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Review

Cellular immunity in ASFV responses

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ABSTRACT

African swine fever virus (ASFV) infection usually results in an acute haemorrhagic disease with a mortality rate approaching 100% in domestic pigs. However, pigs can survive infection with less-virulent isolates of ASFV and may become chronically infected. Surviving animals are resistant to challenge with homologous or, in some cases, closely related isolates of the virus indicating that pigs can develop protective immunity against ASFV. During asymptomatic, non-virulent ASFV infections natural killer cell activity increases in pigs, suggesting this cell type plays a role in ASFV immunity. Furthermore, depletion of $CD8^+$ lymphocytes from ASFV immune pigs demolishes protective immunity against related virulent viruses. This suggests that ASFV specific antibody alone is not sufficient for protection against ASFV infection and that there is an important role for the $CD8^+$ lymphocyte subset in ASFV protective immunity. These results were supported by DNA immunization studies, demonstrating a correlation between the protection afforded against lethal challenge and the detection of a large number of vaccine-induced antigen-specific $CD8^+$ T-cells. Peripheral blood mononuclear cells (PBMCs) from ASF immune pigs protected from clinical disease show higher proportions of ASFV specific $CD4^+CD8^{high+}$ double positive cytotoxic T cells than PBMCs from ASF immune but clinically diseased pig. The frequency of ASFV specific IFNγ producing T cells induced by immunization correlates to the degree of protection from ASFV challenge, and this may prove to be a useful indicator of any potential cross-protection against heterologous ASFV isolates.

Contents

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1. Introduction

As described in other chapters of this special issue, infection with ASFV may influence and/or compromise the immune responses to the virus in domestic pigs. ASFV mainly targets myeloid lineage cells, especially monocyte/macrophages and dendritic cells (Malmquist and Hay, 1960; Wardley and Wilkinson, 1977; González-Juarrero et al., 1992; Carrillo et al., 1994; Gregg et al., 1995a,b; Sánchez-Cordón et al., 2008), which are professional antigen presenting cells (APCs) and have critical roles in the immune system. These cells detect pathogen-associated molecular patterns (PAMPs) through their array of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), initiate immune responses, secrete cytokines/chemokines, present antigens and clear pathogens by phagocytosis. Another characteristic of ASF pathology is the extensive apoptosis of lymphocytes and lymphopenia (Sanchez-Vizcaino et al., 1981; Gómez-Villamandos et al., 1995; Ramiro-Ibáñez et al., 1996, 1997; Oura et al., 1998b; Salguero et al., 2004), which is likely to affect immune responses to ASFV. The ASFV genome encodes a large number of genes that have been identified as playing a role in host immune evasion including the NF-κB and NFAT inhibitor A238L (Neilan et al., 1997a; Miskin et al., 2000; Granja et al., 2006), the apoptosis inhibitors A179L (Brun et al., 1996; Revilla et al., 1997) and A224L (Neilan et al., 1997b; Nogal et al., 2001) and the protein phosphatase 1 activator DP71L (Afonso et al., 1998; Rivera et al., 2007). The multigene family 360 and 505 genes are believed to play a role in inhibiting the induction of IFN α/β (Neilan et al., 2002; Afonso et al., 2004) and the D96R (also referred to as UK) gene is also a potential immune evasion gene, although its mechanism of action is unclear (Zsak et al., 1998). Further information on these genes is available in an other chapter of this special issue. ASFV has a range of genes that are likely to have evolved to carefully manipulate its natural hosts (soft ticks, bushpigs and warthogs) in the sylvatic cycle in Africa. In fact, in stark contrast to the course of infection in domestic pigs, ASFV spread within bushpig tissues is not extensive (Oura et al., 1998a) and replication is generally at least two logs lower in the blood and tissues of bushpigs and warthogs than in domestic pigs (Thomson et al., 1980; Oura et al., 1998a). Furthermore bushpigs and warthogs show no clinical signs of disease. *In vitro* ASFV replicates similarly in cells derived from bushpigs and warthogs as it does in cells from domestic pigs (Anderson et al., 1998), and therefore it can be assumed that *in vivo*, the innate immune response in these animals may be sufficient to limit the replication and spread of ASFV. Unsurprisingly the cellular immune response in bushpigs and warthogs to ASFV has not been investigated in detail, however, like the domestic pig, ASFV infection of bushpigs resulted in immunity to genetically related viruses (Anderson et al., 1998). Sequence analysis of the ASFV isolates used in these experiments showed that genotype I virus could prevent viraemia from subsequent challenge with genotype I virus, but not to challenge with genotype VIII virus and *vice versa*, and neither genotype I nor genotype VIII prevented genotype XIX viraemia (accession numbers JX235333 and JX235334; Bastos et al., 2003).

ASFV infection of domestic pigs generally leads to an acute/peracute haemorrhagic disease with an extremely high chance of mortality, possibly due to the fact that the animals do not have time to develop sufficient immunity to recover from the infection. Therefore, the first question that this review will consider is whether ASFV immunity is simply a matter of time? In other words will ASFV susceptible mammalian hosts develop cellular immunity to ASFV if the animals survive for long enough?

Theoretically immune responses can occur to any antigen, but not all immune responses contribute to protective immunity, and some immune responses may be responsible for pathology. As mentioned above, ASFV infects APCs, codes for a number of immune evasion genes, and induces lymphocyte apoptosis. Thus, the second question that this review will address is, if pigs develop anti-ASFV cellular immune response(s), will these cellular immune responses be sufficient to prevent or reduce clinical disease or, alternatively, does the immune response contribute to ASF pathology? Other important considerations are whether it is possible to generate an immune response that is able to clear the virus completely and so prevent persistent ASFV infection and whether it is possible to induce protective (cellular) immunity against ASFV through the artificial delivery of antigen to naive pigs? If the answer to these questions is "no" then a conventional vaccine against ASF is unlikely to be feasible. This review will mainly focus on the topics related to these questions.

Cytokines are known to be important drivers of the immune response, however to date the number of *in vivo* studies reported is limited and have concentrated on infection with virulent rather than non-virulent ASFV isolates. *In vitro* non-virulent strains induce higher levels of INFα, IL-6, IL-12p40 and TNFα expression than related virulent isolates when analyzed by PCR (Gómez del Moral et al., 1999; Gil et al., 2003, 2008; Afonso et al., 2004; Zhang et al., 2006). In addition, IL-12p40 and IFNα/β are secreted after *in vitro* infection with non-virulent isolates (Afonso et al., 2004; Gil et al., 2008), while TNFα is secreted after infection by both non-virulent and virulent viruses (Gómez del Moral et al., 1999; Gil et al., 2008). It is important to note that *in vivo* large amounts of TNFα and IFNα/β can be detected in the serum of animals infected with virulent ASFV isolates (Gómez del Moral et al., 1999; Salguero et al., 2002; Karalyan et al., 2012) and that intracellular TNFα can be detected extensively in lymphoid tissues (Carrasco et al., 2002; Gómez del Moral et al., 1999; Salguero et al., 2002; Fernández de Marco et al., 2007). Conversely, IL-1β is secreted after *in vitro* infection with virulent ASFV (Zhang et al., 2006) and can be detected readily in serum and tissues (Carrasco et al., 2002; Salguero et al., 2002). These discrepancies highlight the difficulty in extrapolating *in vitro* data to what happens *in vivo* and further studies are required to determine if the cytokines observed *in vitro* can be detected in and around the limited sites of replication of non-virulent viruses *in vivo*. Further discussion of cytokines in this review will be limited to where they may directly influence the outcome of cellular immune responses to ASFV. In addition ASFV causes the modulation of porcine lymphocyte function either directly or indirectly through the actions of cytokines, however this subject is not the focus of this review and has been covered elsewhere (Martins and Leitão, 1994).

There are constraints to studying cellular immune responses to ASFV, principally revolving around the host and host cell specificity. Attempts have been made to use small animals as models in ASFV infection studies, such as SCID or SCID-Beige mice reconstituted with porcine lymphocytes (Revilla et al., 1994) or with bone marrow cells (Takamatsu et al., 1999), or rodents used for immunization/vaccination to detect antibodies (Forman et al., 1982;

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Argilaguet et al., 2011). The high species-specificity of ASFV *in vivo* however contrasts with the relative ease of adapting the virus to grow *in vitro* in established cell lines from other animal species, although this adaptation generally leads to a loss of virulence in pigs. Several attempts to adapt ASFV to replicate in mice have failed, including preliminary attempts using IFN-α/β receptor knockout mice (Rodriguez, Brun and Sevilla, unpublished results), despite the fact that this mouse model is able to sustain *in vivo* replication of other arboviruses such as Rift Valley fever virus (Lorenzo et al., 2010) and bluetongue virus (Calvo-Pinilla et al., 2009). Therefore, experiments to reliably assess cellular immune responses against ASFV have to be performed in the domestic pig. As virulent ASFV isolates usually kill domestic pigs relatively quickly after infection, immunological studies use moderate or non-virulent isolates (such as Dominican Republic, Malta 78) or non-haemoabsorbing, non-virulent isolates (such as NH/P68, OURT88/3), or viruses that have been partially attenuated by passage through tissue culture such as E75a. Although these isolates are now known to be genetically related (genotype I), their pathogenesis and induction of immunity are not uniform. In addition, the breed, age and condition of the pigs used in each experiment were also different; therefore, the results and observations from the different experiments that we review in this chapter may not be directly comparable.

2. Demonstration of ASFV specific porcine cellular immune responses

2.1. ASFV-specific lymphocyte proliferation and helper T cell

As discussed in detail below, pigs that survive inoculation with ASFV isolates with reduced virulence can be protected from challenge with the same or closely related virulent viruses (Malmquist, 1963; Sanchez-Vizcaino et al., 1981; Ruiz Gonzalvo et al., 1983; Martins et al., 1988; Scholl et al., 1989; Canals et al., 1992; Revilla et al., 1992; Martins et al., 1993; Alonso et al., 1997; Leitão et al., 2001; Jenson et al., 2000; 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. It seems therefore that ASFV susceptible mammalian hosts are able to develop protective immunity to ASFV infection, however, the role that cellular immunity plays in this protection still needs to be addressed.

The antigen specific T cell proliferation assay is a simple method used to demonstrate the presence of antigen specific memory T cells in immunized animals and thus was used in ASFV studies from the early 1980s. The first demonstration of ASFV specific lymphocyte proliferation was described by Wardley and Wilkinson (1980) after 10 days of infection 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. Although the specific proliferation (originally described as blastogenic index) was very low, PBMCs from pigs inoculated with viruses that had been partially attenuated by passage through tissue culture proliferated in response to UV-inactivated virus *in vitro*, reaching a maximum response at 4 weeks post infection (stimulation index = 2.1 ± 0.75; against base line of 0.0173 ± 0.01) (Sanchez-Vizcaino et al., 1981). These results indicated that moderate or non-virulent strains of ASFV infection induced ASFV specific memory T cell proliferation against homologous virus. As the proliferation assay alone does not reveal the functions of proliferated lymphocytes, from the end of 1980s to the 1990s more detailed studies, combined with proliferation assays, were published (Casal et al., 1987; Scholl et al., 1989; Canals et al., 1992; Revilla et al., 1992; Alonso et al., 1997). These publications reported cross-reactivity in proliferation assays between ASFV isolates and the production of IL-2 and IFNγ in ASFV after PBMCs from recovered pigs were cultured with virus or virus infected cells *ex vivo*. Canals et al. (1992) also reported that ASFV specific proliferation were blocked equally by anti-CD4 and CD8 mAbs when stimulated with infectious virus, whereas when stimulated with UV-inactivated virus specific proliferations were only 60% inhibited by anti-CD8 mAb, but completely blocked by anti-CD4 mAb. The flow cytometry analysis demonstrated that UV-inactivated virus stimulated cultures induced both $CD4^+$ and $CD8^+$ subsets but also results indicated $CD4^+$ $CD8^+$ double positive lymphocytes were present in such cultures. As porcine memory helper T cells are described as phenotypes of $CD4^+CD8^{lo}$ (or $CD4^+CD8\alpha\alpha$) (Yang and Parkhouse, 1997; Zuckermann, 1999) and from the cytokines produced and proliferation blocking experiments with antibodies, at least ASFV specific memory helper T cells were likely to be induced. In fact, Casal et al. (1987) demonstrated T cell dependent synthesis of ASFV specific antibodies *in vitro* using PBMCs from Ba71 immune pigs. The maximum antibody production *in vitro* was demonstrated at 4 day after the peak of the proliferation response, and by removing T cells from the PBMCs by rosetting or anti-CD4 mAb with complement treatment, the antibody synthesis *in vitro* was demolished.

2.2. In vivo depletion of $CD8^+$ lymphocytes abrogates protective immunity to ASF

The critical importance of $CD8^+$ lymphocytes on protective immunity to ASF was demonstrated by depleting $CD8^+$ lymphocytes from ASF immune pigs *in vivo* (Oura et al., 2005). Pigs immune to ASF were generated by inoculating two sets of outbred pigs with the non-virulent OURT88/3 isolate of ASFV. $CD8^+$ lymphocytes were depleted from these pigs by inoculating ascites containing anti-CD8α mAb (11/295/33; IgG2a) intravenously for 5 or 6 days (between 31 and 36 days post OURT88/3 inoculation) and the pigs were challenged with the virulent virus OURT88/1 on the 2nd day of mAb inoculation. Pigs that were effectively depleted of $CD8^+$ lymphocytes demonstrated severe clinical disease with high viraemia and were required to be euthanized, whereas isotype control mAb inoculated pigs were completely protected from OURT88/1 challenge. As CD8α is expressed on many lymphocyte subsets in pigs, Oura et al. (2005) next attempted to deplete $CD8\beta^+$ lymphocyte subsets by inoculating anti-CD8β mAb PPT22 (IgG1) in ASF immune pigs. However IgG1 is not an ideal antibody isotype for depletion *in vivo*, and only one out of 7 pigs showed a significant depletion of $CD8\beta^+$ lymphocytes. This pig died with acute ASF. The rest of the pigs in which there was no depletion/reduction of CD8β cells were completely protected from challenge. This publication demonstrated the importance of cell mediated immunity in protection from ASFV, especially emphasizing the involvement of lymphocyte subset(s) that express CD8 in protection. It also showed that antibody, developed through the inoculation of non-virulent ASFV OURT88/3 alone, is not sufficient for protection to virulent OURT88/1 challenge.

2.3. Cytotoxic T lymphocytes (CTL)

CTL play a critical role in protection against intracellular pathogens especially virus infection (Barry and Bleackley, 2002) including haemorrhagic fever caused by Filoviruses (Warfield and Olinger, 2011), however they are also known to cause pathology (Barry and Bleackley, 2002; Iannacone et al., 2007). CTL also play an important role in antimicrobial immunity through the secretion of cytokines such as IFNγ and TNF (Wong and Pamer, 2003). As CD8 molecules on the CTL interact with MHC class I, which present peptides from cytosolic pathogens to T cell receptors (TCR), CD8 is an important marker for CTL. However, as CD8 is also expressed on NK cells, NKT cells, subset of γδ T cells and memory helper T cells

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in pigs, CD8 is not a decisive marker of CTL in pigs. Three phenotypically distinguishable porcine CTLs have been reported. These are CD8 single positive, $CD4^+CD8^{dull+}$ (De Bruin et al., 2000) and $CD4^{hi+}CD8^{hi+}$ (Denyer et al., 2006).

ASFV specific cytotoxic lymphocytes were first described in pigs infected with a virulent Uganda isolate of ASFV. Autologous testis cells infected with tissue culture adapted ASFV Uganda virus were used as target cells (Norley and Wardley, 1984). Cytotoxicity against ASFV, but not Aujeszky's disease virus (suid herpesvirus-1), infected cells was observed in PBMCs from 7 to 8 dpi, however the cytotoxicity was not restricted to autologous target cells and the phenotype of the cytotoxic lymphocyte(s) was not identified. As none of the pigs survived virulent Uganda infection, the *in vitro* induction of effector CTL from memory CTL was not tested. In contrast, later experiments (Martins et al., 1988, 1993; Scholl et al., 1989) examined ASFV specific cytotoxic T cells using MHC defined (designated as swine leukocyte antigen [SLA]) inbred miniature swine (SLA aa, cc and dd haplotypes) that had recovered from the non-haemabsorbing ASFV isolate NH/P68. Effector CTLs were induced from memory CTLs recovered from PBMCs by stimulation *in vitro* with NH/P68 virus using blood derived macrophages as target cells. Cytotoxicity was higher in the NH/P68 infected macrophages of the same haplotype, and was blocked by anti-CD8 mAb, but not by anti-CD4 mAb, indicating the involvement of SLA Class I antigen. Cells infected with the Tengani ASFV isolate were killed less effectively than those infected with NH/P68 or the related isolate Lisbon 60. ASFV specific lysis was lost after 4 days of *in vitro* stimulation with antigen, thus the authors suggested that the loss of SLA restricted lysis might have been caused by induction of lymphokine activated killer (LAK) cells. The phenotype(s) of CTL or possible LAK cell was not then examined. Such promiscuous killing was also observed by stimulating lymphocytes with virus for 7 days (Scholl et al., 1989). The presence of virus specific, but non-MHC-restricted, cytotoxic T cells has been reported for other swine diseases (Saalmüller et al., 1994, 1999; Pauly et al., 1996) with CD5 and/or CD6 negative CTLs being identified as causing the non-MHC restricted cytotoxicity in the pigs (Saalmüller et al., 1994, 1999; Pauly et al., 1996; Denyer et al., 2006; Gerner et al., 2009). Thus the observations that cytoxicity was "not restricted to autologous target cells" and was "preferential for the same haplotype", with a "loss of specific lysis" and showed "promiscuous killing" in the above publications was likely to have been due to the presence of CD5 and/or CD6 negative non-MHC-restricted cytotoxic lymphocytes in the effector cell population. After the publication of Martins et al. (1993), most of the work identifying ASFV specific CTLs and the antigens that they recognize, was carried out using dd and/or cc haplotype inbred pigs (Alonso et al., 1997; Leitão et al., 1998, 2000; Jenson et al., 2000; Oura et al., 2005; Denyer et al., 2006). Alonso et al. (1997) demonstrated that only purified $CD8^+$ lymphocytes, but not purified $CD4^+$ lymphocytes, had CTL activity and this activity was blocked by anti-CD8 antibody, though nearly 20% of $CD8^+$ lymphocytes co-expressed CD4 and this proportion was increased after culture (14 days). Whether the $CD4^+CD8^+$ double positive population of cells was involved in cytotoxic activity was discussed but not examined (Alonso et al., 1997). Oura et al. (2005) demonstrated that ASFV immune cc inbred pigs, generated by non-virulent ASFV isolate OURT88/3 inoculation, were not always completely protected from virulent OURT88/1 challenge as they developed transient fever and viraemia that coincided with an increase in the circulating $CD8\beta^+$ lymphocyte subset. It is likely that this subpopulation of $CD8^+$ lymphocytes had a conventional CTL phenotype, as they expressed CD8β chain to form a $CD8\alpha\beta$ heterodimer (Yang and Parkhouse, 1997) and did not co-express CD4. The increase of circulating $CD8\beta^+$ lymphocytes was likely to have been associated with the viraemia, but the function of these $CD8\beta^+$ lymphocytes was not examined.

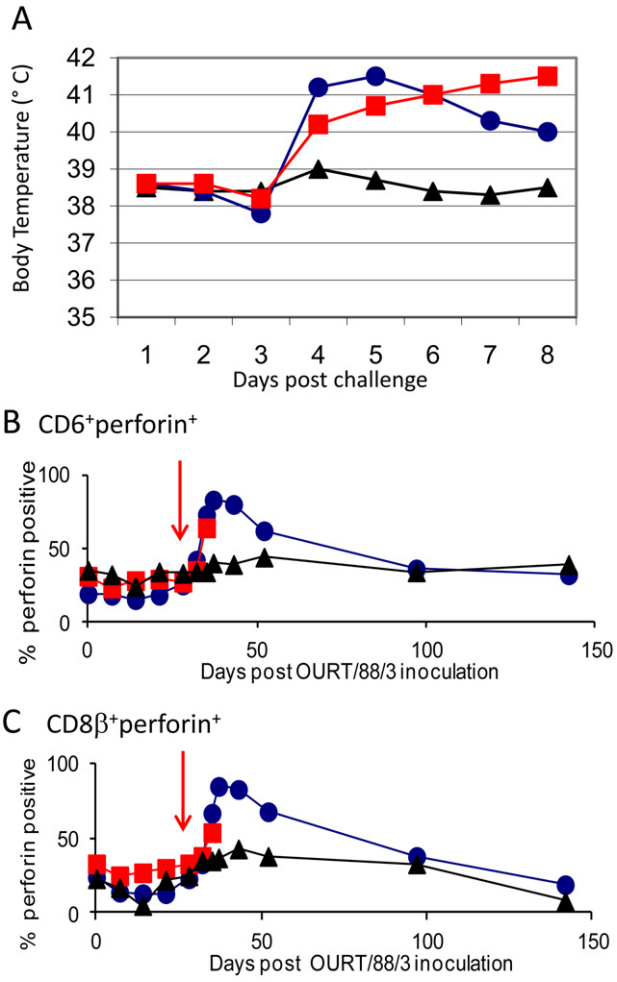

Fig. 1. High levels of circulating perforin$^+$ CTL in ASF immune pig with clinical signs of ASF. All ASF immune pigs, except one (UW81), were completely protected from OURT88/1 ASFV challenge, and no change in circulating lymphocyte subsets were observed (Black triangles in A, B, C, pig UW77). In contrast the diseased (see pyrexia in (A): Blue circles) immune pig (UW81) had high levels of circulating perforin$^+$CD6$^+$ (B) which co-expressed CD8β chain (C) but not express CD4 (not shown) indicating that these cells were a conventional CTL phenotype. One of non-immune pig challenged with OURT88/1which survived for 8 days (UW83: Red square) also showed increasing perforin$^+$CD6$^+$CD8β$^+$ CTL (B and C). The red arrows indicate the time of challenge with OURT88/1.

Functional CTLs possess cytotoxic granules, perforin and granzyme in their cytoplasmic compartment. Therefore, detecting these cytotoxic granules in cells could identify functionally active cytotoxic lymphocytes. By examining the expression of cytotoxic granules in porcine lymphocytes, Denyer et al. (2006) demonstrated the presence of two phenotypically distinctive ASFV specific CTLs, one conventional phenotype (perforin$^+$ $CD2^+CD3^+CD4^-CD5^+CD6^+CD8^+CD16^-$), and a second $CD4^+$ phenotype (perforin$^+$ $CD2^+CD3^+CD4^+CD5^+CD6^+CD8\alpha\beta^+CD16^-$). Both subsets were able to lyze ASFV infected syngeneic target cells.

2.4. Induction of double positive CTLs: critical for protection?

When conventional pigs (Large white × Landrace) were immunized by inoculating non-virulent ASFV isolate OURT88/3 (10^4 $TCID_{50}$, i.m.), and then challenged 4 weeks later with the related but virulent isolate OURT88/1 (10^4 HAD_{50}, i.m.), all immunized pigs were completely protected, except one pig (UW81) which exhibited transient pyrexia and viraemia (supplementary Table 1).

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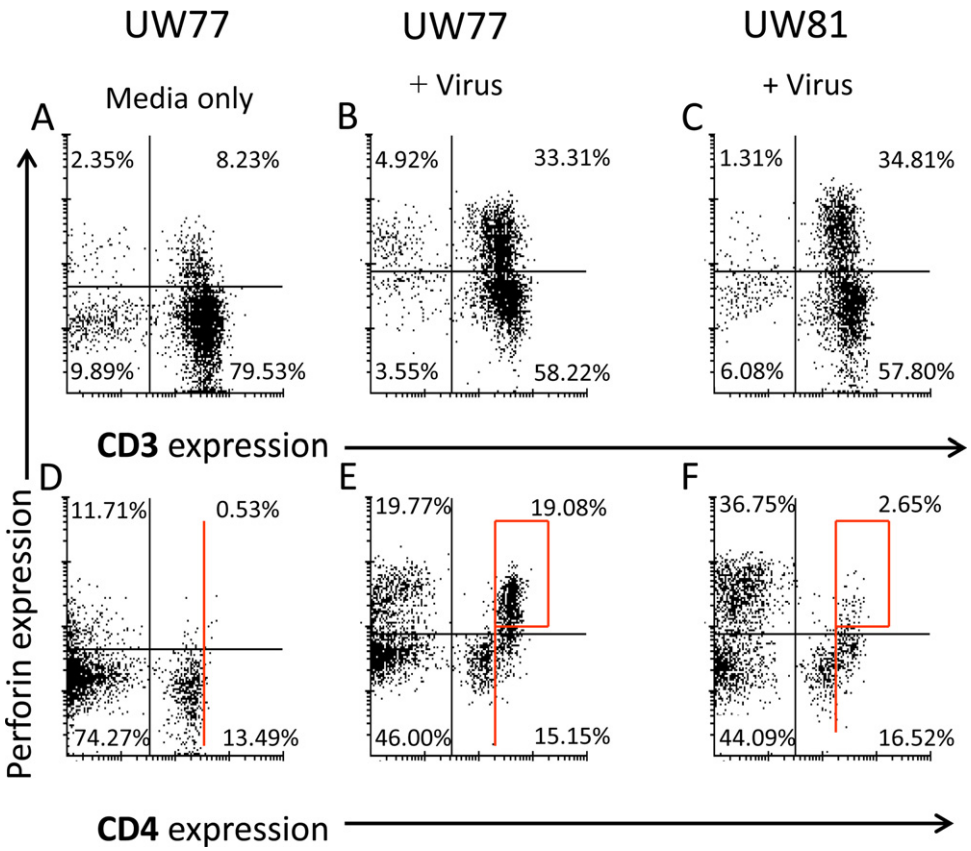

Fig. 2. Induction of double positive ($CD4^+CD8^+$) CTL *ex vivo* from pig UW77, but not from pig UW81. PBMCs from OURT88/3 immunized pigs, before challenge with OURT88/1, were stimulated *ex vivo* with live OURT88/1 virus for 5 days and the phenotypes of proliferated lymphocytes were analyzed by flow cytometry using Ki67 as a proliferation marker. Within proliferated lymphocytes ($Ki67^+$), $perforin^+$ lymphocytes were examined for their expression of surface markers. A, B, and C show expression of CD3 and perforin, D, E, F show expression of perforin and CD4. A and D are medium only controls from pig UW77. C and E are PBMCs from pig UW77 stimulated with ASFV, and C and F are PBMCs from pig UW81 stimulated with ASFV. The proportion of perforin positive T cells ($CD3^+$) between pigs UW77 (B) and UW81 (C) are very similar, whereas pig UW81 induced very low levels of $CD4^+$ CTL (F), compared to pig UW77 (E).

Similar to previous results (Oura et al., 2005), an increase of circulating $CD8^+$ lymphocytes was detected in pig UW81 at the onset of fever (Fig. 1), whereas no such increase was observed in the pigs that did not exhibit clinical signs or viraemia (compare pigs UW77 and UW81 in Fig. 1, also see supplementary Fig. 1). Interestingly, an increase in $CD8^+$ lymphocytes was also detected in one naïve pig (UW83) which survived for 8 dpi with OURT88/1 (Fig. 1). The phenotype of the $CD8^+$ lymphocyte subset was examined in greater detail and was found to be of the conventional CTL phenotype as it expressed perforin, CD5 and CD6, as well as CD3, but did not express CD4, nor CD16, a marker associated with MHC non-restricted cytotoxicity (Denyer et al., 2006).

Before challenge with OURT88/1, PBMCs from immune pigs were stimulated *in vitro* for 5 days with OURT88/1 and the phenotypes of proliferated lymphocytes were analyzed by flow cytometry using Ki67 as a proliferation marker. PBMCs from all pigs, including the pig showing transient fever/viraemia (pig UW81), proliferated to OURT88/1 to similar levels (40–50% of lymphocytes expressed $Ki67^+$, compared to less than 1% without ASFV stimulation). Within the $Ki67^+$ lymphocytes that proliferated to ASFV more than 90% expressed CD3 (and therefore were T cells) and about 37% expressed cytotoxic granule perforin (Fig. 2). There were no differences in the proportions seen in the diseased pig (UW81) and the protected pigs (see pig UW77 as an example, Fig. 2). However, when the phenotypes of the proliferating $perforin^+$ T cells were examined, only 6.7% of the perforin positive T cells from the diseased pig (UW81) were $CD4^+$, whereas 49.1% of the perforin positive T cells expressed CD4 with high intensity from the completely protected pig UW77 (Fig. 2). Put another way, more than half (55.7%) of the proliferating $CD4^+$ cells in the protected pigs (for example UW77) expressed perforin, in contrast to only 13.8% of proliferating $CD4^+$cells from the diseased pig (UW81) (Fig. 3). The composition of lymphocytes that proliferated after stimulation with ASFV from pigs UW77 and UW81 are summarized in Fig. 3, and include the phenotype of memory helper T cells, NK cells, γδ T cells and B cells.

Extrathymic CD4/CD8 double positive T cells have been described in a number of species (see reviews by Zuckermann, 1999; Sullivan et al., 2001). In addition to porcine memory helper T cells which are $CD4^+CD8\alpha\alpha^+$, it has been reported in both humans and mice that the activation of $CD8^+$ T cells through the T cell receptor activates expression of the CD4 gene and hence the cells become double positive T cells (Kitchen et al., 1998; Flamand et al., 1998). CD4 expression on $CD8^+$ T cells could function as an adhesion and chemotactic receptor (Kitchen et al., 2002), providing enhanced T cell function through stronger expression of IFNγ and Fas ligand (Kitchen et al., 2004) and hence modulate CTL function for optimal cell-mediated immunity to viral antigens (Kitchen et al., 2005). If CD4 expression in porcine $CD8^+$ T cells was induced by activation through ASFV *ex vivo* stimulation, as has been observed for other antigens in humans and mice, this would be consistent with the high proportion of double positive CTLs described above, and the observations made by Alonso et al. (1997). However, the proportion of double positive CTLs observed after stimulation was much lower in PBMCs from the diseased pig USW81 than in PBMCs from

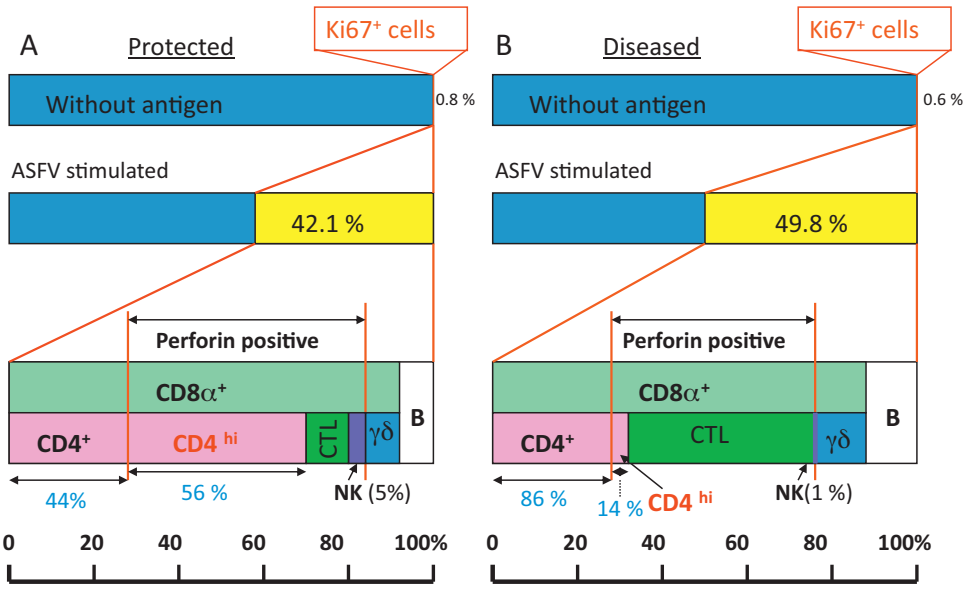

Fig. 3. Comparison of proliferated (Ki67$^+$) lymphocyte subsets induced by ASFV between protected (UW77) and diseased (UW81) pigs. PBMCs from OURT88/3 immunized pigs, before challenge with OURT88/1 isolate, were stimulated *ex vivo* with OURT88/1 virus for 5 days and proliferated (Ki67$^+$) lymphocytes were analyzed by flow cytometry. A: PBMCs from pig UW77, B: PBMCs from pig UW81. Without ASFV stimulation, less than 1% of lymphocytes are Ki67 positive (top columns), whereas with ASFV stimulation 42.1% (UW77) and 49.8% (UW81) are proliferated (Ki67$^+$) (second columns). Third columns illustrate the composition of lymphocyte subsets within the Ki67$^+$ lymphocytes population, and composition of perforin negative lymphocyte subsets. CTL indicates conventional CTL phenotype. Percentages shown in the third column in blue indicate the proportion of CD4$^+$ lymphocytes with or without perforin expression. About 10% of γδ T cells and B cells also proliferated after ASFV stimulation.

the protected pig UW77 (Figs. 2 and 3). Although this is only a single observation it is tempting to speculate that either the CTLs from pig UW81 were unable to express CD4 upon ASFV antigen stimulation, or that the ASFV specific CD4/CD8αβ double positive CTL precursors were not effectively primed in this pig. This may have contributed to the viraemia and pyrexia and hence the increased circulation of single positive CTLs. Further investigation is needed to explore the possible relationship between double positive CTLs and protection.

3. Other lymphocyte subsets and ASF

The basic composition of porcine lymphocytes are similar to other mammalian species, however, the proportions of lymphocyte subsets and their precise phenotypes may differ from other species. For further details, including a discussion of some of the peculiarities of porcine lymphocytes refer to Piriou-Guzylack and Salmon (2008) and Gerner et al. (2009).

3.1. NK cells

NK cells are considered to play a major role in combating viral infection (Żeromski et al., 2011) by directly killing virus-infected cells and producing cytokines (such as IFNγ) and chemokines. NK cells also interact with other lymphocytes, especially dendritic cells (DCs), and enhance both the function of DCs as well as inducing their maturation. In return, DCs enhance NK cell activity which is also augmented by type I IFNs secreted by virus-infected cells. Recent developments in NK cell research indicate that NK cells also play a role in anti-viral immunity through their antigen specific memory (see reviews by Paust et al., 2010; Paust and von Andrian, 2011).

Using a non-haemadsorbing non-virulent isolate of ASFV (NH/P68), Leitão et al. (2001) demonstrated a significant increase in NK cell cytotoxicity at 7 days post infection (dpi) in pigs that remained asymptomatic throughout the experiment, compared to the uninfected control pigs. On the other hand, pigs that presented with clinical signs such as viraemia, pyrexia, lesions consistent with chronic infection, and developed hypergammaglobulinemia demonstrated a less dramatic increase in NK cell activity. The asymptomatic pigs from this experiment were shown to be resistant to subsequent challenge with the virulent Lisbon 60 isolate of ASFV. This observation suggested that activation of NK cells might be an important contributing factor to prevent clinical disease caused by non-haemadsorbing non-virulent isolates of ASFV, and to the effective induction of protective immunity against virulent virus challenge. In contrast, 4 out of 6 pigs inoculated with the moderately virulent Malta 78 ASFV isolate showed marked depression of NK activity between 3 and 6 dpi (Norley and Wardley, 1983). Interestingly, 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. On the other hand, live, but not UV-irradiated, ASFV can inhibit NK cell activity *in vitro* (Mendoza et al., 1991). The virulence of the viral strain may also play a role, as NK cell activity of PBMCs derived from naïve pigs was stimulated *in vitro* by non-virulent NH/P68 virus, but depressed by virulent Lisbon 60 isolate (Martins and Leitão, 1994). In addition, our preliminary experiments with naïve pig lymphocytes from spleen also indicate that non-virulent ASFV OURT88/3 is a better inducer of IFNγ (detected by ELISPOT) *in vitro* than the virulent viruses that we have tested to date. It is likely that the IFNγ detected in these experiments was secreted by NK and/or NKT cells, because firstly the splenocytes were derived from naïve pigs, and secondly depletion of CD16$^+$ lymphocytes greatly reduced IFNγ secretion by these cells. Co-culturing naïve dd pig PBMCs with Max cells, a dd pig derived kidney cell line (Pauly et al., 1995), that had been infected with attenuated Uganda (Hess et al., 1965) resulted in double the proportion of perforin positive lymphocytes, compared to co-culturing with uninfected Max cells. The majority of these perforin positive cells expressed CD16 and therefore were likely to be NK or NK T cells (Fig. 4). Taken together these results indicate that the innate compartment of immunity is able to respond effectively against at least non-pathogenic ASFV.

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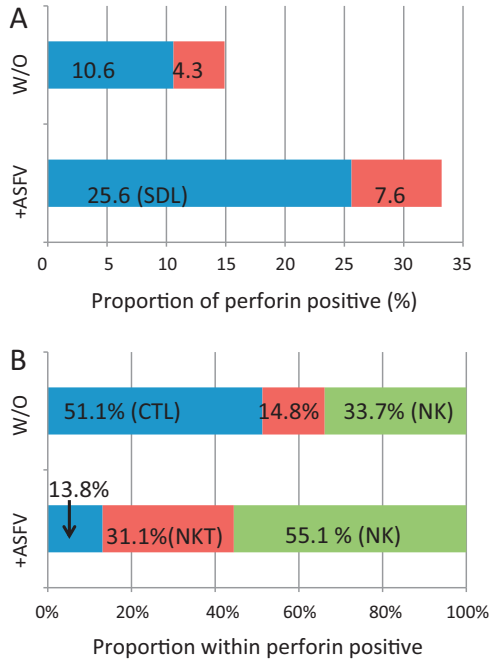

Fig. 4. ASFV induce perforin positive NK and NK T cells in naïve pig PBMC. PBMCs from naïve dd haplotype inbred pig were co-cultured with dd pig derived MAX cells, either uninfected or infected with ASFV attenuated Uganda, for 48 h at 37 °C. Co-cultured PBMCs were then analyzed by flow cytometry for their expression of perforin, and their phenotype. First perforin positive cells were gated and within the perforin positive cells, expression of CD3 against CD6, and CD16 against CD6 were examined. The $CD3^+CD6^+$, and $CD6^+CD16^-$ populations of lymphocytes were composed of CTL. $CD3^-CD6^-$ lymphocytes were NK cells. $CD3^+CD6^-$ lymphocyte populations included NKT cells, but not all the cells expressed the NK marker so adjustment was made from the $CD6^-CD16^+$ population of lymphocytes which contained both NK and NKT cells ([$CD6^-CD16^+$ population] – [NK ($CD3^-CD6^-$) population] = NKT). There were very minor populations of $CD6^+CD16^+$ lymphocytes as previously described by Denyer et al. (2006) which was excluded from calculation. All perforin positive lymphocytes expressed CD8. The results are mean values from two separate experiments. (A) The proportion of perforin positive cells was more than double (33.2%) when co-cultured with ASFV infected MAX cells compared to uninfected MAX cells (15%). The majority of perforin positive cells are small dense lymphocytes (SDL) (shown in blue colour) rather than larger granular cells (shown in brown). (B) Within the perforin positive cells, the majority were CTL phenotype ($CD3^+CD6^+CD8^+CD16^-$) in uninfected MAX cell co-cultured PBMCs. In contrast, ASFV infected MAX cell co-cultured PMBCs were mostly the NK cell phenotype ($CD3^-CD6^-CD8^+CD16^+$), followed by the NKT cell phenotype ($CD3^+CD6^-CD8^+CD16^+$) with the CTL phenotype being the lowest.

3.2. NK T cells

CD1d restricted NKT cells recognize lipid and glycolipid antigens such as α-galactosylceramide (Gumperz and Brenner, 2001), so their role in viral infections may be minimal as viruses, unlike bacteria are not known to generate specific lipids or glycolipids. However, recent studies indicated that NKT cells may play a role in anti-viral responses (see review by Diana and Lehuen, 2009), and moreover, unlike many other viruses, ASFV is composed of a single lipid bilayer (Hawes et al., 2008) and encodes for a prenyltransferase (Alejo et al., 1999). NKT cells are phenotypically T cells but co-express NK cell markers. NKT cells are present in pigs and have been described as $perforin^+CD3^+CD4^-CD5^{\pm}CD6^-CD8^+CD11b^+CD16^+$ (Denyer et al., 2006). Although details of the functional nature of porcine NKT cells have not been reported, pig lymphocytes proliferate and produce IFNγ when co-cultured with α-galatosylceramide, a NKT cell specific stimulator. As described in the NK cell section of this chapter, the proportion of NKT cells increased after PBMCs from naïve pigs were co-cultured with ASFV infected MHC matched cells (Fig. 4), and therefore could be involved in the production of IFNγ following ASFV infection. The elucidation of the role of NKT cells in ASFV immune responses requires further investigation.

3.3. γδ T cells

As described in the introduction, antigen presenting cells such as monocytes/macrophages and DCs are known to support ASFV replication and therefore, ASFV may potentially compromise their ability to present antigen and initiate ASFV specific immune responses. However other types of cells can also present antigens. A number of studies have shown that a subset of γδ T cells can also present antigen including porcine γδ T cells (Takamatsu et al., 2002) and those from other species (Collins et al., 1998; Brandes et al., 2005; Cheng et al., 2008). Importantly porcine γδ T cells incubated with ASFV can present ASFV to ASFV specific T cells (Takamatsu et al., 2006). In addition, porcine γδ T cells can secrete a number of cytokines and chemokines, including IFNγ (Takamatsu et al., 2006), indicating that these cells may play a role during ASFV infection, but their significance requires further investigation.

Other possible alternative APCs are fibrocytes. Fibrocytes are circulating bone marrow derived cells which can enter tissue and differentiate into fibroblasts, but express haematopoietic cell markers such as CD34 and CD45. These cells have been identified in a number of species including the pig (Balmelli et al., 2005) and have been demonstrated to stimulate classical swine fever virus (CSFV) specific cytotoxic T cells. Whether fibrocytes play a role in the cellular immune response to ASFV is not known.

4. ASFV antigens recognized by porcine T cells

ASFV is a large DNA virus with a genome size of 170–190 kbp. Thus screening all potential ASFV antigens by conventional assays is not practical. Therefore a number of groups have employed various strategies to identify antigen recognized by ASF immune porcine T cells. Alonso et al. (1997) generated inbred dd haplotype immune pigs as a source of CTLs by infecting E-75a partially attenuated ASFV *via* the oro-nasal route and then challenging 6 weeks later with the virulent isolate E-75 *via* intramuscular injection. This group used alveolar macrophages as target cells which are susceptible to wild-type and recombinant vaccinia virus (VV). Using this system target alveolar macrophages were infected with wild-type VV or recombinant VV expressing ASFV immediate early protein p32 (VVp32). ASFV specific CTL were able to lyze VVp32 infected target cells at a consistent, but low level (13%) compared to wild-type VV infected target cells (3%).

Usually antigens recognized by CTL are processed and presented through the MHC class I pathway, therefore conventional wisdom would require the antigen to be synthesized endogenously. However, in certain circumstances, exogenous antigen can be presented through MHC class I (antigen cross-presentation), especially when antigen is associated with cell debris that has been taken-up by phagocytosis or endocytosis (Guermonprez and Amigorena, 2005). Leitão et al. (1998) exploited this route to assess CTL antigens by fusing ASFV antigens with the *Pseudomonas aeruginosa* outer membrane lipoprotein I gene. Outer membrane protein preparations were incubated with inbred pig derived macrophages and used as target cells. CTL effector cells were prepared from PBMCs from NH/P68 recovered cc haplotype inbred pigs. Macrophages treated with a lipoprotein containing amino acids 249–273 of the Lisbon 60 VP72 gene (HKPHQSKPILTDENDTQRTCSHTNP) were lyzed by CTL and the lysis was blocked by anti-class I monoclonal antibody. Using the same system, a fusion containing a fragment that was 99% identical to a portion of the G1340L gene from the Ba71v isolate was identified that induced SLA restricted ASFV specific lymphocyte proliferation, and stimulated ASFV specific CTL activity

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in vitro (Leitão et al., 2000). Although co-culture of PBMCs from pigs immunized with fusion lipoprotein containing the G1340L fragment reduced ASFV replication *in vitro* in autologous macrophages, the pigs themselves died from acute ASF within 4–9 days post-challenge with Lisbon 60.

In order to screen more ASFV antigens, Jenson et al. (2000) prepared a random plasmid library of sheared ASFV genomic DNA fragments downstream from the T7 promoter. These random fragments were expressed in cc and dd inbred pig derived fibroblasts, which express MHC class I but not MHC class II, by transfection and infection with a recombinant VV expressing T7 RNA polymerase. PBMCs from ASFV immune cc and dd pigs were generated through the infection of pigs with non-virulent OURT88/3 followed by infection with the virulent OURT88/1 isolate. The PBMCs generated were used to screen 72 randomly selected plasmids in an ASFV specific $CD8^+$ lymphocyte proliferation assay. This proliferation was inhibited by anti-CD8 and anti-MHC class I antibodies, but not inhibited by anti-CD4 or anti-MHC class II antibodies. Fourteen of the 72 randomly selected clones were recognized by ASFV immune pig lymphocytes and subsequent sequencing identified that one of the positive clones coded for part of the putative membrane protein k11L (also known as I329L).

Recently Argilaguet et al. (2012) described a DNA vaccination strategy in which 33% of immunized pigs were protected from ASFV and the protection correlated with the induction of $CD8^+$ T-cells that specifically recognized either one of two previously un-described 9-mer peptides within the ASFV haemagglutinin protein (CD2v, EP402R).

5. ASFV specific IFNγ production

IFNγ was originally called macrophage-activating factor because it up-regulates macrophage functions including antigen processing and presentation (Schroder et al., 2004). Although a number of cell types secrete IFNγ, the main producers are activated T cells, NK and NKT cells. Activation of macrophages by IFNγ enhances direct antimicrobial activity and proinflammatory responses, including cytokine and chemokine production, and promotes IFNγ production at the site of inflammation by recruited lymphocytes such as NK cells. As ASFV mainly targets professional APCs, IFNγ might have a direct impact on ASFV-host cell interactions, especially during the early stages of virus infection. Esparza et al. (1988) reported that porcine IFNγ reduced ASFV replication in porcine macrophages *in vitro*. From this point, IFNγ produced from NK cells might have an impact. PBMCs from inbred pigs immunized by injection with autologous macrophages infected with tissue culture adapted non-virulent Ba71v, produced IFNγ *ex vivo* when stimulated with either virulent or non-virulent Ba 71, but not with heterologous ASFV (Revilla et al., 1992). PBMCs from pigs that survived inoculation with OURT88/3, followed 3 weeks later by OURT88/1, were used to assess cross-reactivity between ASFV isolates in an IFNγ ELISPOT assay (King et al., 2011). PBMCs from pigs inoculated with OURT88/3 and then OURT88/1 had a greater frequency of IFNγ producing lymphocytes than those inoculated with OURT88/3 alone and good correlation to protection. The cross-reactivity experiments demonstrated that a high level of IFNγ was detected when immune PBMCs were incubated with other genotype I isolates, including the West African isolate Benin 97/1 isolate, but not Lisbon 57. There was almost no ASFV specific IFNγ detected in response to a genotype VIII isolate (Malawi Lil 20/1), but surprisingly good cross-reactivity against a genotype X isolate (virulent Uganda, 1965). These results were tested *in vivo* by generating OURT88/3 immunized pigs, boosting with OURT88/1 (both genotype I) and then challenging with the West African isolate Benin 97/1 isolate (genotype I) or virulent Uganda isolate (genotype X). All of the resulting immune pigs were protected from the virulent Uganda isolate challenge, and the majority of pigs were protected from Benin 97/1 challenge (King et al., 2011). As these authors previously observed that OURT88/3-OURT88/1 immune pigs were never protected from Malawi Lil 20/1 challenge and only partially protected from challenge with Lisbon 57, these results showed that IFNγ ELISPOT cross-reactivity correlated well with ASFV isolate cross-protection.

The IFNγ positive lymphocyte population from ASFV stimulated immune PBMC was dominated by the $CD4^+CD8^+$ T cell phenotype, but only a third of these cells were typical memory helper T cell ($CD4^+CD8^{low}$) phenotype and the rest were $CD8^{hi}$ CTL phenotype (Denyer, Stirling & Takamatsu unpublished observation).

6. The importance of T-cells in ASF protection: lessons learned from DNA vaccination

From the studies described above, it seems clear that T-cells play an essential role in protection against ASFV. Thus, an ideal, effective vaccine for the future should not only induce neutralizing antibodies (see other chapter in this special issue), but should also induce specific T-cell responses against ASFV. In an attempt to reach this goal, several vaccine prototypes have been designed using DNA immunization as a tool to stimulate and prime the immune response to ASFV with immunodominant ASFV antigens (Kutzler and Weiner, 2008) or to target these immunodominant antigens for rapid intracellular degradation within the proteasome in order to optimize their SLAI-restricted presentation (Rodriguez and Whitton, 2000). Several lessons have been learned from these studies: (i) DNA immunization with a plasmid encoding PQ, a fusion of two of the most immunodominant ASFV-genes (p30 and p54) (Gómez-Puertas et al., 1998), works in mice but fails in pigs, opening new concerns about the use of small animal models to study ASF (Argilaguet et al., 2011), (ii) driving PQ to $SLAII^+$-APCs increased the induction of anti-p54 and anti-p30 antibodies and specific T-cells in pigs, however these animals were not protected against lethal ASFV challenge, (iii) the lack of protection afforded by this vaccine correlated with the induction of non-neutralizing antibodies and a moderate number of IFNγ-secretory ASFV-specific $CD4^+$ T-cells (Argilaguet et al., 2011), (iv) the immunological outcome induced by a given vaccine and its protective capabilities against ASFV-challenge can be dramatically modified by manipulating the antigenic presentation of the encoded antigens (Argilaguet et al., 2012). DNA vaccines encoding p54 and p30 fused to the extracellular domain of the haemagglutinin (sHA), failed to protect pigs against lethal challenge, in spite of the specific humoral and cellular immune responses that were induced. Whereas, in clear contrast, immunization with pCMV-UbsHAPQ, encoding the same ASFV antigens fused to ubiquitin for rapid proteasomal hydrolysis, protected 33% of the immunized pigs (two out of six in two independent experiments) in an SLA I-restricted manner and in the absence of antibodies prior to challenge. Interestingly, protection correlated with the induction of $CD8^+$ T-cells that specifically recognized previously un-described 9-mer peptides, either F3 and/or A6, within the ASFV haemagglutinin (Argilaguet et al., 2012).

Preliminary immunization experiments using the synthetic F3 and A6 peptides plus Freund's adjuvant confirmed the protective potential of these two peptides (Fig. 5A) and suggests that it may be possible to obtain broader-protective vaccines against ASFV by incorporating new ASFV determinants which cover the SLA I heterogeneous pig population. Apart from their protective capabilities, the identification of the F3 and A6 peptides has allowed the exploration of a very interesting immunological phenomenon that might have implications for future vaccine designs: immunedominance.

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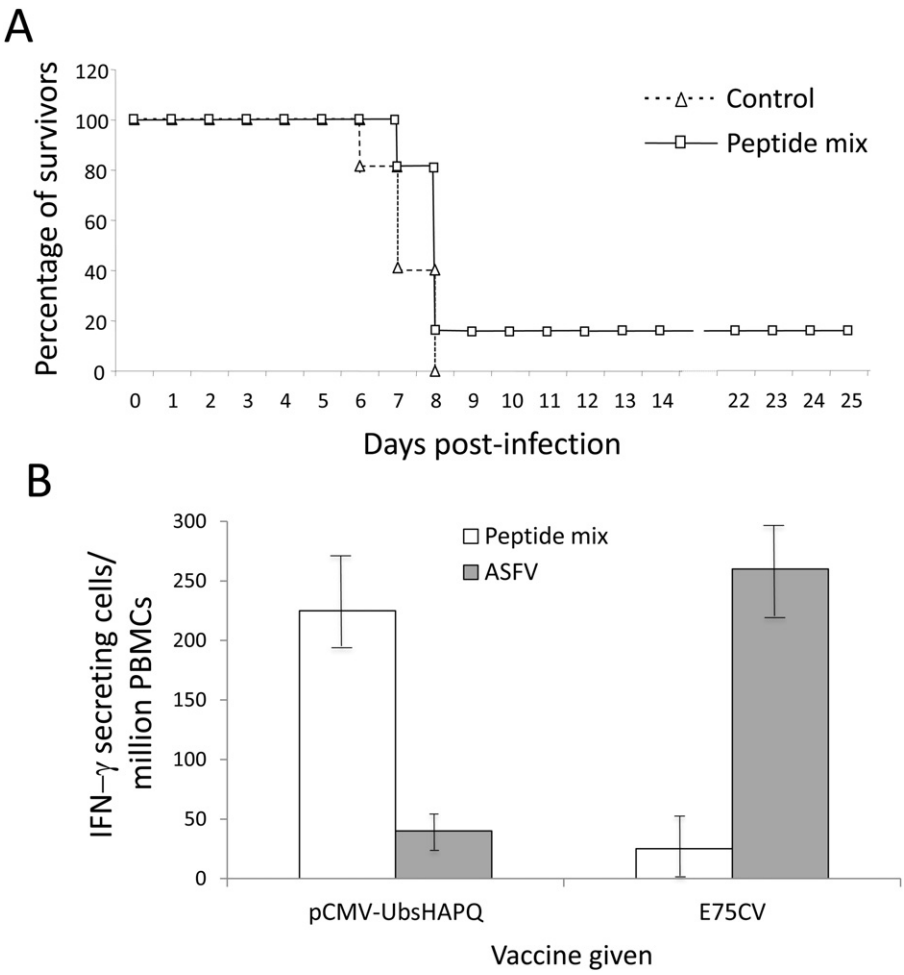

Fig. 5. The identification of CD8-T cell peptides with protective capabilities through DNA immunization. (A) 5 Large white pigs (6 weeks old males) were immunized three times with the F3 and A6 9-mer HA-peptides (50 μg from each peptide per dose) in the presence of Freund's adjuvant (complete, incomplete and no adjuvant, respectively) at 15 days intervals. As controls for this experiment, 5 pigs were immunized with PBS following the same schedule as above (complete, incomplete and no adjuvant, respectively). To evaluate the protective potential of the synthetic peptides, pigs were infected with a lethal dose of 10^4 UHA_{50} of the E75 virulent strain. The figure represents the mortality found in one of two independent experiments, both showing similar survival rates. (B) Groups of pigs were either immunized twice with pCMV-UbsHAPQ or once with 10^4 UHA_{50} of the E75 CV1 attenuated strain. One month later all pigs were challenged with a lethal dose of 10^4 UHA_{50} of the E75 virulent strain. PBMCs from surviving pigs (three animals per group), were obtained 25 days after challenge and were *in vitro* stimulated with media (negative control), with the E75 ASFV isolate (10^5 HAU_{50}/ml), or with a mix of the F3 and A6 peptides (2 μg/ml of each one). Values shown correspond to the average number of IFNγ-secretory cells detected per million of PBMCs obtained after stimulation (no spots were counted in the absence of specific stimuli). Standard deviations found within each group are represented.

Groups of pigs were immunized with either pCMV-UbsHAPQ or with the E75 CV1 attenuated strain and one month later all pigs were challenged with a lethal dose of the homologous E75 virulent strain. PBMCs from surviving pigs were obtained 25 days after challenge (virus was totally cleared from their blood) and were *in vitro* stimulated with media, E75 ASFV isolate or with the F3 and A6 peptides. Interestingly, while surviving pigs pre-immunized with pCMV-UbsHAPQ induced very strong responses against the F3 and/or A6 peptides, little response were seen upon stimulation with live virus (Fig. 5B). Conversely, PBMCs from surviving pigs pre-immunized with the attenuated E75 CV1 strain specifically recognized the entire virus, but did not recognize F3 and A6 (Fig. 5B).

Gene library immunization experiments with random ASFV-genome fragments have confirmed the potential of DNA vaccination to break ASFV immunodominance and induce broader T-cell responses than natural infection (Lacasta et al., unpublished results). This approach may hopefully lead to immunization strategies that confer protection against heterologous viruses in the future.

7. Concluding remarks

Pigs that survive infection with moderately virulent ASFV, immunization with low- or non-virulent ASFV, or deletion mutants (Lewis et al., 2000) can develop protective immunity against closely related ASFV re-infection or challenge. Despite the fact that infection of pigs with some lower virulence isolates can cause chronic infection, this is encouraging for ASF vaccine development as it is proof that protective immunity against ASFV can be induced in domestic pigs. DNA vaccination has been shown to be a useful tool to dissect both the antigens and the mechanisms involved in protection against ASFV. The fact that DNA vaccines encoding only a few ASFV antigens can confer protection to lethal challenge, in the absence of antibodies, seems to confirm the relevance of T-cells to the protection afforded. A number of potential ASFV-antigens, the mechanisms, and the lymphocyte subsets involved in protection were summarized and discussed in this review, but more studies are clearly needed. Identifying which ASFV antigen(s) are involved in the induction of protective immunity remains a considerable challenge. So far successful protective immunity

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has been induced to a limited number of specific ASFV isolates that, except for one report that used a 9GL (also called B119L) deletion mutant of Malawi Lil-20/1 (genotype VIII) to successfully protect against the parental virus (Lewis et al., 2000), all came from Western Hemisphere isolates belonging to genotype I. The current vogue of classifying ASFV genotypes based on partial sequencing of the B646L gene has limited use when trying to predict cross-protection between ASFV isolates (Chapman et al., 2010; King et al., 2011). Hopefully the application of next generation sequencing technologies, in conjunction with a wider program of cross-reaction/cross-protection experiments, will enable scientists to narrow down and identify cross-protective antigen(s) in the future.

The involvement of immune complexes and complement in chronic pneumonia and glomerular pathology associated with ASF has been well documented (Pan et al., 1975; Moulton et al., 1975; Martín-Fernández et al., 1991; Fernandez et al., 1992) and cytokines are also likely to contribute to ASF pathology, for example possibly through lymphocyte apoptosis. Whether the cellular immune response(s) against ASFV contributes to ASF pathology *in vivo* is not known, but potential negative effects of cellular immune response(s) against ASFV require attention in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2012.11.009.

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