

Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection

**C. Sánchez-Torres, P. Gómez-Puertas, M. Gómez-del-Moral, F. Alonso,
J. M. Escribano, A. Ezquerro, and J. Domínguez**

Dpto. Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain

Received March 5, 2003; accepted July 3, 2003
Published online September 16, 2003 © Springer-Verlag 2003

Summary. Monocytes-macrophages, the target cells of *African swine fever virus* (ASFV) are highly heterogeneous in phenotype and function. In this study, we have investigated the correlation between the phenotype of specific populations of porcine macrophages and their permissiveness to ASFV infection. Bone marrow cells and fresh blood monocytes were less susceptible to in vitro infection by ASFV than more mature cells, such as alveolar macrophages. FACS analyses of monocytes using a panel of mAbs specific for porcine monocyte/macrophages showed that infected cells had a more mature phenotype, expressing higher levels of several macrophage specific markers and SLA II antigens. Maturation of monocytes led to an increase in the percentage of infected cells, which correlated with an enhanced expression of CD163. Separation of CD163⁺ and CD163⁻ monocytes demonstrated the specific sensitivity of the CD163⁺ subset to ASFV infection. In vivo experiments also showed a close correlation between CD163 expression and virus infection. Finally, mAb 2A10 and, in a lower extent, mAb 4E9 were able to inhibit, in a dose-dependent manner, both ASFV infection and viral particle binding to alveolar macrophages. Altogether, these results strongly suggest a role of CD163 in the process of infection of porcine monocytes/macrophages by ASFV.

Introduction

African swine fever virus (ASFV) is an enveloped icosahedral deoxivirus that shares structural and genetic characteristics with viruses in the families *Poxviridae* and *Iridoviridae* and has been classified in a new family, named *Asfarviridae*, of which it is the sole member [17]. ASFV replicates mainly in porcine cells belonging to the monocyte/macrophage lineage [16, 36]. Entry of ASFV into swine macrophages involves a mechanism of receptor-mediated endocytosis, in which saturable binding sites have been demonstrated [1]. Moreover, Galindo et al. [21]

have reported virus attachment to still unknown proteins on the membrane of susceptible cell lines, showing that neither carbohydrates nor lipids are involved in the process. *In vivo*, resident macrophages infected by ASFV are concentrated in specific areas within lymphoid organs, while they are virtually absent in others [27, 36]. Likewise, in acute infections, only a limited percentage of blood monocytes (6–30%) become infected by ASFV [41]. These *in vivo* observations suggest a different susceptibility to ASFV among subpopulations of porcine monocytes/macrophages, which has been supported by *in vitro* studies [34, 49]. Similar findings have been also reported for a number of viruses that infect cells of the monocyte/macrophage lineage [15, 20, 22, 43], probably as a consequence of the heterogeneity of these cell populations. The difference in permissiveness among different cell subtypes could be explained either by the expression of virus specific membrane molecules necessary for virus attachment/entry [14] or by intracellular factors [12, 33], essential for viral replication.

Monocyte/macrophages display important differences in morphology, phenotype and functional capabilities depending on multiple factors, such as their origin, maturation or activation stage of the cells, or signals from the local microenvironment [45]. In several species, monoclonal antibodies (mAbs) raised against surface proteins have been shown as useful tools to investigate such heterogeneity. For instance, human blood monocytes can be separated into distinct subpopulations on the basis of CD14, CD16 and CD64 expression, with different degrees of maturation according to the expression of other surface markers (CD33, MHC class II, CD11b, CD11c, etc), as well as diverse functional activities [29, 51].

In swine, only five clusters of differentiation specific of the myeloid lineage have been defined so far, i.e. CD14, CD16, SWC3, CD163 and SWC9, and only the latter two are restricted to macrophages [18, 48]. We have described a panel of mAbs against several membrane proteins of porcine monocytes/macrophages [6, 7], among which 2A10 has been shown to identify porcine CD163 [46]. These mAbs allow definition of subpopulations of tissue macrophages and blood monocytes [7, 13, 46].

In the present study we find a high correlation between the expression of some of these molecules, particularly 2A10, and the susceptibility to infection by ASFV. In addition, mAb 2A10 inhibits the binding of ASFV to macrophages, interfering with virus infection. These results suggest a role of CD163 in the process of infection of porcine monocytes/macrophages by ASFV.

Materials and methods

Cells

Twenty Large-White outbred pigs, were used as cell donors over different periods of time. Alveolar macrophages were collected by alveolar lavage as previously described [11], washed with Hanks buffer containing 2 mM EDTA and frozen until use. Swine PBMC were isolated on Percoll discontinuous gradients after blood sedimentation in dextran, as has been described [26]. Adherent mononuclear cells were obtained by culturing PBMC for 2 h at 37 °C on plastic flasks. Bone marrow cells were obtained perfusing ribs with phosphate-buffered

saline (PBS). Red cells and non-viable cells were removed by centrifugation at $400 \times g$, 10 min on Lymphocyte separation medium (Biowhittaker). Cells recovered from the interphase were washed and resuspended in RPMI1640 medium (Biowhittaker) containing 10% FCS and $50 \mu\text{M}$ 2-mercaptoethanol. Alveolar macrophages, the adherent cells from PBMC, and bone marrow cells were cultured in 6-well plates at 5×10^6 cells/well in RPMI medium containing 10% fetal calf serum, 20% pig serum, 2 mM L-glutamine, $50 \mu\text{M}$ 2-mercaptoethanol, 20 mM HEPES and $30 \mu\text{g/ml}$ gentamycin. For *in vitro* infection assays, cells were incubated with ASFV viruses at a multiplicity of infection (m.o.i) of 10 for 2 h at 37°C . Cells were then washed several times with Hanks buffer, and fresh medium added. Twenty-two hours later, cells were detached with PBS containing 10 mM EDTA and harvested for assay.

Viruses

The virulent ASFV isolate Spain-75 (E75) was obtained from spleen homogenates of infected pigs. Virulent isolates were prepared by one or two passages on buffy coat cell cultures (E75L) as described [44]. E75MS13 isolate was attenuated by 13 passages on MS cells. Virus titration was performed on swine PBMC and expressed as $\text{TCID}_{50}/\text{ml}$ as described elsewhere [26]. Recombinant virus 1207VR Δ TK β -gal, has been described elsewhere [23].

Monoclonal antibodies

Description of mAbs to leukocyte surface antigens used in this study is given in Table 1. All mAbs were developed and produced in our lab [5–9, 19, 40] except TÜK-4, purchased from DAKO, 74-22-15 a gift from J. Lunney, and G7 and PM18-7, gifts from Y. B. Kim [18]. MAb 174F11.8 to ASFV p30 [50] was kindly provided by D.L. Rock.

Immunofluorescence assays

For single-color immunofluorescence analysis, 2×10^5 cells were incubated with $50 \mu\text{l}$ of hybridoma supernatant (primary mAbs) for 30 min at 4°C . They were then washed three times with PBS containing 0.1% BSA and 0.01% NaN_3 (fluorescence buffer, FB) and incubated with FITC-conjugated goat F(ab')₂ anti-mouse Ig (Dako), diluted 1/50 in FB, for 30 min at

Table 1. Monoclonal antibodies used in this work

mAb	Isotype	Specificity	Reference
2A10	G1	CD163	7, 46
2C12	G2b	ND	7
4E9	G1	ND	7
3F7	G1	ND	7
3B11	G1	ND	7
4B7	G2a	SLA I	5
1F12	G2b	SLA II	8
2A5	G1	CD45	9
1H6	G2a	CD5	40
2F4	G1	wCD11r3	6, 19
TÜK-4	G2a	CD14	18
G7	G1	CD16	18
74-22-15	G2b	SWC3	18
PM18/7	G1	SWC9	18

4 °C. Cells were washed four times in FB, fixed in 0.1% paraformaldehyde and analyzed in a FACScan flow cytometer (Becton Dickinson). For two-color immunofluorescence, after adding the secondary FITC-conjugated antibody, cells were incubated 30 min at 4 °C with a biotin-labeled second mAb diluted 1/20 in FB. Then, cells were washed three times and incubated with PE-conjugated streptavidin (Southern Biotechnology) diluted 1/1000 in FB, for 20 min at 4 °C. After washing, cells were fixed and analyzed as described above.

Isolation of CD163⁺ and CD163⁻ cells

Cells were magnetically isolated using the VarioMACS cell sorter technique (Miltenyi Biotec). 10⁸ PBMC were incubated with 10 ml of 2A10 hybridoma supernatant for 45 min on ice. Then, cells were washed twice with PBS containing 5% FCS and 2 mM EDTA (MACS buffer) and incubated for 15 min on ice with 20 µl/10⁷ cells of MACS goat anti-mouse IgG magnetic microbeads. After washing with MACS buffer, PBMC were passed through a MACS separation column, and magnetically labeled cells (CD163⁺ fraction) were collected. The effluent fraction was then incubated with 74-22-15 hybridoma supernatant (anti-SWC3) and MACS microbeads, as described above. Cells were passed through the separation column and CD163⁻ SWC3⁺ cells were harvested (CD163⁻ fraction).

Animal infection procedures

A total of 20 three-month old Large-White pigs were used in two separated experiments. Pigs were i.m. inoculated in the shoulder with 10⁵ TCID₅₀ of the E75L isolate. Blood samples were daily collected from two inoculated animals. Two pigs injected with saline solution were used as controls.

Virus infection blocking assay

For assessing mAb blockade of ASFV infection, alveolar macrophages (10⁵) were incubated during 30 min at 4 °C with various mAb concentrations. Then, cells were infected with 100 PFU of the recombinant ASFV 1207VRΔTKβ-gal. This virus allows determining the number of infected cells by chromogenic dye staining, as described [23]. After 2 h of incubation at 4 °C, cells were washed twice, fresh medium was added and incubated at 37 °C. Fourteen hours after infection, infected macrophages were revealed with X-Gal. Infection reduction data were obtained from three independent experiments and referred to the total infected cells obtained in absence of mAbs.

Virus binding assay

For binding competition experiments 10⁵ macrophages were incubated with antibodies (2 µg) at 4 °C for 30 min; subsequently Percoll purified 1207VRΔTKβ-gal virus, radiolabelled with ³⁵[S]Met/Cys (300 µCi/ml), was added [23]. Cells were maintained at 4 °C and, at various times after virus addition, supernatant was discarded and cells washed three times with fresh medium. Then 2% SDS buffer was added, and radioactivity in the solubilized fraction was measured in a liquid scintillation counter (Wallac).

Dot-blot assay for binding of ASFV to porcine CD163

Percoll purified ASFV, equivalent to 0.5 µg of protein, or equivalent amounts of baculovirus or porcine CD163, which had been purified by affinity chromatography using mAb 2A10 [46], were blotted to nitrocellulose membranes. Membranes were exhaustively washed with PBS and removed from the blotting device. Nonspecific binding sites were blocked with 5% non-fat milk powder in PBS. Dots were then incubated with 0.1 µg of CD163 which was diluted

in PBS containing 5% milk powder and allowed to attach for 4 h at room temperature. A set of duplicated dots was incubated with PBS-5% milk powder, as control. The membranes were washed three times with PBS, and incubated with mAb 2A10 diluted in PBS-5% milk powder for 1 h. Finally, membranes were incubated with rabbit-anti-mouse Ig conjugated with peroxidase and visualized with the ECL detection assay (Amersham) according to manufacturer's indications.

Results

Susceptibility of different populations of porcine monocytes/macrophages to ASFV infection

We first analyzed the susceptibility to ASFV infection of cells representing three different maturation stages within the macrophage cell lineage: bone marrow precursors (SWC3⁺ bone marrow cells), blood monocytes and alveolar macrophages. SWC3, a molecule that is expressed at high levels along all stages of the myeloid differentiation pathway [18, 48], was used throughout this study as a marker to select the analyzed cells. More than 75% of bone marrow cells and all monocytes and alveolar macrophages expressed SWC3. When these cells were infected *in vitro* with ASFV, different degrees of viral infection were found. Level of infection was monitored by flow cytometry using a mAb against viral protein p30, a viral early-late protein that is incorporated in the plasma membrane [50]. At 24 h post-infection (pi), p30 expression was detected on $7 \pm 1\%$ of SWC3⁺ bone marrow cells, $18 \pm 7\%$ of blood monocytes, and $76 \pm 9\%$ of alveolar macrophages. Similar results were obtained using both virulent and attenuated viruses. These data are consistent with a higher permissiveness of mature macrophages to ASFV infection.

Phenotypic analysis of blood monocytes infected by ASFV

Since only a low percentage of circulating monocytes become infected by ASFV, we investigated a possible correlation between infection and a particular phenotypic feature of these cells. To study the phenotype of cells we used mAbs summarized in Table 1. Some of the antigens recognized by these mAbs, such as SWC3, wCD11R3 and CD16, are highly expressed on granulocytes, monocytes and alveolar macrophages; others, such as 4E9, 2A10 and 3F7, are expressed by all alveolar macrophages but only by a subset of blood monocytes, and seem to be associated with the maturation or differentiation stage of the cells in this lineage [7, 13, 46].

Two-colour FACS analysis with mAbs 4E9, 2A10, 3F7, anti-SLA-I, anti-SLA-II, anti-wCD11R3 and anti-SWC3 versus mAb anti-ASFV p30 showed that infected monocytes, expressing p30, coexpressed high levels of 4E9, 3F7 and SLA II antigens (Fig. 1). The majority of CD163⁺ cells expressed p30; however, there was a reduction in the number of CD163⁺ cells upon infection, that was equivalent to the number of CD163⁻ p30⁺ cells, suggesting a downmodulation of this antigen by viral infection. No significant differences were observed in the level of expression of wCD11R3 and SWC3 molecules between p30⁺ and p30⁻

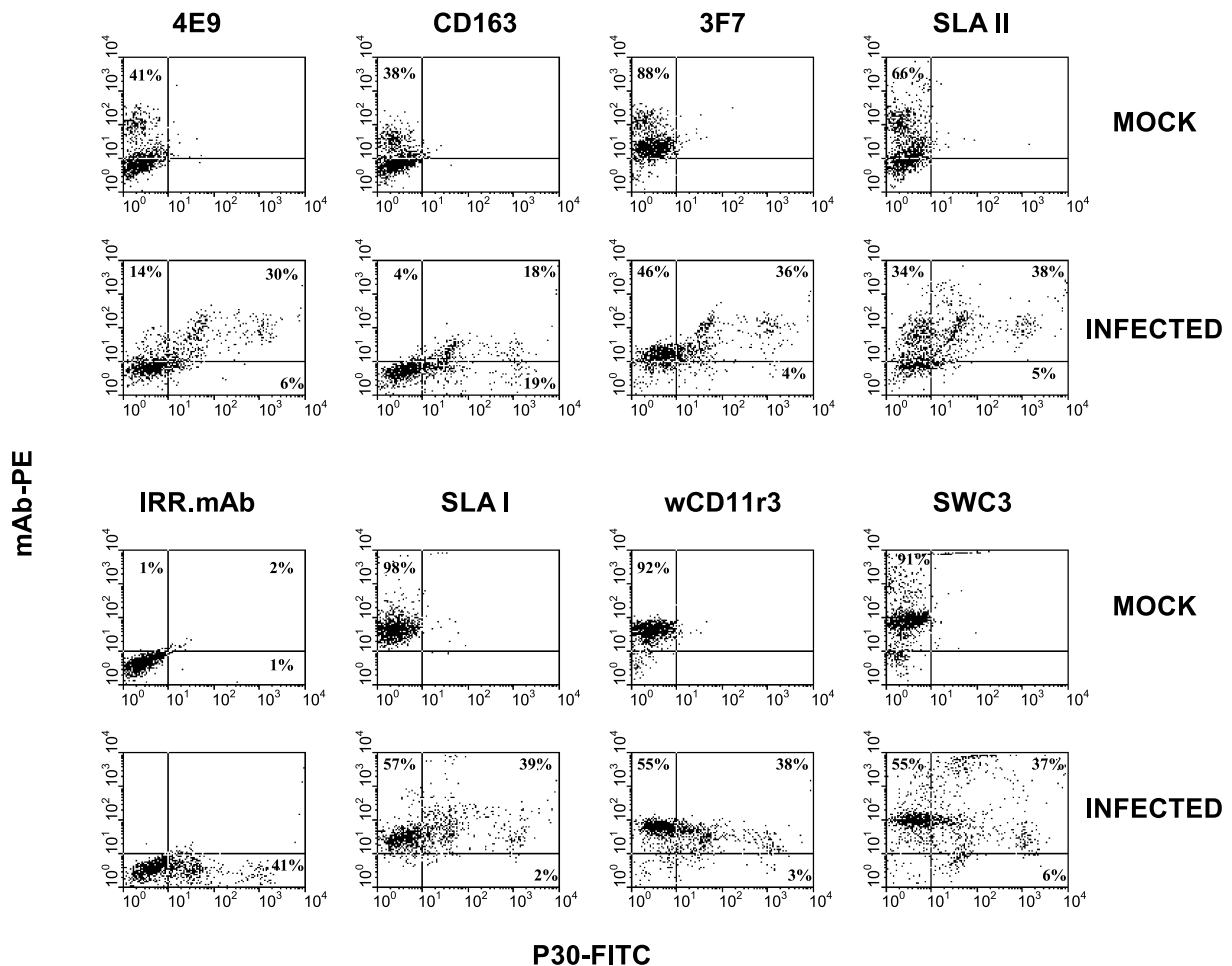


Fig. 1. Two-colour flow cytometric analysis of ASFV-infected (INFECTED) compared to mock treated (MOCK) blood monocytes. Blood monocytes were stained 24 h.p.i with anti-p30 and FITC-conjugated goat anti-mouse Ig (x-axis), followed by labelling with biotin labelled mAbs specific for indicated antigens and streptavidin-PE (y axis). A biotinylated irrelevant mAb of IgG1 isotype (IRR. mAb) was used as negative control. Numbers indicate the percentage of cells in the corresponding quadrants. Results shown are from one representative experiment of three performed

cells. These results suggest that ASFV-susceptible blood monocytes are associated with a $CD163^+3F7^+4E9^+SLA II^{high}$ phenotype.

Since phenotypical changes have been reported in cells upon ASFV infection [27, 34, 42], we compared the fluorescence intensity of different markers on infected *versus* non infected cells. Figure 2 represents the variation in the mean fluorescence intensity of each marker on *in vitro* infected monocytes and alveolar macrophages in comparison to uninfected cells, 24 h after the addition of the virus. The levels of expression of most of these antigens did not vary significantly after infection. However, a clear down-modulation of CD16 and CD14, and an up-regulation of 2C12 antigen was seen. 4E9 antigen increased up to 150% in

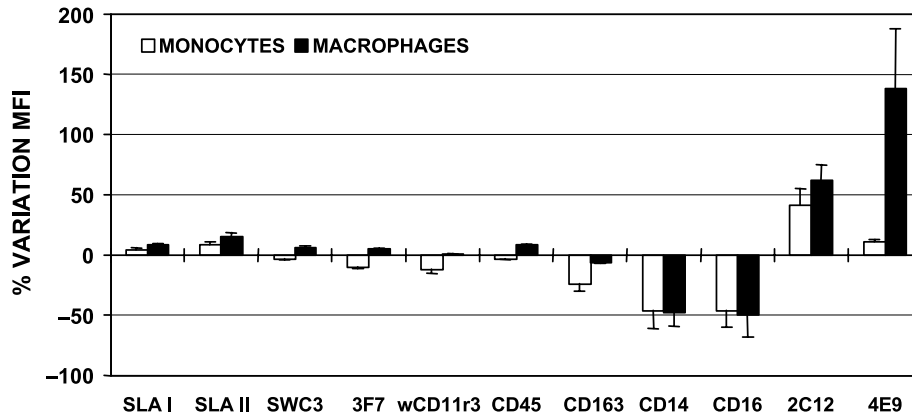


Fig. 2. Effect of ASFV infection on the expression of different leukocyte surface antigens. Blood monocytes and alveolar macrophages were infected with the ASFV isolate E75MS13 (m.o.i. of 10) and 24 h later stained with the different mAbs and analysed by flow cytometry. The values are expressed as % of variation in mean fluorescence intensity (MFI) compared with non-infected cells. Data represent the mean \pm SD of five experiments

infected alveolar macrophages but only slightly in monocytes. As above noted, CD163 antigen decreased consistently its expression in infected blood monocytes, but not in alveolar macrophages.

Susceptibility of cultured blood monocytes to ASFV

Since our results suggested a correlation between a maturation stage and susceptibility to infection, we tested whether blood monocyte-derived macrophages were more susceptible to infection by ASFV than freshly isolated monocytes. Blood monocytes maintained in culture in the presence of homologous serum increased in size and complexity, becoming morphologically similar to tissue macrophages. One, 3 and 6 days-old monocytes, cultured in medium containing 20% pig serum, were infected with ASFV (m.o.i. 10) and p30 expression was measured 24 h after virus addition. The percentage of p30⁺ cells increased progressively with monocyte culture time with values of $22 \pm 5\%$, $44 \pm 5\%$ and $50 \pm 8\%$ in 1, 3 and 6 days-old cultures, respectively. However, infection levels obtained in alveolar macrophages were never reached.

We also analyzed the change in the expression of cell surface antigens resulting from the culture of monocytes with pig serum. The percentage of cells expressing the CD163 and SWC9 antigens increased with the time in culture, whereas the number of cells that expressed the 4E9 and 3F7 antigens tended to diminish. For all the other markers there was no significant change in the percentage of cells expressing them, in spite of some changes in the intensity of fluorescence (not shown). According to these results, CD163 and SWC9 antigens can be considered as maturation markers within the monocyte/macrophage differentiation pathway. Figure 3 shows the expression kinetics of both molecules in comparison to the percentage of virus susceptible cells throughout the time of culture. As can be

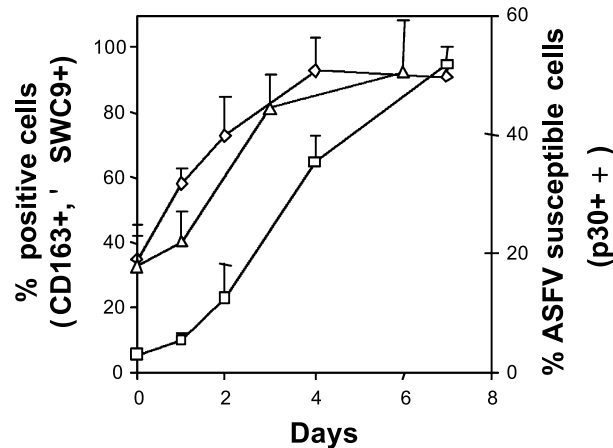


Fig. 3. Correlation between expression of CD163 and SWC9 antigens on monocytes and their susceptibility to ASFV infection. Blood monocytes were infected with ASFV isolate E75MS13 (moi 10), after 0, 1, 3 or 6 days in culture. Infection susceptible monocytes at each time were determined as monocytes stained by mAb 174F11.8 (anti-vp30), 24 h after virus addition to the cultures (Δ , right y-axis). For comparison, % of CD163 (\diamond) and SWC9 (\square) positive monocytes at different culture times is plotted in the left y-axis. Results shown are mean + SD of five independent experiments

observed, there is a higher correlation of the number of ASFV susceptible cells with the expression of CD163, as evidenced by the almost identical slope of both curves ($r = 0.94$), in contrast to the SWC9 expression curve ($r = 0.86$).

ASFV infection of CD163⁺ and CD163⁻ isolated blood monocytes

To further investigate the relationship of the CD163⁺ phenotype with susceptibility to ASFV infection in blood monocytes, the CD163⁺ and CD163⁻ monocyte subpopulations were magnetically sorted from PBMC into CD163⁺ monocytes and CD163⁻ monocytes. This separation rendered typical yields of 2% (CD163⁺) and 8% (CD163⁻) of total PBMC. FACS profiles of CD163⁺ and CD163⁻ subpopulations 12 h after separation are shown in Fig. 4A to illustrate the homogeneity of these preparations. Cells were infected with ASFV, and the percentage of p30 expressing cells determined 24 h later (Fig. 4B). p30 expression was minimal on CD163⁻ cells ($6.5 \pm 0.2\%$), even though a m.o.i. of 10 was used. In contrast, a significant percentage of CD163⁺ cells were clearly infected ($39 \pm 8\%$). When p30 expression was determined 48 h after infection, there was an increase in the number of infected cells within the CD163⁻ population ($12 \pm 2\%$) concomitant with the up-regulation of CD163 antigen expression (40% of CD163⁺ cells after 48 h culture). These data strongly support a direct association between the CD163⁺ phenotype and susceptibility to ASFV infection.

Phenotypic analyses of in vivo ASFV-infected cells

We next examined changes in peripheral blood monocytes expressing CD163 in pigs following experimental infection with the virulent E75L ASFV isolate.

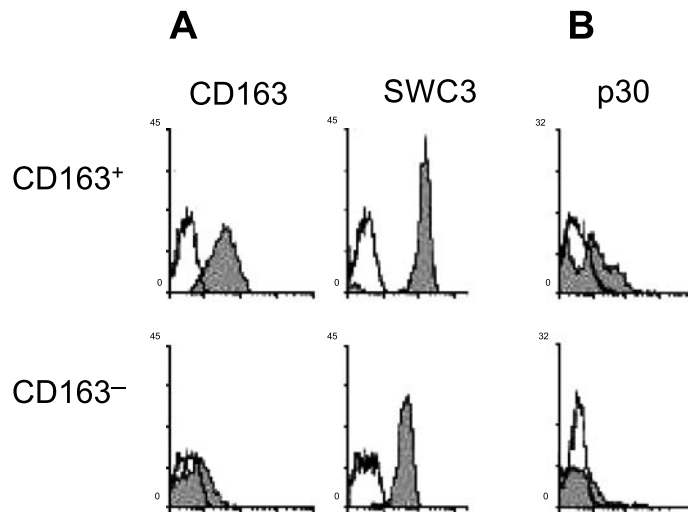


Fig. 4. Susceptibility of CD163⁺ and CD163⁻ monocyte subsets to ASFV infection. Blood monocytes magnetically fractionated into CD163⁺ and CD163⁻ subpopulations were infected with ASFV isolate E75L (moi of 10). **A** Expression of CD163 and SWC3 antigens in both subpopulations 12 h after the separation procedure. **B** Expression of p30 in both fractions 24 h after infection. Open histograms correspond to background staining with fluorescent conjugate alone. Results shown are from one representative experiment of three performed

A marked decrease in the absolute number of monocytes, identified as SWC3⁺ SSC^{low} was observed at day 3–4 pi that persisted until death of animals. This decrease was more evident in the CD163⁺ cells, with a reduction higher than 80% at day 6 pi, respect to the pre-infection levels (Fig. 5A). ASFV p30 expression was detected at day 2 pi in 9–19% of monocytes, and peaked at day 3–4 pi, when 30–40% of monocytes were p30⁺. Moreover, as assessed by two-color flow cytometry, the majority of these p30⁺ cells were CD163⁺ (Fig. 5B).

Inhibition of ASFV infection and cell binding using mAbs

We next tested the ability of mAb 2A10 to interfere ASFV infection of alveolar macrophages. Cells were incubated with a panel of antibodies against porcine leukocyte surface antigens, prior to infection with a recombinant ASFV expressing the β -gal gene. The number of infected cells was determined fourteen hours later, by staining with X-Gal. Only mAbs 2A10 and 4E9 had an effect on infection, causing a significant reduction in the number of infected cells, in a dose-dependent manner (Fig. 6A and B). This reduction was specific and not due to a non-specific blocking effect, since antibodies of the same isotype and/or against molecules expressed at higher levels than CD163 and 4E9, such as wCD11R3, 2C12 or SLA-I antigens, had no effect.

To determine the role of the antigens recognized by mAbs 2A10 and 4E9 during viral infection, binding competition experiments were performed with radiolabeled virus and these mAbs. Results demonstrated that 120–240 min after

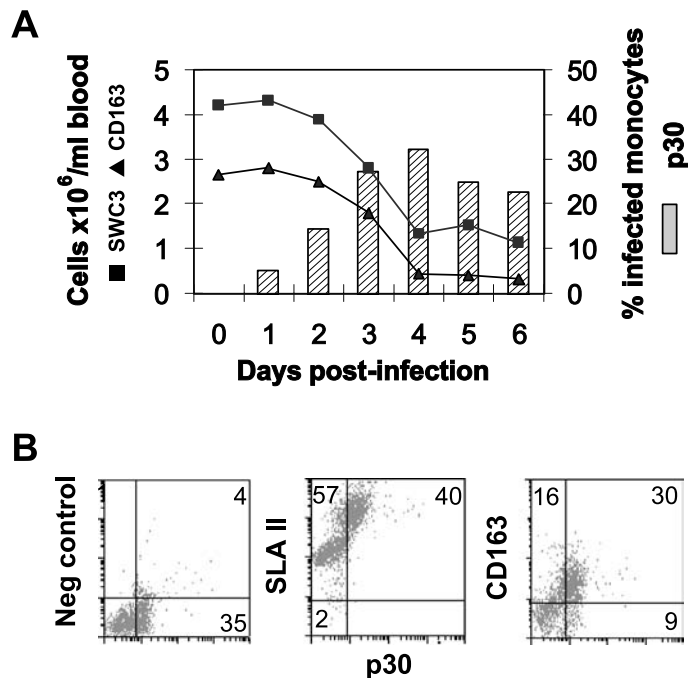
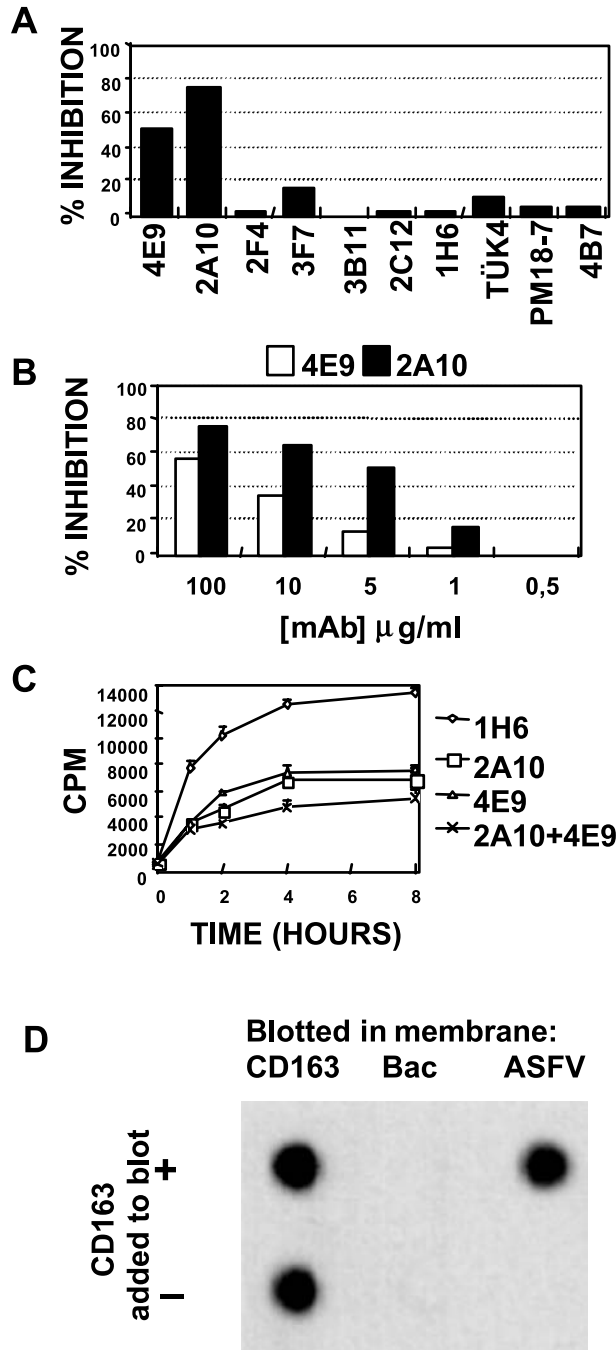


Fig. 5. **A** Changes in the absolute numbers of SWC3⁺ (■) and CD163⁺ (▲) monocytes (left axis) in pigs inoculated with ASFV isolate E75. Bars represent the percentage of monocytes expressing ASFV p30. **B** Two-colour flow cytometric analysis of blood monocytes from pigs inoculated with ASFV isolate E75 at 3 dpi. Monocytes were gated from PBMC by their side and forward light scatter. Cells were stained with anti-p30 and FITC-conjugated goat anti-mouse Ig (x-axis) and with biotinylated mAbs anti-CD163, and anti-SLA-II-DR and streptavidin-PE (y-axis). Irrelevant isotype-matched mAbs were used as negative controls. Numbers indicate the percentage of cells in the corresponding quadrants

Fig. 6. **A** MAb inhibition of ASFV infection in alveolar macrophages. Cells (10⁵/well) were incubated with the different mAbs (hybridoma supernatant) prior to infection with 100 pfu of a recombinant ASFV expressing the β-gal gene. After 14 h incubation, infected cells were revealed with X-Gal. **B** Cells were incubated with different concentrations of mAbs 2A10 or 4E9, prior to infection as described in **A**. In **A** and **B**, results are expressed as the mean percentage of inhibition of ASFV infection relative to the control (cells not preincubated with mAb). **C** Blocking of ASFV binding to alveolar macrophages by mAbs. Cells (10⁵) were incubated with mAbs (2 μg) prior to the addition of purified (³⁵S)-methionine/cysteine-labeled ASFV. An irrelevant isotype-matched mAb was used as negative control. Results are expressed as the mean ± SD cpm bound to cells at different times after virus addition, from three independent experiments performed in triplicate. **D** Binding of CD163 antigen to ASFV particles. Membrane was blotted with affinity chromatography purified CD163 antigen, baculovirus or ASFV and incubated with 0.1 μg of CD163 antigen or buffer alone for 4 h at room temperature. Dots were revealed with anti-CD163 mAb 2A10 plus rabbit-anti-mouse Ig conjugated with peroxidase and visualized using the ECL reagent

virus addition, inhibition of virus binding to macrophages by mAb 4E9 and 2A10 was higher than 50% as compared to an isotype-matched antibody control (Fig. 6C). Simultaneous incubation of cells with a mixture of 2A10 and 4E9 mAbs resulted in a slight increase of binding inhibition with respect to that obtained with the



individual mAbs. These data suggest that CD163 and 4E9 antigens are involved in initial steps of the ASFV infection related to virus-cell attachment.

ASFV binding to CD163 molecule was further examined by using a dot-blot assay. Percoll purified ASFV was blotted onto nitrocellulose membranes and incubated with affinity purified CD163 antigen. Binding of CD163 to the virus was revealed with mAb 2A10. As shown in Figure 6D, purified CD163 antigen was able to bind specifically to ASFV but not to baculovirus, that was used as a negative control. No signal was observed when incubation with CD163 antigen was omitted. These findings reinforce the conclusion that CD163 is able to bind ASFV particles, and therefore could be involved in the first stages of ASFV infection.

Discussion

African swine fever virus infects preferentially cells of the mononuclear phagocyte system although remarkable differences in the susceptibility to infection have been reported [16, 36, 49]. Mononuclear phagocytes show a high heterogeneity in morphology and function, that has been attributed to factors such as the differentiation/maturation stage, activation state, or the presence of specific cytokines or extracellular matrix components within a particular anatomical microenvironment [28, 45]. These morphological and functional differences are accompanied by differences in the expression of surface molecules that may contribute to the binding of virus to the cell, playing therefore a determinant role in the degree of permissiveness to virus entry and replication. To approach the analysis of the heterogeneity of the mononuclear phagocytic cells in the pig we have developed a panel of monoclonal antibodies against porcine monocyte/macrophage surface antigens. Using these antibodies we have been able to distinguish and extensively characterize two subsets of blood monocytes as well as to identify different subsets of tissue macrophages [7, 13, 46]. In the present study we investigate the relationship between the phenotype of mononuclear phagocytes, and the susceptibility of these cells to ASFV infection.

Our results show an increased susceptibility to infection by ASFV with the degree of maturation of cells of the monocyte/macrophage lineage. Thus, while few bone marrow myeloid cells are susceptible to infection, most alveolar macrophages are infected. Furthermore, *in vitro* maturation of blood monocytes makes these cells more susceptible to infection, rising the percentage of susceptible cells from around 20% for freshly isolated monocytes to more than 50% after 6 days in culture. These results are similar to those of McCullough and coworkers [3, 35], although some significant differences were noted, so they did not find susceptible cells in freshly isolated blood monocytes, and more strikingly they found only a small fraction of alveolar macrophages susceptible to infection. These discrepancies could be due to the use of different strains of ASFV. In fact, differences in the tropism of different ASFV isolates have been previously reported [38].

By using two colour FACS, we found that most of the infected monocytes expressed CD163, a marker that has been associated with maturation [46]. Since in previous studies SWC1⁺SWC9⁺ intermediate monocytic cells were identified

as the earlier stage of cells of monocyte lineage susceptible to ASFV infection [35], we analyzed the correlation of CD163 and SWC9 expression with susceptibility to infection during *in vitro* maturation of blood monocytes into macrophages. In these experiments we found a better correlation with the expression of CD163 ($r = 0.94$ vs $r = 0.86$). Moreover, as previously mentioned, around 20% of freshly isolated monocytes, which are SWC9 negative, were found to be susceptible to infection. Furthermore, in a previous report we have shown that SWC9 expression is subsequent to that of CD163 [13]. Definitive demonstration of the CD163⁺ phenotype of the ASFV susceptible population came from the experiments of *in vitro* infection of separated CD163⁺ and CD163⁻ subsets of blood monocytes, which showed that only the former contained infection susceptible cells.

Besides this, other factors, not yet identified, seem also to play a role in determining the susceptibility of the cells, as far as only a fraction of the CD163⁺ blood monocytes were infected. These additional factors could also explain the differences previously mentioned of our results with those from other authors, i.e., in the infection of alveolar macrophages [3, 35], as they can influence the ability to infect of the distinct ASFV isolates.

Other interesting fact observed in our experiments is the modulation of cell surface molecules following ASFV *in vitro* infection. CD14 and CD16 reduced their expression in both monocytes and alveolar macrophages; similarly the expression of CD163 antigen on infected monocytes was slightly lower than that of non-infected monocytes. The reduction in the expression of these markers may be the result of a downregulation of these antigens directly induced by virus-cell interaction, as it has been reported in other viral models [37], or a consequence of soluble mediators or cytokines released by infected cells [4]. However, this reduction is not due to a general blockade of protein synthesis, since the expression of other molecules such as SWC3, CD45 or SLA I was not affected or, as in the case of the 2C12 and 4E9 antigens, even increased. This later antigen experiences the biggest increase in expression of all the molecules analyzed, however this increase was only found in alveolar macrophages.

2A10 and 4E9 mAbs inhibited ASFV infection and binding to alveolar macrophages suggesting a role for CD163 and 4E9 antigens in the initial steps of the infection process, related to the viral attachment to cells. In fact, by using an immunodot assay, we have been able to demonstrate binding of CD163 to ASF virus. Many viruses use multiple receptors to enter the cell [30, 47]. In some cases these receptors act sequentially, so binding to the first receptor is required for binding to the second [47]. The first ASFV protein reported to be involved in virus attachment to the host cell was p12 [2]. Afterwards, other ASFV proteins (p72, p54 and p30) have been involved in virus neutralization. Antibodies to p72 and p54 inhibit viral attachment to the host cell, whereas antibodies to p30 inhibit viral internalization [24]. Moreover, p54 and p30 proteins have been shown to bind to porcine alveolar macrophages and compete the virus binding and internalization, respectively [25]. Thus, it is possible that the initial binding of ASF virus to a specific cellular receptor facilitates the subsequent interaction of additional viral proteins with other cell receptors. Coexpression

of these receptors would increase the efficiency of virus binding and entry [47]. In this context it is tempting to think of CD163 and 4E9 antigens as components of a putative receptor complex. Although our results are compatible with such hypothesis we do not know at present whether CD163 and 4E9 act as true ASFV receptors and, if so, if they function together as part of the same receptor complex or separately. In addition, other molecules may also contribute to viral entry in these cells since combinations of 2A10 and 4E9 mAbs were unable to completely block ASFV binding and replication in macrophages. Alternatively, 2A10 and 4E9 mAbs may have lower binding affinity than the viral attachment protein, resulting in an incomplete blocking. The ability of ASFV to enter and replicate in cells that do not express the 2A10 and 4E9 molecules may indicate the existence of alternative receptors. The lack of high affinity receptors on cells such as hepatocytes, endothelial cells or renal tubular cells could explain their lower susceptibility to infection, that occurs only at the end of the disease, when most macrophages have already been destroyed and viral load is very high [39].

CD163 belongs to the scavenger receptor cysteine-rich (SRCR) superfamily, and has been recently identified as a scavenger receptor for hemoglobin-haptoglobin complexes [31, 32]. The molecule recognized by mAb 4E9 is more widely expressed in tissue macrophages than CD163 and is detected, although at low levels, on other cell types such as granulocytes, platelets or renal tubule epithelial cells [7]. This finding might explain, at least in part, the susceptibility to ASFV infection of some CD163⁻ cells [10]. In order to confirm the potential role of CD163 and 4E9 molecules as cellular receptors for ASFV we are currently cloning the genes that encode them. Transfection of nonpermissive cells with these genes will help to determine whether expression of 2A10 and/or 4E9 can render susceptible to ASFV binding and infection. Construction of mutant molecules to perform binding experiments with recombinant viral attachment proteins would be also interesting for further characterization of their role in ASFV infection. Precise characterization of host receptors for ASFV can have important implications in our understanding of the pathogenesis of the disease, as well as in the design of an effective vaccine. It may also lead to the development of competitive chemical compounds capable of blocking virus binding and subsequent infection of the cells.

Acknowledgements

This work was supported by CICYT grants BIO-2000-1636 and AGL2000-1441, and INIA SC98-090. We thank Drs. R. Blasco and I. Galindo for critical reading of manuscript.

References

1. Alcamí A, Carrascosa AL, Viñuela E (1990) Interaction of African swine fever virus with macrophages. *Virus Res* 17: 93–104
2. Angulo A, Viñuela E, Alcamí A (1993) Inhibition of African swine fever virus binding and infectivity by purified recombinant virus attachment protein p12. *J Virol* 67: 5463–5471

3. Basta S, Knoetig SM, Spagnuolo-Weaver M, Allan G, McCullough KC (1999) Modulation of monocytic cell activity and virus susceptibility during differentiation into macrophages. *J Immunol* 162: 3961–3969
4. Buechler C, Ritter M, Orso E, Langmann T, Klucken J, Schmitz G (2000) Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* 67: 97–103
5. Bullido R, Ezquerra A, Alonso F, Gómez del Moral M, Domínguez J (1996a) Characterization of a new monoclonal antibody (4B7) specific for porcine MHC (SLA) class I antigens. *Invest Agr Prod Sanid Anim* 11: 29–37
6. Bullido R, Alonso F, Gómez del Moral M, Ezquerra A, Alvarez B, Ortuño E, Domínguez J (1996b) Monoclonal antibody 2F4/11 recognizes the α – chain of a porcine β 2 integrin involved in adhesion and complement mediated phagocytosis. *J Immunol Methods* 195: 125–134
7. Bullido R, Gómez del Moral M, Alonso F, Ezquerra A, Zapata A, Sánchez C, Ortuño E, Alvarez B, Domínguez J (1997a) Monoclonal antibodies specific for porcine monocytes/macrophages: macrophage heterogeneity in the pig evidenced by the expression of surface antigens. *Tissue Antigens* 49: 403–413
8. Bullido R, Domenech N, Alvarez B, Alonso F, Babín M, Ezquerra A, Ortuño E, Domínguez J (1997b) Characterization of five monoclonal antibodies specific for swine class II major histocompatibility antigens. Crossreactivity studies with leukocytes of domestic animals. *Dev Comp Immunol* 21: 311–322
9. Bullido R, Gómez del Moral M, Domenech N, Alonso F, Ezquerra A, Domínguez J (1997c) Monoclonal antibodies to a high molecular weight isoform of porcine CD45: biochemical and tissue distribution analyses. *Vet Immunol Immunopathol* 56: 151–162
10. Carrasco L, Gómez-Villamandos JC, Bautista MJ, Martín de las Mulas J, Villeda CJ, Wilkinson PJ, Sierra MA (1996) In vivo replication of African swine fever virus (Malawi 83) in neutrophils. *Vet Res* 27: 55–62
11. Carrascosa A, Santarén J, Viñuela E (1982) Production and titration of African swine fever virus in porcine alveolar macrophages. *J Virol Methods* 3: 303–310
12. Carrascosa AL, Bustos MJ, Galindo I, Viñuela E (1999) Virus-specific cell receptors are necessary, but not sufficient, to confer cell susceptibility to African swine fever virus. *Arch Virol* 144: 1309–1321
13. Chamorro S, Revilla C, Alvarez B, Lopez-Fuertes L, Ezquerra A, Dominguez J (2000) Phenotypic characterization of monocyte subpopulations in the pig. *Immunobiology* 202: 82–93
14. Cheng-Mayer C, Liu R, Landau NR, Stamatatos L (1997) Macrophage tropism of immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. *J Virol* 71: 1657–1661
15. Cirino NM, Panuska JR, Villani A, Taraf H, Rebert NA, Merolla R, Tsivitse P, Gilbert IA (1993) Restricted replication of respiratory syncytial virus in human alveolar macrophages. *J Gen Virol* 74: 1527–1537
16. Colgrove GS, Haelterman EO, Coggins L (1969) Pathogenesis of African swine fever in young pigs. *Am J Vet Res* 30: 1343–1359
17. Dixon LK, Costa JV, Escribano JM, Rock DL, Viñuela E, Wilkinson PJ (2000) Asfarviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Cartens E, Estes M, Lemon S, Maniloff J, Mayo MA, McCleoch D, Pringle CR, Wickner RB (eds) *Virus Taxonomy*. Seventh report of the International Committee on Taxonomy of Viruses, pp 159–165. Academic Press, New York
18. Domínguez J, Ezquerra A, Alonso F, McCullough K, Summerfield A, Bianchi A, Zwart RJ, Kim YB, Blecha F, Eicher S, Murtaugh M, Pampusch M, Burger K (1998)

- Porcine myelomonocytic markers: summary of 2° CD porcine workshop. *Vet Immunol Immunopathol* 60: 329–341
19. Domínguez J, Alvarez B, Alonso F, Thacker E, Haverson K, McCullough K, Summerfield A, Ezquerro A (2001) Workshop studies on monoclonal antibodies in the myeloid panel with CD11 specificity. *Vet Immunol Immunopathol* 80: 111–119
 20. Duan X, Nauwynck HJ, Pensaert MB (1997) Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). *Arch Virol* 142: 2483–2497
 21. Galindo Y, Viñuela E, Carrascosa AL (1997) Protein cell receptors mediate the saturable interaction of African swine fever virus attachment protein p12 with the surface of permissive cells. *Virus Res* 49: 193–204
 22. Gendelman HE, Narayan O, Kennedy-Stoskopf S, Kennedy PG, Ghotby Z, Clements JE, Stanley J, Pezeshkpoor G (1986) Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. *J Virol* 58: 67–74
 23. Gómez-Puertas P, Rodríguez F, Ortega A, Oviedo JM, Alonso C, Escribano JM (1995) Improvement of African swine fever virus neutralization assay using recombinant viruses expressing chromogenic marker genes. *J Virol Methods* 55: 271–279
 24. Gomez-Puertas P, Rodriguez F, Oviedo JM, Ramiro-Ibañez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM (1996) Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *J Virol* 70: 5689–5694
 25. Gomez-Puertas P, Rodriguez F, Oviedo JM, Brun A, Alonso C, Escribano JM (1998) The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology* 243: 461–471
 26. González S, Mendoza C, Sánchez-Vizcaíno JM, Alonso F (1990) Inhibitory effect of African swine fever virus on lectin-dependent swine lymphocyte proliferation. *Vet Immunol Immunopathol* 26: 71–80
 27. Gonzalez-Juarrero M, Lunney JK, Sánchez-Vizcaíno JM, Mebus C (1992) Modulation of splenic macrophages, and swine leukocyte antigen (SLA) and viral antigen expression following African swine fever virus (ASFV) inoculation. *Arch Virol* 123: 145–156
 28. Gordon S, Clarke S, Greaves D, Doyle A (1995) Molecular immunobiology of macrophages: recent progress. *Curr Opin Immunol* 7: 24–33
 29. Grage-Griebenow E, Flad HD, Ernst M (2001) Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol* 69: 11–20
 30. Haywood AM (1994) Virus receptors: binding, adhesion strengthening, and changes in viral structure. *J Virol* 68: 1–5
 31. Kristiansen M, Graverson JH, Jacobsen C, Sonne O, Hoffman HJ, Law SKA, Moestrup SK (2001) Identification of the haemoglobin scavenger receptor. *Nature* 409: 198–201
 32. Law SKA, Micklem KJ, Shaw JM, Zhang XP, Dong Y, Willis AC, Mason DY (1993) A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *Eur J Immunol* 23: 2320–2325
 33. Lewin SR, Lambert P, Deacon NJ, Mills J, Crowe SM (1997) Constitutive expression of p50 homodimer in freshly isolated human monocytes decreases with in vitro and in vivo differentiation: a possible mechanism influencing human immunodeficiency virus replication in monocytes and mature macrophages. *J Virol* 71: 2114–2119
 34. McCullough KC, Schaffner R, Fraefel W, Kihm U (1993) The relative density of CD44-positive porcine monocytic cell populations varies between isolations and upon culture and influences susceptibility to infection by African swine fever virus. *Immunol Lett* 37: 83–90

35. McCullough KC, Basta S, Knotig S, Gerber H, Schaffner R, Kim YB, Saalmuller A, Summerfield A (1999) Intermediate stages in monocyte-macrophage differentiation modulate phenotype and susceptibility to virus infection. *Immunology* 98: 203–212
36. Minguez I, Rueda A, Dominguez J, Sánchez-Vizcaíno JM (1988) Double labeling immunohistological study of African swine fever-infected spleen and lymph nodes. *Vet Pathol* 25: 193–198
37. Naniche D, Wild TF, Rabourdin-Combe C, Gerlier D (1993) Measles virus hemagglutinin induces downregulation of gp57/67, a molecule involved in virus binding. *J Gen Virol* 74: 1073–1079
38. Oura CA, Powell PP, Parkhouse RM (1998) African swine fever: a disease characterized by apoptosis. *J Gen Virol* 79: 1427–1438
39. Perez J, Rodríguez F, Fernández A, Martín de las Mulas J, Gómez-Villamandos JC, Sierra MA (1994) Detection of African swine fever virus protein VP73 in tissues of experimentally and naturally infected pigs. *J Vet Diagn Invest* 6: 360–365
40. Pescovitz MD, Book BK, Aasted B, Domínguez J, Bullido R, Trebichavsky I, Novikov B, Valpotic I, Tomaskovic M, Nielsen J, Arn S, Sachs DH, Lunney JK, Boyd P, Walker J, Lee R, Saalmuller A (1998) Analyses of monoclonal antibodies reacting with porcine CD5. *Vet Immunol Immunopathol* 60: 269–273
41. Ramiro-Ibañez F, Escribano JM, Alonso C (1995) Application of a monoclonal antibody recognizing protein p30 to detect African swine fever virus-infected cells in peripheral blood. *J Virol Methods* 55: 339–345
42. Ramiro-Ibañez F, Ortega A, Ruiz-Gonzalvo F, Escribano JM, Alonso C (1997) Modulation of immune cell populations and activation markers in the pathogenesis of African swine fever virus infection. *Virus Res* 47: 31–40
43. Rich EA, Chen IS, Zack JA, Leonard ML, O'Brien WA (1992) Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1). *J Clin Invest* 89: 176–183
44. Ruiz-Gonzalvo F, Carnero ME, Caballero C, Martínez J (1986) Inhibition of African swine fever infection in the presence of immune sera in vivo and in vitro. *Am J Vet Res* 47: 1249–1252
45. Rutherford MS, Witsell A, Schook LB (1993) Mechanisms generating functionally heterogeneous macrophages: chaos revisited. *J Leukoc Biol* 53: 602–618
46. Sanchez C, Domenech N, Vazquez J, Alonso F, Ezquerro A, Dominguez J (1999) The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. *J Immunol* 162: 5230–5237
47. Shafren DR, Dorahy DJ, Ingham RA, Burns GF, Barry RD (1997) Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry. *J Virol* 71: 4736–4743
48. Thacker E, Summerfield A, McCullough K, Ezquerro A, Dominguez J, Alonso F, Lunney J, Sinkora J, Haverson K (2001) Summary of workshop findings for porcine myelomonocytic markers. *Vet Immunol Immunopathol* 80: 93–109
49. Wardley RC, Wilkinson PJ (1977) The growth of virulent African swine fever virus in pig monocytes and macrophages. *J Gen Virol* 38: 183–186
50. Whyard TC, Wool SH, Letchworth GJ (1985) Monoclonal antibodies against African swine fever viral antigens. *Virology* 142: 416–420
51. Ziegler-Heitbrock HWL (1996) Heterogeneity of human blood monocytes: the CD14⁺ CD16⁺ subpopulation. *Immunol Today* 17: 424–428

Author's address: A. Ezquerro, Dpto. Biotecnología. INIA, Ctra de la Coruña km. 7,5, 28040, Madrid, Spain; e-mail: ezquerr@inia.es