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## Review

# Pathology of African swine fever: The role of monocyte-macrophage

J.C. Gómez-Villamandos\*, M.J. Bautista, P.J. Sánchez-Cordón, L. Carrasco

Department of Comparative Pathology, Veterinary Faculty, University of Córdoba-Agrifood Campus of International Excellence (ceiA3), Edificio Sanidad Animal, Campus de Rabanales, 14014 Córdoba, Spain

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### ABSTRACT

African swine fever (ASF) is a viral hemorrhagic disease with different clinical and lesional changes depending of virulence of strains/isolates and immunological status of pigs. In acute and subacute forms of ASF, severe vascular changes are present, with hemorrhages in different organs (mainly melena, epistaxis, erythema, renal petechiae and diffuse hemorrhages in lymph nodes), pulmonary edema, disseminate intravascular coagulation and thrombocytopenia. Lymphopenia and monocytopenia are developed during acute and subacute ASF. Lymphopenia is associated with lymphoid depletion in primary and secondary lymphoid organs, which is caused by apoptosis. All these lesions are not related to viral replication in endothelial cells or lymphocytes. Monocytes-macrophages show viral replication and cytopathic effect, including hemadsorption. The more significant changes in these cells are increased number and secretory activation (increased levels of proinflammatory cytokines) in targets organs. Proinflammatory activation is the initial cause of clinical and lesional pictures in ASF, including fever and changes in levels of acute phase proteins. Levels of IFN- $\beta$  and - $\gamma$  are increased from initial phase of acute ASF. Anti-inflammatory response, represented by increased level of IL-10, is observed also, although in the final phase of acute ASF only.

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### 1. Introduction

The course of the African swine fever (ASF) may vary depending on a number of factors, including the host, the virus, and the immunological status of the farm. In acute and subacute forms,

the more significant lesions are vascular changes – and particularly bleeding – prompting a high mortality rate (Anderson, 1986; Villeda et al., 1993a, 1995). In clinical terms, acute ASF develops over a 7-day period, compared with 10–20 days for the subacute form of the disease. Chronic ASF cause clinical lesions, they are just not specific and infection is detected by serological screening for anti-ASF virus antibodies (Pan and Hess, 1984). Moderate-to-low virulence ASF virus isolates have been reported to trigger persistent viral infections (Mebus and Dardiri, 1979; McVicar, 1984), helping

\* Corresponding author. Tel.: +34 957218162; fax: +34 957212018.  
E-mail address: [jcgomez@uco.es](mailto:jcgomez@uco.es) (J.C. Gómez-Villamandos).

the disease to persist in endemic areas; the virus may be isolated from blood and lymphoid organs over a period of several months (McVicar, 1984).

Animals with acute ASF display fever and a tendency to crowding; they also exhibit loss of appetite, inactivity, apathy and early leukopenia, induced by lymphopenia and changes in monocyte numbers (Colgrove et al., 1969; Pan and Hess, 1984). Despite the presence of bleeding and bone-marrow lesions, platelet counts are not significantly impaired (Villeda et al., 1993a).

Affected pigs show erythema (particularly affecting the skin of the ears, tail, distal extremities, chest, abdomen and perianal area), together with vascular lesions, which tend to be more apparent in white pigs. Pigs also develop cyanosis of the skin in these areas 1–2 days prior to death. Mucosal nasal discharge and nasal hemorrhage (epistaxis) may also be observed. Other symptoms include vomiting, abdominal pain, constipation, and diarrhoea, which is initially mucoid but may later become bloody (melena) (Moulton and Coggins, 1968; Mebus and Dardiri, 1979).

While clinical signs tend to be less marked in subacute ASF, vascular lesions are often more severe. The subacute form of the disease carries an additional risk: animals that recover may continue to carry the virus over a long period (McVicar, 1984). These clinical signs in acute and, especially, subacute ASF may be confused with other swine hemorrhagic diseases including classical swine fever and erysipelas.

Together with the vascular lesions observed in live animals developing acute ASF (sometimes only renal petechiae), other characteristic changes include hyperemic splenomegaly, pulmonary edema, hemorrhages and disseminated intravascular coagulation (DIC). The pathogenesis of these changes has been widely studied; research into the mechanisms involved in virus spread and lymphopenia has highlighted the major modulatory role played by monocytes-macrophages (m-MØ).

## 2. Virus replication and cytopathic effect

In infected pigs, monocytes and macrophages appear to be the main ASF virus target cells. Virus replication causes cell swelling and rounding, together with margination of nuclear chromatin (Fig. 1A–D). On semithin sections stained with toluidine blue, virus replication is identified by these changes in cell and nucleus size and morphology, and by the presence of intracytoplasmic juxtanuclear inclusion bodies – identifiable by their pale coloring – in which ASF virus replication is taking place. Viral infection/replication can be detected by immunohistochemical techniques (Fig. 1A and B), with diffuse cytoplasmic staining and cytoplasmic inclusion body with dark staining in some cells (Fig. 1B). Ultrastructural examination reveals nuclear changes, dilated membranous structures in the cytoplasm, and ASF virus replication sites; the latter are evident as rounded organelle-free areas containing elongated membranous structures and both mature and immature viral particles. Mature viral particles are seen as icosahedral structures (175–190 nm in diameter) containing an electron-dense nucleoid. Mature and immature viral particles are associated with ribosomes and helical polyribosomes in viral replication sites. Virus particles are released from infected cells by budding (Fig. 1C and D) (Sierra et al., 1987; Brookes et al., 1998; Gómez-Villamandos et al., 1997a,b). Virus replication induces necrosis of infected cell and cytoplasmic non-enveloped viral particles are free and they can be observed in blood, lymph and interstitial tissue. Apoptosis of porcine macrophage cultures during *in vitro* infection with the high- and low-virulence isolates have been studied and significant proportion of the infected cell populations remained non-apoptotic cell have been observed with highly virulent strain, but not with moderately virulent strain (Portugal et al., 2009).

Following oral-nasal or intramuscular infection, the virus replicates mainly in mononuclear phagocytic cells in tonsils and in mandibular and other regional lymph nodes, spreading through lymph and blood to secondary organs of replication, where it may be detected within 2–3 dpi (Colgrove et al., 1969). In these secondary organs, replication is mostly observed in macrophages, but in the middle and final phases of acute ASF (5–7 dpi), evidence of virus replication is found in non-m-MØ cells, epithelial and/or mesenchymal cells (Table 1), a phenomenon that may play a minor role in the pathogenesis of the disease.

The abundant replication and necrosis observed in macrophages in various organs leads to the presence of numerous free virions in the interstitial space (Carrasco et al., 1996b). This, together with the destruction of ASF virus target cells in these areas, probably favours the infection of other cell populations during later stages of the disease (Fernández et al., 1992; Gómez-Villamandos et al., 1995c, 1999). However, the observation of virus replication in non-macrophage cells when m-MØ are still present and undergoing intense proliferation suggests the existence of a different, more complex, mechanism in the infection of these cells (Gómez-Villamandos et al., 1999). This mechanism may involve the activation of m-MØ and chemical mediators secreted by them, possibly leading to the expression of an ASF virus-specific membrane receptor (Gómez-Villamandos et al., 1999). This hypothesis is borne out by the fact that fibroblasts, smooth muscle cells, megakaryocytes, fat-storing cells and reticular cells have little phagocytic capacity; the virus is therefore unlikely to enter these cells along with phagocytosed cell debris (Alcami et al., 1990).

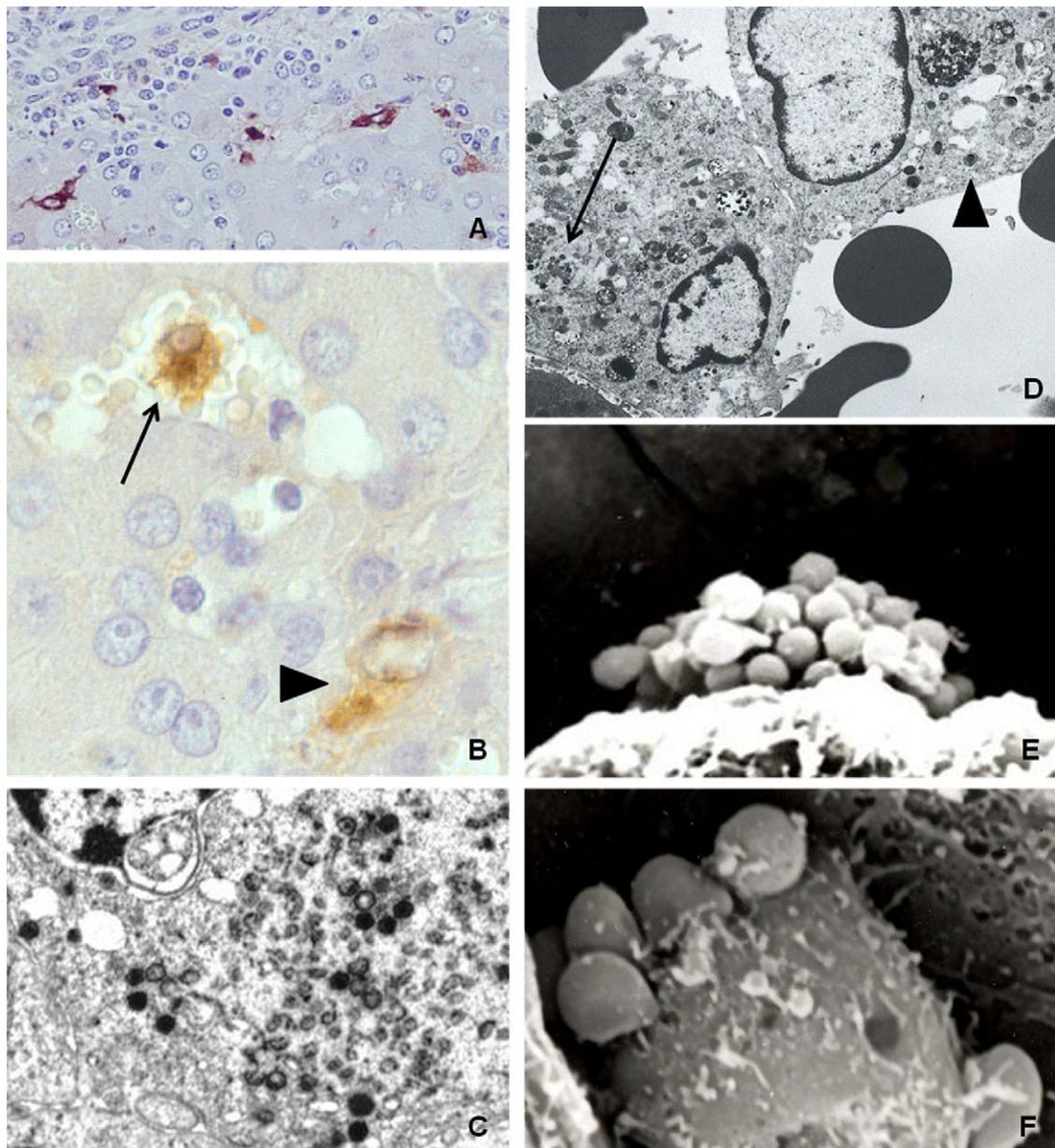
Hemadsorption is a characteristic feature of ASF, first reported *in vitro* and subsequently used for diagnostic purposes. In cell cultures, erythrocytes appear in a crownlike arrangement surrounding infected cells. Similar findings have been reported *in vivo* in ASF-infected pigs (Sierra et al., 1991), where pear-shaped erythrocytes have been observed surrounding infected macrophages (Fig. 1E) and monocytes (Fig. 1F). There are close links between hemoadsorption, virus budding and the presence of viral particles adhering to erythrocytes in small invaginations of the cytoplasmic membrane, a characteristic feature of ASF virus spread from the earliest stages of the disease.

Virus particles, but not virus replication, have been observed in lymphocytes (Carrasco et al., 1996d) and platelets, as a consequence of virus replication in megakaryocytes (Gómez-Villamandos et al., 1997a). These elements act as passive vehicles for the spread of the virus throughout the body; their function is thus similar to, though less intense than, that of erythrocytes (Anderson, 1986; Sierra et al., 1991), and becomes apparent only in advanced stages of the disease.

## 3. Macrophage activation

In addition to the phenomena described above (replication, hemoadsorption and necrosis), m-MØ also undergo a number of changes which play a major role in the pathogenesis of ASF: phagocytic activation, increased numbers and secretory activation. Infected and uninfected macrophages show morphological changes indicative of phagocytic activation, characterized by increased cell size, lysosome proliferation and presence of phagocytosed cell debris.

Research suggests that despite virus replication in macrophages and their subsequent necrosis during ASF, there is an increase in macrophage numbers in all organs in which virus replication takes place, involving different populations of fixed and interstitial macrophages. This increase is associated with enhanced macrophage secretory activity and the release of cytokines, demonstrated in pigs infected with virulent ASF virus isolates using a range



**Fig. 1.** Immunohistochemical detection (ABC technique) of gP55 ASF virus in Kupffer cells and interstitial macrophages (A), in a monocyte with hemadsorption and dark brown inclusion body (arrow) and in a hepatocyte (arrowhead) (B). Ultrastructural detail of viral replication site and viral budding in an interstitial macrophage (C). Ultrastructural evidences of viral replication in Kupffer cells with slight signs of hemadsorption (arrow) and activation (arrowhead) (D). Scanning electron microscopy pictures showing hemadsorption in a Kupffer cell (E) and in a monocytes circulating in a hepatic sinusoid (F).

**Table 1**  
 Viral replication and infection in non-m-MØ.

Cells	Technique	Authors
Hepatocytes	TEM	Sierra et al. (1987)
Capillary endothelium (kidney, liver)	TEM	Sierra et al. (1989), Gómez-Villamandos et al. (1995a,b)
Epithelium of collector renal ducts	TEM/IHC	Fernández et al. (1992), Gómez-Villamandos et al. (1995c)
Tonsillar epithelium	TEM	Gómez-Villamandos et al. (1997b)
Fibroblasts	TEM	Gómez-Villamandos et al. (1995d)
Reticular cells	TEM	Gómez-Villamandos et al. (1997a)
Smooth muscle cells	TEM	Gómez-Villamandos et al. (1995d)
Pericytes	TEM	Carrasco et al. (1996a)
Ito cells	TEM	Gómez-Villamandos et al. (1995a)
Glomerular mesangial cells	TEM	Gómez-Villamandos et al. (1995c)
Megakaryocytes	TEM	Gómez-Villamandos et al. (1997a, 1998)
Neutrophils (and precursor)	TEM	Carrasco et al. (1996b)
Lymphocytes	TEM/ISH	Carrasco et al. (1996c), Ballester et al. (2010)

TEM: transmission electron microscopy, IHQ: immunohistochemical techniques ISH: in situ hybridisation.

of techniques including TNF- $\alpha$  and IL-1 immunolabelling and in situ hybridization for the detection of IL-1. Cytokine expression has been observed in both uninfected and infected cells, demonstrating that virus replication does not inhibit secretory activity (Gómez-Villamandos et al., 1999). The role of this secretory activation in the pathogenesis of ASF will be discussed in this paper.

With regard to the numerical changes undergone by m-M $\phi$ , it should be noted that research in the 80s and even into the 90s (e.g. Ramiro-Ibáñez et al., 1997) pointed to the destruction of m-M $\phi$ , and subsequent immunodepression favouring the appearance of secondary infection, as a major factor in the pathogenesis of ASF. However, later studies (Sierra et al., 1989; Gómez-Villamandos et al., 1995a) showed that intense m-M $\phi$  necrosis takes place only at a late stage of the disease, by which time the lesions characteristic of ASF are evident and the pathogenic mechanisms involved have already been activated. Studies on acute ASF have shown a sharp increase in macrophage numbers in target organs, linked to the arrival of the virus (e.g. at 3 dpi in the liver and 5 dpi in the kidney). Thereafter, numbers are significantly depleted at a late stage in disease due to the necrosis induced by virus replication; even then, numbers of macrophages remain higher than in healthy controls, although the marked phagocytosis taking place at this stage may lead to impaired macrophage function (Sánchez-Vizcaíno et al., 1981; Martins et al., 1988).

Subcellular findings, coupled with the negative results obtained using techniques to detect the chromatin fragmentation prompted by apoptosis, leave little doubt as to the necrosis induced by virus replication; the involvement of apoptosis in m-M $\phi$  depletion during virus infection can be ruled out (Ramiro-Ibáñez et al., 1996).

It has been suggested that secretory activation of proinflammatory cytokines m-M $\phi$  during the early stages of infection plays a key role in the pathogenesis of ASF (Gómez-Villamandos et al., 1995c, 1995d). This activation has been detected by ultrastructural study (Fig. 2A and B) and immunohistochemically in lymphoid organs (Salguero et al., 2002, 2005), lung (Carrasco et al., 2002), liver (Fig. 3A and B) and kidney (Fig. 3C and D). A significant increase in

serum TNF- $\alpha$  and IL-1 $\beta$  levels has been observed from 2 dpi by us in different experiments with pigs infected with highly-virulent strains. The increase in TNF- $\alpha$ -specific mRNA is correlated with the expression of viral antigens encoding proteins p30 or p52. However, TNF- $\alpha$  mRNA decreased to baseline levels at 2 dpi, whereas viral mRNA persisted or increased until death. Similarly, the number of TNF- $\alpha$ -producing cells was always lower than that of ASF virus-infected cells. This may reflect the highly-regulated expression of this cytokine, due to products released by neighboring cells, including IL-10, observed in our experiments, TGF- $\beta$ , and prostaglandins, capable to induce TNF- $\alpha$  production. These results contrast with those reported by Powell et al. (1996), who found that ASF virus inhibited the production of TNF- $\alpha$  in infected macrophages. The discrepancy between the findings of these studies could be due to the different ASF virus isolates tested (Gómez del Moral et al., 1999; Gil et al., 2008; Salguero et al., 2008).

Early signs of fever and the increase of TNF- $\alpha$  and IL-1 $\beta$  in serum levels coincide with elevated cytokine expression in lymphoid organs from 1 to 2 dpi (Salguero et al., 2002, 2005) a slight increased expression of these cytokines in the liver. These facts suggest that the onset of fever and the increase in these cytokine production in the initial stage of acute ASF is linked to the activation of various macrophage populations in the spleen and lymph nodes mainly, and of Kupffer cells secondarily, which also show expression of IL-6. In this way, increased cytokine expression would appear to depend on the virus reaching the organ in question, rather than being the result of a uniform, synchronized systemic response. On the other hand, acute-phase-protein synthesis by the liver is associated to activation of Kupffer cells (Risalde et al., 2011; Bode et al., 2012). In acute ASF, a medium increase in serum amyloid A, and slight increase C-reactive protein (classified as type 1 acute phase proteins, and predominantly induced by IL-1 and TNF- $\alpha$ ) and haptoglobin (classified as type 2 acute phase proteins, and mainly induced by IL-6) have been detected, related to viral replication in Kupffer cells and activation of these cells. (Carpintero et al., 2007; Sánchez-Cordón et al., 2007).

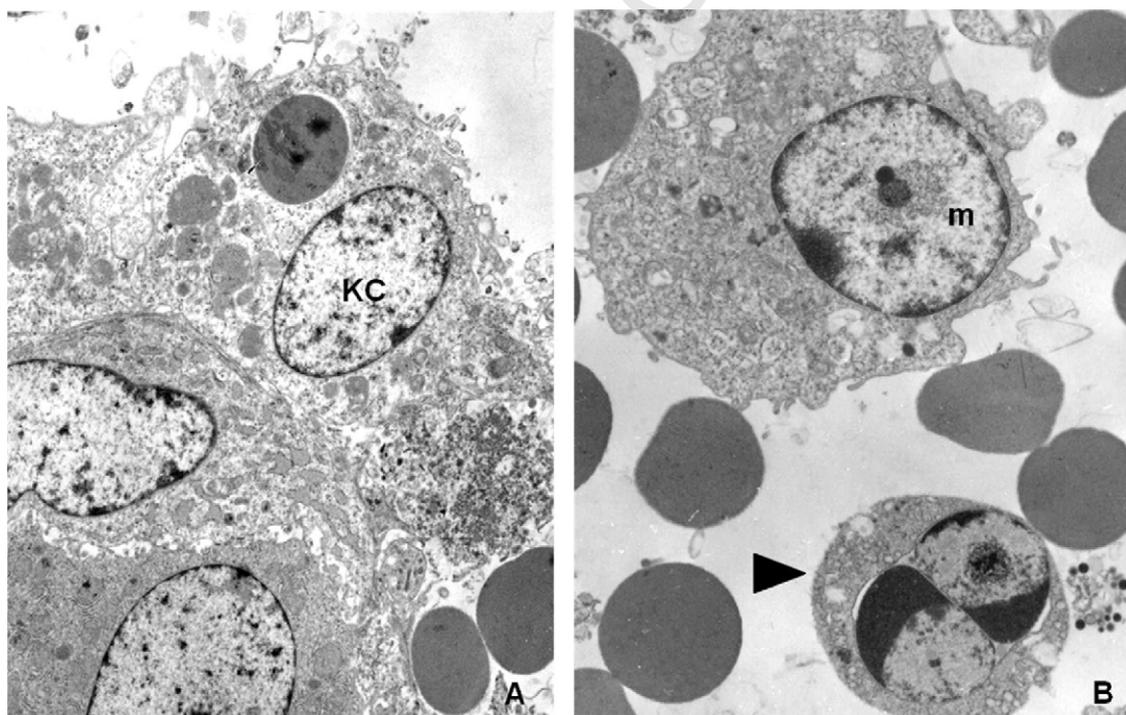
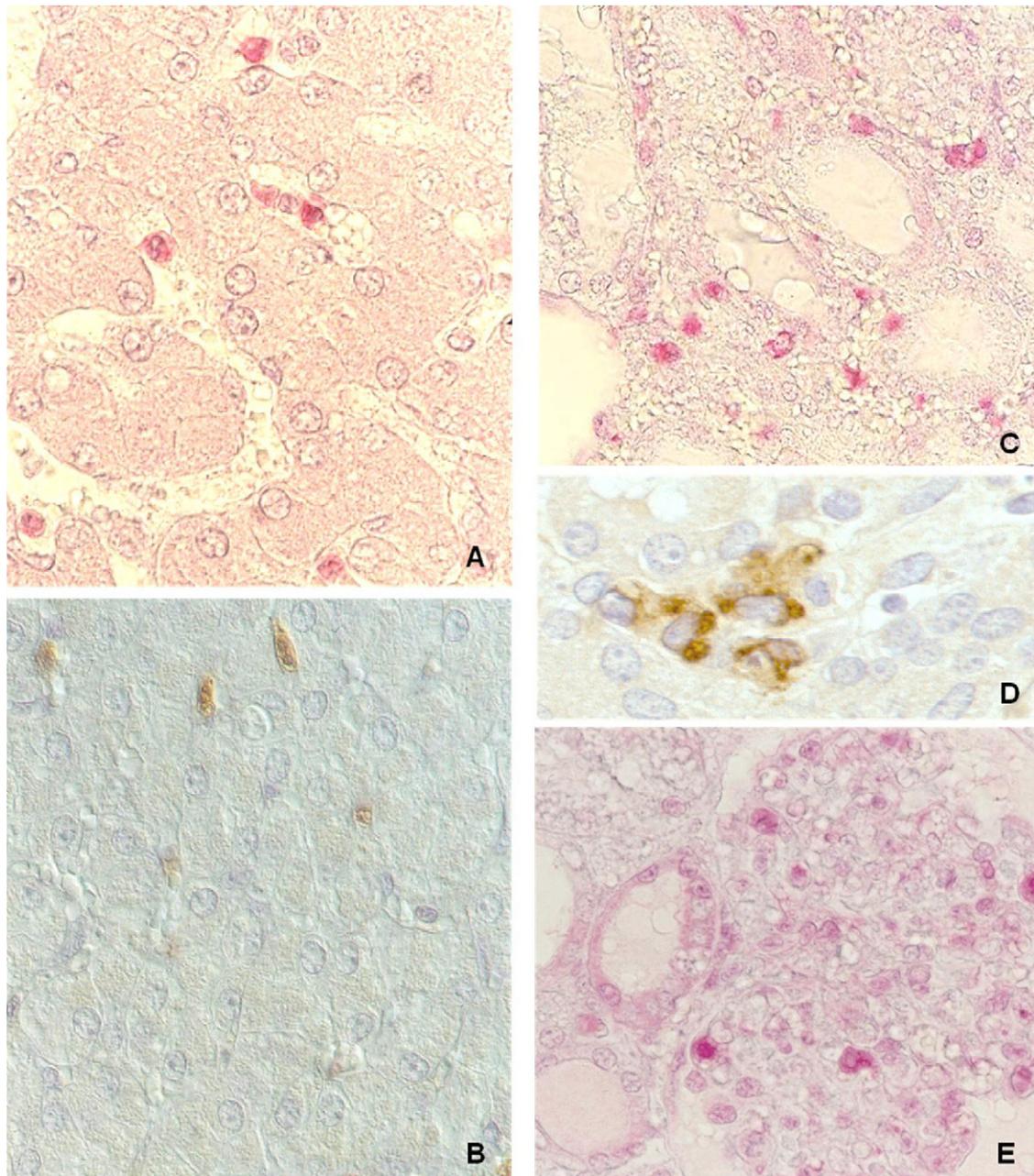


Fig. 2. Kupffer cell (A) and a monocyte (B) with ultrastructural changes indicative of secretory activation. An apoptotic circulating lymphocyte (arrowhead) (B).



**Fig. 3.** Immunohistochemical expression of TNF- $\alpha$  (A, C, E) (sABFA technique) and IL-1 $\alpha$  (B, D) (ABC technique) in liver (A, B), renal interstitium (C, D) and renal glomerulus, which shows mesangial proliferation (E).

242 While cells staining positive to the various antibodies used  
 243 for cytokine detection are primarily m-M $\phi$ , positive staining  
 244 has also been observed in other cell populations, including  
 245 proinflammatory-cytokine-secreting stromal cells, in the final  
 246 stages of ASF. These cells may modulate the clinical symptoms  
 247 and lesions characteristic of the disease through the release of  
 248 chemical mediators, inducing changes in non-infected neighboring  
 249 cells. Virus replication or infection in non-m-M $\phi$  cells, particularly  
 250 endothelial cells or lymphocytes, can be ruled out as the main cause  
 251 of clinical signs and lesions in ASF, since infection or replication  
 252 in these cells takes place only in the final stages of the disease  
 253 (Gómez-Villamandos et al., 1995b, 1995d).

254 It should also be noted that macrophages have been observed  
 255 in areas of organs where they are not normally found, such  
 256 as the glomerular mesangium. Their presence may be responsible  
 257 for some of the changes taking place at local level, such as

258 mesangioproliferative glomerulonephritis, a lesion characterized  
 259 by the proliferation of mesangial cells. The pathogenesis of this  
 260 lesion generally involves growth factors including IL-6, identified  
 261 as an autocrine growth factor for mesangial cells (Rampino  
 262 et al., 2011), which has been detected by the authors in the  
 263 glomerular mesangium coinciding with the onset of mesangiopro-  
 264 liferative glomerulonephritis (Fig. 3E). The production of IL-6 may  
 265 lie either in the mesangial cells themselves or in a small number of  
 266 macrophages infiltrating this area of the kidney.

267 Macrophages may be responsible for the changes observed in IL-  
 268 8 and IL-10 levels. IL-8 increases from 3 dpi, coinciding with slight  
 269 inflammatory lesions in lungs characterized by presence of neutro-  
 270 phils. Increased level of IL-10 is detected in the final stages of  
 271 acute ASF. The last cytokine has an inhibitory effect on the pro-  
 272 duction of proinflammatory cytokines (Iyer and Cheng, 2012), and  
 273 could have an important role in the pathogenesis of subacute form

of ASF or it could be a switch to determine the course of the disease. Further studies about the regulation of inflammation (expression of proinflammatory and anti-inflammatory cytokines) in subacute ASF should be developed.

#### 4. Vascular changes

Characteristic vascular changes during acute ASF include petechial hemorrhage, bleeding of varying intensity in gastrohepatic and renal lymph nodes, hyperemic splenomegaly, pulmonary edema and DIC. Edema and hemorrhages are more intense in subacute than acute ASF. Ascites, hydropericardium, perirenal edema and edemas of the gall bladder and bile-duct walls are also more common, and partial hyperemic splenomegaly is often observed. The kidneys display more intense bleeding (petechiae and ecchymoses) in the cortex, medulla and renal pelvis in subacute ASF than in the acute form. Bleeding may also be observed in gastrohepatic and renal lymph nodes, as well as in submandibular, retropharyngeal, mediastinal, mesenteric and inguinal lymph nodes.

##### 4.1. Hyperemic splenomegaly

The spleen is widely considered the chief target organ of ASF after the lymph nodes. The most characteristic splenic lesion in ASF is a hyperemic splenomegaly, which varies in severity depending on the virulence of the inoculated strain (Mebus and Dardiri, 1979; Carrasco et al., 1995). In acute ASF, the spleen crosses the entire abdominal cavity, from one side to the other; it is very large (up to 6 times larger than normal), with rounded edges, friable in consistency and purplish-black in color. In contrast, following infection with a moderately-virulent strain or isolate, initial hyperemic splenomegaly is progressively reversed, leaving some focal damage which eventually disappears; low-virulence strains may prompt no more than increased spleen firmness.

Like gross lesions, microscopic lesions depend on the virulence of the strain and on the effect of the virus on the abundant m-MØs in the spleen. ASF virus primarily targets splenic red pulp, the marginal zone and sheathed capillaries; lesions tend to be most intense in these areas, where m-MØs are usually abundant. Acute ASF is characterized by hyperemic vascular changes, lymphoid depletion and virus replication in macrophages leading to their necrosis, while chronic ASF is more often marked by proliferative lesions.

Splenic red pulp is hyperemic, and may be completely filled by erythrocytes, platelet thrombi, fibrin networks and cell debris (Carrasco et al., 1997a), leading to structural disruption. It is in fact the structure of splenic red pulp that gives rise to the hyperemic splenomegaly characteristic of acute ASF. Red pulp comprises a mesh of fibers and smooth muscle cells surrounded by a fixed population of splenic-cord macrophages. Virus replication and cytopathic effect are observed in these macrophages, inducing loss of intercellular junctions and the progressive detachment of macrophages from smooth muscle cells. Macrophages subsequently disappear due to virus replication. Exposure of the *lámina externa basal lamina* of muscle cells to blood induces activation of the coagulation system, involving platelet activation and aggregation and fibrin deposition; this in turn gives rise to the accumulation of erythrocytes within splenic cords. Blood clearance, one of the main functions of the spleen, is therefore impaired, and hypoxia induces lymphocyte necrosis, thus enhancing lymphoid depletion. (Carrasco et al., 1995, 1996b, 1997a). In the subacute form of the disease, the mechanism is similar but of lower intensity thus allowing the action of those enzymes liberated after the destruction of macrophages.

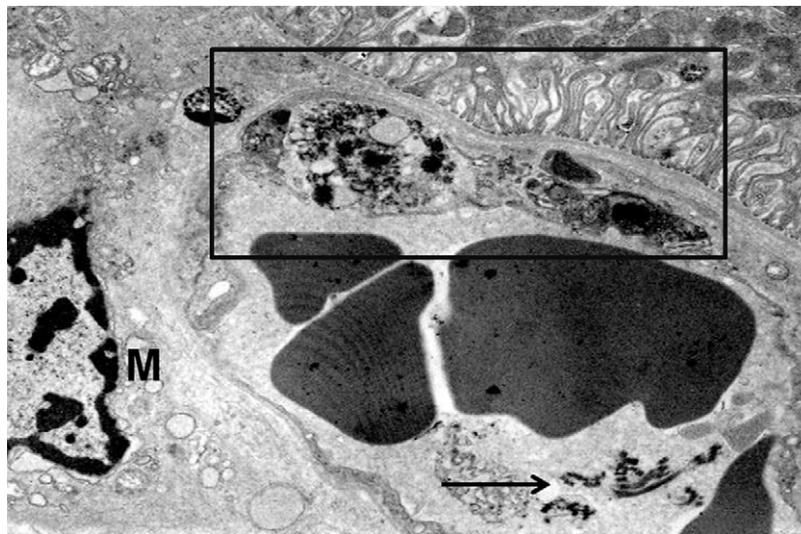
##### 4.2. Hemorrhage

Hemorrhage is among the lesions most consistently reported in a range of organs in acute and subacute forms of ASF; for that reason, ASF is classified as a viral hemorrhagic disease (Anderson, 1986; Sierra et al., 1987). The vascular changes observed in subacute forms of ASF, mainly hemorrhage and edema, are more intense than those reported in acute forms of the disease. The pathogenic mechanisms giving rise to hemorrhage differ depending on the virulence of the ASF virus isolate and on the presence or absence of fixed vascular macrophages in affected organs. Hemorrhages are more common in organs not containing a fixed vascular macrophage population, particularly gastrohepatic and renal lymph nodes, but they may also occur in other organs including the intestine, the heart and the lung, although the latter does contain fixed macrophages. The final stages of the disease are marked by extensive bleeding and severe alterations to the coagulation system, with increased bleeding time, impaired blood clot retraction and decreased platelet aggregation (Nesser and Kotzé, 1987).

Endothelial damage, as cause of hemorrhages, was initially attributed to direct action of the virus on endothelial cells (Colgrove et al., 1969; Sierra et al., 1989). However, ultrastructural examination has shown that when bleeding is first observed in the lymph nodes and kidneys of pigs infected with virulent ASF strains, there is no evidence of infection or virus replication in the endothelial cells of these organs (Gómez-Villamandos et al., 1995a; Carrasco et al., 1996b, 1997b); virus replication in these cells can thus be ruled out as the initial cause of hemorrhage in ASF, although it may contribute to bleeding in the final stages of the disease, when replication has been observed in the capillary endothelium.

However, bleeding coincides with another phenomenon which may account for the endothelial lesions: phagocytic activation of capillary endothelial cells, accompanied by lysosome proliferation and the presence of phagocytosed cell debris, gives rise to endothelial hypertrophy (Fig. 4) which may lead to complete occlusion of some capillary lumina, increasing intravascular pressure. Affected capillaries display a loss of endothelial cell junctions, cell debris is observed between basement membrane and endothelial cells, and there is a generalized loss of endothelium; due to endothelial disruption, cell debris and erythrocytes appearing in the interstitium give rise to hemorrhages. At the same time, this loss of endothelial cells results in exposure of the capillary basement membrane, to which activated platelets can adhere (Fig. 5). This phenomenon, coupled with the necrosis of splenic cord macrophages described earlier, prompts activation of the coagulation system, leading to the DIC characteristic of ASF (Villeda et al., 1993a,b). However, these lesions do not induce severe thrombocytopenia, probably due to the short course of the disease.

Why are not endothelial activation and hemorrhages present in all organs? The absence of a resident macrophage population in lymph nodes and renal interstitial capillaries may be a decisive factor in the phagocytic activation of endothelial cells in these vessels. Research has highlighted the absence of phagocytic activity in endothelial cells in the liver (Gómez-Villamandos et al., 1995c; Sánchez-Cordón et al., 2008), spleen and lung (Sierra et al., 1990; Pérez et al., 1994; Carrasco et al., 1996d) in ASF, since these organs have resident macrophage populations which clear cell debris from vascular lumina. Moreover, endothelial stimulation is not observed in lymphoid sinuses, which also have a resident macrophage population (Hoshi et al., 1988a,b). The cell debris phagocytosed by macrophages arises from the intense apoptosis and necrosis taking place in the liver, spleen and lymph nodes. These phenomena, coupled with the secretory activation of local interstitial macrophages (Fig. 4), may help to trigger the phagocytic activation of endothelial cells: macrophage numbers increase in the early and middle stages of the disease, thereafter declining in the late stages due to the



**Fig. 4.** Phagocytic activation of endothelium in an interstitial renal capillary (square), associated to a interstitial edema and with close relationship with a macrophage showing cellular changes indicative of secretory activation (M). Fibrin network (arrows).

direct action of the virus; they express proinflammatory cytokines (TNF- $\alpha$  and IL1 $\alpha$ ) which are evidence of secretory activation. This hypothesis is borne out by changes reported in classical swine fever (CSF), in which cell debris is also transported in the bloodstream but there is not a intense proinflammatory activation of macrophages near capillaries (Gómez-Villamandos et al., 2000).

The endothelial lesions described above are the morphological manifestation of blood biochemistry values indicative of endothelial dysfunction reported in acute ASF, including elevated Factor VIII levels (Edwards et al., 1984) and a fall in prostacycline levels (Anderson et al., 1987). Endothelial cell activation may be the cause of the increased tissue plasminogen activator levels detected in pigs infected with virulent ASF strains from 4 to 5 dpi (Villeda et al., 1995). This increase suggests over activation of the fibrinolytic system, which – together with the vasodilator effects of PGE<sub>2</sub> (Anderson et al., 1987) – may exacerbate the bleeding prompted by endothelial lesions.

Research into bleeding in pigs infected with moderately-virulent strains has shown that renal hemorrhage is more intense (petechiae and ecchymoses) and more extensive (cortex, medulla and pelvis) than in acute forms, but is not linked to the endothelial lesions reported in acute ASF (Gómez-Villamandos et al., 1995d). Morphological and ultrastructural examinations aimed

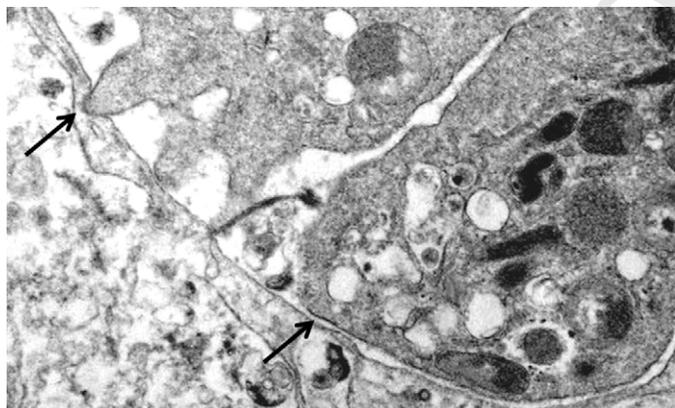
at ascertaining the origin of hemorrhage in subacute ASF suggest that the marked bleeding reported may be linked to intense vasodilation and increased vascular permeability, leading to severe interstitial edema and hemorrhage due to erythrodiapedesis (Gómez-Villamandos et al., 1995d). We have measured area of capillaries and we have detected a severe increase (vasodilatation). Moreover, numerous leukocytes and erythrocytes adhered to endothelium and crossing wall vascular by diapedesis, together intense perivascular edema (increased vascular permeability), have been observed. Mononuclear cell diapedesis gives rise to an intense perivascular infiltrate composed of macrophages, lymphocytes and plasma cells. The transmigration of cells from capillaries may be mediated by immune mechanisms, given the lymphoplasmacytic nature of the cell infiltrate and the existence of mesangioproliferative glomerulonephritis, a lesion associated with the deposition of immunoglobulins and complement C<sub>3</sub> (Hervás et al., 1996) and the elevated immunoglobulin levels recorded in pigs inoculated with attenuated ASF strains (Sánchez-Vizcaíno et al., 1981).

#### 4.3. Thrombocytopenia

The presence of thrombocytopenia appears to vary depending on the virulence of the ASF strain: following the experimental inoculation of a virulent strain (Villeda et al., 1993a,b) we have observed slight thrombocytopenia only at a late stage, once hemorrhage had been detected, and in many cases following the worsening and sudden death of affected pigs, although severe bone-marrow lesions and virus replication in stromal and hematopoietic cells are observed previously (Gómez-Villamandos et al., 1997a).

In subacute ASF, by contrast, an intense though transitory thrombocytopenia is observed in the early and middle stages of the disease (Villeda et al., 1993a,b), and may play a major role in the development of hemorrhage. It appears to arise as the result of ultrastructural changes in megakaryocytes, and particularly of the appearance of denuded megakaryocytes (apoptotic megakaryocytes).

Some soluble chemical mediators are potent modulators of megakaryocyte maturation (Hoffmann, 1989), and may play a major role in the appearance of denuded megakaryocytes in ASF. This hypothesis is supported by the fact that replication is observed in bone-marrow macrophages until 7dpi and that there is an increase in plasma monokine levels (Gómez-Villamandos et al., 1998). These monokines are able to trigger cytokine secretion by



**Fig. 5.** Platelets with different degranulation stages (arrows) attached to endothelium in an interstitial renal capillary.

stromal cells and T lymphocytes; the cytokines in turn regulate megakaryocyte maturation (Bagby, 1987; Hoffmann, 1989).

Peripheral platelet consumption observed from 3 dpi (Villeda et al., 1993a,b; Carrasco et al., 1996d) in subacute ASF may be correlated to the expression and secretion of procoagulant factors by activated macrophages (Gómez-Villamandos et al., 1997a; Bautista et al., 1998). Megakaryocytes may react to early peripheral platelet consumption by producing abundant platelets; this would prevent thrombocytopenia at 3–5 dpi, while the resulting megakaryocyte “fatigue” would give rise to the appearance of numerous denuded megakaryocytes and thence to the intense thrombocytopenia observed from 6 dpi in pigs inoculated with a moderately-virulent ASF strain (Gómez-Villamandos et al., 1998).

#### 4.4. Pulmonary edema

Severe pulmonary edema, giving rise to respiratory changes, is a characteristic finding in pigs infected with highly-virulent ASF strains; affected animals die in shock, and foam is generally observed in the mouth and nose.

The mononuclear phagocyte system of the lung in pigs comprises interstitial macrophages, free rounded alveolar macrophages (AMs) and spindle-shaped pulmonary intravascular macrophages (PIMs) located in pulmonary capillaries adjacent to the endothelium. This latter cell population is found only in certain species, notably pigs, cows and humans. Research has highlighted an increase in AM and particularly PIM numbers from the early stages of ASF, probably induced by chemical mediators and circulating cell debris from various organs. Although ASF virus replication has been reported in various lung macrophage populations, PIMs appear to be the main ASF virus target cell in the lung; they tend to be enlarged and display secretory activation. (Carrasco et al., 1996a). The appearance of the most severe vascular changes coincides with intense proinflammatory cytokine secretion by PIMs, many of which exhibit signs of viral infection. These cytokines are known to develop chemotactic activity and to be involved in mechanisms leading to increased endothelial permeability (Carrasco et al., 2002). The fact that increased numbers and secretory activation of macrophages appears before the virus is in the lung at 3 dpi suggests that these changes are initially induced by circulating chemical mediators released into the blood from primary replication organs.

At the same time, cell debris induces marked phagocytic activation of PIMs, which are seen as larger, and rounded rather than spindle-shaped; they also display proliferation of lysosome structures and phagocytosis of cell debris. This in turn leads to increased intravascular pressure, prompting a number of vascular changes, including interstitial edema; separation of the alveolar epithelium from the capillary endothelium then gives rise to alveolar edema (Carrasco et al., 1996a).

#### 5. Lymphoid organs and lymphopenia

Acute ASF is characterized by early leukopenia due mainly to a decline in lymphocyte and monocyte numbers. Lymphopenia coincides with marked lymphoid depletion – considered a characteristic feature of ASF – affecting not only the spleen and lymph nodes but also a number of lymphoid organs displaying no characteristic ASF lesions; in the thymus, for example, lymphoid depletion gives rise to a slight “starry sky” pattern (Salguero et al., 2004). We have measured the level of chemical mediator released by lymphocytes by enzyme-linked immunosorbent assay in serum of pigs with acute ASF. IL-2 showed increased level only at 4 dpi suggesting a slight and insufficient type 1 immune response against ASFv (Boyman and Sprent, 2012). However, IFN- $\gamma$  showed the level

increased from the 2 and 4 dpi. Similar results in IFN- $\beta$  and - $\gamma$  have been recently published by Karalyan et al. (2012) associated to atypical lymphocytes, which could be one cause of the changes observed in the lymphocytes during acute ASF, including proliferation of these cells and apoptosis by lytic activity of NK cells.

Using a range of techniques, including the TUNEL method, immunolabeling of caspase and transmission electron microscopy lymphoid depletion and the death of infiltrated lymphocytes have been attributed to massive apoptosis (Gómez-Villamandos et al., 1995b, 1997b; Carrasco et al., 1997a,b; Salguero et al., 2004; Fernández de Marco et al., 2007; Sánchez-Cordón et al., 2008).

A number of mechanisms have been put forward to account for the programmed cell death observed among lymphocytes in viral diseases, a phenomenon which may be directly or indirectly linked to infection of these cells. Although ASF virus may infect lymphocytes, virus replication has not been observed in these cells, and may thus be ruled out as a possible cause of lymphocyte apoptosis (Carrasco et al., 1996c). It has been suggested that activated m-M $\phi$  and/or macrophages containing virus replication sites may synthesize and secrete monokines, which trigger lymphocyte apoptosis (Salguero et al., 2005).

Lymphopenia would be aggravated in the final stages of the disease by the destruction of lymphoid structures resulting from hypoxia induced by vascular damage and DIC.

#### 6. Conclusions

Data reported for the various studies show that the virus replication in m-M $\phi$  and secretory activation of these cells are the modulator element if the pathogenesis of the ASF. Thus, the phenomena of hemadsorption in vivo around m-M $\phi$  with viral replication are responsible for organic spread by virus-associated to erythrocytes. Replication and necrosis of splenic cords macrophages are the cause of the hyperemic splenomegaly. The secretory activation of m-M $\phi$  (TNF- $\alpha$ , IL-1 $\alpha$ , IL-1b, IL-6, IL-8) has a pro-inflammatory, pro-coagulant and pro-apoptotic profile, leads to pulmonary edema, hemorrhage (by phagocytic activation of endothelial cells) and lymphoid depletion (by apoptosis of lymphocytes) that characterize the disease. The direct action of the virus on other cell populations is secondary in the pathogenesis of the disease.

In animals with subacute ASF, the activation profile of these cells is different, with changes indicative of anti-inflammatory activity (IL-10). These changes are conjugated with immunologic phenomena, causing the clinic and lesions of the subacute form of the disease, with intense edema and haemorrhages (by vasodilatation and increased permeability) and transient thrombocytopenia, in which peripheral consumption of platelets is the first step.

Future studies about this reaction of the macrophage to different virulence isolates and the elucidation of the regulatory mechanism of the immune response during chronic ASF, could shed light on this devastating disease.

#### Uncited references

Gómez-Villamandos et al. (1996) and McVicar et al. (1981).

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