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Characterization of the interaction of African swine fever virus with monocytes and derived macrophage subsets.

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HIGHLIGHTS

- Responses of monocytes and macrophage subsets to virulent and avirulent ASF viruses
- Virulent isolate showed an enhanced capacity to replicate in monocytes and moM1
- Infection with the avirulent strain induced MHC I down-regulation in moMΦ/moM2
- Infection with the virulent strain induced lower release of IL-18, IL-1β, IL-1α

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 Sardinian isolate (22653/14) and a tissue culture adapted avirulent strain (BA71V) of ASFV with porcine monocytes, un-activated (moMΦ), classically (moM1) and alternatively (moM2) activated monocyte-derived macrophages was conducted in an attempt to better understand this relationship. 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. No differences were observed in the expression of CD163 between ASFV infected and uninfected bystander cells. ASFV down-regulated CD16 expression but did not modulate MHC class II levels in monocytes and macrophage subsets. BA71V-infected but not 22653/14-infected moMΦ and moM2 presented with a reduced expression of MHC class I compared to the mock-infected controls. Higher levels of IL-18, IL1-β and IL-1α were released from moM1 after infection with BA71V compared to 22653/14 or mock-infected control. These results revealed differences between these ASFV strains, suggesting that virulent isolates have evolved mechanisms to counteract activated macrophages responses, promoting their survival, dissemination in the host and so ASF pathogenesis.

Keywords: ASFV; monocyte-derived macrophages; polarization; porcine; flow cytometry; cytokines.

Introduction

African swine fever (ASF) is a contagious and often fatal disease of domestic pigs and wild boar, for which there is no vaccine or treatment available (Sanchez-Vizcaino, 2006). It is currently present in many sub-Saharan African countries, Russian Federation, Trans-Caucasus, part of East Europe and Sardinia (OIE, WAHIS interface). The aetiological agent is the African swine fever virus (ASFV), a large, enveloped double-stranded DNA virus, which is the only member of the *Asfarviridae* family (Dixon et al., 2005). ASFV mainly targets immune cells of the myeloid lineage, especially monocytes and macrophages, which are 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, since they express anti-apoptotic proteins that allow infected cells to survive and disseminate the virus through the body (Dixon et al., 2013). In contrast, the tissue-culture adapted BA71V strain is able to infect macrophages and to synthesise viral late proteins, but induces early cell death, thereby limiting the production of infectious viral progeny (Zsak et al., 2001). Infection of monocytes and macrophages with virulent ASFV strains induces the synthesis of pro-inflammatory cytokines such as TNF-α and IL-1α (Gómez del Moral et al., 1999, Gil et al., 2008) and it has been speculated that the lymphopenia observed during ASF is driven by pro-inflammatory cytokine release from infected macrophages (Oura et al., 1998).

Monocytes are blood-borne circulating primary immune cells, which migrate to tissues and differentiate into inflammatory dendritic cells and/or macrophages during inflammation and, less efficiently, in the steady state (Geissman et al., 2010). Tissue-resident macrophages maintain tissue homeostasis and have an important role in immune response to pathogens (Geissman et al., 2010). Macrophages may be differentially activated resulting in their polarisation into different functional subsets, referred to as M1 and M2 macrophages (Mosser, 2003). Classical activation with IFN-γ and

LPS polarises M1 macrophages, which mediate defence to intracellular pathogens, by killing intracellular viruses, bacteria and protozoa and driving Th1 cellular immune responses (Mosser, 2003). In contrast, alternative activation by IL-4 or IL-13 induces M2 macrophages, which produce high levels of the anti-inflammatory cytokine IL-10, fail to make nitric oxide and are primarily associated with mechanisms of immunosuppression and wound repair (Mosser, 2003). Few studies have described classical and alternative macrophage activation in pigs (Singleton et al., 2016, Garcia-Nicolas et al., 2014, Sang et al., 2014). As described in humans, generation of porcine M1 macrophages can be achieved *in vitro* by exposure to IFN-γ and LPS (Singleton et al., 2016, Sang et al., 2014). Nevertheless in pigs there is not a standardized protocol and IFN-γ alone was used to achieve classical activation (Garcia-Nicolas et al., 2014). *In vitro* exposure of macrophages to IL-4 have been adopted in pigs to polarize monocyte-derived (Singleton et al., 2016, Garcia-Nicolas et al., 2014) or alveolar (Sang et al., 2014) macrophages to an M2 phenotype.

To date, few studies have analysed the effects of ASFV on monocytes and macrophages in terms of their expression of functional surface markers (Sánchez-Torres et al., 2003; Lithgow et al., 2014) or cytokine responses (Gómez del Moral et al., 1999, Gil et al., 2008, Gil et al., 2003, Zhang et al., 2006) and none have compared responses of differentially activated macrophage subsets. Considering the central importance of macrophages for ASFV pathogenesis and the polarising effects of classical and alternative activation on macrophage phenotype/function, we hypothesized that cells in distinct activation statues will respond differently to ASFV. We further hypothesised that responses would differ depending on the virulence of the ASFV strain; virulent isolates might have evolved mechanisms to modulate activated macrophages responses in order to promote their survival and dissemination. To address these questions, we conducted a detailed *in vitro* analysis of the interaction of monocytes, un-activated and activated monocyte-derived macrophages with a virulent (22653/14) and a non-pathogenic (BA71V) ASFV strain.

Materials and Methods

2.1 Animals

Seven healthy ASFV-naïve cross-bred pigs (*Sus scrofa*), 6-18 months of age, were used in the study. The ASFV seronegative status of the animals was confirmed by a commercial ELISA test (Ingenasa, Madrid, Spain), according to the manufacturer's protocol, and with an immunoblotting test (OIE, 2012). The animals were housed at the experimental facility of IZS della Sardegna (Sassari, Italy) and animal housing and handling procedure were performed in accordance with the local ethics committee, in agreement with the guide of use of laboratory animals of the Italian Ministry of Health. Heparinized blood was collected by puncture of the cranial vena cava, using a 50 mL syringe containing sodium heparin connected to a 2.0x45 mm 14-gauge needle (Delta Med, Mantova, Italy).

2.2 Viruses

The avirulent ASFV BA71V strain (kindly provided by the EU ASF Reference Laboratory CISA-INIA, Madrid, Spain) was propagated *in vitro* by inoculation of sub-confluent monolayers of Vero cells; 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). The virulent Sardinian field strain 22653/14 was isolated from the spleen of a naturally infected pig collected from a 2014 outbreak in the province of Cagliari (Exotic Disease Laboratory ASF Virus Archive, IZS of Sardinia, Sassari, Italy); it is placed in the p72 genotype I and cluster within sub-group X of the B602L gene, as with the other 57 Sardinian ASFV isolates collected during 2002-2014 (Sanna et al., 2016). It was propagated *in vitro* by inoculation of sub-confluent monolayers of porcine monocytes/macrophages for no more than six passages and viral titres were obtained by serial dilution of the virus suspension on monocyte/macrophages followed by observation for hemadsorption (Malmquist and Hay, 1960).

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

2.3 Purification of monocytes, macrophage differentiation and activation

PBMC were prepared by layering 30 ml of heparinized blood diluted 2:1 in PBS (Phosphate Buffered Saline, Sigma-Aldrich, St Louis, MO, USA) over 20 ml of Histopaque-1077 (Sigma-Aldrich) and centrifuged at 600 x *g* for 20 minutes at 4°C without breaking. PBMC were aspirated from the plasma-Histopaque interface and washed three times in PBS, by centrifugation at 1000 x g for 5 minutes at 4°C. PBMC were re-suspended in RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin (monocyte medium) (Berg *et al*., 2013). Porcine monocytes were isolated from PBMC using flasks (Corning, NY, USA) pre-incubated with autologous porcine plasma, according to a previous method but with slight modifications (Berg *et al*., 2013). In brief, flasks were incubated for 1 hour with autologous porcine plasma at 37°C, 5% CO_2, after which plasma was removed and PBMC resuspended in monocyte medium with 0.1% BSA (Sigma-Aldrich) were added. After 1 hours at 37°C, non-adherent cells were removed by 4 washes with un-supplemented RPMI-1640 medium and adherent cells were incubated overnight at 37°C, 5% CO_2, in monocyte medium. The following morning adherent cells were detached by placing the flasks on ice for 1 hour. Detached cells were centrifuged at 200 x g for 8 minutes at 4°C and re-suspended in fresh monocyte medium; an aliquot was used to count and to assess cells viability using a Countess Automated Cell Counter (Thermo Fisher Scientific). 7-10x10^5 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. To differentiate monocytes into macrophages (moMΦ), cells were cultured for 5 days in monocyte medium supplemented with 50 ng/ml of recombinant human M-CSF (hM-CSF) (eBioscience, Vienna, Austria) (Singleton et al., 2016). Porcine moMΦ were further stimulated for 24 hours with activators to achieve classical (moM1) or alternative (moM2) activation, as previously described in humans and pigs (Mosser, 2003, Garcia-Nicolas et al, 2014, Singleton et al., 2016). For classical activation 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) were added to MoMΦ, for alternative activation 20 ng/ml of recombinant porcine IL-4 (R&D Systems, Minneapolis, MN, USA) was added to moMΦ.

2.4 Confocal microscopy

Differentiation of monocytes into moMΦ and polarization in moM1 and moM2 were observed by confocal microscopy. Monocytes were grown on two-well chamber slides (Thermo Fisher Scientific) and were stained immediately or differentiated in moMΦ, moM1 and moM2, as described above. Cells were fixed with 4% paraformaldehyde and labelled with Hoechst for nuclear staining and Alexa Fluor 488 conjugated phalloidin (Invitrogen) to visualize actin cytoskeleton. Microscopy was performed using a Leica SP5 Confocal Microscope (Leica Microsystem) 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.

2.5 ASFV infection of monocytes/macrophages subsets and growth curves

Culture medium from monocytes, moMΦ, moM1 and moM2 cultures were removed and replaced with an MOI of 1 of the virulent 22653/14 or the attenuated BA71V ASFV, diluted in fresh monocyte medium free of growth factors and/or cytokines. Mock-infected controls were included in every experiment. Cells were incubated at 37ºC, harvested after 18 hours and infection was assessed by intracytoplasmic p72 expression using flow cytometry. To evaluate BA71V and 22653/14 growth kinetics in moMΦ, moM1 and moM2, cells were infected with an MOI of 0.01 and after 90 minutes the inoculum was removed, cells were washed with unsupplemented RPMI-1640 medium and fresh monocyte medium added to the wells. In defined experiments, culture supernatants were collected to evaluate viral copies numbers or cytokine release in response to ASFV infection. Culture supernatants were collected 18 or 0, 24, 48, 72 hours post-infection (pi), cleared by centrifugation at 2000 x *g* for 3 minutes and stored at -80°C until analysed.

2.6 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 µM 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 µM 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 µl containing 5 µl 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 (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.

2.7 Multi-parameter cytofluorometric analysis of monocytes and macrophage subsets

Cells were harvested from cultures using ice cold PBS with 10 mM EDTA, centrifuged at 2000 x g for 3 minutes, washed in PBS and transferred to wells of a 96-well round bottom plate. 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 then washed twice with PBS supplemented with 2% FBS. Immunofluorescence staining of cells was performed to detect surface markers by incubating cells with the following monoclonal antibodies (mAbs) for 10 min at RT: MHC class II DR (2E9/13; Bio-Rad Antibodies, Kidlington, United Kingdom), MHC class I (JM1E3; Bio-Rad Antibodies), CD16-RPE (G7, Thermo Scientific Pierce, Rockford, IL, USA), CD163-RPE (2A10/11, Bio-Rad Antibodies). After incubation cells were washed with PBS supplemented with 2% FBS. Staining with MHC class II DR and MHC class I mAbs was visualized by subsequent staining with rat anti-mouse IgG2b PE-conjugated secondary antibody (332723; R&D Systems) or polyclonal goat anti-mouse IgG Fc cross adsorbed RPE-conjugated secondary antibody (Thermo Scientific Pierce) for 10 minutes at RT and then cells were washed with PBS supplemented with 2% FBS. Surface stained cells were fixed and permeabilized using Leucoperm, according to manufacturer's protocol (Bio-Rad Antibodies). 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. Irrelevant isotype control mAbs were used to control staining with surface markers: mouse

PE-IgG1 isotype control (ZX3, Thermo Scientific Pierce), unconjugated IgG1 and unconjugated and IgG2b isotype controls (both Bio-Rad Antibodies). All the washes used centrifugation at 836 x *g* for 3 minutes. Flow cytometry analysis were performed on a FACSCalibur (BD Biosciences) and at least 5000 live monocytes/macrophages were acquired. Analysis of data was performed by Cell Quest Pro Software (BD Biosciences), by gating on viable cells (Live/Dead Fixable Dead Cell Stain negative) in the monocyte/macrophage population, and then their expression of surface markers/ASFV proteins were assessed. Gates for surface markers were set using the corresponding isotype controls, whereas gates for ASFV proteins were set using the mock-infected controls.

2.8 Analysis of the cytokine levels in culture supernatants after macrophage activation and in response of monocytes and macrophage subsets to ASFV infection

18 hours after ASFV-infection, culture supernatants were collected, cleared by centrifugation at 2000 x *g* for 3 minutes and stored at -80°C until analysed. The simultaneous measurement of GM-CSF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α were performed using Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine assay (Merck Millipore, Darmstadt, Germania) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

2.9 Data analysis and statistics

All experiments were performed in triplicates 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 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.

Results

3.1 Monocytes differentiation into macrophages and their activation

Macrophages (moMΦ) were differentiated from monocytes by culture with 50 ng/ml of hM-CSF for 5 days and activated by the classical or alternative method for a further 24 hours. Macrophage differentiation and activation was assessed by confocal microscopy and flow cytometry (Figure 1). 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 1A). In addition, they presented higher dimension (forward scatter; FSC) and granularity (side scatter; SSC) as well as expressing higher levels of CD163, and MHC II-DR than monocytes (Figure 1B and Supplementary Figure S1). No significant differences in terms of dimension (FSC) and granularity (SSC) were observed between macrophage subsets, whereas these populations differed in terms of surface marker expression. In accordance with previous studies (Garcia-Nicolas et al., 2014), classical activation increased MHC II-DR expression, and alternative (IL-4) activation resulted in a decrease in CD163 expression (Figure 1B).

3.2 Susceptibility of monocytes and macrophage subsets 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 cytometric staining, using the gating strategy displayed in Figure 2A. Cells were mock-infected or infected with the tissue-culture adapted BA71V or the virulent Sardinian isolate 22653/14, using an MOI of 1. For both

isolates, macrophages were more susceptible to ASFV infection than freshly isolated monocytes (Figure 2). Classical activation resulted in a reduced susceptibility to ASFV infection with differences between strains, which were not observed with moMΦ and moM2. 22653/14 presented a greater ability to infect moM1, and monocytes, compared to BA71V (Figure 2). To assess whether the differences observed between 22653/14 and BA71V in monocytes and moM1 were due to inhibition to late viral protein synthesis, we assessed the levels of the early protein p30 in monocytes and macrophage subsets (Figure 3A). Despite differences were observed between BA71V and 22653/14 infected monocytes in terms of p72 expression, the same levels of $p30^+$ cells were detected in monocytes infected with these strains. Instead, 22653/14 infection resulted in higher levels of $p30^+$ moM1 than BA71V. We next assessed if pre-treatment of macrophages with IFN-γ or LPS alone was able to reduce susceptibility to ASFV. Activation with these agents alone decreased BA71V but not 22653/14 infection (Figure 3B). 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 3B).

3.2 ASFV growth in macrophage subsets

A kinetic analysis of moMΦ, moM1 and moM2 infection with BA71V and 22653/14 was performed using an MOI of 0.01. Replication was assessed by the intracellular levels of p30 and p72 and the viral genome copies numbers in cell culture supernatants. By 24 hours post-infection with the virulent 22653/14, both moM1 and moM2 displayed lower levels of ASFV proteins than moMΦ, but at 48 hours almost all (>90%) of the live un-activated and activated macrophages were $p30^+$ or $p72^+$. At 72 hours it was not possible to analyse sufficient numbers of viable cells. Very low levels of ASFV proteins were detected after infection of all cell types with the attenuated BA71V strains, even after 48 hours. A small but statistically significant population of p30+ and p72+ M2

cell population was observed at 24 hours. At each time point (0, 24, 48, 72 hours), the viral copies numbers in culture supernatants were evaluated. Very low BA71V viral copy numbers were detected in culture supernatants. Nevertheless, a statistically significant higher number of viral copies was observed in moM2 culture supernatants compared to other macrophage subsets at 48 and 72 hours pi. In contrast, high levels of 22653/14 viral copies detected in the supernatants from all three macrophage subsets by 72 hours.

3.3 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 (Figure 5), analysing differences between BA71V and 22653/14 strains and comparing infected cells with both uninfected bystander cells and mock-infected cells. Due to the fact that the numbers of $p72^{+}$ monocytes and moM1 after BA71V infection using an MOI of 1 were less than 10%, it was unreliable to analyse BA71V infected cells for these subsets. First we 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. We observed no differences in marker expression levels between the 'mock Vero' or 'mock monocyte-macrophage' infection and cells cultured in monocyte medium (data not shown), and consequently all mock-infection controls were performed using monocyte medium. As displayed in Figure 5 and Supplementary Figure S2, ASFV infection down-regulated the expression of CD16 in porcine monocytes and macrophage subsets. Statistically significant differences were observed between infected and both uninfected bystander and mock-infected cells. 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 5 and

Supplementary Figure S3). Very little difference in percentages of MHC II^+ cells were also observed between infected and bystander monocytes and moM2 (Figure 5 and Supplementary Figure S4). As displayed in Figure 5 and Supplementary Figure S5, for both strains infected and bystander monocytes/macrophages displayed similar percentages of $CD163^+$ cells. However after infection with BA71V, but not 22653/14, both bystander and infected monocytes, moMΦ and moM2 displayed lower levels of CD163 compared to the mock-infected controls.

3.4 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 we assessed if mock-infection with clarified Vero cell or monocyte-macrophage cell lysates for 18 hours induced cytokine responses. Since we observed no differences between co-culture with 'mock Vero' or 'mock monocyte-macrophage' and monocytes medium in the levels of GM-CSF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, TNF-α (data not shown), 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-α 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-α, IL-1β and IL-18: moM1 released higher levels of IL1-α, IL-1β and IL18 in response to BA71V infection compared to uninfected control (Figure 6). Differences were also observed in the levels of IL-1α between mock-infected and BA71V-infected monocytes and in the levels of IL-1β between mock-infected and BA71V-infected moM2 and monocytes, albeit the latter difference was without statistical significance.

Discussion

This study aimed to provide a better understanding of the interaction of monocytes and derived macrophage subsets with ASFV. Macrophages in antithetic polarized states might react differently to ASFV infection, with further differences between isolates of diverse virulence, influencing the development of disease rather than acquisition of protective immunity.

We first focused on determining the susceptibility of monocytes and derived macrophage subsets to ASFV infection. 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). ASFV 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. 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 full replicative cycle in monocytes, which *in vivo* probably results in its ability to disseminate within the host. ASFV 22653/14 presented also greater ability to infect moM1 compared to the avirulent strain and pre-treatment of macrophages with IFN-γ or LPS alone resulted in an increased resistance to BA71V but not 22653/14 infection. A previous study reported that IFN-γ 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). Nevertheless, we observed differences between strains varying in virulence. A recent study reported similar results in the sensitivity of ASFV strains to IFN-α. Pre-treatment of alveolar macrophages with IFN-α reduced replication of attenuated but not virulent ASFV strains (Golding et al., 2016). BA71V and other attenuated strains present deletions in the region containing 360/530 multi-gene families (Zsak et al., 2001), which suppress type I IFN responses (Afonso et al., 2004) and might also interfere with antiviral genes

induced by IFN-γ. Our data also showed that pre-treatment of macrophages with LPS alone resulted in an increased resistance to BA71V-infection but not 22653/14. It may be hypothesised that LPS engagement with TLR4 leads to IFN-β 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 studies on the susceptibility of activated monocyte-derived macrophages to porcine reproductive and respiratory syndrome virus (PRRSV): IFN-γ activation almost completely prevented infection by a low virulence PRRSV strain and to a lesser extent with virulent field isolates (Garcia-Nicolas et al., 2014), whereas M2 polarization did not affect susceptibility to PRRSV infection (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 cells were infected, suggesting that activation only delayed and did not inhibit 22653/14 replication in these cells. Interestingly, using an MOI of 0.01 very low levels of ASFV viral proteins were detected after BA71V-infection. Probably using a low MOI replication of the avirulent strain in macrophages is inhibited soon after infection, 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 24 hours pi between BA71V-infected macrophage subsets: moM2 display higher intracellular levels of ASFV proteins than moMΦ and moM1, suggesting that alternative macrophage activation is negatively correlated with ASFV resistance.

The effect of ASFV-infection on surface markers expression was next investigated. In accordance with previous publications, ASFV down-regulated expression of the low affinity Fc receptor CD16 on monocytes and macrophages (Sánchez-Torres et al., 2013, Lithgow et al., 2014), and this effect has not been lost during BA71V attenuation. The effect of ASFV on MHC class I and II expression

was assessed since this can affect antigen presentation and thus the development of adaptive immune responses. 22653/14-infected monocytes/macrophages presented similar MHC-I levels to bystander or mock-infected cells, instead BA71V-infected moMΦ and moM2 displayed a lower percentage of MHC I^+ 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. Only slight 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).

Finally, we characterised the cytokine responses of porcine monocyte and derived macrophage substes to ASFV infection. Previous studies reported that macrophage infection with ASFV resulted in an enhanced expression of mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, 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-α in culture supernatants (Gómez del Moral et al., 1999, Zhang *et al.* 2006) and in accordance to the latter study no TNF-α 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-1β release was observed in response to ASFV infection, but we observed higher IL-1β levels from monocytes/moM1 mainly in response to the avirulent strain. Also higher levels of IL-1α were

detected after BA71V infection in comparison to 22653/14. IL-1β has a pro-apoptotic role (Friedlander et al., 1996), so it could be speculated that the release of IL-1β after BA71V infection may contribute to the early monocyte/macrophage apoptosis, which *in vivo* probably limits viral replication and pro-inflammatory dysregulation. The virulent 22653/14 might have developed mechanism to inhibit IL-1β release, promoting 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, whereas we did not observe IL-12 production after ASFV. This difference might related to different isolates tested, or because IL-12p40 is also a subunit of IL-23 (Duque and Descoteaux, 2014), which might be released in response to ASFV infection. Nevertheless, we detected increase in the levels of another potent IFN-γ inducer in the supernatants of BA71V infected macrophages: IL-18 (Duque and Descoteaux, 2014). Its release might be implicated in the acquisition of a TH1 cell response, correlated to protection against ASFV (Takamatsu et al., 2013), and the virulent 22653/14 might have developed mechanism to counteract the induction of this response.

Conclusion

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 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.

Conflict of interest statement

The authors declare no conflict of interests.

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Figure Captions

Figure 1. Differentiation of monocytes into macrophages and their classical and alternative activation. Differentiation of monocytes into macrophages and their activation was morphologically assessed by confocal microscopy (original magnification 40X) (A). Differences between monocytes, moMΦ, moM1 and moM2 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 +/- SD from three independent experiments utilizing different animals are shown. Values were compared using a one-way ANOVA followed by a Kruskal-Wallis test; ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Figure 2. Susceptibility to infection by monocytes and derived macrophage subsets. Monocytes, moMΦ, moM1 and moM2 were infected with the avirulent BA71V or with the virulent 22653/14 strains using an MOI of 1, alongside mock-infected controls. 18 hours pi percentages of $p72^+$ 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. 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 5 independent experiments utilizing different animals are shown. For each conditions (MOCK, BA71V and 22653/14) values of moM1 and moM2 were compared to the corresponding un-activated control (moMΦ), using a one-way ANOVA followed by a Kruskal-Wallis test; ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Figure 3. Different expression of early and late proteins in ASFV-infected monocytes and macrophage subsets. In panel A expression of early protein p30 is represented. Monocytes,

moMΦ, moM1 and moM2 were infected with the avirulent BA71V or the virulent 22653/14 strain using an MOI of 1, alongside mock-infected controls. 18 hours pi percentages of $p30^+$ cells and MFI of p30 were evaluated using flow cytometry. The mean data +/- SD from three independent experiments utilizing different animals are shown. In panel B 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 22653/14. 18 hours pi 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 BA71V-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. In both panels A and B, 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. In panel A, for each population differences in the p30 expression between BA71V and 22653/14 were compared using a Mann-Whitney test.

Figure 4. Growth characteristic of the ASFV strains on different macrophage subsets. moMΦ, moM1 and moM2 were infected with the avirulent BA71V or the virulent 22653/14 ASFV strain, using an MOI of 0.01. At 0, 24, 48, 72 hours pi triplicate samples were collected: intracytoplasmic levels of p30 and p72 were assessed by flow cytometry and 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 those of moMΦ, using a one-way ANOVA followed by a Kruskal-Wallis test; ***p<0.001, **p<0.01, *p<0.05.

Figure 5. Effect of ASFV on the surface markers expression of monocytes and macrophage subsets. Monocytes, moMΦ, moM1 and moM2 were infected with the attenuated BA71V or the virulent 22653/14 strain using an MOI 1, alongside mock-infected controls. 18 hours pi, expression of p72 and surface markers (CD16, MHC II, MHC I, CD163) were evaluated using flow cytometry. The mean data +/- SD from three independent experiments utilizing different animals are shown. 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 mock-infected control, using a one-way ANOVA followed by a Kruskal-Wallis test; ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Figure 6. Investigation of cytokines release by monocytes and macrophage subsets in response to ASFV infection. Monocytes, moMΦ, moM1 and moM2 were infected with the attenuated BA71V or the virulent 22653/14 strain using an MOI of 1, alongside mock-infected controls. 18 hours pi, the levels of IL-1α, IL-1β and IL-18 in culture supernatants were evaluated. The mean data +/- SD from three independent experiments utilizing different animals are shown. Values were compared using a one-way ANOVA followed by a Kruskal-Wallis test; ***$p<0.001$, **$p<0.01$, *$p<0.05$.