African swine fever (ASF) is a highly contagious acute haemorrhagic disease of domestic pigs caused by a large icosahedral DNA virus, which produces a wide range of syndromes. As yet, there is no vaccine for ASF, so the only method of control is through strict sanitary measures. Many different African swine fever virus (ASFV) isolates occur, ranging in pathogenicity from highly virulent to avirulent (reviewed by Plowright et al., 1992). The mechanism of protective immunity, however, is far from clear. Early attempts to demonstrate ASFV-neutralizing antibodies in sera from recovered pigs were unsuccessful (De Boer, 1967). However, more recent attempts (Ruiz-Gonzalvo et al., 1986; Zsak et al., 1993) described an antibody-mediated reduction of infectivity of virulent ASFV isolates in both Vero cell cultures and swine macrophages in vitro, but not complete neutralization of the virus. The role of antiviral antibodies in homologous protective immunity to a virulent ASFV isolate was examined by passive antibody transfer experiments (Wardley et al., 1985; Onisk et al., 1994). These experiments showed a reduction in mortality, reduced virulence and a delayed onset of infection in pigs treated with an antiASFV immunoglobulin (Ig). Wardley et al. (1985) suggested that the reduction in viraemia and increased survival times seen in these experiments were mediated by complement-dependent, antibody-mediated cytotoxicity or antibody-dependent, cell-mediated cytotoxicity. Pigs immunized with baculovirus-expressed ASFV haemagglutinin (Ruiz-Gonzalvo et al., 1996) or the structural proteins p30 (involved in ASFV internalization) and p54 (involved in ASFV attachment) (Gomez-Puertas et al., 1998) have shown different degrees of protection ranging from delay in onset of disease to complete protection. A more recent study demonstrated, however, that neutralizing antibodies to p30, p54 and p72 (involved in ASFV attachment) did not protect pigs from ASFV challenge, but did delay the onset of disease (Neilan et al., 2004). Anti-ASFV antibodies clearly play an important role in ASF protection and pathology, but for complete protection there is a requirement for other active components of the immune response such as cellular immunity. The presence of ASFV-specific cytotoxic T lymphocytes has been demonstrated (Martins et al., 1993); these are CD8+ and MHC class I-dependent and may recognize VP32 (Martins et al., 1993; Ramiroibanez et al., 1997; Jenson et al., 2000). Other studies have described high levels of IFN-γ production by ASFV-immune lymphocytes stimulated in vitro with ASFV (Revilla et al., 1992) and elevated natural killer (NK) cell activity during infection (Leitao et al., 2001); however,
Fig. 1. Depletion of CD8⁺ lymphocytes correlates with abrogation of immunity to ASFV. In two separate experiments, outbred pigs were infected with the avirulent OUR/T88/3 virus isolate, given anti-CD8 mAb and then challenged with the virulent OUR/T88/1 virus isolate. Longitudinal blood samples were taken for determination of CD8⁺ lymphocyte depletion efficacy by FACS analysis (a) and measurement of systemic ASFV viraemia in the CD8⁺-depleted, isotype control, protected control and unprotected control groups of pigs (b).
the significance of these cellular components of immunity in relation to ASF protection is not known.

In this study, we directly addressed the question of whether cellular components of immunity, in addition to anti-ASFV antibodies, are required to protect pigs from ASFV challenge. As a model for protective immunity we exploited the fact that outbred pigs infected with the avirulent Portuguese non-haemadsorbing tick ASFV isolate OUR/T88/3 (Boinas et al., 2004) are immune to subsequent challenge with the virulent Portuguese tick isolate of ASFV, OUR/T88/1 (Boinas et al., 2004). Using this model, we carried out a series of in vivo depletion experiments. In the first experiment, 12 healthy outbred pigs of similar body weight (20–25 kg) were inoculated intramuscularly with 10^4 TCID50 of the avirulent OUR/T88/3 and the pigs were then divided into three groups of four according to treatment. Group A received anti-CD8 (IgG2a) monoclonal antibody (mAb) [a mixture of anti-CD8 mAb 76-2-11 (Pescovitz et al., 1985) and anti-CD8 mAb 11-295-33 (Saalmüller, 1996)]. Group B received isotype control IgG2a anti-bovine WC1 mAb (CC15), which does not cross-react in pigs. Group C received no mAb treatment (protected control group). An additional group (group D) consisting of four naive pigs (without previous exposure to OUR/T88/3) was an unprotected control group set up in order to check challenge virus efficacy. As pigs infected with OUR/T88/3 do not exhibit viraemia or any evidence of persistence post-infection (p.i.) (Boinas et al., 2004), pigs in groups A and B were injected intravenously with clarified mouse ascitic fluid (10 ml per animal per day) daily for 5 days from day 31 to day 35 after OUR/T88/3 infection. On the second day of ascitic fluid inoculation (day 32), all four groups of pigs were challenged intramuscularly with 50% haemadsorbing dose (HAD50) of 10^4 of the virulent OUR/T88/1. All animals were then monitored daily for clinical signs, viraemia (using a haemadsorption assay; Malmquist & Hay, 1960) and temperature, and the phenotype of circulating lymphocytes was analysed by two-colour flow cytometry with the following mouse mAbs: anti-porcine CD4 (74-12-4, IgG2b; Saalmüller, 1996), anti-porcine CD8α (11-295-33, IgG2a; Saalmüller, 1996), anti-porcine γδ T cells (PPT27, IgG1, and PPT16, IgG2b; Yang & Parkhouse, 1996, 2000), anti-porcine CD8β (PPT22, IgG1; Yang & Parkhouse, 1997). These mAbs were used in conjunction with fluorescent-labelled goat anti-mouse isotype-specific secondary antibodies (Southern Biotechnology) and analysed on a FACS Calibur (Becton Dickinson). Serum samples were taken every 7 days throughout the course of the experiment and antibody titres were measured by ELISA (Office International des Epizooties, 1996). The second in vivo depletion experiment was identical to the first except that the isotype control IgG2a mAb used was anti-bovine CD8 (CC63), which does not cross-react with pigs, and the ascitic fluid was given for 6 rather than 5 days from day 31 to 36 days after infection with OUR/T88/3. Also, in the second experiment only three pigs were used in the protected control group.

In the first experiment, clear depletion of CD8+ lymphocytes was observed in only two of the four pigs (Fig. 1a, Experiment 1). The two pigs (C96 and C99) in which depletion of CD8+ lymphocytes was observed developed high viraemia (Fig. 1b) and raised body temperatures, but survived challenge with the virulent ASFV isolate OUR/T88/1. The remaining two pigs in the group (C97 and C98) were not depleted of CD8+ lymphocytes, exhibited no viraemia (Fig. 1b) or clinical signs and were completely protected from challenge with OUR/T88/1. This experiment showed that CD8+ lymphocytes play an important role in the protective immune response to ASFV infections, although as the two CD8+ lymphocyte-depleted viraemic pigs survived challenge with the virulent OUR/T88/1 isolate, this indicated that either the depletion of CD8+ lymphocytes was not complete or other factors such as antibodies are playing a role in the protective immune response to ASFV.

To confirm the above observations and to attempt to improve the efficacy of CD8+ lymphocyte depletion, a second in vivo depletion experiment was carried out with injections of mAb for 6 rather than 5 days. This procedure greatly improved the efficacy of depletion in that CD8+ lymphocytes from three of the four pigs were depleted by the fifth day of anti-CD8 mAb treatment (day 3 post-challenge) and the remaining pig (D89) showed a substantial reduction of CD8+ lymphocytes in the circulation (Fig. 1a, Experiment 2). All four pigs showed high levels of viraemia after OUR/T88/1 challenge (Fig. 1b, Experiment 2), and three of the pigs (D87, D90 and D93) developed severe disease and were euthanized in order to avoid unnecessary suffering according to Home Office requirements. Interestingly, the remaining pig (D89), which was not fully depleted of CD8+ lymphocytes (Fig. 1a, Experiment 2), survived the challenge. This observation supports the importance of CD8+ lymphocytes in protection against or survival from ASFV challenge. The isotype control groups from both experiments were completely protected from OUR/T88/1 challenge, showing no reduction or depletion of CD8+ lymphocytes, and no viraemia or clinical symptoms of ASF were observed. All non-immune animals (unprotected control group) died of acute ASF following challenge with OUR/T88/1. One of the three pigs in the control protected group from the second experiment (D95) had a subclinical infection with low viraemia (Fig. 1b, Experiment 2); however, this pig showed no fever or clinical signs of ASF. All four animals in this group, including pig D95, were protected from OUR/T88/1 challenge. Interestingly, analysis of the circulating CD8+ lymphocytes in pig D95 (Fig. 2) showed a clear increase in CD8+ lymphocytes just after detection of viraemia on day 3 post-challenge. This increase in CD8+ lymphocytes was caused by an increase of CD8β+ expressing lymphocytes and not the CD4+CD8+ or the CD8+γδ+ lymphocyte subpopulations (Fig. 2). This result indicated that CD8β+ expressing lymphocytes may play a role in viral clearance.

Unlike most outbred pigs, inbred cc haplotype pigs (Sachs
et al., 1976) infected with OUR/T88/3 are not always fully protected from subsequent challenge with OUR/T88/1 (unpublished observations). Therefore, in the next experiment we examined whether inbred pigs of the cc haplotype, infected with OUR/T88/3, demonstrated increased CD8\(^+\) lymphocytes in the circulation when viraemia was induced by challenge with OUR/T88/1. Groups of six cc and six dd haplotype inbred pigs were infected by intramuscular injection with \(10^4\) TCID\(_{50}\) of the avirulent OUR/T88/3 isolate and were challenged at 35 days p.i. intramuscularly with \(10^4\) HAD\(_{50}\) of the virulent OUR/T88/1 isolate. All six pigs of the dd haplotype were completely protected from lethal challenge, exhibited no viraemia (data not shown) and had no detectable changes in the relative levels of circulating lymphocyte subsets, including CD8\(^+\) lymphocytes. Representative data from three of the dd haplotype pigs is illustrated in Fig. 3(d–f). In contrast, three of the six pigs of the cc haplotype developed severe acute ASF symptoms and were euthanized in order to avoid unnecessary suffering. Interestingly, the three surviving cc haplotype pigs developed viraemia (3.3–6.6 log\(_{10}\) HAD\(_{50}\)) lasting for 7–14 days and this viraemia was accompanied by an increasing number of circulating CD8\(^+\) lymphocytes (Fig. 3a). The CD8\(^+\) lymphocytes increased from a mean value of 33% of the total lymphocyte population before viraemia was detected in the pigs (day 41 p.i. with OUR/T88/3; day 6 post-challenge with OUR/T88/1) to a mean value of 69% after viraemia was detected in the pigs (day 49 p.i. with OUR/T88/3; day 13 post-challenge with OUR/T88/1). This increase of 36% in the CD8\(^+\) lymphocytes was attributable

Fig. 3. Increased numbers of CD8\(^+\) lymphocytes in the circulation of cc haplotype pigs (a–c) but not dd haplotype pigs (d–f) following challenge with the virulent ASFV isolate OUR/T88/1. Inbred pigs of the cc and dd haplotype were infected with the avirulent OUR/T88/3 virus isolate (day 0) and subsequently challenged (arrow) with the virulent OUR/T88/1 isolate on day 35 p.i. Longitudinal peripheral blood samples were taken for staining by FACS analysis. The figure shows total CD8\(^+\) lymphocytes (a, d), total CD8\(^{+}\)CD8\(^{+}\) lymphocytes (b, e) and CD4\(^+\)CD8\(^+\) lymphocytes (c, f). Data from three representative dd haplotype pigs (d–f) and the three surviving cc haplotype pigs (a–c) are presented.
to a rise in the number of lymphocytes expressing CD8β (Fig. 3b), but not to a rise in the CD4+CD8+ subpopulation (Fig. 3c). The CD8β+ lymphocytes increased from a mean value of 18% of the total lymphocyte population in the three pigs before viraemia was detected (day 41 p.i. with OUR/T88/3; day 6 post-challenge with OUR/T88/1) to a mean value of 55% of the total lymphocytes in the circulation after viraemia was detected in the pigs (day 49 p.i. with OUR/T88/3; day 13 post-challenge with OUR/T88/1). This 37% rise in total CD8β+ lymphocytes in the circulation in response to viraemia was equivalent to a 200% rise in CD8β+ lymphocytes in the circulation. Thus, there was a strong correlation between viraemic pigs and increased CD8β+ lymphocytes in the circulation, indicating that CD8β+ lymphocytes may play an important role in virus clearance.

We next attempted to deplete CD8β+ lymphocyte subsets by inoculating pigs with asctic fluid containing anti-pig CD8β mAb (PPT22; Yang & Parkhouse, 1997). The depletion conditions in this experiment were not ideal as the isotype of mAb PPT22 is IgG1, which is not efficient at fixing complement. In the first CD8β depletion experiment, only one of four pigs demonstrated a reduction in the number of CD8β+ lymphocytes and this animal did in fact die 6 days post-challenge of acute ASF. The remaining three pigs survived the infection without showing any clinical signs of disease (data not shown). The second experiment was identical to the first except that six rather than four pigs were inoculated with the anti-pig CD8β mAb. No depletion of CD8β+ lymphocytes was seen in any of the six pigs and there was no effect on ASF protective immunity, as the six pigs survived challenge with OUR/T88/1 without showing any clinical signs of disease or viraemia (results not shown). The fact that the only pig with successfully reduced/depleted CD8β+ lymphocytes in these two experiments died of acute ASF indicated that CD8β+ lymphocytes may play a role in ASF protective immunity. However, because the depletion of CD8β+ lymphocytes was ineffective in the majority of pigs, we could not conclude that this was definitely the case.

In this paper, we have demonstrated the importance of CD8+ lymphocytes in the protective immune response to ASFV infection. In young pigs, a large proportion of CD8+ lymphocytes are NK cells (Yang & Parkhouse, 1996, 1997) and a possible contribution of NK cells in the immune response to ASFV infection was suggested by Leitao et al. (2001). However, very low levels of NK cells were seen in these experiments, as the combined number of CD8β− and CD4+CD8+ lymphocytes was roughly the same as the total CD8+ lymphocyte population (Figs 2 and 3). In addition, porcine NK cells do not express CD8β and therefore NK cells were not responsible for the increased CD8+ lymphocyte populations seen as a response to viraemia. Unique to the pig, porcine memory helper T cells also express CD8 (Saalmüller et al., 2002). As we did not perform specific depletion of helper T-cell subsets in this study, we could not eliminate the possibility that the total CD8+ lymphocyte depletion, including the depletion of memory CD4+CD8+ helper T cells, may have resulted in failure of the secondary antibody response, which could abrogate protective immunity to ASFV challenge. However, due to the following reasons, we could conclude that the involvement of CD4+CD8+ helper T cells or secondary antibody responses in the protective immune response in this system was unlikely. Firstly, the anti-ASFV antibody titres in pigs in all OUR/T88/3-infected groups (CD8 depleted, protected control and isotype control groups) were raised before OUR/T88/1 challenge (28 days p.i. with OUR/T88/3) and remained at a similar level during ascitic fluid inoculation and after the challenge with OUR/T88/1 (data not shown). The pigs in the CD8+ lymphocyte-depleted group developed viraemia and clinical symptoms very soon after the challenge infection (by 3 days post-challenge) and importantly there were no differences in antibody titres seen in the CD8+ lymphocyte-depleted unprotected group compared with the isotype-protected and control-protected groups at this time point (data not shown). If an effective secondary antibody response was protecting the pigs, one would expect to see increased amounts of antibody in the protected compared with the unprotected groups of pigs, which was not the case. Also, viraemic pigs without CD8+ lymphocyte depletion (Figs 2 and 3) all showed an increased number of circulating CD8β+ lymphocytes after challenge with OUR/T88/1, whereas memory helper T cells of the CD4+CD8+ phenotype remained at a low steady level and did not increase after challenge. An increase in the number of CD4+CD8+ memory T cells associated with activation would be expected if this cell population were involved in the development of a secondary antibody response. Therefore, we could conclude that anti-ASFV antibodies alone, from OUR/T88/3 infection, were not sufficient to protect pigs from OUR/T88/1 challenge and there was no evidence of a protective secondary antibody response at 3 days post-challenge when the pigs developed the disease.

Observations from these experiments and others (M. S. Denyer, T. Wileman, C. Stirling & H. Takamatsu, unpublished data) indicate that the population of CD8β+ lymphocytes observed in this paper is likely to belong to a CD3+CD4−CD5+CD6+CD8α+CD8β+ T-cell subset expressing the cytotoxic granule perforin internally and this T-cell subset is therefore likely to be cytotoxic. If this is the case, this cytotoxic T-cell population may contribute to the elimination of ASFV-infected cells, resulting in reduced levels of viraemia. Although further phenotypic and functional analyses of CD8+ lymphocyte subpopulations involved in protection from ASFV infections are needed in the future, this paper demonstrates an important role for CD8+ lymphocytes in the protective immune response to ASFV infection. This information, combined with work
showing a role for antibodies in ASFV protection, will aid in the development of new strategies aimed at the production of an effective ASF vaccine. Finally, the experiments with cc haplotype inbred pigs have provided a first insight into the possible involvement of MHC in resistance to ASF.

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References


