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

Giulia Franzoni[a,b,*], Simon P. Graham[c,d], Silvia Dei Giudici[b], Piero Bonelli[b], Giovannantonio Pilo[b], Antonio G. Anfossi[a], Marco Pittau[a], Paola S. Nicolussi[b], Alberto Laddomada[b], Annalisa Oggiano[b]

[a] Department of Veterinary Medicine, University of Sassari, Sassari, 07100, Italy
[b] Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, 07100, Italy
[c] School of Veterinary Medicine, University of Surrey, Guildford, GU2 7AL, United Kingdom
[d] The Pirbright Institute, Ash Road, Pirbright, GU24 0NF, United Kingdom

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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 Sardinian isolate (22653/14) and a tissue culture adapted avirulent strain (BA71V) of ASFV with porcine monocytes, unactivated (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.

1. 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

* Corresponding author at: Department of Veterinary Medicine, University of Sassari, Via Vienna, Sassari, 07100, Italy.
E-mail addresses: gfranzoni@uniss.it (G. Franzoni), s.graham@surrey.ac.uk (S.P. Graham), Silvia.DeiGiudici@izs-sardegna.it (S.D. Giudici), piero.bonelli@izs-sardegna.it (P. Bonelli), gantonio.pilo@izs-sardegna.it (G. Pilo), aanfossi@uniss.it (A.G. Anfossi), pittau@uniss.it (M. Pittau), paola.nicolussi@izs-sardegna.it (P.S. Nicolussi), alberto.laddomada@izs-sardegna.it (A. Laddomada), annalisa.oggiano@izs-sardegna.it (A. Oggiano).

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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 polarization 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 hypothesised 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, unactivated and activated monocyte-derived macrophages with a virulent (22653/14) and a non-pathogenic (BA71V) ASFV strain.

2. 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 2.0 × 45 mm 14-gauge needle (Delta Med, Mantova, Italy).

2.2. Viruses

The avirulent ASFV BA71 V 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 min 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 min 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 h 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 h 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 h. Detached cells were centrifuged at 200 x *g* for 8 min 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). 7–10 × 10^5 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