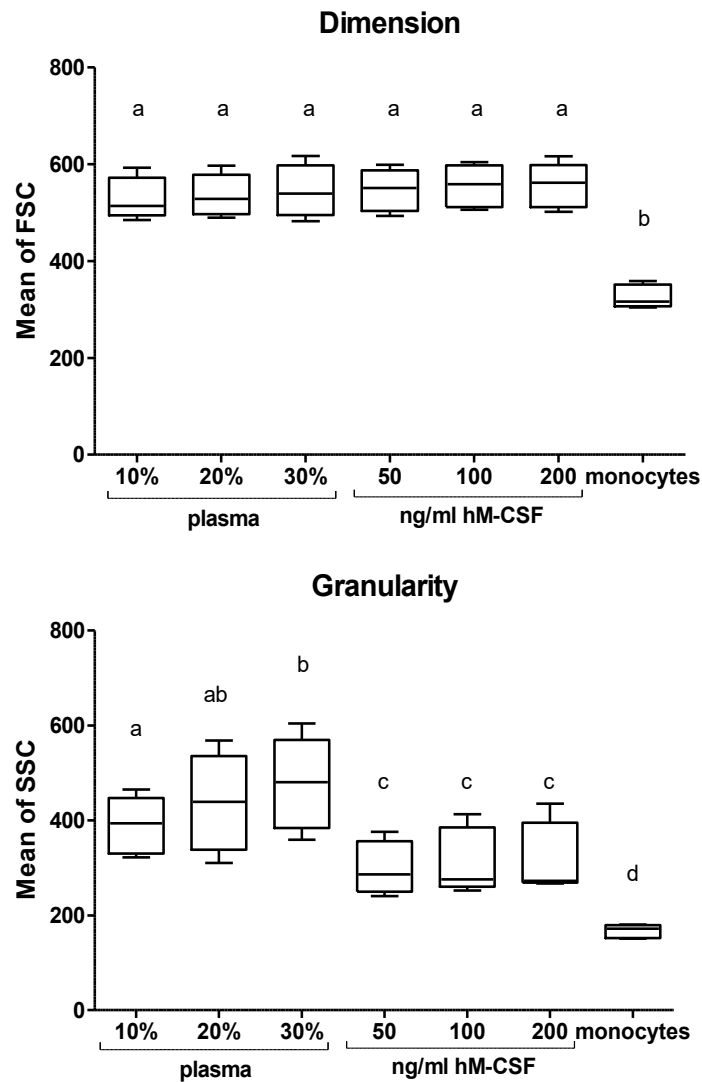
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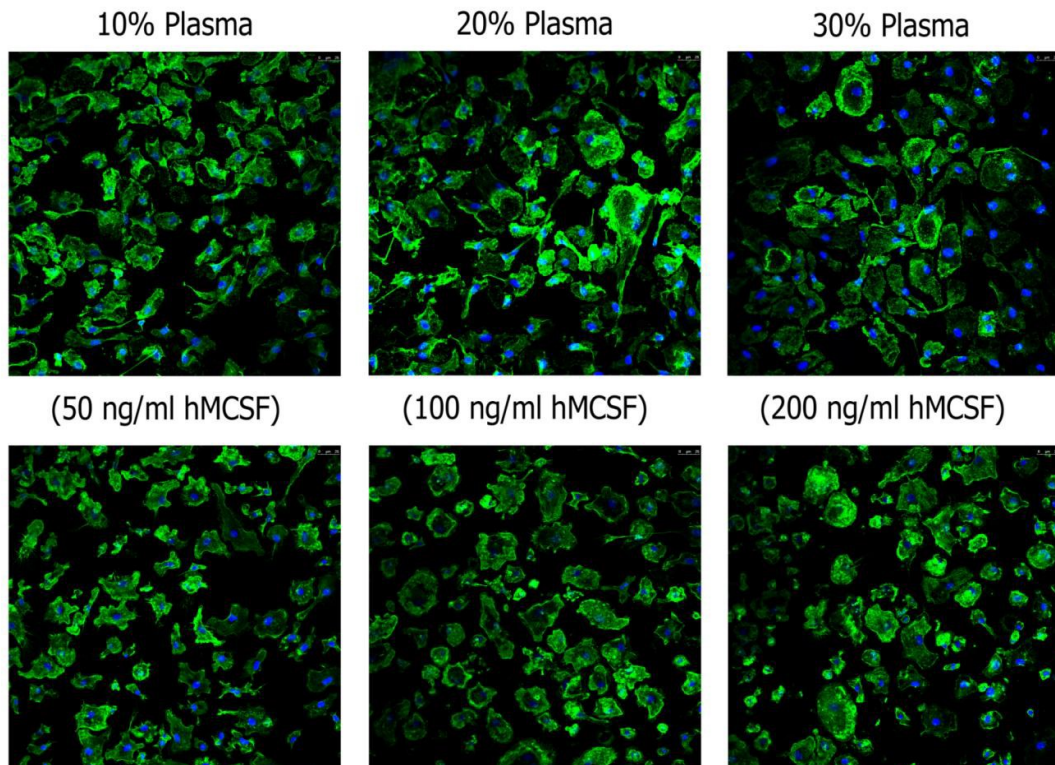
terms of FSC, instead SSC was increased in AP-moMΦ compared to M-CSF-moMΦ. Increasing porcine plasma concentrations produced a dose dependent increase in granularity as shown by the SSC values. Confocal microscopy showed that AP-moMΦ appeared more pleomorphic and presented an increased number of elongated projections protruding from cell surfaces compared to M-CSF-moMΦ, as represented in Figure 3.2.

Changes in surface marker expressions were then evaluated by flow cytometry. moMΦ differentiation by either method induced a cell surface immunophenotype significantly dissimilar from monocytes, with a significant up-regulation in expression of CD163, MHC-II DR and CD203a, as shown in Figure 3.3. CD163 was more highly expressed in M-CSF-moMΦ compared to AP-moMΦ. Percentages of MHC-II DR<sup>+</sup> and CD203a<sup>+</sup> cells were similar between protocols with the only exception of moMΦ differentiated with 30% of autologous plasma, which presented significantly higher proportions of CD203a<sup>+</sup> cells compared to M-CSF-moMΦ.

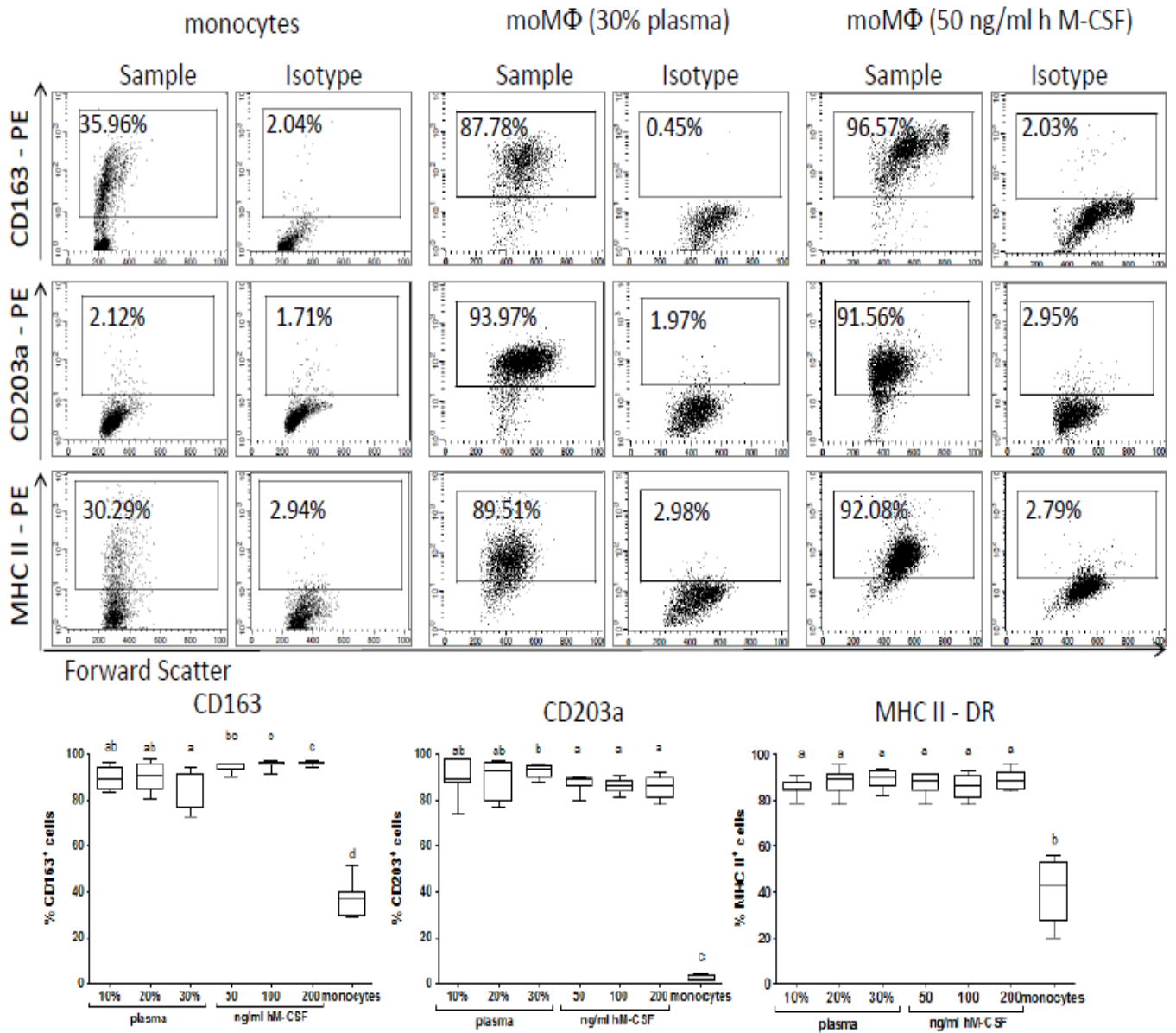




**Figure 3.1. Morphological analyses of monocytes and moMΦ populations using flow cytometry.** Blood derived monocytes were analysed immediately or differentiated into macrophages through incubation with 10, 20 or 30% (v/v) of porcine plasma, or using 50, 100 or 200 ng/ml of hM-CSF for 5 days at 37°C in 5% CO<sub>2</sub>. Differences between populations in terms of dimension (FSC, forward scatter) and complexity (SSC, side scatter) were evaluated using flow cytometry. Boxplots indicate the median (middle line), 25<sup>th</sup> and 75<sup>th</sup> percentiles (boxes), maximum and minimum (whiskers) of FSC/SSC of three independent experiments performed in triplicate. Values for each marker were compared using an ANOVA followed by a Kruskal-Wallis test. Different letters indicate significant differences between moMΦ populations ( $p < 0.05$ ).



**Figure 3.2. Morphological analyses of moM $\Phi$  populations using confocal microscopy.** Blood derived monocytes were differentiated into macrophages through incubation with 10, 20 or 30% (v/v) of porcine plasma, or using 50, 100 or 200 ng/ml of hM-CSF for 5 days at 37°C in 5% CO<sub>2</sub>. Confocal microscopy observations after staining nuclei in blue with Hoechst 33342 and cytoskeleton in green with Alexa Fluor 488-conjugated Phalloidin. Magnifications are 40X.



**Figure 3.3. Expression of cell surface markers by monocytes and moMΦ populations.** Monocytes and moMΦ populations were analysed for surface markers expression and differences between monocytes and moMΦ populations in terms of CD163, MHC II-DR and CD203a are displayed. Representative dot plots of monocytes and moMΦ differentiated using 30% of plasma or 50 ng/ml of h M-CSF are shown. Boxplots indicate the median (middle line), 25<sup>th</sup> and 75<sup>th</sup> percentiles (boxes), maximum and minimum (whiskers) of positive cells of three independent experiments performed in triplicate. Values of MHC II-DR, CD163 and CD203a expression were compared using a Mann-Whitney Test. Different letters indicate significant differences between moMΦ populations ( $p < 0.05$ ).

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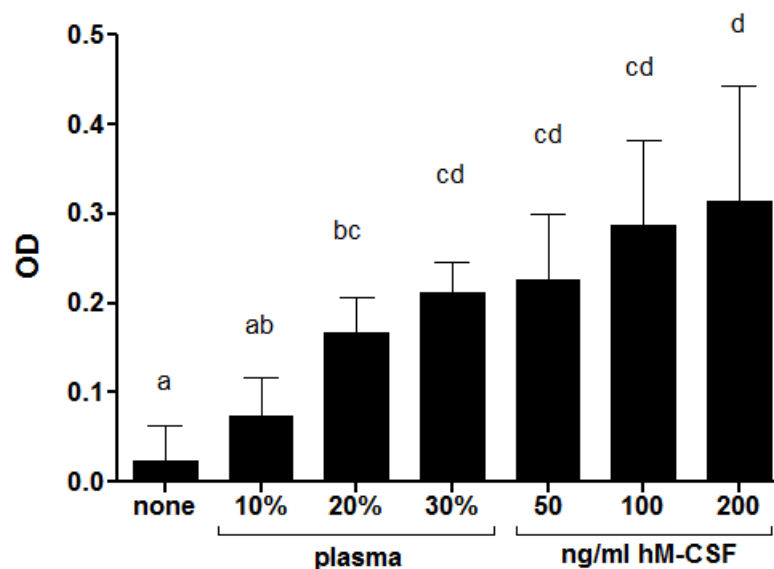
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### 3.3 Assessment of metabolically active cells

The metabolic activity of different moMΦ populations was evaluated using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay. As displayed in Fig 3.4, moMΦ differentiated using hM-CSF as well as 20% and 30% of autologous porcine plasma showed enhanced metabolic activity compared to monocytes cultured using monocyte medium (RMPI + antibiotics + 10% FBS) alone. M-CSF-moMΦ, and the 200 ng/ml cultures in particular, presented increased activity compared to AP-moMΦ. Significant differences in the metabolic activity were also found between macrophages produced with 10% compared to 30% of autologous plasma.



**Figure 3.4 Assessment of the metabolic activity of moMΦ populations.** Monocytes were seeded into a 96 well plate and differentiated into moMΦ through incubation with un-supplemented monocyte medium (RMPI + antibiotics + 10% FBS; 'none') or in media supplemented with 10-20-30% (v/v) porcine plasma or monocyte medium with 50-100-200 ng/ml of hM-CSF for 3 days at 37°C in 5% CO<sub>2</sub>. Metabolic activity was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay. Background absorbance was subtracted and graph represents the mean data +/- SD from three independent experiments utilizing different animals. Values were compared using ANOVA followed by a Kruskal-Wallis test. Different letters indicate significant differences between moMΦ populations (p<0.05).

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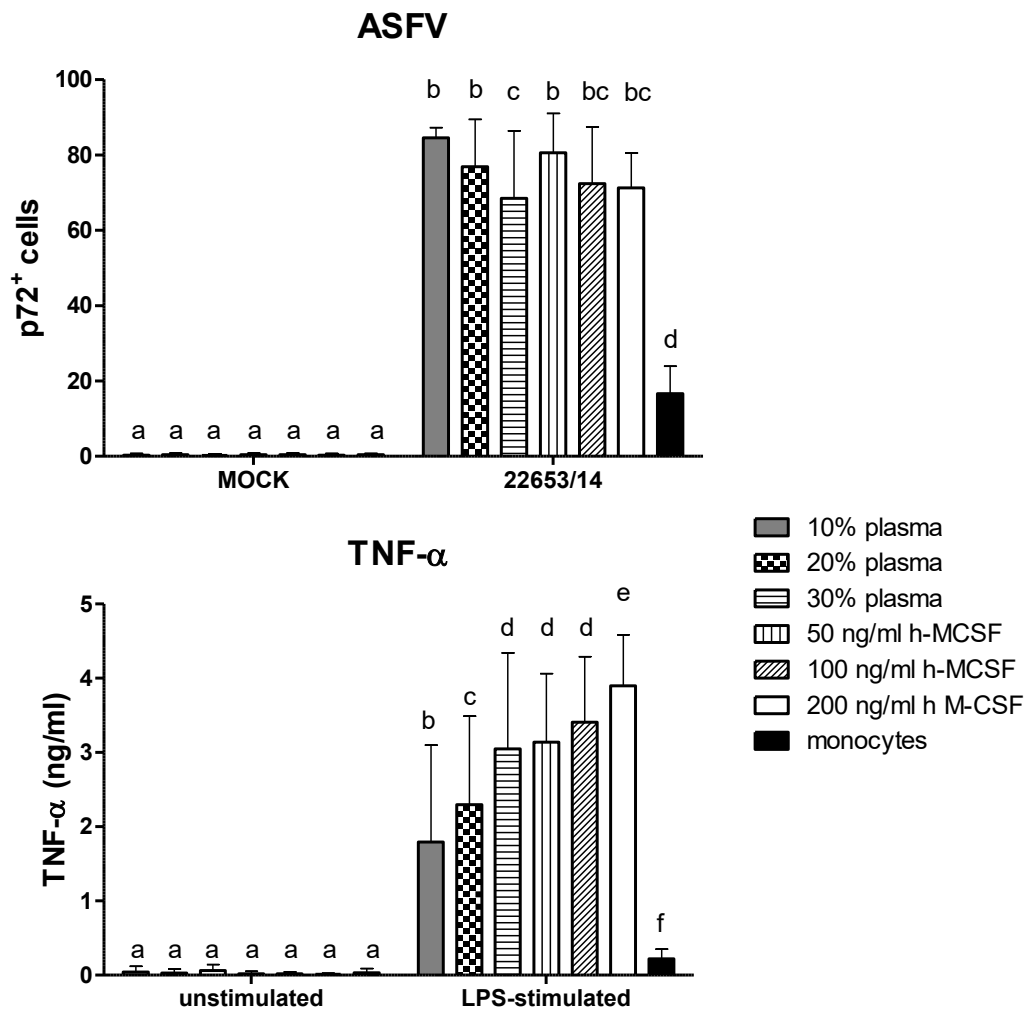
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### **3.4 Susceptibility to ASFV**

The susceptibility of moMΦ differentiated with the different techniques to ASFV infection was also analysed. The intracytoplasmic expression of late ASFV p72 protein was measured to evaluate if the differentiation method could influence viral tropism. Our results showed that all moMΦ populations expressed p72 at higher levels compared to monocytes, with minimal differences being observed between moMΦ differentiated using the different culture conditions (Figure 3.5). Using 30% of autologous plasma a slightly lower susceptibility to ASFV infection was observed (Figure 3.5).

### **3.5 LPS-stimulated release of TNF- $\alpha$**

The ability to release TNF- $\alpha$  in response to LPS stimulation was also determined. As displayed in Figure 3.5, all the six moMΦ populations displayed increased TNF- $\alpha$  production compared to monocytes. MoMΦ differentiated using 200 ng/mL M-CSF secreted the greatest amount of TNF- $\alpha$ . No significant differences between those cultured in 50, 100 ng/mL M-CSF and 30% of porcine plasma were found. Macrophages differentiated in 10% of porcine plasma produced the lowest amount of TNF- $\alpha$  (Figure 3.5).



**Figure 3.5. Susceptibility to ASFV infection and TNF- $\alpha$  release in response to LPS stimulation by monocytes and moM $\Phi$ .** On the top, susceptibility to ASFV is displayed. Monocytes and moM $\Phi$  populations were mock-infected or infected with the virulent ASFV isolate 22653/14 using a MOI of 1. 24 hours pi cells were harvested and infection was evaluated by intracytoplasmic detection of the viral p72 in flow cytometry. The mean data  $\pm$  SD from three independent experiments utilizing different animals are shown. Below, TNF- $\alpha$  release in response to LPS stimulation is displayed. Monocytes and moM $\Phi$  differentiated with different methods were left untreated or stimulated with 100 ng/ml of LPS for 6 hours. Supernatants were collected and stored at  $-80^{\circ}\text{C}$  till analysis. The mean data  $\pm$  SD from five independent experiments utilizing different animals are shown. TNF- $\alpha$  amounts in culture supernatants were evaluated using an ELISA assay. For both panels, differences between groups were compared using an ANOVA followed by a Kruskal-Wallis test. Different letters indicate significant differences between moM $\Phi$  populations ( $p < 0.05$ ).

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### 3.6 Patterns of cytokine secretion

The basal release of a panel of cytokines from moMΦ differentiated under the different culture conditions was assessed using a multiplex immunoassay. No differences in the levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$  were observed between macrophage populations (data not shown). Instead statistically significant differences were detected in the levels of IL-10, IL-12, IL-1RA (Figure 3.6). MoMΦ differentiated using 100 and 200 ng/mL hM-CSF released a statistically significantly higher amount of IL-10 compared to AP-moMΦ. Instead the supernatants of AP-moMΦ contained statistically significant higher amounts of IL-12, but this was also present in the background control (RPMI-1640 supplemented with 30% of porcine plasma), suggesting that the IL-12 was derived from the autologous plasma as opposed to being released by the AP-moMΦ. Higher levels of IL-1RA were detected in the supernatant of AP-moMΦ compared to M-CSF-moMΦ or the background control.

Finally, differences in the ability of moMΦ produced under selected differentiation culture conditions (30% of plasma and 50 ng/ml of hM-CSF) to respond to classical and alternative activation were assessed. Differentiating cells under these conditions resulted in the same levels of metabolic activity (Figure 3.4) and so ensured that the number of live cells in culture would not influence the results. Culture media was discarded before macrophage stimulation. As represented in Figure 3.7, using both protocols, classical activation resulted in release of IL-12 and pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ . Higher levels of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$  were observed in moM1 derived from 50 ng/ml M-CSF-moMΦ compared to 30% AP-moMΦ, instead there were no differences in the other cytokines tested. Almost

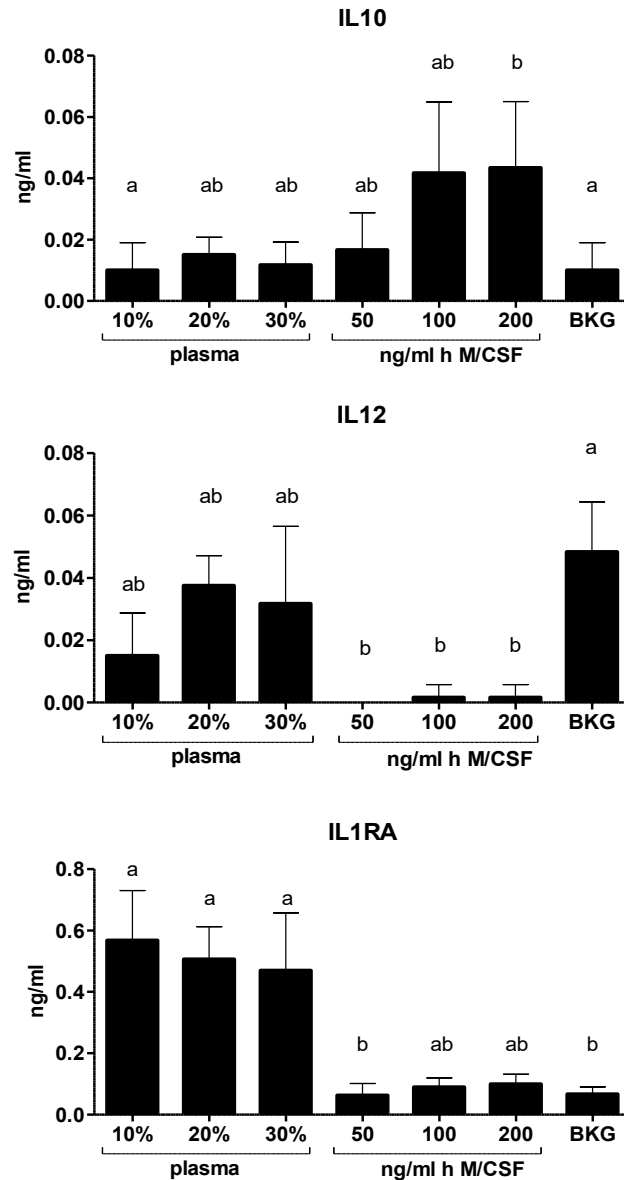
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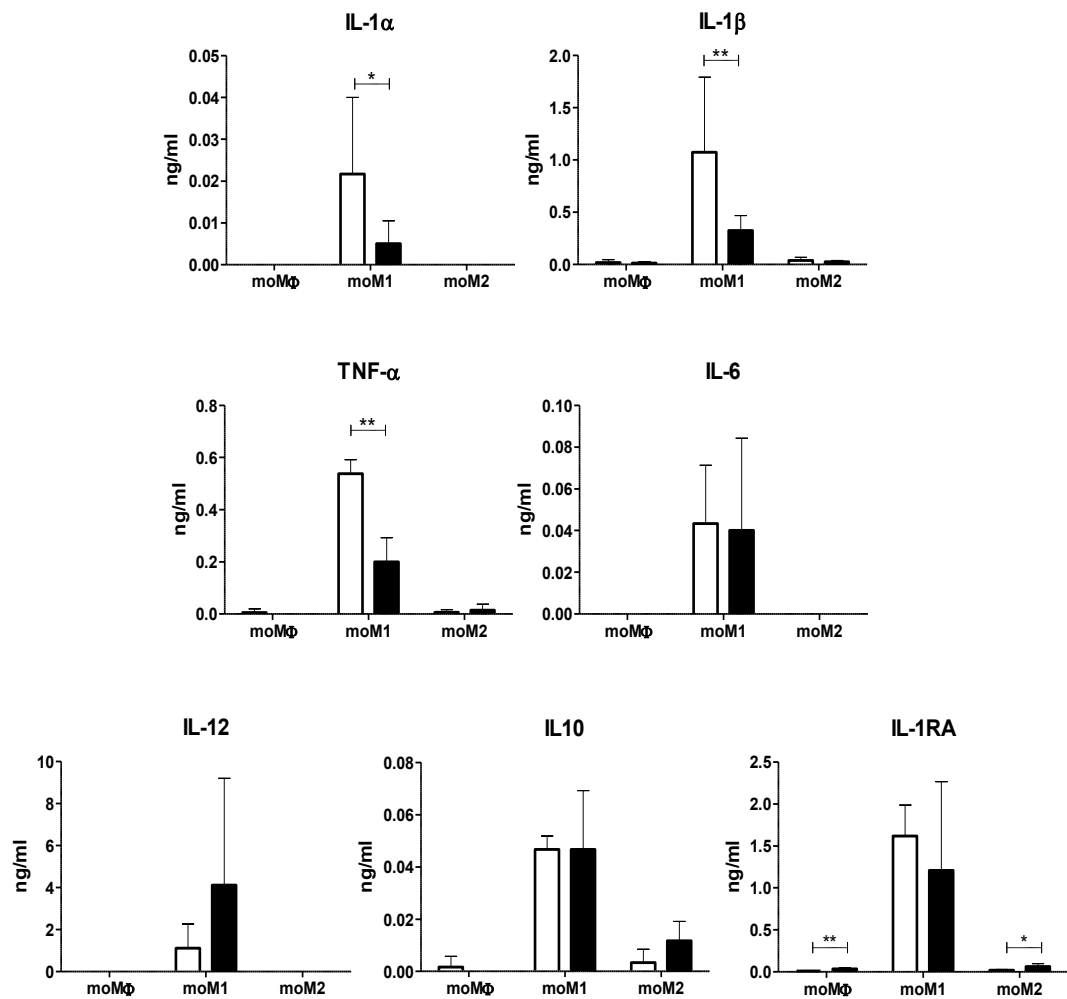
undetectable levels of cytokines were observed after alternative activation of either moM $\Phi$  population.



**Figure 3.6. Basal cytokine release from moM $\Phi$  differentiated in different culture conditions.** Blood derived monocytes were differentiated into moM $\Phi$  through incubation with 10, 20 or 30% (v/v) of porcine plasma, or using 50, 100 or 200 ng/ml of hM-CSF for 5 days at 37°C in 5% CO<sub>2</sub>. IL-1 $\alpha$ , IL-1 $\beta$ , IL-RA, IL-6, IL-10, IL-12 and TNF- $\alpha$  in culture supernatants and background control (BKG) were evaluated using a Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine, according to manufacturer's protocol. The mean data  $\pm$  SD from three independent experiments utilizing different animals are shown. Values for each cytokine were



compared using an ANOVA followed by a Kruskal-Wallis test. The different letters indicate significant differences between moMΦ populations ( $p < 0.05$ ).



**Figure 3.7. Cytokines release by classically and alternatively activated moMΦ.** MoMΦ differentiated through incubation with 30% (v/v) of porcine plasma (white bar) and using 50 ng/ml of hM-CSF (black bar) were both classically (moM1) and alternatively (moM2) activated. For classical activation macrophages were stimulated with 100 ng/ml of recombinant porcine IFN- $\gamma$  and 100 ng/ml of LPS while for alternative activation with 20 ng/ml of recombinant porcine IL-4 for 24 hours at 37°C in 5% CO<sub>2</sub>. IL-1 $\alpha$ , IL-1 $\beta$ , IL-RA, IL-6, IL-10, IL-12 and TNF- $\alpha$  were detected in culture supernatants using a Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine, according to manufacturer's protocol. The mean data +/- SD from three independent experiments utilizing different animals are shown. Values for each cytokine were compared using a Mann-Whitney Test. Different letters indicate significant differences between macrophage populations ( $p < 0.05$ ).

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**CHAPTER 4. RESULTS: CHARACTERIZATION OF THE INTERACTION  
OF ASFV WITH MONOCYTES AND MACROPHAGE SUBSETS**

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## 4.1 Introduction

ASFV mainly targets cells of the myeloid lineage, especially monocytes and macrophages, thought to be crucial for viral persistence and dissemination (Sierra *et al.*, 1991, Sánchez-Cordón *et al.*, 2008). Infection with virulent ASFV isolates evolves towards cell lysis at very late time of infection (Breese and DeBoer, 1966), since they express proteins (such as A224L, A179L, EP153R) that prevent apoptosis, allowing infected cells to survive and disseminate the virus through the body (Nogal *et al.*, 2001, Galindo *et al.*, 2008, Hurtado *et al.*, 2004). On the contrary, the avirulent tissue-culture adapted BA71V strain is able to infect macrophages and to synthesise viral late proteins, but induces early cell death, preventing the production of infectious viral progeny (Zsak *et al.*, 2001).

To date few studies analysed the effects of ASFV on monocytes and macrophages in terms of their expression of surface markers (Sánchez-Torres *et al.*, 2003, Lithgow *et al.*, 2014) or cytokine and chemokine responses (Gómez del Moral *et al.*, 1999, Gil *et al.*, 2008, Gil *et al.*, 2003, Zhang *et al.*, 2006, Fishbourne *et al.*, 2013) and none have compared responses of activated macrophage subsets. Considering the central role of M $\Phi$  for ASFV pathogenesis and the effects of classical and alternative activation on M $\Phi$ , it was tested if cells in distinct activation statues reacted differently to ASFV, with differences between isolates varying in virulence. Virulent isolates might have developed mechanisms to affect activated macrophages responses, to promote their survival and dissemination in the host. To address these questions, in the second part of the work a detailed *in vitro* analysis of the interaction of monocytes and derived macrophage subsets with a virulent (22653/14) and an avirulent (BA71V) ASFV strains was conducted.

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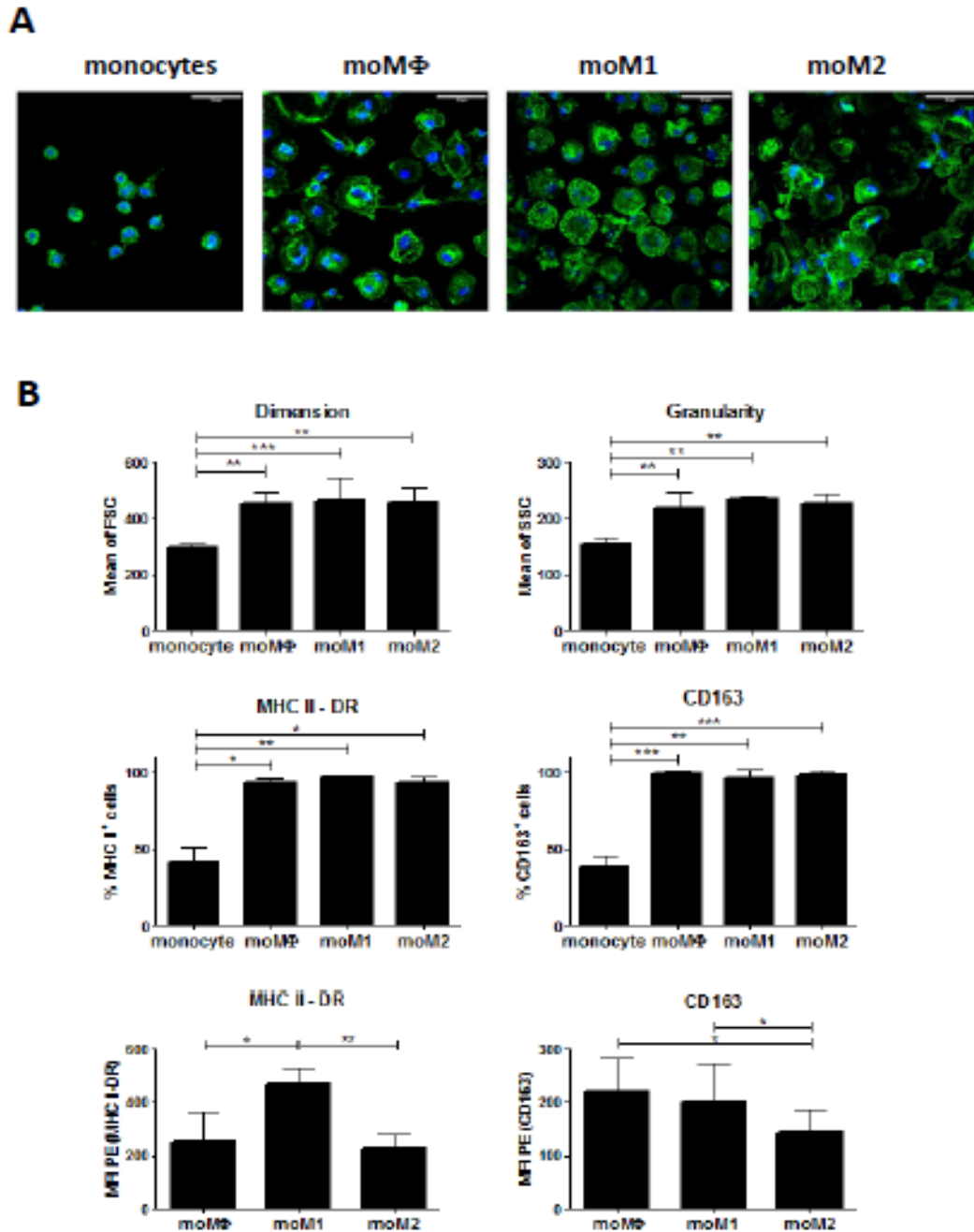
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## 4.2 Generation of monocyte-derived macrophage subsets

Considering the results generated in the first phase of the work, porcine moMΦ were generated by culturing porcine monocytes in media supplemented with 50 ng/ml of hM-CSF for 5 days. These cells were activated by the classical or alternative method for a further 24 hours. Monocytes differentiation into macrophages and their activation was assessed by confocal microscopy and flow cytometry, as displayed in figure 4.1 and 4.2. All monocyte-derived macrophage subsets were larger than their monocyte precursors and presented with a spherical shape with short hairy protrusions on their surface (Figure 4.1A). In addition, they presented higher dimension (forward scatter; FSC) and granularity (side scatter; SSC) as well as expressed higher levels of CD163, and MHC II-DR than monocytes (Figure 4.1B and 4.2). No differences in terms of dimension (FSC) and granularity (SSC) were observed between macrophage subsets, whereas these populations differed in terms of surface markers expression. In accordance with previous studies (Garcia-Nicolas *et al.*, 2014), classical activation induced increase in the MHC II-DR expression, and IL-4 pre-treatment resulted in a little reduction of the MFI of CD163 (Figure 4.1B).



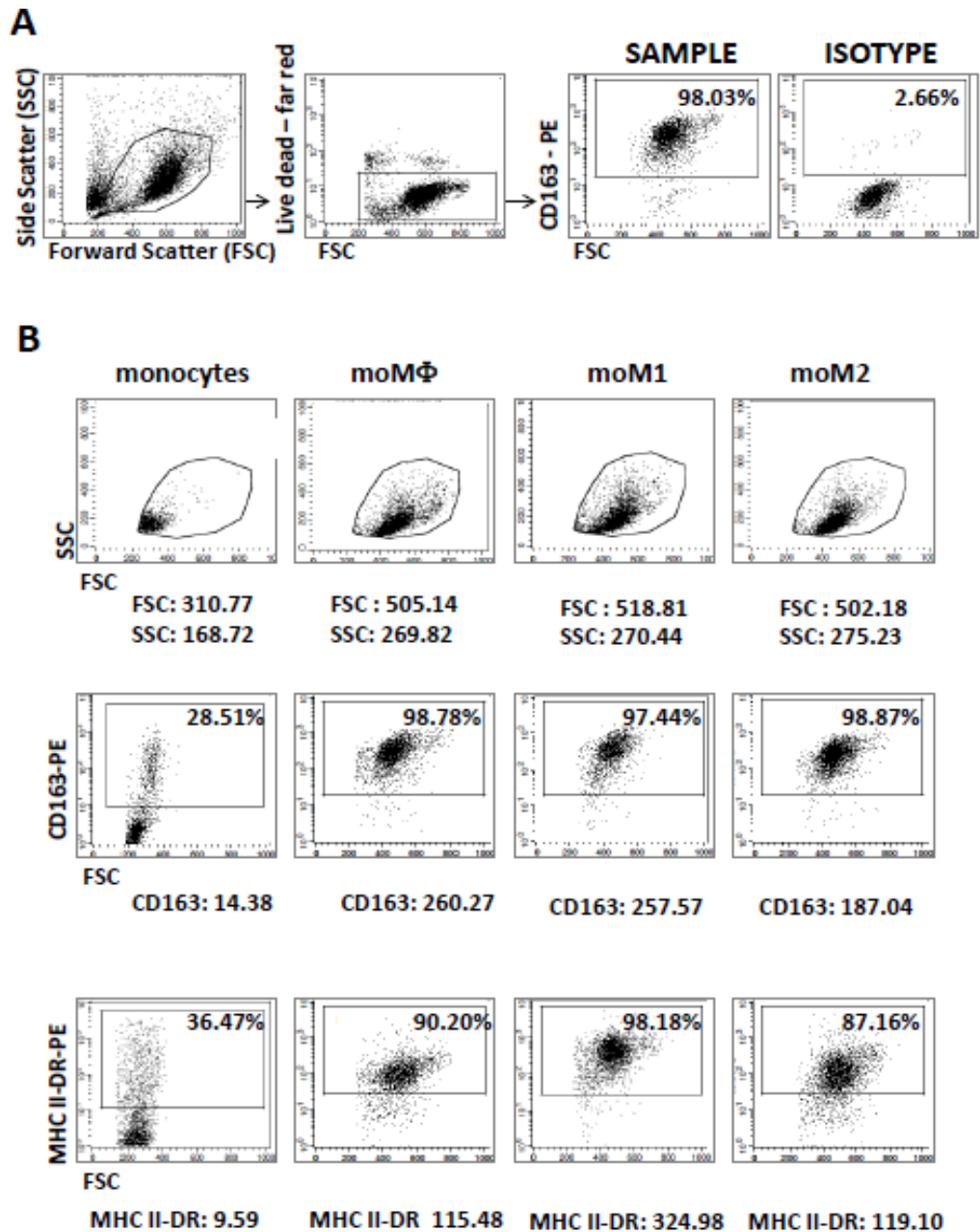
**Figure 4.1. Differentiation of monocytes into macrophage subsets.** Blood derived monocytes were analysed directly or differentiated into macrophages using hM-CSF. Un-activated macrophages (moMΦ) were left un-treated or activated for 24 hours in a classical (IFN- $\gamma$  and LPS, moM1) or alternative (IL-4, moM2) way. Differentiation of monocytes into macrophages and their activation was morphologically assessed by confocal microscopy (original magnification 40X) (A). Differences between monocytes and derived macrophage subsets in terms of FSC (forward scatter), SSC (side scatter) and expression of CD163 and MHC II-DR were assessed by flow cytometry (B). The mean data  $\pm$  SD from three independent experiments utilizing different animals are shown. Values were compared using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\* $p$ <0.001, \*\* $p$ <0.01, \* $p$ <0.05.

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**Figure 4.2 Representative dot plots of monocytes and derived macrophage subsets.** Differences between monocytes and monocyte-derived macrophage subsets in terms of FSC (forward scatter), SSC (side scatter) and expression of CD163 and MHC II-DR were assessed by flow cytometry. In panel A gating strategy used to investigate surface marker expression on monocytes/macrophages are displayed: cells with FSC and SSC typical for monocytes/macrophages and viable (Live/Dead Fixable Dead Cell Stain<sup>+</sup>) were analysed for surface expression of surface markers. Gating were set by comparing staining with an isotype control mAb. In panel B representative dot plots of monocytes and macrophage subsets are displayed. Below each plot, MFI values for SSC/FSC or surface markers are reported, while inside each plot % of CD163<sup>+</sup> or MHC II-DR<sup>+</sup> cells are indicated.

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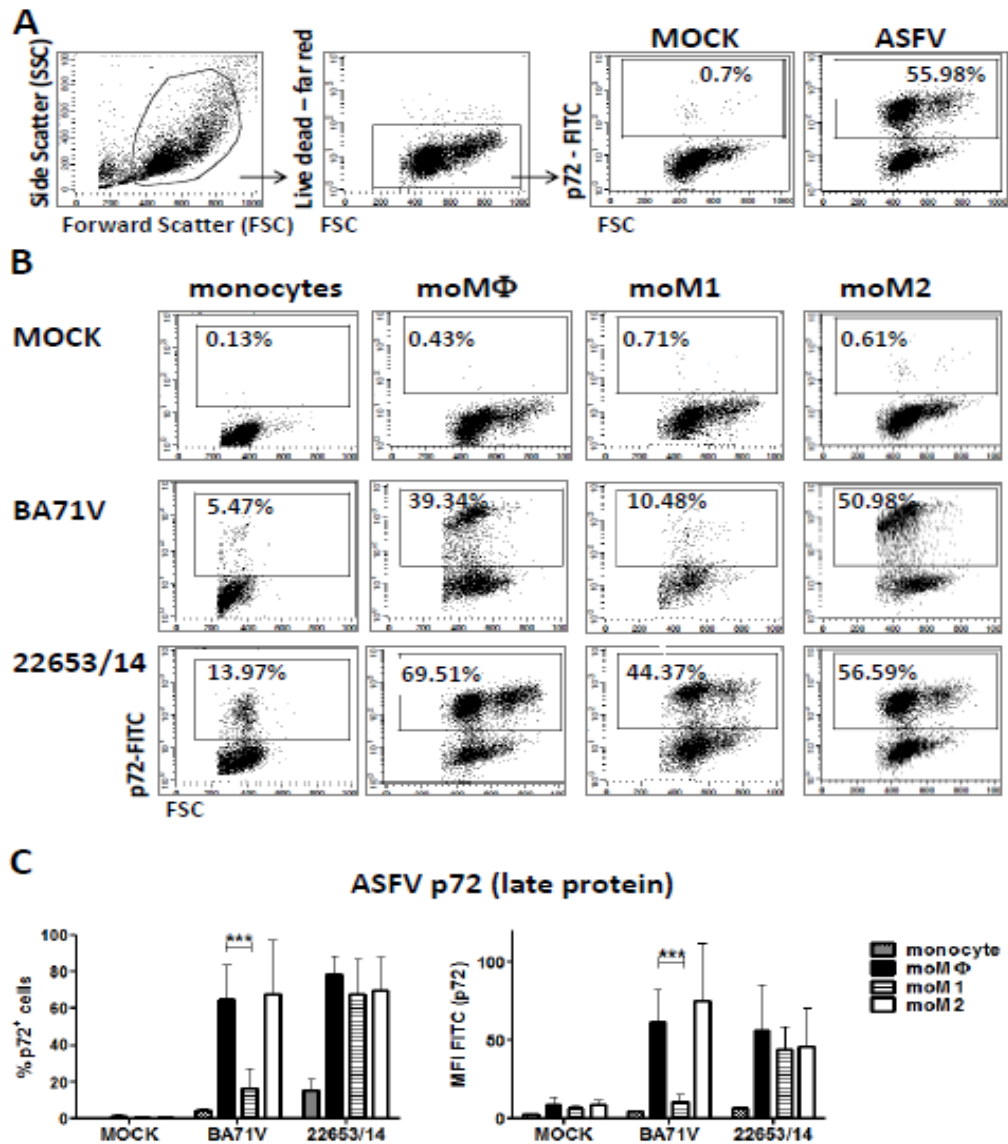
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### **4.3 Monocytes and macrophage subsets susceptibility to ASFV infection**

Susceptibility of monocytes and macrophage subsets to ASFV infection was assessed by quantification of the intracellular levels of the late protein p72 by flow cytometry, using the gating strategy displayed in Figure 4.3A. Cells were mock-infected or infected with the tissue-culture adapted BA71V or the virulent Sardinian isolate 22653/14, using a MOI of 1. As displayed in Figure 4.3, for both isolates, macrophages were more susceptible to ASFV infection than freshly isolated monocytes. Classical activation resulted in a higher resistance to ASFV infection, with differences between isolates varying in virulence, whereas for both BA71V and 22653/14 similar levels of p72 proteins were observed between moM $\Phi$  and moM2. 22653/14 presented greater ability to infect monocytes and moM1 than the avirulent strain, in fact only 5-10% of p72<sup>+</sup> monocytes and moM1 were detected after BA71V infection (Figure 4.3). Using a MOI of 1, after 22653/14 infection, statistically significant differences between moM $\Phi$  and moM1 were observed only in some of the pig analysed (data not shown). In order to check if the differences observed between 22653/14 and BA71V in monocytes and moM1 were due to inhibition to viral protein synthesis, levels of the early protein p30 in monocytes and macrophage subsets were evaluated (Figure 4.4). Despite differences observed between BA71V and 22653/14 infected monocytes in terms of p72, the same levels of p30<sup>+</sup> cells were detected in monocytes infected with these strains. Whereas, as displayed in Figure 4.4, 22653/14 infection resulted in higher levels of p30<sup>+</sup> moM1 than BA71V.



**Figure 4.3. Susceptibility to infection by monocytes and derived macrophage subsets.** Monocytes and macrophage subsets were infected with the avirulent BA71V or virulent 22653/14 ASFV strain using a MOI of 1, alongside mock-infected control. 18 hours post-infection cells were harvested and % of p72<sup>+</sup> cells and MFI of p72 were evaluated using flow cytometry. In Panel A gating strategy used to investigate ASFV infection on live monocytes/macrophages is displayed: cells with FSC and SSC typical for monocytes/macrophages and viable (Live/Dead Fixable Dead Cell Stain<sup>™</sup>) were analysed for their intra-cytoplasmic levels of late viral ASFV protein p72; gating were set by comparing staining with mock-infected controls. In Panel B representative dot plots of BA71V-infected or 22653/14-infected monocytes, moMΦ, moM1, moM2 are displayed. In Panel C the mean data +/- SD from five independent experiments utilizing different animals are shown. For each virus-stimulated conditions values of activated macrophages (moM1 and moM2) were compared to the corresponding un-activated control (moMΦ), using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\*p<0.001, \*\*p<0.01, \*p<0.05.

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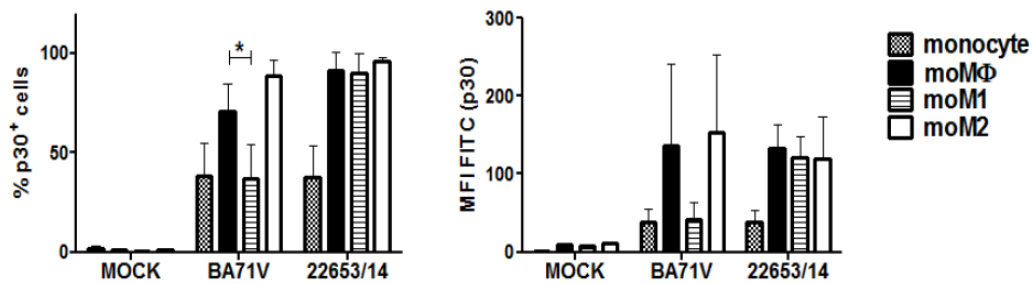
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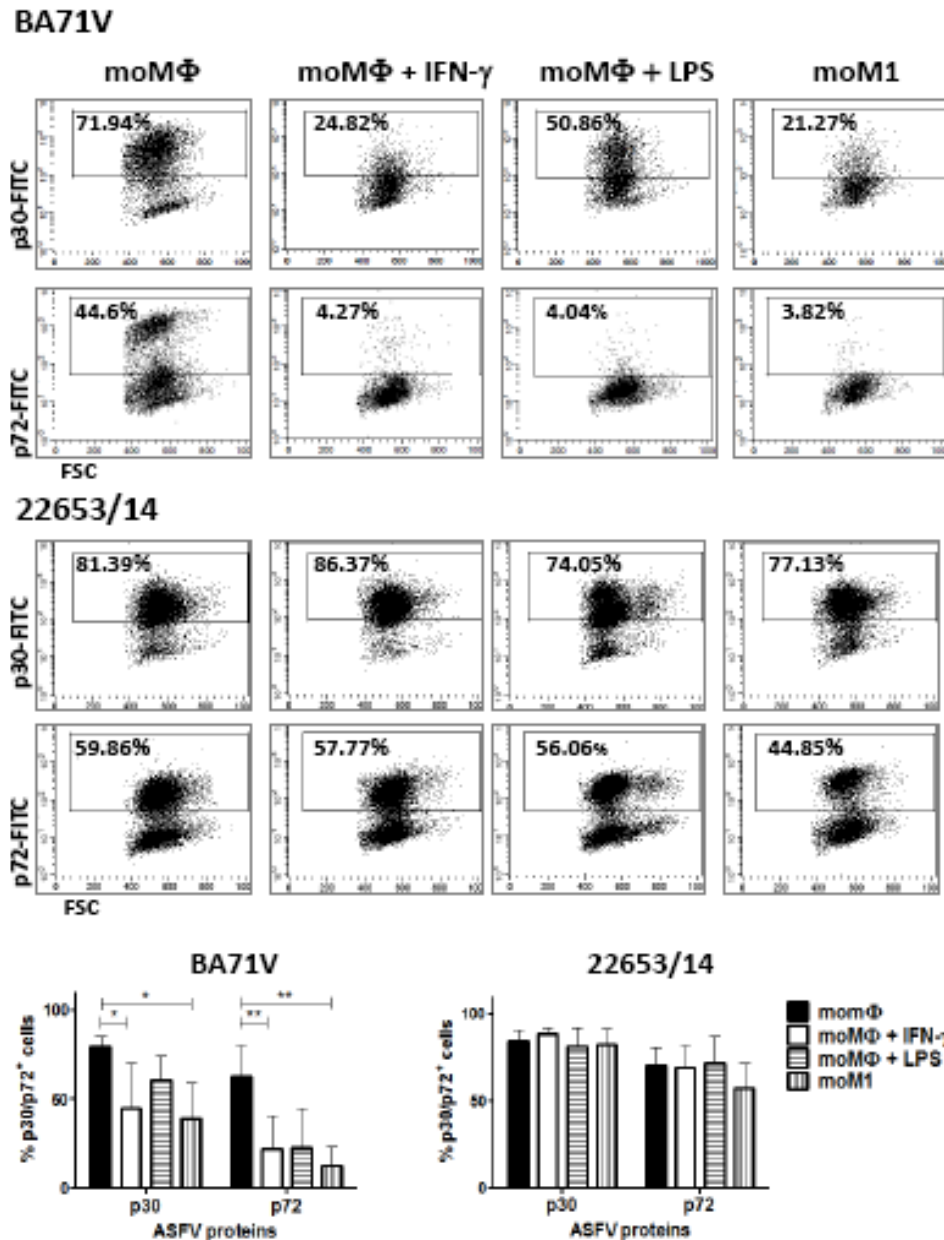


### ASFV p30 (early protein)



**Figure 4.4 Expression of the early protein p30 in ASFV-infected monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the avirulent BA71V strain or with the virulent 22653/14 field strain using a MOI of 1. Mock-infected control cells were included for each cell type. 18 hours pi cells were harvested and percentages of p30<sup>+</sup> cells and MFI of p30 were evaluated using flow cytometry. The mean data +/- SD from three independent experiments utilizing different animals are shown. Values of activated macrophages (moM1 and moM2) were compared to the corresponding un-activated control (moMΦ), using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\*p<0.001, \*\*p<0.01, \*p<0.05. Differences in the p30 expression between BA71V and 22653/14 were compared using a Mann-Whitney test.

Then it was assessed if pre-treatment of macrophages with IFN-γ or LPS alone was able to reduce susceptibility to ASFV. Activation with these cytokine alone decreased BA71V but not 22653/14 infection (Figure 4.5). In addition, pre-treatment of macrophages with M1-polarizing factors resulted in a lower expression of the early ASFV protein p30 in BA71V but not 22653/14 infected cells (Figure 4.5).



**Figure 4.5 Different expression of early and late proteins in macrophages activated with IFN-g +/- LPS.** Un-activated macrophages (moMΦ) were left untreated or activated with IFN-γ or/and LPS for 24 hours and then mock-infected or infected with the avirulent BA71V or the virulent field isolate 22653/14. 18 hours post-infection cells were harvested and expression of p30 and p72 were assessed by flow cytometry. The mean data +/- SD from three independent experiments utilizing different animals are shown. Representative dot plots of ASFV-infected un-activated and activated macrophages are displayed and below the effect of macrophage activation with IFN-γ +/- LPS on expression of p30 and p72 ASFV proteins is reported. Values of activated macrophages were compared to the corresponding un-activated control (moMΦ), using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\*p<0.001, \*\*p<0.01, \*p<0.05.

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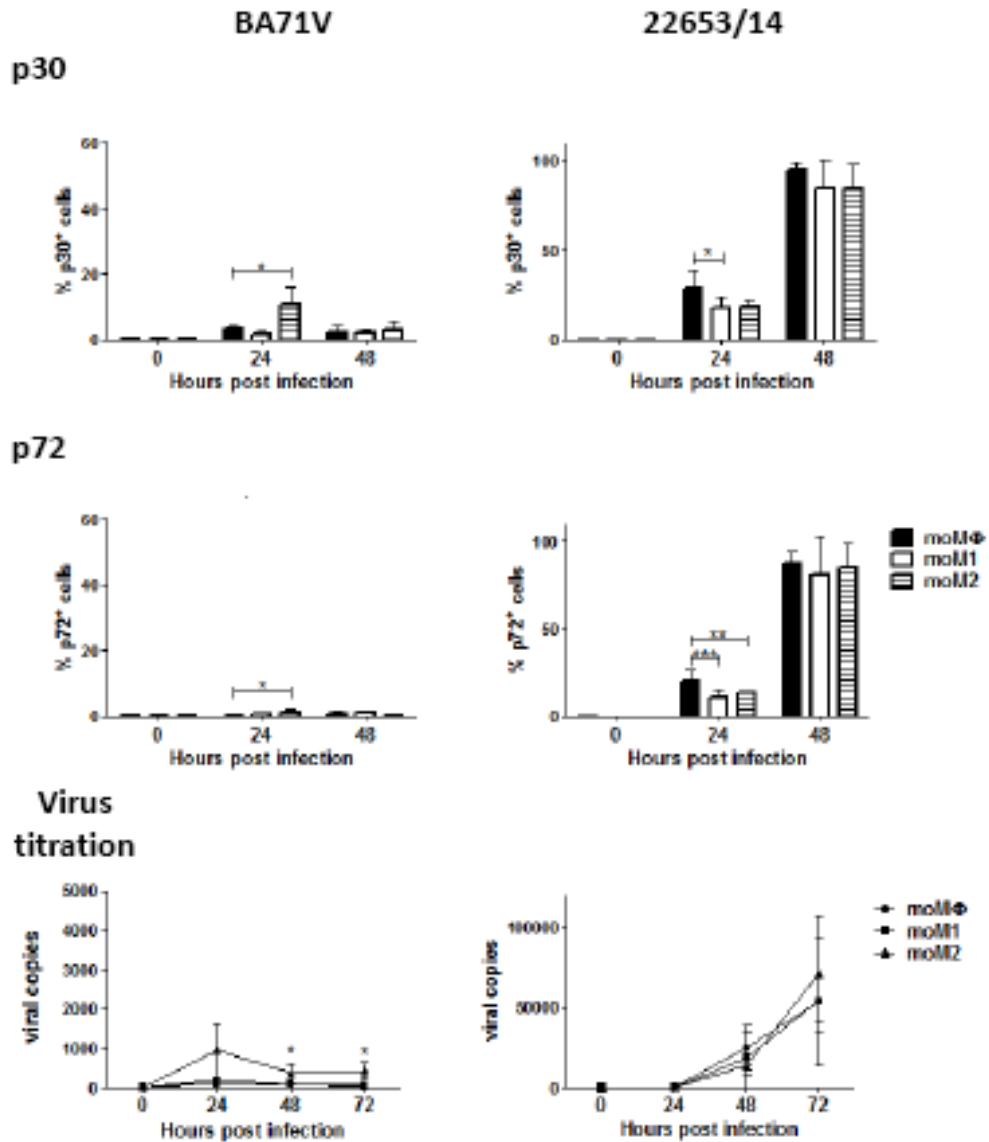
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#### 4.4 ASFV growth in macrophage subsets

A kinetic analysis of BA71V and 22653/14 replication in moMΦ, moM1 and moM2 was conducted by assessing the intracellular levels of p30 and p72 with flow cytometry and the viral copies number in cells supernatants with RT-PCR. As displayed in Figure 4.6, using a MOI of 0.01, 24 hours pi with the virulent 22653/14 both moM1 and moM2 displayed lower levels of ASFV proteins than moMΦ, but at 48 hours pi almost all (around 90%) of the live un-activated and activated macrophages were p30<sup>+</sup> or p72<sup>+</sup>. 72 hours pi it was not possible to acquire 5000 live macrophages in flow cytometry, since the majority of infected macrophages were dead. Using a MOI of 0.01, very low levels of ASFV proteins were detected after infection with the attenuated BA71V strain, even 48 hours pi. 24 hours pi, moM2 displayed little but statistically significantly higher levels of both p30 and p72 proteins than other macrophage subsets, not observed at the later time point. At each time points (0, 24, 48, 72 hours pi), the viral copies numbers in culture supernatants were evaluated. As displayed in Figure 4.6, using a MOI of 0.01 even 72 hours pi with the avirulent BA71V a very low number of viral copies were detected in culture supernatants. Nevertheless, a statistically significant higher number of viral copies was observed in moM2 culture supernatants at 48 and 72 hours pi. On the contrary, using a MOI of 0.01 the virulent 22653/14 isolate was able to actively replicate in all the macrophage subsets, with high levels of viral copies detected 72 hours pi. Despite differences in the percentages of ASFV viral proteins detected 24 hours pi using flow cytometry, no statistically significant differences between macrophage populations in

terms of viral proteins in culture supernatants were observed at any time pi (Figure 4.6).



**Figure 4.6 Growth characteristic of the ASFV strains on different macrophage subsets.** Un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the avirulent BA71V or the virulent 22653/14 ASFV strains, using a MOI of 0.01. After 1.5 hour of incubation cells were washed twice and cultured in fresh monocyte medium. At 0, 24, 48, 72 hours pi triplicate samples were collected: intracytoplasmic levels of p30 and p72 were assessed by flow cytometry. In parallel, at each time points the viral levels in culture supernatants were assessed by qPCR. The mean data +/- SD from three independent experiments utilizing different animals are shown. At each time-point values for moM1 and moM2 were compared to the corresponding un-activated control

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(moMΦ), using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

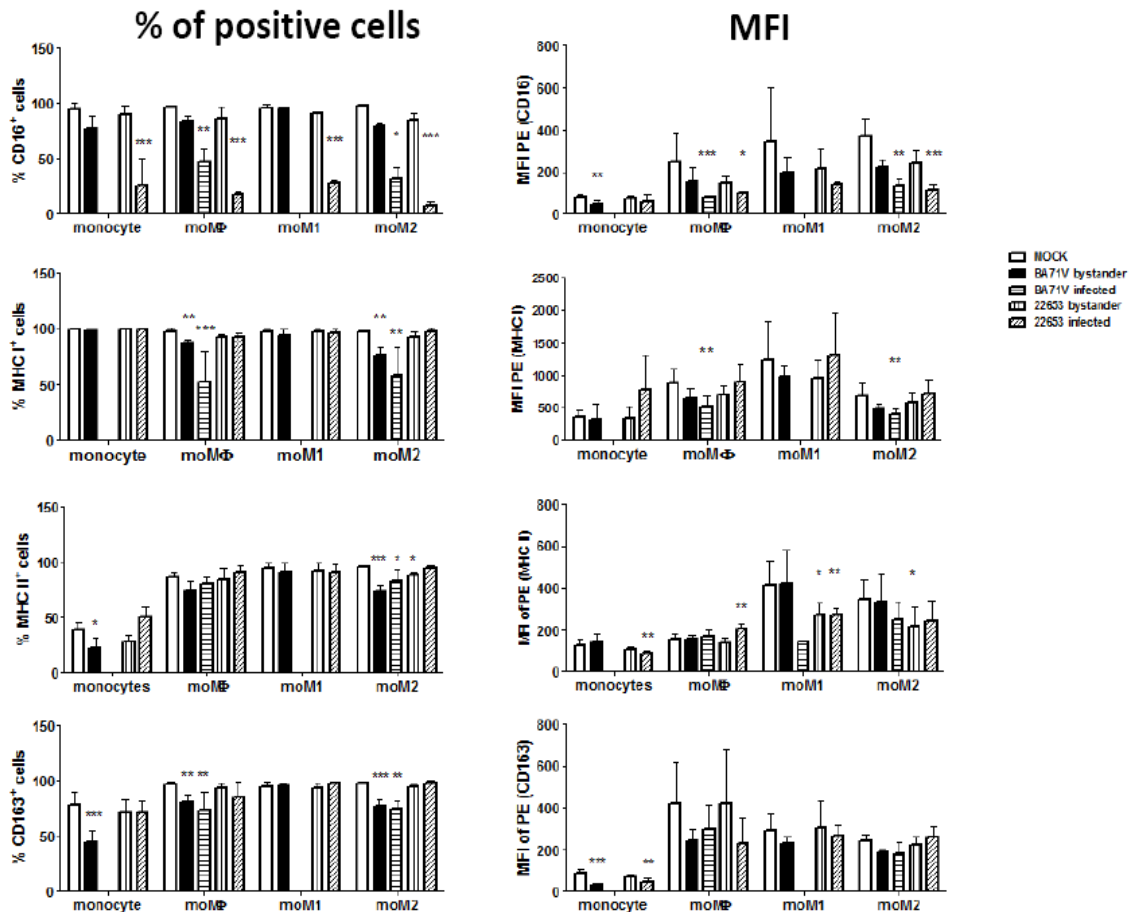
#### **4.5 Effect of ASFV infection on surface marker expression on monocytes and derived macrophage subsets**

The effect of ASFV infection on expression of CD16, MHC I, MHC II, CD163 was assessed using flow cytometry, analysing differences between BA71V and 22653/14 strains and comparing infected cells with both uninfected bystander and mock-infected cells (Figure 4.7). Because the levels of p72<sup>+</sup> monocytes and moM1 after BA71V infection using a MOI of 1 were less than 10% (Figure 4.3), it was unreliable to compare infected and bystander cells for these subsets. First it was assessed the effect of mock-infection with a Vero cell lysate and a monocyte-macrophage cell lysate for 18 hours on the expression of CD16, MHC II, MHC I and CD163. No differences in marker expression levels between the ‘mock Vero’ or ‘mock monocyte-macrophage’ infection and cells cultured in monocyte medium (data not shown) were observed, and consequently mock-infection controls were performed using monocyte medium. As displayed in Figure 4.7 and 4.8, ASFV infection down-regulated the expression of CD16 in porcine monocytes and macrophage subsets. Statistically significant differences in the % of positive cells were observed between infected and both uninfected bystander and mock-infected cells. Infected cells showed a lower MFI of positive cells compared to the mock-controls. MHC I levels on monocytes/macrophages did not change after 22653/14 infection, instead BA71V-infected moMΦ and moM2 had a lower expression (both percentages and MFI) of this marker than bystander and mock-infected cells (Figure 4.7 and 4.9). Very little difference in MHC II percentages were also detected between infected and bystander

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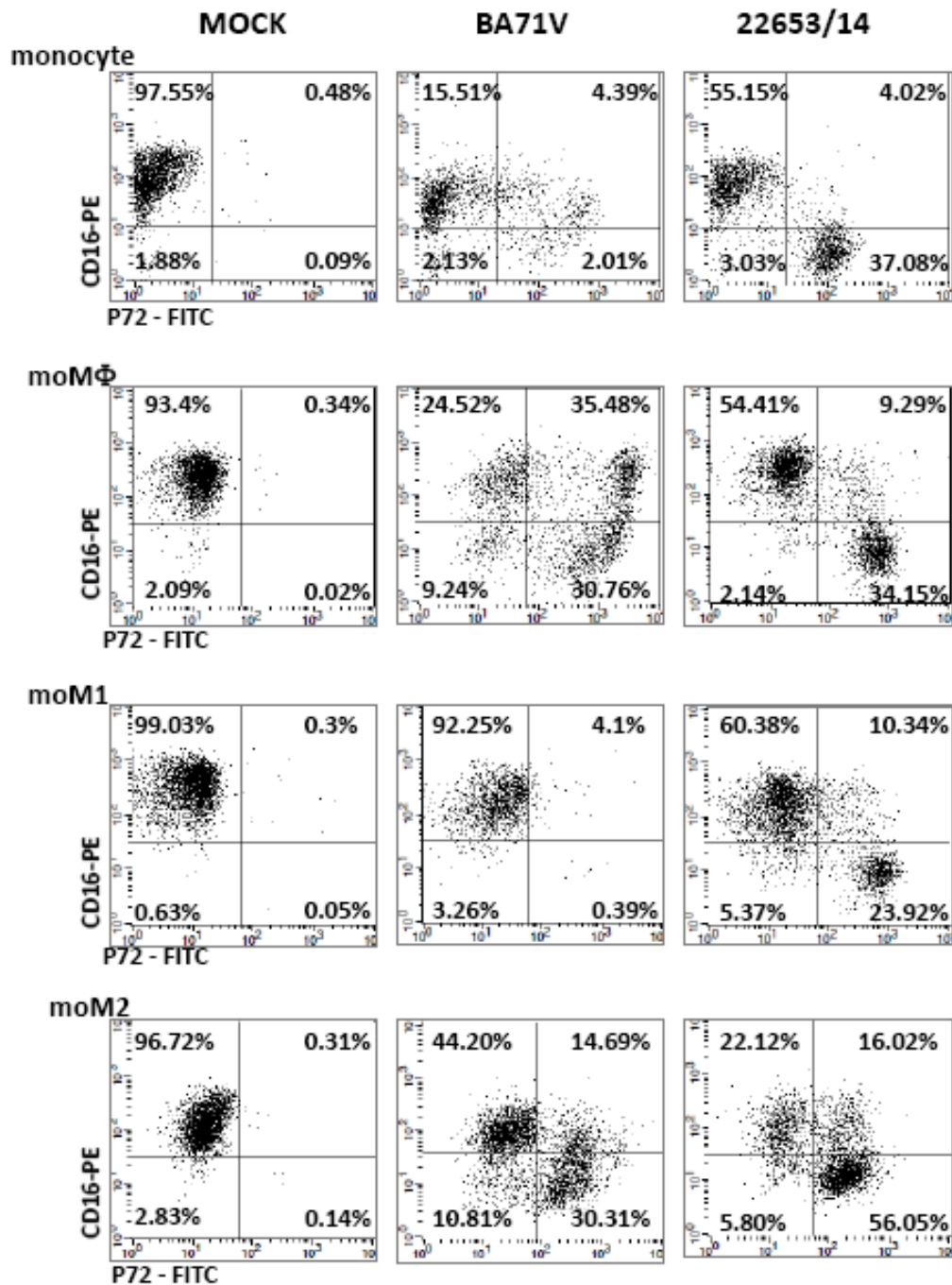
monocytes and moM2, but not correlated with their MFI (Figure 4.7 and 4.10). As displayed in Figure 4.7 and 4.11, for both strains infected and bystander monocytes/macrophages displayed similar percentages of CD163<sup>+</sup> cells. However after infection with BA71V both bystander and infected monocytes, moMΦ and moM2 displayed lower levels of CD163 compared to the mock-infected controls. Infection with the virulent 22653/14 virus did not affect CD163 expression in macrophages, nevertheless little but statistically significant differences were observed in the MFI of positive cells between 22653/14-infected and mock-infected monocytes (Figure 4.7).



**Figure 4.7. Effect of ASFV on the surface markers expression of monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V or the virulent 22653/14 strain using a MOI 1, alongside mock-infected controls. 18 hours pi, cells were harvested and expression of p72 and surface markers (CD16, MHC II, MHC I, CD163) were evaluated using flow cytometry. The mean data  $\pm$  SD from three independent experiments utilizing different animals are shown. Differences between mock-infected (white) and BA71V-bystader (black), BA71V-infected (horizontal bars), 22653/14-bystander (vertical bars), 22653/14-infected (oblique bars) cells for each marker were evaluated. On the right, differences in terms of % of positive cells are displayed, while on the left MFI of positive cells are reported. Values for each virus-stimulated condition were compared to the corresponding un-stimulated control (mock) using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

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**Figure 4.8. Effect of ASFV on CD16 expression of monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V strain or with the virulent 22653/14 field strain using a MOI 1. Mock-infected control cells were included for each cell type. 18 hours pi, cells were harvested and expression of p72 and CD16 were evaluated using flow cytometry. For each mock or virus-stimulated condition representative dot plots of monocytes, moMΦ, moM1 and moM2 are displayed.

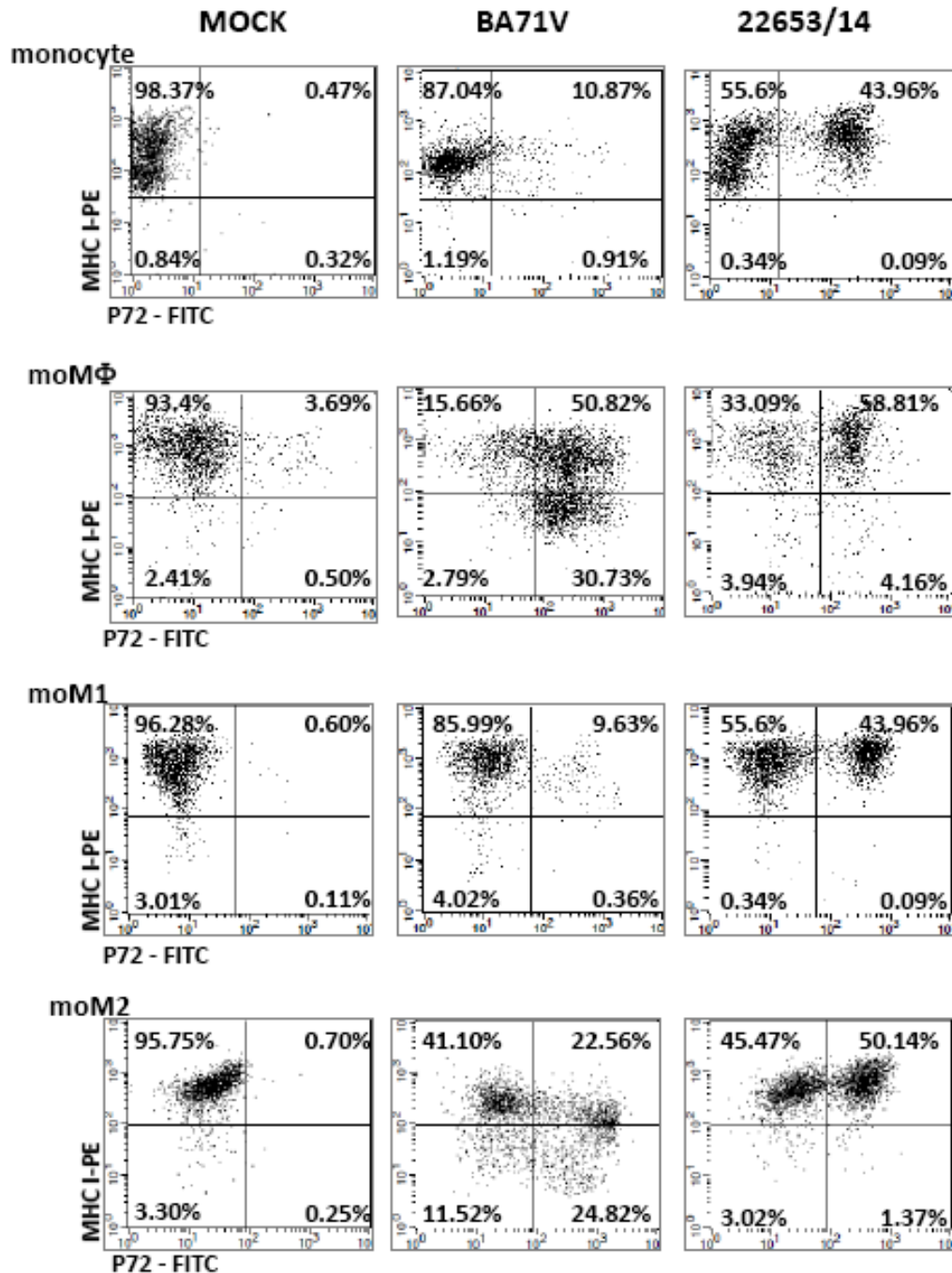
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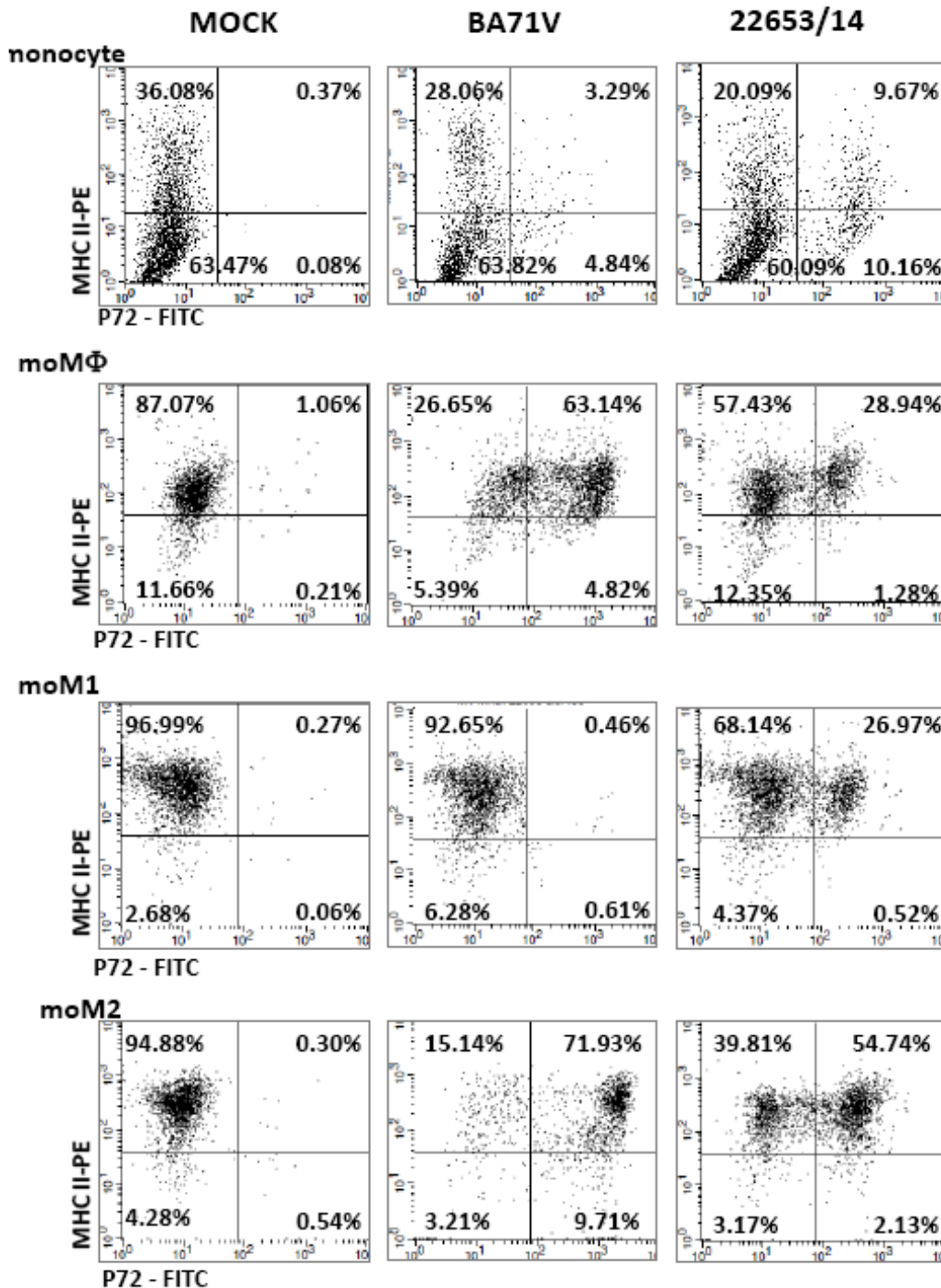
**Figure 4.9. Effect of ASFV on MHC I expression of monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V strain or with the virulent 22653/14 field strain using a MOI 1. Mock-infected control cells were included for each cell type. 18 hours pi, cells were harvested and expression of p72 and MHC I were evaluated using flow cytometry. For each mock or virus-stimulated condition representative dot plots of monocytes, moMΦ, moM1 and moM2 are displayed.

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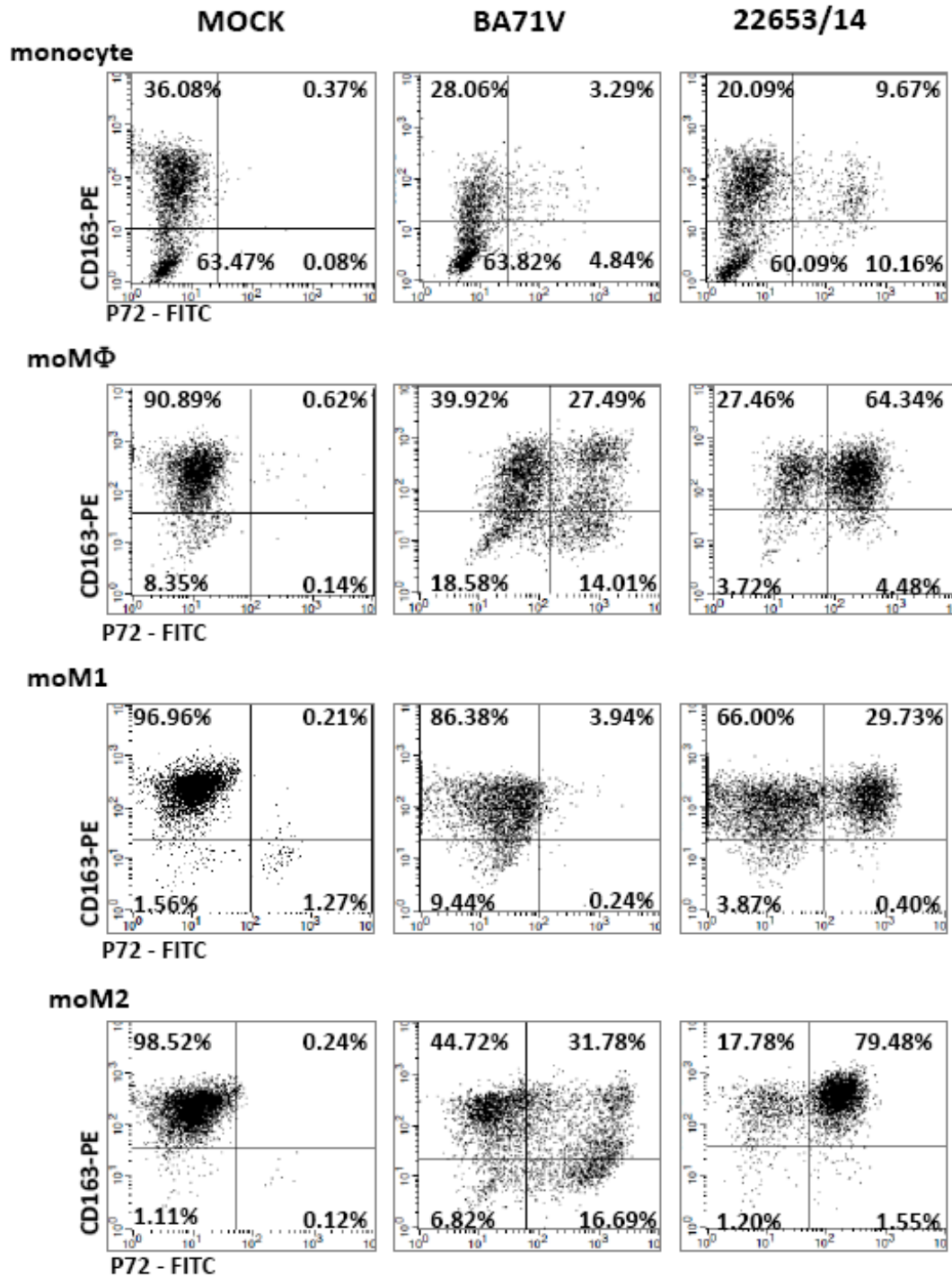
**Figure 4.10. Effect of ASFV on MHC II-DR expression of monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V strain or with the virulent 22653/14 field strain using a MOI 1. Mock-infected control cells were included for each cell type. 18 hours pi, cells were harvested and expression of p72 and MHC II-DR were evaluated using flow cytometry. For each mock or virus-stimulated condition representative dot plots of monocytes, moMΦ, moM1 and moM2 are displayed.

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**Figure 4.11. Effect of ASFV on CD163 expression of monocytes and macrophage subsets.** Monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V strain or with the virulent 22653/14 field strain using a MOI 1. Mock-infected control cells were included for each cell type. 18 hours pi, cells were harvested and expression of p72 and CD163 were evaluated using flow cytometry. For each mock or virus-stimulated condition representative dot plots of monocytes, moMΦ, moM1 and moM2 are displayed.

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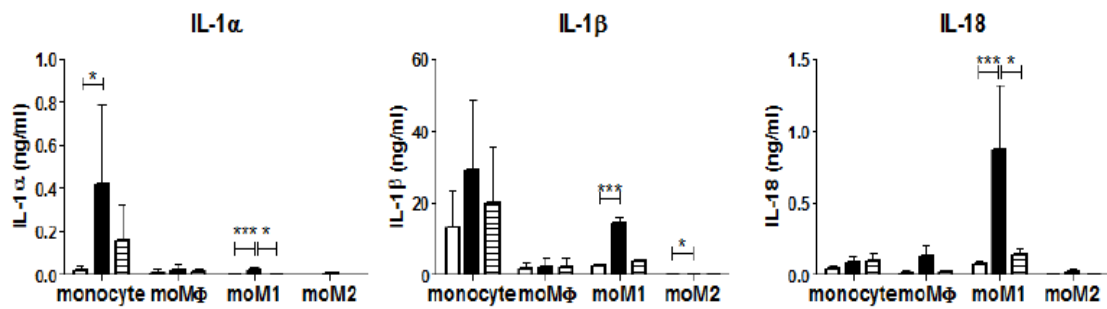
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#### **4.6 Production of cytokines in ASFV-infected monocytes/macrophage subsets**

Finally, the cytokine responses of monocytes and macrophage subsets to BA71V and 22653/14 were investigated. First it was assessed if mock-infection with clarified Vero cell or monocyte-macrophage cell lysates for 18 hours induced cytokine responses. Since no differences between co-culture with ‘mock Vero’ or ‘mock monocyte-macrophage’ and monocytes medium in the levels of GM-CSF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, TNF- $\alpha$  (data not shown) were detected, monocyte medium was used as the negative control in subsequent experiments. No statistically significant GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-12 or TNF- $\alpha$  responses were observed from monocytes and derived macrophage subsets infected with either strain (data not shown). Instead differences were observed in the levels of IL1- $\alpha$ , IL-1 $\beta$  and IL-18: moM1 released higher levels of IL1- $\alpha$ , IL-1 $\beta$  and IL18 in response to BA71V infection compared to uninfected control (Figure 4.12). Differences were also observed in the levels of IL-1 $\alpha$  between mock-infected and BA71V-infected monocytes and in the levels of IL-1 $\beta$  between mock-infected and BA71V-infected moM2 and monocytes, albeit the latter difference was without statistical significance (Figure 4.12).



**Figure 4.12. Investigation of cytokines release by monocytes and macrophage subsets in response to ASFV infection.** Monocytes, un-activated (moM $\Phi$ ), classically (moM1) and alternatively (moM2) activated macrophages were infected with the attenuated BA71V strain or with the virulent 22653/14 field strain using a MOI of 1. Mock-control cells were included for each cell type. 18 hours pi, the levels of IL-1 $\alpha$ , IL-1 $\beta$  and IL-18 in culture supernatants were evaluated. The mean data +/- SD from three independent experiments utilizing different animals are shown. Values for MOCK (white), BA71V (black) and 22653/14 (horizontal bars) were compared using a one-way ANOVA followed by a Kruskal-Wallis test; \*\*\*p<0.001, \*\*p<0.01, \*p<0.05.

## CHAPTER 5. DISCUSSION

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As previously stated, the first phase of the work focused on the identification of the optimal method to *in vitro* differentiate porcine monocyte into moMΦ. MoMΦ have been utilised in numerous studies of the porcine immune system, especially in the context of myelotropic viruses such as ASFV and PRRS (Thacker *et al.*, 1998, Sanchez-Torres *et al.*, 2003, Vincent *et al.*, 2005, Fishbourne *et al.*, 2013, Garcia-Nicolas *et al.*, 2014, Singleton *et al.*, 2016), but currently there is no standardized protocol for their generation. The availability of a reliable standard technique would add value to efforts to both understand basic porcine macrophage biology and to study pathogen interactions with this cell population. To address this need, in the first phase of the work a comparison of commonly described methods to *in vitro* generate moMΦ was performed: addition of porcine plasma (10 or 20 or 30 % v/v) or hM-CSF (50 or 100 or 200 ng/ml).

Clear phenotypic differences between moMΦ produced under the different conditions were found. moMΦ generated using autologous plasma presented increased granularity compared to those differentiated using hM-CSF, as estimated by their SSC properties in flow cytometry analysis, particularly evident when using plasma concentrations of 30%. moMΦ generated with porcine plasma displayed a more elongated phenotype and, as already noted by other authors (Sánchez *et al.*, 1999), were characterized by the presence of numerous cytoplasmic projections. Using autologous plasma, a greater inter-animal variability was observed, whereas the use of hM-CSF produced macrophage populations characterized by phenotypic homogeneity. Differentiation of monocytes into macrophages is accompanied by the acquisition of new cell surface antigens, whose detection has been widely employed to identify different maturation stages within the monocyte/macrophage lineage. In

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particular the expression of CD163 (Sanchez *et al.*, 1999), a 120kD single pass type 1 transmembrane cell surface glycoprotein, and CD203a, previously clustered as SWC 9 (Dominguez *et al.*, 1998), helps to identify mature macrophages (Sánchez-Torres *et al.*, 2003). Previous studies reported that CD163, CD203a and MHC II-DR are up-regulated during *in vitro* MoM $\Phi$  differentiation (Kristiansen *et al.*, 2001, Chamorro *et al.*, 2004, Wang *et al.*, 2011) and we observed up-regulation of these surface markers with each of the protocols tested. Nevertheless, the use of higher concentrations of porcine plasma led to a reduced expression of CD163, whereas no significant differences were observed in the levels of CD203a and MHC II-DR between moM $\Phi$  populations. A pronounced individual variability in terms of cell surface markers expression, especially evident when analysing CD163 expression, was observed in moM $\Phi$  differentiated using porcine autologous plasma. These results suggest that hM-CSF is able to promote the production of a more homogeneous cell population.

Addition of plasma or hM-CSF was able to promote porcine monocyte/macrophage survival in culture, as previously reported by other authors (Wang *et al.*, 2011). A lower number of metabolically active cells were detected when cells were grown in monocyte medium (RPMI + antibiotics + 10% FSB) alone rather than in media supplemented with porcine plasma or h-MCSF. Supplementation with hM-CSF resulted in a higher number of metabolically active cells, particularly evident using the highest concentration tested (200ng/ml). These results are in accordance with previous publications, where the mitogen activity of hM-CSF for porcine monocytes was described (Fairbairn *et al.*, 2013). Monocytes cultured in un-supplemented



serum-free RPMI did not survive, as previously reported in humans (Becker *et al.*, 1897) and pigs (Basta *et al.*, 1999).

Then the susceptibility of moM $\Phi$  differentiated with different techniques to ASFV infection was assessed, analysing the intracellular levels of the late ASFV p72 protein. Using flow cytometry, it was evaluated if the differentiation method influenced viral tropism. In accordance with previous publications, moM $\Phi$  were more susceptible to ASFV infection than freshly isolated monocytes (Basta *et al.*, 1999, McCullough *et al.*, 1999, Sanchez-Torres *et al.*, 2003), but moM $\Phi$  showed similar permissiveness to ASFV infection notwithstanding the different media in which they were grown. MoM $\Phi$  differentiation was also assessed by analysing TNF- $\alpha$  release in response to LPS (Gessani *et al.*, 1993). Previous work reported that moM $\Phi$  released high levels of this cytokine in response to LPS stimulation with no differences between various pig breeds (Kapetanovic *et al.*, 2013). In our study macrophages differentiated using all 6 protocols released higher levels of TNF- $\alpha$  than freshly isolated monocytes, suggesting that with all the methods cells acquire a macrophage-like functionality. Differences were detected between different concentrations of porcine plasma (10 vs 20 vs 30%) and hM-CSF (50 and 100 vs 200 ng/ml), nevertheless these were probably related to the higher number of viable moM $\Phi$  present in culture at time of stimulation.

Then, basal release of cytokines from moM $\Phi$  populations and ability to respond to activation were assessed. Using different protocols, small differences were detected in the basal levels of IL-10 and IL-12 in culture supernatants, although IL-12 was also detected in the background plasma control. Despite the immunosuppressive role of IL-10, the levels detected were extremely low and they might not influence the *in*

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*in vitro* responses of macrophages to external stimuli. In fact, moMΦ differentiated using hM-CSF responded efficiently to classical activation. Greater differences were detected in the levels of IL-1RA; moMΦ differentiated using porcine plasma release higher levels of this cytokine although diversity amongst pigs was observed. IL-1RA, a member of the IL-1 family and inhibitor of the pro-inflammatory effect of IL-1 $\alpha$  and IL1 $\beta$ , is stimulated by many substances including adherent IgG, other cytokines and bacterial or viral component (Arend *et al.*, 1998). In humans and mice it is implicated in the polarization of macrophages in moM2b, in combination with LPS, immune complexes and apoptotic cells (Duque *et al.*, 2014). Our results suggest that some plasma components induce IL-1-RA release which might affect macrophage phenotype and potentially skew their response to external stimuli. Few studies have described classical and alternatively macrophage activation in pigs (Garcia-Nicolas *et al.*, 2014, Sang *et al.*, 2014, Singleton *et al.*, 2016). As previously stated, in pigs classical activation induced release of inflammatory cytokines and IL-12. In response to activation with IFN- $\gamma$  and LPS, moMΦ differentiated using hM-CSF released higher levels of IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  compared to those generated with autologous plasma and in the latter a pronounced inter-animal variability in terms of IL-12 was observed. Considering the important role of moM1 in host defence to viruses, intracellular bacteria and protozoa (Mosser and Edwards, 2008), our data suggest that in studies on macrophages-intracellular pathogens interactions the use of recombinant hM-CSF for moMΦ generation might be the more suitable protocol. As previously stated, in humans exposure to IL-4 polarize cells toward an M2a phenotype, with production of high levels of IL-10, TGF- $\beta$ , IL-1RA and chemokines that promotes recruitment of Th2 cells (Mosser, 2003; Duque *et al.*, 2014). In pigs,

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independently of the protocols used to mature macrophages, no release of IL-10 or IL-1RA from moM2 was detected. Further studies should be performed to analyse alternative macrophage activation in pigs, but that was beyond the scope of the present work.

In conclusion, data generated in the first phase of the work suggest that all the protocols tested can be considered as suitable to differentiate porcine monocytes into moMΦ. However, the adoption of autologous porcine plasma, especially at high concentrations, yielded to a pronounced cell pleomorphism and the use of 30% of porcine plasma produced moMΦ with basal release of IL-1RA, reduced expression of CD163, and lower ability to respond to classical activation, suggesting that these cells are developing toward a M2b-like phenotype. Instead supplementation with hM-CSF produced macrophage populations characterized by phenotypic and functional homogeneity, provided a better reproducibility between experiments and a slightly higher responsiveness to M1 polarization, even if a little but statistically significant basal release of IL-10 was detected. Nevertheless, the higher proliferation rate found in macrophages cultured in media supplemented with high concentration of hM-CSF could impair *in vitro* study where a constant number of cells is required. The information generated suggested the adoption of low concentration of hM-CSF for porcine moMΦ generation, so in the second part of the work moMΦ were generated using 50 ng/ml of hM-CSF.

The second phase of the work aimed to provide a better understanding of the interaction of monocytes and derived macrophage subsets with ASFV. We consider crucial to better characterise the interaction of ASFV with the porcine immune system, and its target cells in particular, in order to generate information useful to underpin

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vaccine development efforts. An efficient vaccine against ASFV would be an important tool to fight this disease, which is now threatening the pig industry in Russia and Europe.

Differences in the expression of functional surface markers and cytokine release on monocytes and derived macrophage subsets after *in vitro* infection with ASFV isolates of differing virulence was performed. Few studies have described classical and alternatively macrophage activation in pigs (Singleton *et al.*, 2016, Garcia-Nicolas *et al.*, 2014, Sang *et al.*, 2014) and no investigation of the interaction of ASFV with activated macrophage subsets was conducted. As previously stated, macrophages in antithetic polarize status might react differently to ASFV infection, with differences between isolates of diverse virulence, influencing the development of disease rather than acquisition of protective immunity.

First, the susceptibility of monocytes and derived macrophage subsets to ASFV infection was determined. Differentiation of monocytes into macrophages resulted in an increased susceptibility to ASFV infection, in accordance with previous publications (Sánchez-Torres *et al.*, 2013, McCullough *et al.*, 1999, Basta *et al.*, 1999). 22653/14 displayed greater ability to infect monocytes than the avirulent BA71V, with differences in the levels of late (p72) but not early (p30) proteins. These results suggest that BA71V replication in these cells is inhibited in the early stage of replication, whereas the virulent isolate has developed mechanisms to promote its growth in monocytes, which *in vivo* probably results in its ability to disseminate in the host. 22653/14 presented also greater ability to infect moM1 than the avirulent strain and pre-treatment of porcine macrophages with IFN- $\gamma$  or LPS alone resulted in an increased resistance to BA71V but not 22653/14 infection. Also

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a previous study reported that IFN- $\gamma$  reduced ASFV replication in porcine monocytes and alveolar macrophages, with inhibition of expression of late (p220 and p72) but not early (p27) proteins (Esparza *et al.*, 1988). The authors did not observe differences in the anti-viral potencies of type II IFN between a virulent (CC83) and an avirulent (BA71V) strains, whereas our data revealed differences between strains varying in virulence. A recent study reported similar results in the sensitivity of ASFV strains to another IFN: IFN- $\alpha$ . Pre-treatment with IFN- $\alpha$  of alveolar macrophages reduced replication of attenuated isolates containing MGF 360/530 deletions but not virulent ASFV strains (Golding *et al.*, 2016). BA71V and other attenuated strains present deletions in the region containing 360/530 MGFs (Zsak *et al.*, 2001), which suppress type I IFN responses (Afonso *et al.*, 2004, Correia *et al.*, 2013) and might also interfere with antiviral genes induced by type II IFN. Our data also showed that pre-treatment of porcine macrophages with LPS alone resulted in an increased resistance to BA71V-infection. Pre-treatment of macrophages with LPS can affect macrophage phagocytotic activity, and thus susceptibility to ASFV infection (Basta *et al.*, 2001), but differences were observed between the avirulent BA71V and the virulent 22653/14 strains. It may be hypothesised that LPS engagement with TLR4 leads to IFN- $\beta$  expression (Malmgaard, 2014), inducing an antiviral state whose effects are significantly stronger against the avirulent isolate. Both isolates were able to infect moM2, without statistically significant differences using an MOI of 1. Similar results were observed in the other two studies on activated monocyte-derived macrophages: IFN- $\gamma$  activation almost completely prevented moM $\Phi$  infection by a low virulent PRRSV, but not or only partially to virulent field isolates (Garcia-Nicolas *et al.*, 2014), whereas M2 polarization did not

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interfere with PRRSV replication (Singleton *et al.*, 2016, Garcia-Nicolas *et al.*, 2014).

Using an MOI of 0.01, the ability of the two ASFV strains to grow in macrophage subsets was evaluated. Macrophage activation, especially classical, resulted in an initial reduction of 22653/14 replication; nevertheless 48 hours pi almost all (>90%) of the live moM1 and moM2 were p30<sup>+</sup>/p72<sup>+</sup> and no differences between macrophage subsets were observed in the levels of viral copies number at any time points, suggesting that activation only delayed and did not inhibit 22653/14 replication in these cells. Interestingly, using an MOI of 0.01 only little levels of ASFV viral proteins were detected after BA71V-infection. Probably using a low MOI the replication of the avirulent strain in macrophages is inhibited soon after infection, before the synthesis of early viral proteins, whereas after high MOI infection BA71V is able to perform initial stages of replication, but then infected cells undergo apoptosis, preventing the production of infectious viral progeny. Little but statistically significant differences were observed between macrophage subsets in the intracellular levels of ASFV proteins 24 hours pi with the avirulent strain. moM2 display higher permissiveness to BA71V-infection than moM $\Phi$  and moM1, suggesting that alternative macrophage activation is negatively correlated with ASFV resistance.

The effect of ASFV-infection on cell surface markers was next investigated. In accordance with previous publication, our results showed that ASFV down-regulates CD16 expression on monocytes and macrophages (Sanchez-Torres *et al.*, 2003, Lithgow *et al.*, 2014). CD16 is a low-affinity receptor for the IgG Fc, that mediates phagocytosis and antibody-dependent cellular cytotoxicity (Dato *et al.*, 1992). Both

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isolates down-regulated the expression of this marker, so this effect has not been lost during BA71V attenuation. Our data suggested that down-regulation is a direct consequence of ASFV infection and occurs after late protein synthesis. Down-regulation of CD16 could impact the function of infected cells and ASFV might modulate the expression of surface markers on these cells in order to reduce their anti-microbial/viral activity, promoting the distribution of cellular sources to synthesize new viral proteins. The effect of ASFV on MHC class I and II expression was assessed. MHC II present antigenic peptides to CD4 T cells, whereas CD8 T cells recognize antigens presented by MHC I (Trombetta and Mellman, 2005), so modulation of their expression can thus affect antigen presentation and development of adaptive immune responses.

22653/14-infected monocytes/macrophages presented similar MHC-I levels to bystander or mock-infected cells, instead BA71V-infected moM $\Phi$  and moM2 displayed a lower percentage of MHC I<sup>+</sup> cells than uninfected cells. This down-regulation might be due to a general blockade of the protein synthesis, a consequence of pro-apoptotic signals that occurs after infection with the avirulent strain, or by other mechanisms commonly used by viruses to inhibit MHC class I expression, such as cellular transport inhibition or active removal from the cell surface (Tortorella *et al.*, 2000). In a previous study ASFV infection was associated with down-regulation of IFN- $\alpha$ -induced MHC class I expression on aortic endothelial cells, where activation of the apoptotic pathway occurred rapidly after infection (Valee *et al.*, 2001). Probably in the absence of the mechanisms developed by virulent ASFV strains to survive in myeloid cells, ASFV-infected cells undergo apoptosis and down-regulate MHC class I, activating NK cell killing (Lanier, 2005). Another hypothesis

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might be that infection with BA71V and not with 22653/14 leads to a higher release of some IFN- $\alpha$  subtypes, with inhibitory action on MHC class I expression. Even if the release of type I IFN in response to ASFV infection was not assessed, a previous study reported that a low virulent ASFV isolate induced enhanced expression of IFN- $\alpha$  than virulent one (Gil *et al.*, 2008) and some IFN- $\alpha$  subtypes have shown inhibitory control on MHC I expression on porcine cells (Zanotti *et al.*, 2015). The absence of MHC class I down-regulation in moM1 might be due to the fact that viral replication is inhibited soon after infection, so the signals that induce MHC class I down-regulation in BA71V-infected moM $\Phi$  do not occur. Extremely little differences were observed in the MHC-II expression of monocytes/macrophages, suggesting that ASFV does not modulate the expression of this marker, as previously described for monocytes, alveolar (Sánchez-Torres *et al.*, 2003) and bone marrow derived macrophages (Lithgow *et al.*, 2014). Finally, we analysed the effect of ASFV on CD163. A role of this molecule in the process of infection of porcine monocytes/macrophages by ASFV has been suggested, but a recent study suggested that this marker was not essential in ASFV infection (Sánchez-Torres *et al.*, 2013, Lithgow *et al.*, 2014). Using p72 mAb to determine the intracellular levels of ASFV, infected monocytes/macrophages expressed similar percentage of CD163 to bystander cells, supporting the results of Lithgow *et al.* (2014). Levels of CD163 on BA71V-infected cells were always lower than the mock-infected control, perhaps because its synthesis was inhibited, as a consequence of pro-apoptotic signals, or because its extracellular portion was released after infection, in response to inflammatory or other stimuli (Ezquerro *et al.*, 2009).

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In the final part of the study the cytokine responses of porcine monocyte/macrophages to ASFV infection were characterised. Previous studies reported that macrophage infection with ASFV resulted in an enhanced expression of mRNA levels of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-15) (Gómez del Moral *et al.*, 1999, Gil *et al.*, 2003, Zhang *et al.* 2006), with differences between isolates of different virulence (Gil *et al.*, 2008). Contrasting results were reported on the levels of TNF- $\alpha$  in culture supernatants: Gomez del Moral *et al.* (1999) observed an increased in the levels of this cytokine in culture supernatant after infection with the virulent E-75, instead Zhang *et al.* (2006) could not detect it, despite the increase of its mRNA in the cells soon after infection with the virulent Malawi LIL120/1 and the low virulent OURT88/3. Authors speculated that post-transcriptional events involved in protein stability could affect the levels of the protein accumulated. In accordance to the latter study no TNF- $\alpha$  release in response to ASFV infection was detected in our work. These differences might be related to the different strains adopted. In accordance with Zhang *et al.* (2008) IL-1 $\beta$  release was observed in response to ASFV infection, but we observed higher IL-1 $\beta$  levels from monocytes/activated macrophages mainly in response to the avirulent BA71V strain. Also higher levels of IL-1 $\alpha$  were detected after BA71V infection in comparison to the virulent 22653/14. Both IL-1 $\alpha$  and IL-1 $\beta$  are strongly pro-inflammatory cytokines and perform their functions through binding of the IL-1 receptor (IL-1R) (Duque and Descoteaux, 2014). IL-1 $\beta$  is synthesized as a biologically inactive precursor inside the cell and it is cleaved by the enzyme caspase-1 to the biologically active mature form that is released from cells (Arend *et al.*, 2008). IL-1 $\beta$  has a pro-

apoptotic role (Friedlander *et al.*, 1996), so it could be speculated that the release of IL-1 $\beta$  after BA71V infection may contribute to the early apoptosis of porcine monocyte/macrophages, which *in vivo* probably limits viral replication and pro-inflammatory dysregulation. The virulent 22653/14 might have developed mechanism to inhibit IL-1 $\beta$  release, in order to promote its replication inside monocyte/macrophages. Gil *et al.* (2008) reported an increase of the IL-12p40 levels in macrophage supernatants after ASFV infection, with differences between isolates of different virulence (Gil *et al.*, 2008). IL-12 release after ASFV infection of monocytes/macrophage subsets was not detected in our experiments, and this difference might due to the fact that an avirulent (BA71V) and not a low virulent (NH/P68) isolate was tested, or because IL-12p40 is also a subunit of IL-23 (Oppmann *et al.*, 2000), which might be released in response to infection with low virulent ASFV strains. Nevertheless, an increase in the levels of another potent IFN- $\gamma$  inducer was detected in the supernatants of BA71V infected macrophages: IL-18 (Dinarello, 1999). This cytokine is a member of the IL-1 family and, like IL-1 $\beta$ , is synthesized as a inactive precursor molecule which is cleaved by caspase-1 before or during the released from cells (Dinarello, 1999; Arend *et al.*, 2008). IL-18 synergizes with IL-12 to promote the development of a TH1 response and it activates T cells to synthesize IL-2, IFN- $\gamma$  and TNF- $\alpha$  (Dinarello, 1999). Release of IL-18 may be implicated in the acquisition of a TH1 cell response, correlated to protection against ASFV (Takamatsu *et al.*, 2013), and the difference observed between isolates of different virulence might suggest that 22653/14 have developed mechanism to counteract the induction of this response.

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In summary, our detailed *in vitro* analysis of the interaction of ASFV isolates varying in virulence with monocytes and derived macrophage subsets revealed that compared to the avirulent BA71V strain, the field isolate 22653/14 showed an enhanced capacity to replicate in monocytes and moM1, did not induced MHC I down-regulation in infected moMΦ/moM2 and induced lower release of IL-18, IL-1β and IL-1α. It is hoped that the observed strains differences will be valuable to aid our understanding of the pathogenesis and immunomodulation of host cell responses by ASFV.

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