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Interaction of porcine monocyte-derived dendritic cells with African swine fever viruses of diverse virulence

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ABSTRACT

African swine fever (ASF) is a devastating disease for which there is no vaccine. The ASF virus (ASFV) can infect dendritic cell (DC), but despite the critical role these cells play in induction of adaptive immunity, few studies investigated their response to ASFV infection. We characterized the *in vitro* interactions of porcine monocyte-derived DCs (moDC) with a virulent (22653/14), a low virulent (NH/P68) and an avirulent (BA71V) ASFV strain. At a high multiplicity of infection (MOI = 1), all three strains infected immature moDC. Maturation of moDC, with IFN-α/TNF-α, increased susceptibility to infection with 22653/14 and other virulent strains, but reduced susceptibility to NH/P68 and BA71V. The reduced moDC susceptibility to BA71V/NH/P68 was IFN-α mediated, whereas increased susceptibility to 22653/14 was induced by TNF-α. Using an MOI of 0.01, we observed that BA71V replicated less efficiently in moDC compared to the other isolates and we detected increased replication of NH/P68 compared to 22653/14. We observed that BA71V and NH/P68, but not 22653/14, downregulated expression of MHC class I on infected cells. All three strains decreased CD16 expression on moDC, whereas ASFV infection resulted in CD80/86 down-regulation and MHC class II DR up-regulation on mature moDC. None of the tested strains induced a strong cytokine response to ASFV and only modest IL-1α was released after BA71V infection. Overall our results revealed differences between strains and suggest that ASFV has evolved mechanisms to replicate covertly in inflammatory DC, which likely impairs the induction of an effective immune response.

1. Introduction

African swine fever (ASF) is a contagious and often fatal disease of domestic and wild pigs, for which there is no vaccine or treatment available (Sanchez-Vizcaino et al., 2006). It is currently present in many sub-Saharan African countries, Russian Federation, Trans-Caucasus, East and Central 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 infects immune cells of the myeloid lineage. While monocytes and macrophages are considered the primary target cells (Sánchez-Cordón et al., 2008), ASFV can infect dendritic cells (DC) (Gregg et al., 1995a). DC are the most potent antigen-presenting cells responsible for the induction of adaptive immune responses against pathogens (Banchereau and Steinman, 1998). After detection and antigen uptake, DCs migrate into secondary lymphoid tissues to present the processed antigen to T lymphocytes. A maturation step, often achieved by the engagement of pattern recognition receptors with pathogen-associated molecular patterns, is essential for DC to execute their role in an efficient manner (McCullough and Summerfield, 2009). DC can be broadly divided into two subpopulations: conventional DC (cDC), whose main function is antigen-presentation, and plasmacytoid DC (pDC), specialized in rapidly secreting large amounts of IFN-α (McCullough and Summerfield, 2009). Due to the low frequency of DC in the circulation and lymphoid organs, myeloid DC can be generated *in vitro* from

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monocytes, through incubation with GM-CSF and IL-4 (Carrasco et al., 2001). These monocyte-derived DC (moDC) represent a third population of DC, termed inflammatory DC, which are thought to differentiate *in vivo* during infection and inflammation (Hespel and Moser, 2012). Porcine moDC present a $CD1^+CD14^{+/-}CD16^+CD80/86^+CD172^+MHC\text{-}I^+MHC\text{-}II^+CD4\text{-}CD3\text{-}CD8\text{-}$ phenotype, possess a strong T-cell stimulatory capacity, and are in an immature state (Carrasco et al., 2001, McCullough and Summerfield, 2009). Stimulation of moDC with recombinant IFN-α and TNF-α induces phenotypic maturation, with increased MHC II and CD80/86 surface expression (Carrasco et al., 2004).

To date, few studies have analysed the interaction of ASFV with DC and none have assessed moDC/ASFV interaction. Previous studies have demonstrated that ASFV can infect dermal DC, resulting in interference with subsequent infection with foot-and-mouth disease virus (Gregg et al., 1995a). It has been observed that virulent ASFV is able to infect cDC *in vivo* leading to a reduction in the number of interdigitating cDC in mandibular lymph nodes from three days post-infection, which might limit the development of a protective immune response (Gregg et al., 1995b). A recent study reported that *in vitro* infection of enriched blood DC with ASFV induced high levels of type I interferon (IFN), suggesting that ASFV-infected pDC could be a potential source of type I IFN in animals undergoing acute ASF (Golding et al., 2016). Infection of DC with ASFV is likely to play a critical role in modulating the immune response. Differences might exist also between ASFV strains varying in virulence, since we and others have demonstrated that macrophage cytokine responses differ between attenuated and virulent strains (Gil et al., 2008, Reis et al., 2016, Franzoni et al., 2017).

Considering the central role of DC in initiating a protective immune defence against pathogens, we hypothesized that moDC would respond differently to ASFV strains of spanning the spectrum of virulence, with more pathogenic strains possessing mechanisms to modulate moDC responses in order to evade immune defences. To address these issues, we conducted a thorough *in vitro* analysis of the interaction of immature and mature moDC with a virulent (22653/14), a low virulent (NH/P68) and an avirulent (BA71V) ASFV strain.

2. Materials and methods

2.1. Animals

Healthy ASFV-naïve cross-bred pigs (*Sus scrofa*), 4–12 month old, were used in the study. The ASFV negative status of the animals was confirmed by real-time PCR (King et al., 2003), a commercial antibody ELISA test (Ingenasa, Madrid, Spain) and an immunoblotting test (OIE, 2012). The animals were housed at the Experiment Station of IZS della Sardegna (Sassari, Italy) and animal housing and handling procedures were performed in accordance with the local ethics committee, in agreement with the guide of use of laboratory animals issued by 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 2.0 × 45 mm 14-gauge needle (Delta Med, Mantova, Italy).

2.2. Viruses

The tissue-culture adapted 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 in 96-well plates, followed by observation for cytopathic effect and crystal violet staining to identify infection rates, as previously described (Carrascosa et al., 2011). Mock-virus supernatants were prepared in identical manner from uninfected Vero cell cultures ('Mock Vero'). The virulent ASFV field strains 22653/14, 28784/16 (Exotic Disease Laboratory ASF Virus Archive, IZS of Sardinia, Sassari, Italy), Italy/83 (kindly provided by Dr Gian Mario De Mia, Italian ASF Reference Laboratory, CEREP, Italy), Arm07 and Ken06.Bus (kindly provided by the EU ASF Reference Laboratory CISA-INIA, Madrid, Spain), and the low virulent NH/P68 (kindly provided by the EU ASF Reference Laboratory CISA-INIA, Madrid, Spain) were propagated *in vitro* by inoculation of sub-confluent monolayers of porcine monocytes/macrophages, as previously described (Malmquist and Hay, 1960). Titres of 22653/14, 28784/16, Italy/83, Arm07, Ken06.Bus ASFV strains were obtained by serial dilution of the virus suspension on monocyte/macrophages in 96-well plates followed by haemadsorption (Malmquist and Hay, 1960). Viral titres of 22653/14 and the non-haemoadsorbing NH/P68 were obtained by serial dilution of the virus suspension on monocyte/macrophages in 96-well plates followed by immunofluorescence staining (OIE, 2012). In brief, medium was removed from each well, cell monolayers washed with PBS and fixed by addition of 30% acetone-70% methanol for 15 min. After two washes with PBS, cells were incubated 1 h at 37 °C 5% CO_2 with FITC-conjugated anti-ASFV polyclonal antibody (kindly provided by Dr Gian Mario De Mia, Italian ASF Reference Laboratory, CEREP, Italy) diluted 1:200 in PBS and then washed twice with PBS before viewing under a fluorescent microscope (OIE, 2012). Viral titres were determined using the Spearman–Kärber formula. Mock-virus supernatants were prepared in identical manner from uninfected monocyte/macrophage cultures ('Mock macro'). Partial p72 gene characterization demonstrated that the Sardinian field strains 28784/16 and Italy/83 belonged to genotype I (Bastos et al., 2003). Additional genotype I strains tested were BA71V, NH/P68, 22653/14 (Gallardo et al., 2009, Franzoni et al., 2017), whereas Arm07 and Ken06.Bus belong to genotype II and IX, respectively (Gallardo et al., 2014, Gallardo et al., 2009).

2.3. Generation of porcine monocyte-derived dendritic cells and activation

Porcine leukocytes were obtained from heparinized blood, as previously described with slight modifications (OIE, 2012). In brief, blood was centrifuged at 700 g for 30 min at 4 °C with no breaks, buffy coat was collected, washed first in red blood cell lysis buffer (distilled water with 0.5 mM Na EDTA, 310 mM NH_4Cl, 24 mM $NaHCO_3$) and then in PBS. Leukocytes were then re-suspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (monocyte medium). Monocytes were enriched using flasks pre-incubated with autologous plasma, as previously described (Franzoni et al., 2017). Monocytes were detached by placing flasks on ice for 1 h, centrifuged, counted and viability was assessed using a Countess Automated Cell Counter (Thermo Fisher Scientific). Cells were re-suspended in monocyte medium and were seeded in a 12 well plates (Greiner CELLSTAR, Sigma) (5×10^5 live cells/well, 1.5 ml/well).

To differentiate monocytes into dendritic cells (moDC), cells were cultured for 4 days at 37 °C 5% CO_2 in monocyte medium supplemented with 20 ng/ml of recombinant porcine GM-CSF and 50 ng/ml of recombinant porcine IL-4 (both R&D Systems, Minneapolis, USA) (Kyrova et al., 2014). At day 4 media was replaced and cells were cultured at 37 °C 5% CO_2 for further 2 days in monocyte medium supplemented with IL-4/GM-CSF alone or with 1000 U/ml recombinant porcine IFN-α (PBL Assay Science, Piscataway, NJ, USA) and 10 ng/ml recombinant porcine TNF-α (R&D System) to induce maturation (Carrasco et al., 2004). In defined experiments, cells were activated using IFN-α or TNF-α alone.

2.4. Confocal microscopy

Differentiation of monocytes into moDC were observed by confocal microscopy, as previously described (Kyrova et al., 2014, Franzoni et al., 2017). Monocytes were cultured on two-well chamber slides (Thermo Fisher Scientific) and were stained immediately or differentiated in moDC and then left untreated or matured with IFN-α/TNF-α. Cells were fixed with 4% paraformaldehyde and labelled with