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Role of hepatic macrophages during the viral haemorrhagic fever induced by African Swine Fever Virus

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Summary. To ascertain the role played by the various liver monocyte-macrophage populations in the course of a viral hemorrhagic fever, fifteen pigs were inoculated intramuscularly with the highly virulent isolate of African Swine Fever Virus (ASFV) España-70 and slaughtered at 1-7 days post-inoculation (dpi). Samples of liver were fixed in different solutions and routinely processed for morphological, immunohistochemical and ultrastructural studies. Viral antigen (vp73) was detected from 3 dpi onward, mainly in circulating monocytes of sinusoid and Kupffer's cells (KC), as well as in portal macrophages and hepatocytes from 5 dpi. Anti-SWC3 immunolabelled cells were increased from 1 dpi, peaking between 3 and 5 dpi, thereafter declining until the end of the experiment. The significant increase in the number of sinusoidal circulating monocytes and KC expressing IL-1α, TNFα and IL-6 from 1 dpi, confirmed the secretory activation of these cells. The results show that in the course of an ASFV-induced hemorrhagic syndrome, hepatic macrophage populations undergo major quantitative and biosynthetic changes prior to virus detection, suggesting the existence of a mechanism by which the virus concentrates infectable cells, which subsequently spread the virus around the body.

Key words: African Swine Fever, Cytokines, Kupffer's cells, Liver

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Introduction

The Kupffer's cells (KC), a resident population of hepatic phagocytes, represent the first line of defence against viruses entering the liver through the portal circulation (Decker, 1990). Cytokines secreted by the KC play an important role in the regulation of the immune response and stimulate the synthesis of acute phase proteins by hepatocytes (Heinrich et al., 1990; Gabay and Kushner, 1999). Moreover, soluble mediators such as interferon (IFN)α, IFNγ and tumour necrosis factor (TNF)α have shown their influence in both the gene expression and replication of the hepatitis viruses, and in evasion of the innate immune response (Shultz et al., 1999; Granja et al., 2006).

The inflammatory and immune responses developing in the liver of pigs during african swine fever (ASF) could be a useful tool to better understand the pathogenetic mechanisms of viral hepatitis which appears in human hemorrhagic fevers, such as dengue, yellow fever or Lassa fever. In the course of these diseases, cells of the mononuclear phagocyte lineage, especially KC, are targets for virus replication, inducing secretory changes that may interfere with the virus life cycle and/or initiate an inflammatory process, changes that remain unclear (Deubel et al., 1997; Marianneau et al., 1999; Huerre et al., 2001; Lukashevich et al., 2003).

ASF is a fatal viral hemorrhagic disease of pigs characterized clinically by haemorrhages in lymphoid organs and severe lymphoid depletion (Wilkinson et al., 1981; Gómez-Villamandos et al., 1995a,b; Salguero et al., 2004, 2005). The main target cells of ASF virus (ASFV) are the monocytes-macrophages (m-MØs) (Sierra et al., 1987, 1991; Mebus, 1988). A number of studies focussing on the liver, which plays a crucial role in the inflammatory response, report hepatitis with

mononuclear cell infiltrate (Sierra et al., 1987; Gómez-Villamandos et al., 1995a,b), together with an early myofibroblastic transformation of fat-storing cells unrelated to virus replication in these cells (Gómez-Villamandos et al., 1995b), and similar to that detected in other forms of hepatitis (Inuzuka et al., 1990; Gressner, 1995). Ultrastructural examination has suggested ASFV replication in KC and possible biosynthetic activation of these cells (Alcami et al., 1990; Gómez-Villamandos et al., 1995a,b).

Recent studies showed that the increase of TNFα expression during ASF could play a critical role in some of the major clinical manifestations of acute ASF, such as intravascular coagulation, shock and apoptosis, attributing these lesions to indirect mechanisms and ruling out a direct viral effect on different cell populations (Gómez del Moral et al., 1999; Salguero et al., 2004, 2005).

The aim of this study was to ascertain the role played by the various liver m-MØ populations in the duration of hepatitis, and to chart the morphological and biosynthetic changes taking place in the course of viral hemorrhagic fever caused by the ASF virus.

Materials and methods

Animals, Virus and experimental design

Eighteen clinically healthy 3-month-old Large White x Landrace pigs of either sex were used. Fifteen animals were each inoculated intramuscularly with 105 haemagglutinating doses (HAD)50 of the highly virulent isolate of ASFV España-70. Three pigs used as controls received only phosphate-buffered saline (PBS), pH 7.2. After inoculation, clinical signs and rectal temperature were monitored daily.

The inoculated pigs were sedated with azaperone (Stresnil®, Janssen Animal Health, Beerse) and anaesthetized with thiopental-sodium (Thiovet®, Vet Limited, Leyland). Finally, they were euthanazed by overdosing with thiopental-sodium in batches of three at 1, 3, 5, 6 and 7 days post-inoculation (dpi). Control animals were slaughtered at the end of the experiment. This experiment was performed in the Centro de Investigación en Sanidad Animal (Valdeolmos, Madrid, Spain), in accordance with the Code of Practice for the Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community Union (86/609/EEC amended by the directive 2003/65/EC).

Processing of specimens

Samples of liver were fixed in 10% buffered formalin solution 0.01 M, pH 7.2 for 24h or Bouin's solution for 8h. Samples were then processed by routine methods and embedded in paraffin wax. Sections of 3 mm were cut and stained with haematoxylin and eosin (HE) for histopathological study or processed for immunohistochemical studies.

Moreover, samples were fixed in glutaraldehyde 2.5% in 0.1 M phosphate buffer, pH 7.4, and were postfixed in 2% osmium tetroxide, dehydrated in acetone and embedded in Epon 812 (Fluka Chemie AG, Buchs). Sections were cut 1 µm thick and stained with 1% aqueous toluidine blue solution for structural study. For transmission electron microscopy (TEM), sections of 50 nm were counterstained with uranyl acetate and lead citrate, and viewed under a Philips CM-10 transmission electron microscope.

Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) technique previously described (Salguero et al., 2002), was used for the immunohistochemical detection of viral protein 73 (vp73) of ASFV and for the identification of m-MØs and cells expressing TNFα, IL-1α and IL-6. Details of the primary antibodies employed in this study, including dilutions and antigen retrieval procedures are summarized in Table 1. Specific primary antibodies were replaced by mouse or rabbit non-immune serum in negative control sections. Tissue sections from control specific-free pigs not exposed to ASF were also used as controls.

Cell counts and statistical analysis

One paraffin-wax block from the liver of each animal (three per timepoint) was selected and serial

Table 1. Details of the immunolabelling reagents.

Primary antibodies	Fixation	Specificity	Dilution	Pretreatment
Monoclonal Anti-ASFV (Ingenasa, Madrid, Spain)	10% Buffered formalin solution	Viral protein vp73	1:10	Pronase*
Monoclonal SWC3 (BioVet-UCO, Córdoba, Spain)	Bouin´s solution	Porcine myeloid marker	1:10	Microwave†
Polyclonal Anti-TNFα (Genzyme, Cambridge CA, USA)	Bouin's solution	Anti-human TNFα	1:500	Microwave
Polyclonal Anti-IL-1α (Endogen, Woburn CA, USA)	Bouin´s solution	Anti-human IL-1α	1:100	Tween-20‡
Polyclonal Anti-IL-6 (Endogen, Woburn CA, USA)	Bouin's solution	Anti-porcine IL-6	1:10	Tween 20

*: Protease type XIV (Sigma-Aldrich Chemie, Steinheim, Germany), 0.1% in PBS for 10 minutes. †: Tri-sodium citrate dihydrate (Merck, F.R., Germany), 0.01 M (pH 3.2) in a microwave oven for 10 minutes. ‡: Tween® 20 (Merck, München, Germany), 0.1% in PBS for 10 minutes.

sections were used for the different immunohistochemical studies. Cells immunolabelled with specific antibodies were counted. Cell counts were made in 25 consecutive areas of 0.2 mm^2 which were chosen randomly in four diagonally positioned squares. Cellular identification of monocytes, neutrophils, macrophages and Kupffer's cells was based on morphological features, location and size of cells. Therefore, neutrophils displayed nucleus with multilobulated shape as compared with the large bilobate nucleus of circulating monocytes. Within the hepatic tissue, macrophages and Kupffer's cells were located in interstitium and sinusoids respectively, showing an abundant cytoplasm and kidney shape nucleus. The results were expressed in cells per 0.2 mm^2. Data on immunopositive cells was assessed to calculate means and standard deviations (SD) using the statistical analysis program GraphPad InStat, version 3.0 (GraphPad Software, Inc.). Differences between the values in control and inoculated animals were tested for significance ($P \leq 0.05$) by Mann-Whitney's *U*-test for non-parametric distributions.

Results

Clinical findings, gross lesions and blood analysis

Inoculated animals showed unspecific signs from 2 dpi consisting of pyrexia (39.5-41.5°C), loss of appetite, respiratory and gastrointestinal disorders. From 3 dpi, haemorrhagic lesions characteristic of ASF appeared in different organs. Noteworthy was the absence of macroscopical changes in the liver. Total leukocytes, mainly lymphocytes, decreased significantly from 3 dpi while cytokines in serum (TNFα and IL-1ß) were significantly increased from 2 dpi onwards. These findings were quoted from previously published articles (Salguero et al., 2002, 2004).

Morphological study of the liver

From 3 dpi, there was evidence of hyperemia, edema in portal spaces and interlobular septa, and a sparse cell infiltrate comprising neutrophils, a few lymphocytes and a moderate number of enlarged macrophages (Fig. 1). Sinusoids displayed a larger number of circulating cells, particularly neutrophils, monocytes and occasional lymphocytes, together with abundant enlarged KC containing engulfed cell debris. Many KC and some monocytes displayed evidence of the cytopathic effect of ASFV: rounded nuclei, peripheral margination of chromatin and intracytoplasmic inclusion bodies.

From 5 dpi to the end of the experiment, lesions appeared even more intense; some perilobular hepatocytes displayed peripheral margination of chromatin and intracytoplasmic inclusion bodies. Infiltrate cells, circulating cells and KC exhibited signs of pyknosis and karyorrhexis, which increased the presence of cell debris. In cells displaying alterations indicative of viral infection, ultrastructural examination disclosed intense cytoplasmic and nuclear lesions, as well as cell rupture characteristic of necrosis. Circulating lymphocytes, and those in cell infiltrate, showed evidence of apoptosis (Fig. 2).

Only towards the end of the experiment, at 6 dpi, did some sinusoidal endothelial cells appear swollen. Ultrastructural analysis disclosed marked detachment of endothelial cells, which in some areas of the sinusoid had even disappeared; however, they displayed no evidence of viral infection. Some of these cells were surrounded by platelets and monocytes. The perisinusoidal space was filled with abundant

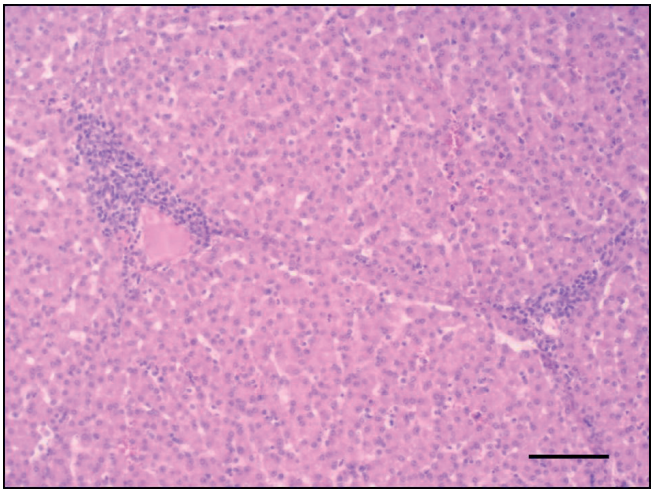

Fig. 1. Edema and cell infiltrate comprising mainly mononuclear cells in portal and perilobulillar spaces. (HE; 6 dpi; bar: 100 µm).

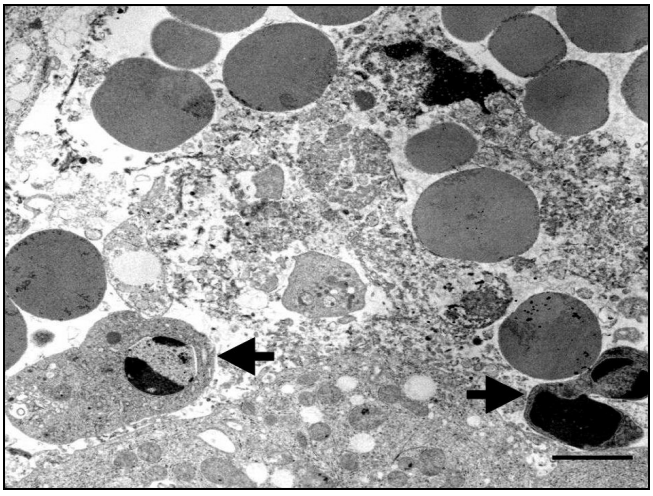

Fig. 2. Subcellular changes in circulating mononuclear cells (arrows) showing evidence of apoptosis with condensed and marginated chromatin. Note the presence of non-phagocytized cell debris (TEM; 5 dpi; bar: 3.3 µm).

erythrocytes, platelets and cell debris, together with fibrillar material identified as fibrin deposits; these changes were confirmed at ultrastructural examination. Fat-storing cells, though showing no evidence of viral infection, displayed certain alterations – reported earlier (Gómez-Villamandos et al., 1995b) – indicative of fibroblastic and myofibroblastic transformation, characterized by enlarged cells with long cytoplasmic projections, proliferation of dilated cisternae of rough endoplasmic reticulum and the presence of microfilament packets in the cell periphery, displaying a linear density similar to that observed in myofibroblasts and smooth muscle fibres. Microscopic and subcellular lesions were not observed in the liver of uninoculated animals

Detection of virus antigen in liver

Control samples from uninoculated animals were stained negatively to ASF viral protein 73 (vp73). In inoculated animals, virus antigen was detected from 3 dpi onward, mainly in circulating monocytes of sinusoid and KC (Fig. 3). The number of cells staining positive for virus antigen peaked at 5 dpi (Fig. 4), by which time signs of viral infection were detected in portal macrophages, hepatocytes and cells in the perisinusoidal space.

Ultrastructural examination disclosed virus replication in KC and circulating monocytes from 3 dpi onwards, and in portal macrophages and hepatocytes from 5 dpi. Towards the end of the experiment (6 dpi), virus particles (enveloped and unenveloped) were detected alongside cells and cell debris in the perisinusoidal space. Virus replication also appeared to be responsible for the cytopathic effect and for hemoadsorption.

Immunohistochemical analysis

Anti-SWC3 antibody stained myeloid cells, mainly m-MØs, KC and neutrophils (Fig. 5). The significant increase in circulating monocytes in sinusoids and portal vessels compared to uninoculated animals, visible from 1 dpi, was maintained until the end of the experiment. However, there was no significant increase in KC and portal macrophages until 3 dpi; numbers peaked between 3 and 5 dpi, thereafter declining until the end of the experiment (Fig. 6).

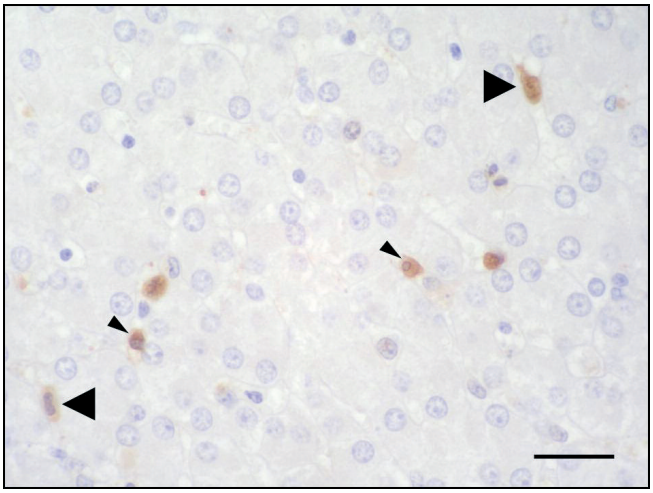

Fig. 3. Circulating monocytes of sinusoids (small arrowheads) and KC (arrowheads) showing positive reaction to viral protein vp73. (ABC; 5 dpi; bar: 7.3 µm).

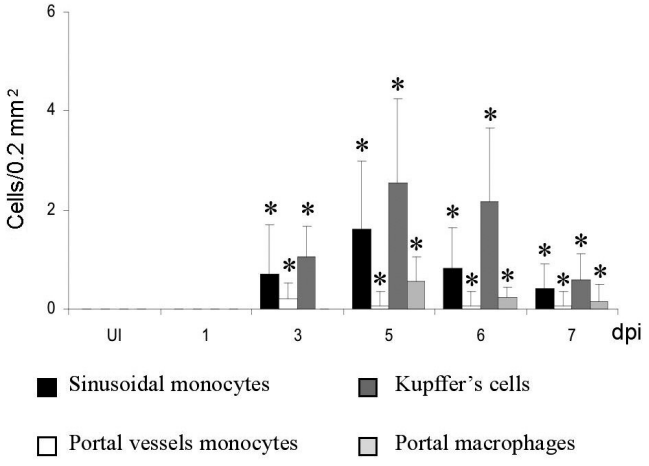

Fig. 4. Number (±SD) of vp73 positive cells in sinusoidal monocytes, portal vessels monocytes, Kupffer's cells and portal macrophages in the liver of uninoculated (UI) animals (*n*=3) and pigs inoculated with ASFV (n=3 pigs per time-point). *: Difference from controls statistically significant, P≤ 0.05.

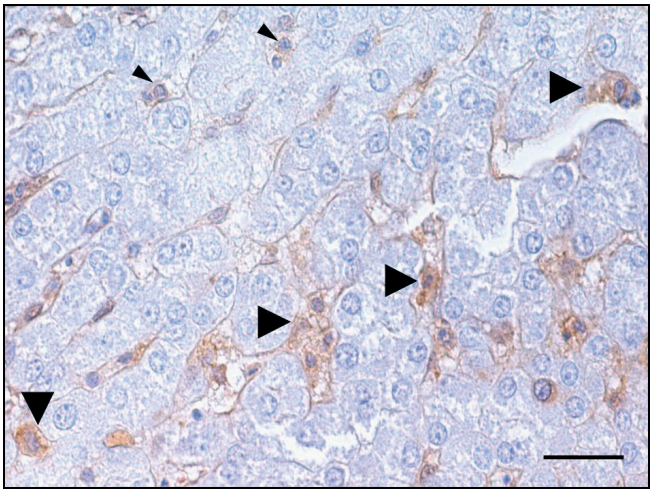

Fig. 5. Kupffer´s cells showing an increase in size (arrowheads) and circulating monocytes (small arrowheads) immunolabelled against SWC3 marker (ABC, 3 dpi; bar: 7.3 µm).

As well as these quantitative changes, KC, portal macrophages and circulating monocytes undergoing virus replication showed ultrastructural changes associated with intense phagocytic activity, characterised by increased cell size, proliferation of lysosomes and presence of engulfed cell debris. Phagocytic activation was sometimes accompanied by changes indicative of secretory activation: decreased heterochromatin, proliferation and dilation of rugous endoplasmic reticulum (RER) cisternae and Golgi-complex proliferation (Fig. 7).

Immunohistochemical analysis confirmed secretory activation, disclosing from 1 dpi a significant increase in the number of sinusoidal circulating monocytes expressing IL-1α (Fig. 8) and TNFα (Fig. 9); this increase did not become significant for KC until 3 dpi. The number of sinusoidal circulating monocytes and KC reacting to IL-1α and TNFα peaked at 5 dpi. Thenceforth, a progressive decrease in reactive cells was observed until end of the experiment, although numbers remained significantly higher than in uninoculated controls (Fig. 6).

From 3 dpi onwards, there was a significant increase in the number of portal-vessel monocytes and macrophages reacting to TNFα. However, the number of cells reacting to IL-1α showed a significant increase only between 3 and 5 dpi. The smaller number of reactive cells in these locations, and the numerical changes observed, may be due to the smaller number of portal spaces analysed per field.

Some circulating neutrophils expressed IL-1α from the start of the experiment, while some endothelial cells

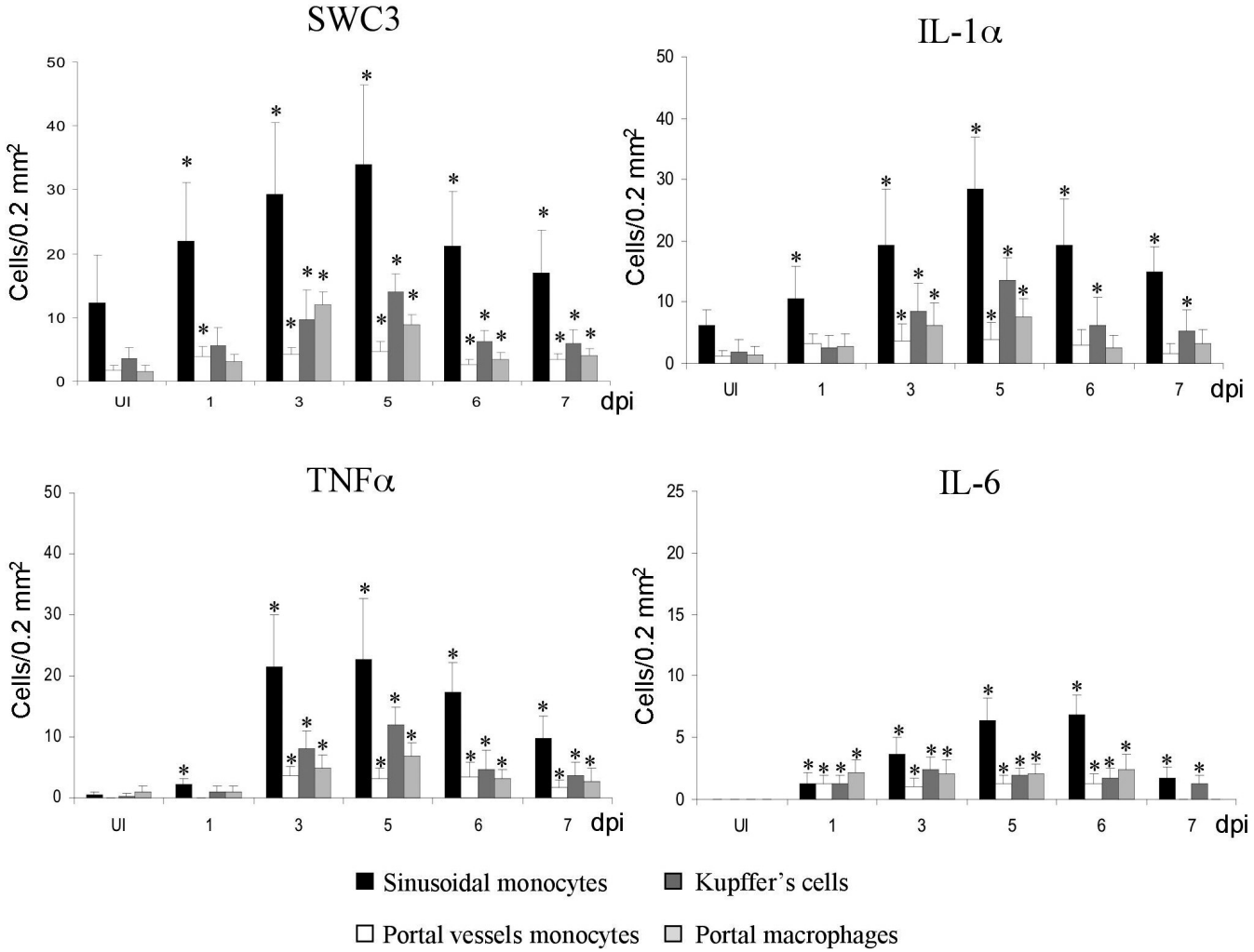

Fig. 6. Counts (±SD) of cells marked against the myeloid marker SWC3 and the cytokines IL-1α, TNF-α and IL-6 in sinusoidal monocytes, portal vessels monocytes, Kupffer's cells and portal macrophages in the liver of uninoculated (UI) animals (n=3) and pigs inoculated with ASFV (n=3 pigs per time-point). *: Difference from controls statistically significant, P≤ 0.05.

in reactive sinusoids did so towards the end of the experiment, at 7 dpi. Similarly, some bile-duct epithelial cells stained positive to TNFα, as did occasional endothelial cells in reactive sinusoids at 7 dpi, although there was no significant increase in the number of reactive cells compared to uninoculated controls.

In control animals, IL-6 was expressed mainly by fibroblasts and endothelial cells; no mononuclear phagocytic system (MPS) cells were found to be reactive to this cytokine. In experimental animals, however, both circulating monocytes and positive-staining KC and macrophages were observed from 1 dpi onwards. These cells increased significantly in number, peaking between 5 and 6 dpi; sinusoid circulating monocytes were the most numerous.

Discussion

Results suggest that in the course of an ASFV-induced hemorrhagic virus syndrome, hepatic macrophage populations – and particularly KC – undergo major quantitative and biosynthetic changes, prompting increased secretion of chemical mediators. These changes coincided, both in time and in space, with the onset of mild hepatitis, with virus replication in liver hepatocytes and with fibroblastic and myofibrobastic transformation of fat-storing cells. The results also point to a clear correlation between the changes taking place in hepatic macrophages and the variations in serum acute-phase protein levels recently observed (Sánchez-Cordón et al., 2007).

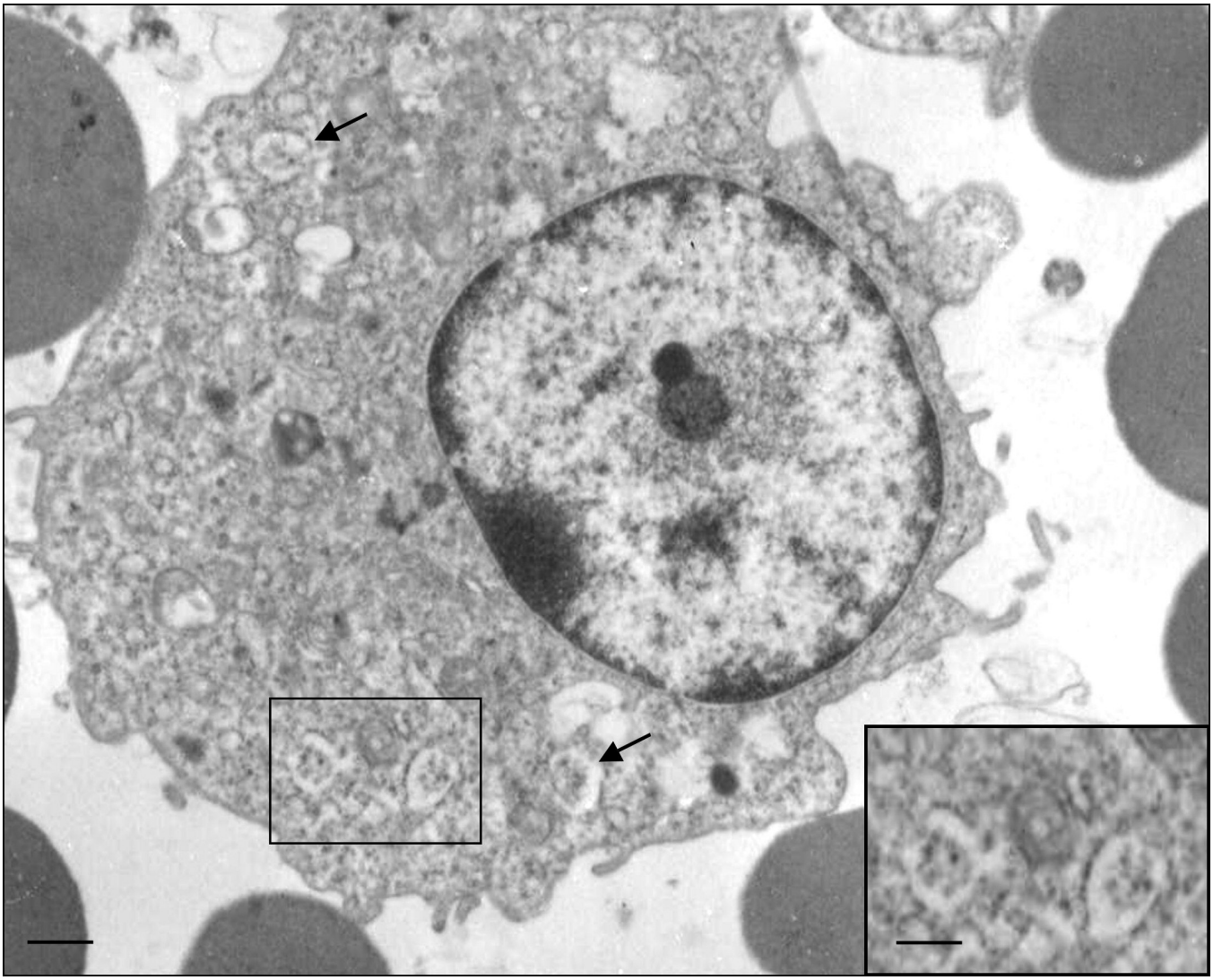

Fig. 7. Secretory activation of a circulating monocyte, including dilated rough endoplasmic reticulum cisternae (arrows), in the sinusoidal space (TEM; 5 dpi; bar: 1 µm). Inset: Rough endoplasmic reticulum cisternae containing moderate electron-dense material (Bar: 750 nm).

MPS cells are the main target cells for ASFV in the liver; here, they increased in number during the initial and middle phases of the disease. This increase took place at the same time as ASFV replication in non-MPS cells such as hepatocytes, suggesting that virus replication in non-MPS cells is not due to the destruction of MPS cells and the presence of numerous free virions (Fernández et al., 1992). These cells do not undergo phagocytic activation that might entail saturation of KC cleaning functions (Shayakhmetov et al., 2004), thus facilitating the infection of other cell elements. The clear relationship between virus replication in non-MPS cells and the secretory activation of chemical mediators in m-MØs suggests that these might induce the expression of the surface receptors (Alcami et al., 1990) required for virus infection and replication in non-MPS cells. This mechanism may come into play in endothelial cells, such as those located in renal capillaries, in which virus replication has been detected in association with the biosynthetic activation of perivascular macrophages (Gómez-Villamandos et al., 1995c). This contrasts with the absence of replication in hepatic sinusoid endothelial cells. This difference suggests that endothelial cells at different sites behave differently in response to ASFV infection. The relationship between endothelial cells and macrophages may play a major role in these differing behavior patterns, since hepatic sinusoid macrophages (KC) are attached to the endothelium, whilst in the renal capillaries there is no such attachment. Biosynthetic activation of KC may induce the activation of endothelial cells and block transendothelial transport of the virus (Schiedner et al., 2003). Here, however, virions were observed in endothelial cells, but there was no evidence of virus replication, suggesting that these cells do not express the specific receptors enabling virus replication in infected cells.

From 1 dpi, there was evidence of virus replication and biosynthetic acivation in m-MØs at other sites, including the spleen, and both renal and gastrohepatic lymph nodes (Salguero et al., 2002). Mediators released by infected cells might activate non-infected neighboring cells, which would migrate to virus-free areas such as the liver. This may account for the fact that, although no cells showing signs of infection were observed in the liver until 3 dpi, from 1 dpi onwards there was a significant increase in the number of circulating m-MØs staining positive for IL-1α, TNFα and IL-6. The increase – prior to virus detection – in the number of non-infected cells expressing IL-1α and TNFα, mediators with chemotactic properties able to act synergically (Le and Vilcek, 1987; Sánchez-Cordón et al., 2002, 2005), suggests the existence of a mechanism by which the virus concentrates infectable cells, which subsequently spread the virus around the body. As the virus reached the liver, there was a significant increase in the number of m-MØs and KC, and in TNFα, IL-1α and IL-6 expression by these cells. This activation of KC would enhance recruitment of immune cells and effector molecules to the infection site (Lieber et al., 1997; Klocker et al., 2000), establishing a feedback mechanism which would enable the concentration of a large number of infectable cells.

Transcription of IL-6 in KC, hepatocytes and other cells is activated by TNFα (Shimizu et al., 1990); IL-6 prompts the appearance of cell infiltrate and the activation of cytotoxic T lymphocytes (Ginsberg et al., 1991). This would account for the fact that changes in circulating monocytes, KC and IL-6-expressing macrophages coincided with TNFa expression by these cells, thus confirming that IL-6 is a major modulator of the immune response in ASF.

The fibroblast and myofibroblast transformations

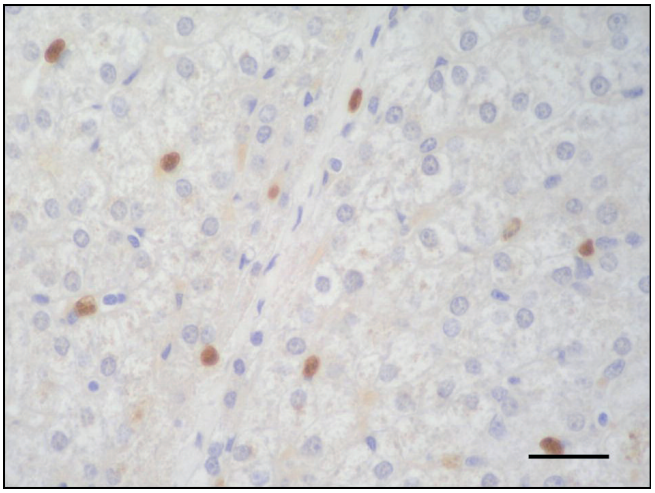

Fig. 8. Circulating monocytes with sinusoidal location expressing IL-1α. (ABC; 3 dpi; bar: 7.3 µm).

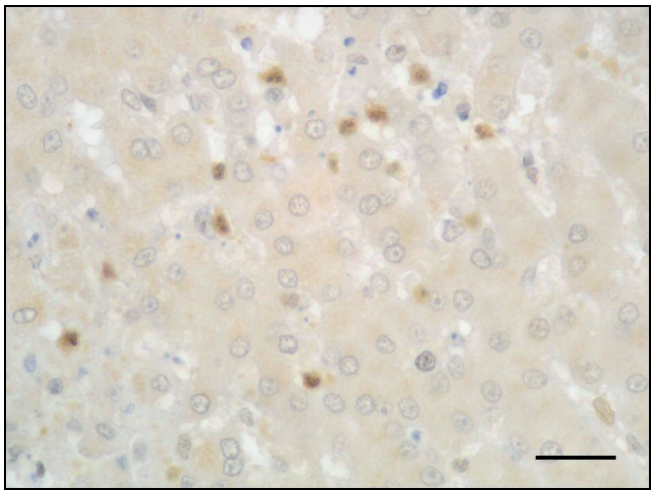

Fig. 9. Immunoreactive cells against TNF-α in sinusoids. (ABC; 7 dpi; bar: 7.3 µm)

observed in fat-storing cells, have been described in viral hepatitis (Inuzuka et al., 1990). These changes could be related to virus replication in these cells, and may either have been the result of an inflammatory stimulus with the release of soluble chemical mediators, or the reaction of the organism to an oxygen deficit which would be produced by intravascular coagulation (Takana, 1985; Gressner, 1992). In the present study, fibroblast and myofibroblast transformations was not directly related to virus replication or intravascular coagulation (Gómez-Villamandos et al., 1995b). It may be assumed that the joint presence of activated KC and TNFα- and IL-1α-expressing macrophages prompted fibroblastic and myofibroblastic transformation of fat-storing cells (Knittel et al., 1997; Da Silva et al., 2003; Han et al., 2004). These results suggest that next to the transforming growth factor-beta 1 (TGF-b1), which appears to be the main mediator involved in myofibroblastic transformation during liver fibrogenesis (Knittel et al., 1996; Hayashi et al., 2000), the pro-inflammatory cytokines could play an important role in liver repair.

Proinflammatory cytokines induce acute phase protein (APP) synthesis by hepatocytes (Gabay and Kushner, 1999), a mechanism in which KC play an intermediate role (Knolle et al., 1995). Some authors have suggested that serum concentration of serum amyloid A (SAA) protein and C-reactive protein (CRP) (classified as type 1 acute phase proteins) are predominantly induced by IL-1 and TNFα (synergistically with IL-6), while type 2 acute phase proteins such as haptoglobin (Hp), are mainly induced by IL-6 (Petersen et al., 2004). Results showed that changes in the number of KC and other liver macrophages expressing IL-1α and TNFα in the course of ASF coincided with variations in serum SAA and CRP levels in samples from pigs inoculated with the España-70 strain (Sánchez-Cordón et al., 2007). Moreover, serum Hp levels have been reported to rise right from the start of the experiment (Sánchez-Cordón et al., 2007), peaking at the same times as KC and IL-6-secreting macrophages. This sheds light on the role of cytokines secreted by liver macrophage populations in ASF, and their possible influence on variations in APP levels. Further research is required to ascertain the role of cytokines released elsewhere.

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