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# Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines

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**Abstract** – Apoptosis was studied in the lungs of pigs during an infection with a European strain of porcine reproductive and respiratory syndrome virus (PRRSV) and it was examined if cytokines were involved in the induction of apoptosis. Twenty-two 4- to 5-week-old gnotobiotic pigs were inoculated intranasally with  $10^{6.0}$  TCID<sub>50</sub> of the Lelystad virus and euthanised between 1 and 52 days post inoculation (PI). The lungs and broncho-alveolar lavage (BAL) cells were assessed both for virus replication and apoptosis; BAL fluids were examined for interleukin (IL)-1, tumour necrosis factor-alpha and IL-10. Double-labellings were conducted to determine the relation between virus replication and apoptosis and to identify the apoptotic cells. Apoptosis occurred in both infected and non-infected cells. The percentages of infected cells, which were apoptotic, ranged between 9 and 39% in the lungs and between 13 and 30% in the BAL cells. The majority of apoptotic cells were non-infected. Non-infected apoptotic cells in the lungs were predominantly monocytes/macrophages, whereas those in the broncho-alveolar spaces were predominantly lymphocytes. The peak of apoptosis in the lungs at 14 days PI was preceded by a peak of IL-1 and IL-10 production at 9 days PI, suggesting a possible role of these cytokines in the induction of apoptosis in non-infected interstitial monocytes/macrophages. However, the latter hypothesis was not confirmed in vitro, since blood monocytes or alveolar macrophages did not undergo apoptosis after treatment with recombinant porcine IL-1 or IL-10.

**pig / lung / porcine reproductive and respiratory syndrome virus / apoptosis / cytokine**

## 1. INTRODUCTION

Apoptosis is a physiological mechanism of cell death important for normal cellular

turnover that is characterised by internucleosomal DNA degradation and pronounced morphological changes [35]. It also occurs during viral infections and

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there is mounting evidence that it can contribute directly to viral pathogenesis [8].

Recently, it has been demonstrated that the porcine reproductive and respiratory syndrome virus (PRRSV) induces apoptosis in the lungs [7, 17, 24, 27]. On a morphological basis, the apoptotic cells are assumed to be alveolar and pulmonary intravascular macrophages and mononuclear cells in the alveolar septa. Double-labelling experiments indicate that most apoptotic cells are non-infected [24, 27].

An infection of the lungs with PRRSV has some peculiar cellular features that may be attributed to apoptosis. Firstly, it is characterised by changes in the population of broncho-alveolar monocytes/macrophages, consisting of a reduction of the population of well-differentiated macrophages and an influx of new monocytes [16]. Since apoptosis is an important mechanism of the cell population control in normal tissue homeostasis, it may be involved in the regulation of the number of monocytes/macrophages during this massive influx of new monocytes. Secondly, PRRSV causes only minimal lung inflammation [21] and neutrophil infiltration [16, 31], which also may be attributed to the involvement of apoptotic processes, since apoptotic bodies are phagocytised by resident macrophages without provoking an inflammatory response.

Viral glycoprotein GP5 has been shown to be involved in apoptosis of the PRRSV-infected cell [26]. However, the mechanism of apoptosis in the non-infected cell is unknown. It has been demonstrated that cytokines, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [18], interferon- $\alpha$  (IFN- $\alpha$ ) [28], interleukin (IL)-1 [6, 11] and interleukin-10 [12, 33] can induce apoptosis. A PRRSV infection generally fails to induce substantial amounts of IFN- $\alpha$  [1, 30, 32]. This argues against a possible role of this cytokine in PRRSV-induced apoptosis. IL-1, however, is produced at high levels in the lungs of PRRSV-infected pigs from 3 till 10 days after inoculation [31]

and a weak TNF- $\alpha$  production has been demonstrated at 12 and 13 days after inoculation [32]. Furthermore, the induction of IL-10 mRNA in alveolar macrophages has been reported in PRRSV-infected pigs [29].

In the present study, the kinetics of apoptosis both in the lungs and broncho-alveolar lavage (BAL) cells was investigated in gnotobiotic pigs upon infection with a European strain of PRRSV, with the purpose of having a better understanding on its potential role in the pathogenesis. Furthermore, it was examined if IL-1, TNF- $\alpha$  and IL-10 may be involved in the induction of apoptosis during a PRRSV infection.

## 2. MATERIALS AND METHODS

### 2.1. Virus strain

A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV [34] was used.

### 2.2. Pigs and experimental design

A total of twenty-nine caesarean-derived colostrum-deprived (CDCD) pigs were used. They were housed in isolation facilities. Twenty-two pigs were inoculated intranasally at the age of 4 to 5 weeks with  $10^{6.0}$  TCID<sub>50</sub> of the Lelystad virus in 3 mL phosphate-buffered saline (PBS) (1.5 mL in each nostril). The remaining seven pigs were left non-inoculated and served as negative controls. One to three of the PRRSV-inoculated pigs were euthanised at 1 ( $n = 1$ ), 3 ( $n = 2$ ), 5 ( $n = 2$ ), 7 ( $n = 3$ ), 9 ( $n = 3$ ), 14 ( $n = 3$ ), 20 ( $n = 1$ ), 25 ( $n = 2$ ), 30 ( $n = 1$ ), 35 ( $n = 2$ ), 40 ( $n = 1$ ) and 52 ( $n = 1$ ) days post inoculation (PI) by intraperitoneal injection with an overdose of barbiturates (Natriumpentobarbital® 20%, IC KELA, Hoogstraten, Belgium). The control pigs were euthanised at 4 ( $n = 2$ ),

5 ( $n = 1$ ), 6 ( $n = 1$ ), 8 ( $n = 2$ ) and 10 ( $n = 1$ ) weeks of age.

The right lung was used for bronchoalveolar lavage and samples from the left apical, cardiac and diaphragmatic lung lobes were collected for quantification of the infected cells and detection of apoptosis.

### 2.3. Collection of samples

The right lung was lavaged using a previously described method [16]. The BAL fluid was centrifuged ( $400 \times g$ , 10 min,  $4^\circ\text{C}$ ) to separate cells and cell-free lavage fluid.

Cell pellets were resuspended in PBS. The total number of BAL cells and their differentiation were determined and the results were published earlier [16]. Cyto-centrifuge preparations of BAL cells were made by centrifuging at  $140 \times g$  for 5 min. Preparations were fixed in acetone for 20 min at  $-20^\circ\text{C}$  for quantification of the infected cells, or in 4% paraformaldehyde for 10 min at room temperature for detection of apoptosis.

Cell-free BAL fluids were concentrated 10 times by dialysis against a 20% w/v solution of polyethylene glycol (mol. mass 20 000), cleared of residual virus by ultra-centrifugation at  $100\,000 \times g$  [31], aliquoted and stored at  $-70^\circ\text{C}$ , until analysis in cytokine bioassays (IL-1, TNF- $\alpha$ ) or cytokine ELISA (IL-10).

Tissue samples from the left lung lobes were embedded in methylcellulose medium and frozen at  $-70^\circ\text{C}$ . Cryostat sections (5 to  $8\ \mu\text{m}$ ) were made and fixed in acetone for 20 min at  $-20^\circ\text{C}$  for quantification of infected cells, or in 4% paraformaldehyde for 10 min at room temperature for detection of apoptosis.

### 2.4. Virological examinations

PRRSV-infected cells in the lung tissue and BAL cells were quantified using a streptavidin-biotin immunofluorescence

technique [16]. In the lung tissue, a distinction was made between single infected cells and viral antigen-positive foci. Viral antigen-positive foci were defined as areas in the lung tissue containing groups of three or more PRRSV-infected cells and cellular debris [16].

### 2.5. Detection of apoptosis

Cyto-centrifuge preparations of BAL cells and cryostat sections of lung tissue were processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Belgium, Brussel, Belgium) according to the manufacturer's instructions. Briefly, cyto-centrifuge preparations and cryostat sections, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at  $4^\circ\text{C}$  for 2 min. Then, the preparations were subjected to an enzymatic incorporation of digoxigenin-labelled nucleotide with TdT. Finally, the preparations were washed with PBS, mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazobicyclo(2.2.2)octane (DABCO) (Janssen Chimica, Beerse, Belgium) and apoptotic cells were detected and enumerated by fluorescence microscopy (Leica DM RBE, Wild Leitz GmbH, Heidelberg, Germany). One cyto-centrifuge preparation and three cryostat sections (one of each lung lobe; standard deviation  $\leq 500$  cells per  $100\ \text{mm}^2$  lung tissue) were examined per pig. At least 1 000 cells per cyto-centrifuge preparation and  $20\ \text{mm}^2$  lung tissue per cryostat section were analysed.

### 2.6. Double-labelling experiments

Double-labelling experiments were conducted to determine the relation between PRRSV replication and apoptosis. Briefly, cyto-centrifuge preparations and cryostat sections, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at  $4^\circ\text{C}$  for 2 min. The preparations

were first incubated with a pool of monoclonal antibodies (MAb) against the PRRSV nucleocapsid protein (dilution 1/100 of WBE1 and WBE4-6) [9]. Subsequently, the preparations were subjected to an enzymatic incorporation of digoxigenin-labelled nucleotide with TdT and then incubated with goat anti-mouse TexasRed (dilution 1/100) (Amersham Biosciences, Buckinghamshire, United Kingdom).

Double-labelling experiments were conducted to determine the percentages of apoptotic cells in the subpopulations of 41D3<sup>+</sup> cells (monocytes/macrophages), CD2<sup>+</sup> cells (T lymphocytes and natural killer (NK) cells), CD4<sup>+</sup> cells (T helper cells), CD8<sup>+</sup> cells (cytotoxic T cells and NK cells) and wCD1<sup>+</sup> cells (B lymphocytes). The percentages in the subpopulation of monocytes/macrophages were determined using MAb 41D3, directed against the putative PRRSV receptor, a 210-kDa protein expressed in macrophages [10]. Briefly, cytocentrifuge preparations of BAL cells and cryostat sections of lung tissue, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at 4 °C for 2 min. The preparations were first incubated with MAb 41D3 (dilution 1/100). Subsequently, the preparations were subjected to an enzymatic incorporation of digoxigenin-labelled nucleotide with TdT and then incubated with goat anti-mouse TexasRed (dilution 1/100) (Amersham Biosciences, Buckinghamshire, United Kingdom). The percentages of apoptotic cells in the subpopulations of CD2<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells were determined using MAbs MSA4 [19], 74-12-4 [20] and 76-2-11 [36], respectively. The percentages in the subpopulation of B lymphocytes were determined using MAb 76-7-4, directed against wCD1 [4]. Briefly, cytocentrifuge preparations of BAL cells were first incubated with the respective MAbs and subsequently with goat anti-mouse TexasRed (dilution 1/100) (Amersham Biosciences, Buckinghamshire, United Kingdom). Then, the prepa-

rations were treated with Triton X-100 (0.1%) at 4 °C for 2 min. Subsequently, the preparations were subjected to an enzymatic incorporation of digoxigenin-labelled nucleotide with TdT.

## 2.7. Cytokine bioassays and ELISA

IL-1 was determined in a proliferation assay using D10(N4)M cells [14, 15]. One unit of IL-1 activity was defined as the reciprocal of the dilution producing 50% proliferation of D10(N4)M cells. To assign bioactivity to IL-1, monoclonal rat anti-mouse IL-1 receptor type 1 antibodies (R&D Systems, Abingdon, United Kingdom) were included in the assay.

TNF- $\alpha$  was determined in a cytotoxicity assay in PK(15) subclone 15 cells [3]. One unit of TNF- $\alpha$  activity was defined as the reciprocal of the dilution producing 50% cytotoxicity. Cytotoxic activity was confirmed to be induced by TNF- $\alpha$  by neutralisation of the samples with rabbit anti-human TNF- $\alpha$  (Innogenetics, Zwijnaarde, Belgium).

IL-10 was determined in a commercial ELISA for porcine IL-10 (Biosource Europe, Nivelles, Belgium) according to the manufacturer's instructions.

## 2.8. In vitro experiments

Porcine peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by Ficoll-Paque<sup>TM</sup> Plus (Amersham Biosciences, Buckinghamshire, United Kingdom) density sedimentation. PBMCs were seeded in 24-well cell culture plates (Nunc A/S, Roskilde, Denmark) at a concentration of 10<sup>7</sup> cells per well. Monocytes were isolated from PBMCs by adherence on a culture flask for 2 h at 37 °C, 5% CO<sub>2</sub>. Non-adherent cells were removed by washing and the adherent cells were cultured at 37 °C overnight. After an additional washing, the final adherent population was used as the source of blood monocytes. Porcine

alveolar macrophages were obtained by means of a broncho-alveolar lavage and seeded in 24-well cell culture plates (Nunc A/S, Roskilde, Denmark) at a concentration of  $10^6$  cells per well. One day after seeding, blood monocytes and alveolar macrophages were treated with recombinant porcine IL-1 $\beta$  (gift from A. Billiau, Leuven, Belgium) at a concentration of 200 U/mL ( $75 \times 10^3$  ng/mL) or with recombinant porcine IL-10 (Biosource Europe, Nivelles, Belgium) at concentrations of 1, 10, or 100 ng/mL. Functional activity of both recombinant porcine IL-1 $\beta$  and recombinant porcine IL-10 had been proven in a proliferation assay using D10(N4)M cells [14, 15] and the mouse mast cell line D36 [23], respectively. Untreated cells were included as controls. Morphological analysis of the cells was performed daily with a contrast-phase Olympus microscope (Hamburg, Germany). The cells were harvested at 96 h after treatment. Cytocentrifuge preparations were made, fixed in 4% paraformaldehyde for 10 min at room temperature and processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Belgium, Brussel, Belgium) according to the manufacturer's instructions. Apoptotic cells were detected and enumerated by fluorescence microscopy (Leica DM RBE, Wild Leitz GmbH, Heidelberg, Germany)

### 3. RESULTS

#### 3.1. Quantification of PRRSV-infected and apoptotic cells in lung tissue (Fig. 1)

PRRSV-infected cells were observed from 3 until 25 days PI with a maximal number of 61 cells/100 mm<sup>2</sup> at 7 days PI. Viral antigen-positive foci were observed between 3 and 14 days PI with a maximal

number of 37 foci/100 mm<sup>2</sup> at 9 days PI (not shown).

Apoptotic cells were detected in the lung tissue of both non- and PRRSV-inoculated pigs. In the seven non-inoculated control pigs, the mean number was  $1350 \pm 263$  cells/100 mm<sup>2</sup>. In PRRSV-inoculated pigs, mean numbers were similar to those of non-inoculated pigs until 3 days PI. The mean numbers increased significantly from  $1800 \pm 424$  cells/100 mm<sup>2</sup> at 3 days PI to a level of  $4800\text{--}4967 \pm 1054\text{--}1097$  cells/100 mm<sup>2</sup> at 7–9 days PI ( $P < 0.05$ ; one-way ANOVA) and then peaked at  $8330 \pm 1070$  cells/100 mm<sup>2</sup> at 14 days PI. From 20 days PI, the mean numbers returned to those of non-inoculated pigs.

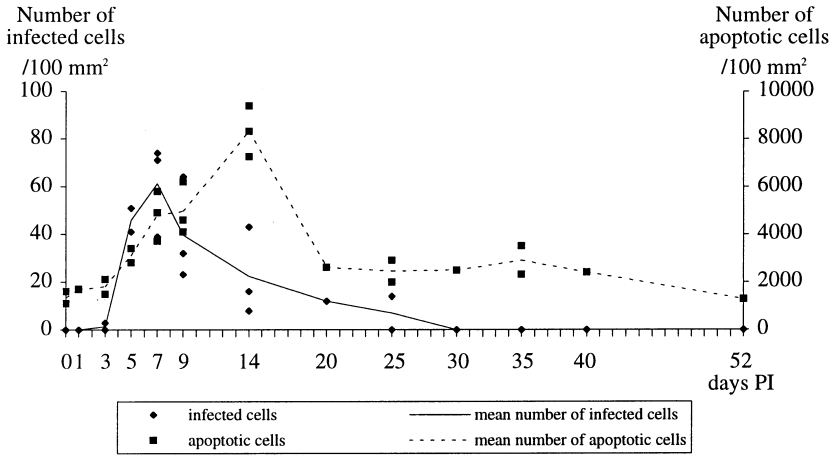
Double-labellings revealed that within the population of infected cells, apoptosis was detected from 5 until 25 days PI with percentages ranging between 9 and 39%. The majority of apoptotic cells (> 99%) were non-infected. There was a spatial correlation between the sites of PRRSV replication and apoptosis. Non-infected apoptotic cells were more abundant in viral antigen-positive foci and they were frequently seen in close proximity to infected cells (not shown).

Double-labelling experiments with MAb 41D3 revealed that 76 to 88% of the apoptotic cells were of the monocyte/macrophage lineage.

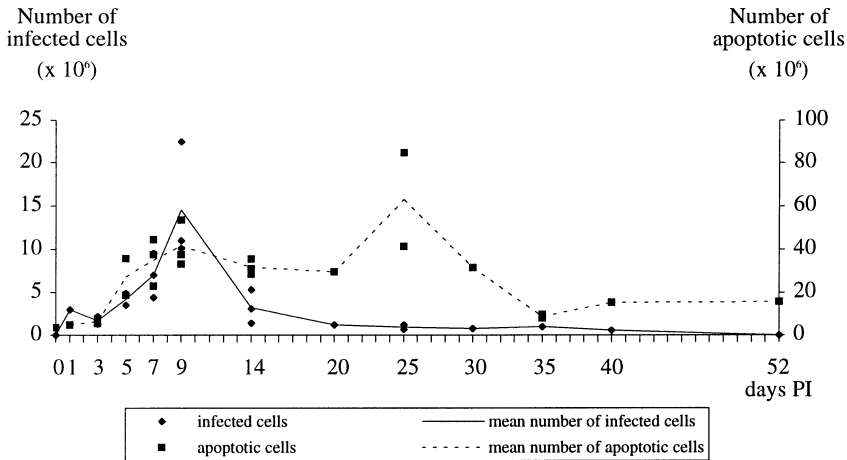
#### 3.2. Quantification of PRRSV-infected and apoptotic cells in the broncho-alveolar spaces (Fig. 2)

PRRSV-infected cells were first observed at 1 day PI ( $3 \times 10^6$  cells), increased to a maximum of  $15 \times 10^6$  cells at 9 days PI, decreased to  $3 \times 10^6$  cells at 14 days PI and remained at levels of  $0.5\text{--}1 \times 10^6$  cells until 40 days PI.

Apoptotic cells were detected in the broncho-alveolar spaces of both non- and PRRSV-inoculated pigs. In the seven non-inoculated control pigs, the mean number was  $5.3 \pm 1.8 \times 10^6$ . In PRRSV-inoculated



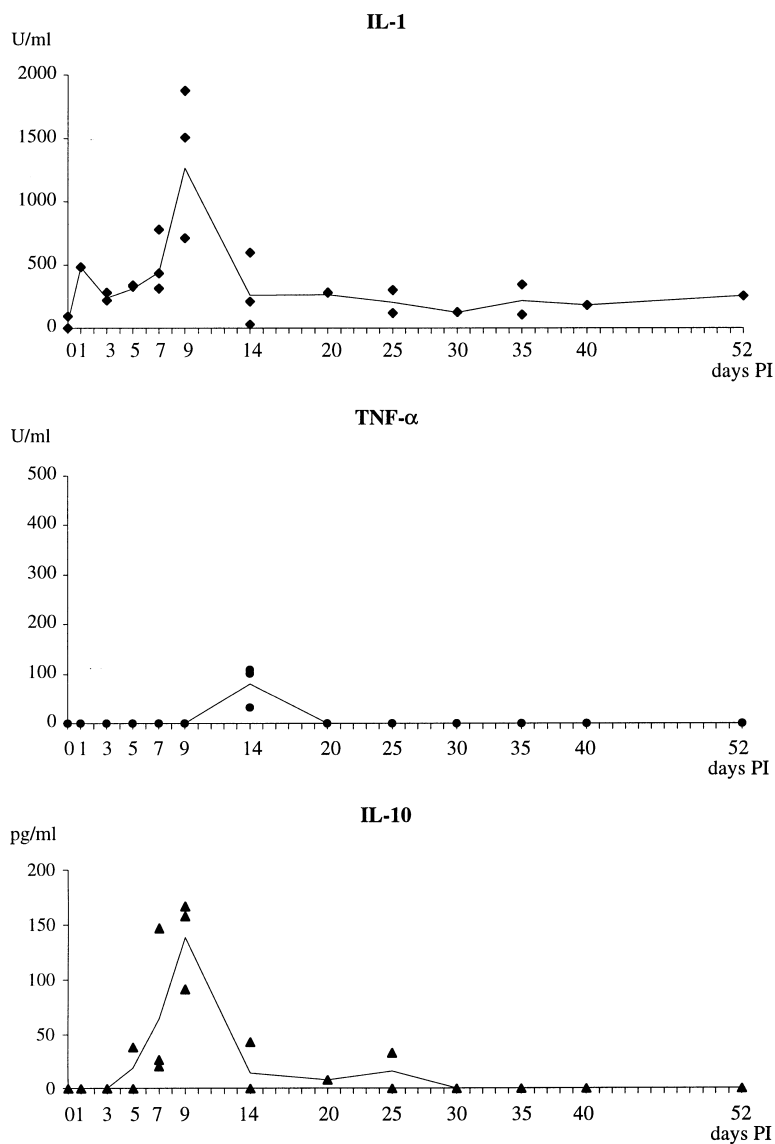
**Figure 1.** Quantification of infected and apoptotic cells in lung tissue (/100 mm<sup>2</sup>) throughout a PRRSV infection. Values at day 0 represent the numbers of infected and apoptotic cells of two non-inoculated control pigs euthanised at 4 weeks of age.



**Figure 2.** Quantification of infected and apoptotic broncho-alveolar lavage (BAL) cells ( $\times 10^6$ ) throughout a PRRSV infection. Values at day 0 represent the numbers of infected and apoptotic cells of two non-inoculated control pigs euthanised at 4 weeks of age.

pigs, the mean numbers were similar to those of non-inoculated pigs until 3 days PI. The mean numbers increased significantly from  $6.6 \pm 1.7 \times 10^6$  at 3 days PI to a first maximum of  $41.4 \pm 10.8 \times 10^6$  at

9 days PI ( $P < 0.05$ ; one-way ANOVA), then remained at the same level until 20 days PI and reached a second maximum of  $63.0 \pm 31.0 \times 10^6$  at 25 days PI. From 35 days PI onwards, the mean numbers



**Figure 3.** Interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-10 (IL-10) levels in broncho-alveolar lavage (BAL) fluids throughout a PRRSV infection. The dots represent individual cytokine levels; the lines represent means at each time point. Values at day 0 represent the cytokine levels of two non-inoculated control pigs euthanised at 4 weeks of age.



of apoptotic cells of PRRSV-inoculated pigs were not different from those of non-inoculated pigs.

Double-labellings revealed that within the population of infected BAL cells, apoptosis was detected until 25 days PI with percentages ranging between 13 and 30% and that the majority of apoptotic cells (> 99%) were non-infected.

Mean percentages of apoptotic cells remained constant in the total population of BAL cells and in the subpopulation of monocytes/macrophages (41D3<sup>+</sup> cells). In the subpopulation of T lymphocytes and NK cells, mean percentages reached a maximum at 7 (CD8<sup>+</sup> cells) or 9 days PI (CD2<sup>+</sup> and CD4<sup>+</sup> cells), sharply decreased thereafter and then remained at constant levels until 52 days PI. In the subpopulation of B lymphocytes (wCD1<sup>+</sup> cells), apoptotic cells peaked at 5 to 9 days PI and decreased sharply thereafter.

### 3.3. IL-1, TNF- $\alpha$ and IL-10 production in BAL fluids (Fig. 3)

A low level of IL-1 (71 U/mL) was found in one out of the seven non-inoculated pigs. The other control pigs had no detectable amounts of IL-1. All PRRSV-inoculated pigs had detectable IL-1 production. The pig euthanised at 1 day PI had an IL-1 titre of 483 U/mL. Mean IL-1 titres increased from 240 U/mL at 3 days PI to a maximum of 1265 U/mL at 9 days PI, decreased to 263 U/mL at 14 days PI and remained at levels of 121–267 U/mL until 52 days PI.

All non-inoculated control pigs were negative for TNF- $\alpha$ . After PRRSV inoculation, TNF- $\alpha$  was only found in the three pigs euthanised at 14 days PI, at low levels (32–109 U/mL).

All non-inoculated control pigs were negative for IL-10. After PRRSV inoculation, IL-10 was found starting from 5 days PI (19 pg/mL). Mean IL-10 levels reached a maximum of 139 pg/mL at 9 days PI,

decreased to 14 pg/mL at 14 days PI and remained at levels of 8–17 pg/mL until 25 days PI.

### 3.4. Quantification of apoptotic cells after in vitro treatment with recombinant porcine IL-1 $\beta$ or IL-10

No morphological features of apoptosis, such as cell shrinkage and cytoplasm hyper-vacuolisation, were observed in the monocyte and macrophage cultures throughout the experiment. Four days after treatment with recombinant porcine IL-1 $\beta$  or IL-10, the relative numbers of apoptotic blood monocytes and alveolar macrophages did not differ from those of untreated cells. Only 2.0% of the untreated blood monocytes and 6.5% of the untreated alveolar macrophages showed apoptosis. After treatment with recombinant porcine IL-1 $\beta$ , percentages of apoptotic blood monocytes and alveolar macrophages were 2.4 and 5.3%, respectively. Similar results were obtained after treatment with the different concentrations of recombinant porcine IL-10. Between 2.5 and 4.0% of blood monocytes and between 5.1 and 6.9% of alveolar macrophages were apoptotic.

## 4. DISCUSSION

The present study showed a clear relation between the peak of virus replication and the onset of apoptosis in the lungs of PRRSV-infected pigs. Apoptosis occurred both in virus-infected and in non-infected cells. Most apoptotic cells were non-infected, which is in agreement with previous studies [7, 24, 27].

The pattern of apoptosis in the non-infected cells was different in lung tissue compared to broncho-alveolar spaces. In lung tissue, apoptosis peaked at 14 days PI and occurred predominantly in monocytes/macrophages. Earlier studies showed that a PRRSV infection induces a two- to

fivefold increase of the number of broncho-alveolar monocytes/macrophages [16]. Since apoptosis is a homeostatic process that serves primarily to eliminate redundant cells during normal development and to eradicate defective cells, apoptosis of interstitial monocytes/macrophages throughout a PRRSV infection may be a mechanism of cell population control. However, the process of apoptosis is apparently not sufficient to completely control the massive influx of new monocytes since abundant monocytes/macrophages cross the interstitium towards the broncho-alveolar spaces [16].

The mechanism of apoptosis in non-infected interstitial monocytes/macrophages is not known. It has been suggested that TNF- $\alpha$  may be responsible for the induction of apoptosis in these cells [7]. However, in the present study only a very weak TNF- $\alpha$  production was detected at 14 days PI. Our *in vivo* data suggest that IL-1 and IL-10 could be involved in the induction of apoptosis in non-infected interstitial monocytes/macrophages. IL-1 and IL-10 levels peaked at 9 days PI and coincided with a significant increase in apoptotic cell numbers. Even though, both cytokines probably do not play a role in the induction of apoptosis in non-infected interstitial monocytes/macrophages, since we failed to induce apoptosis in blood monocytes or alveolar macrophages *in vitro* with recombinant porcine IL-1 $\beta$  or IL-10. Using similar *in vitro* experiments, both cytokines were shown to induce apoptosis in human cells. IL-1 $\beta$  was shown to induce apoptosis in a glioblastoma-derived human cell line when concentrations between 1 and 200 U/mL were used [6]. Similarly, human monocytes/macrophages underwent apoptosis following treatment with 10 ng/mL of IL-10 [33]. Thus, the exact mechanism by which PRRSV triggers apoptosis in non-infected interstitial monocytes/macrophages is still under debate. In addition to cytokines, macrophages may produce a lot of other apoptogenic mediators, such as nitric oxide and

reactive oxygen species, and any or all of these mediators may contribute to the induction of apoptosis in non-infected monocytes/macrophages. Besides these apoptogenic mediators, viral glycoprotein GP5, incorporated in cellular debris, may also be responsible for the induction of apoptosis in non-infected cells. This glycoprotein has clearly been shown to be involved in the induction of apoptosis [26]. Perhaps, induction of apoptosis can also be alternatively accomplished by attachment of whole virus particles to the cell without penetration and ensuing viral infection. Furthermore, the phagocytic clearance of cellular debris in viral antigen-positive foci by monocytes/macrophages may also be responsible for the apoptosis of non-infected cells. It has been demonstrated that phagocytosis triggers macrophage release of the Fas ligand and induces apoptosis in bystander leukocytes [5]. Since monocytes/macrophages themselves express Fas, cross-linking of Fas with the soluble Fas ligand may indeed induce apoptosis in non-infected bystander monocytes/macrophages. Both GP5 and phagocytosis may explain why the highest number of apoptotic cells was found in the proximity of viral antigen-positive foci. Furthermore, in these regions, apoptotic cells may have been wrongly defined as non-infected, since some of these cells may have been in the early stage of the PRRSV replication cycle and thus not yet expressing viral antigens.

In the broncho-alveolar spaces, two peaks of apoptosis were detected. The first peak at 9 days PI involved mainly lymphocytes. Both T helper cells (CD2<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>), cytotoxic T cells and NK cells (CD2<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>), and B cells (wCD1<sup>+</sup>) underwent apoptosis. The percentage of apoptotic cells in T lymphocytes was the highest at the peak of virus replication in BAL cells. This suggests that the PRRSV infection induces apoptosis in broncho-alveolar T lymphocytes and NK cells. This is not surprising since T lymphocytes, activated during the antiviral immune

response, are predisposed to undergo apoptosis [22]. The second peak of apoptosis at 25 days PI can be explained by the fact that the total BAL cell numbers were the highest at that time point, as demonstrated in a previous study [16].

Apoptosis was detected in virus-infected macrophages during the persistent stage of the PRRSV infection. Previous studies have demonstrated that the appearance of a PRRSV-specific immunity causes a significant decrease in the number of viral antigen-positive cells starting at 9 days PI. Nevertheless, a low number of single viral antigen-positive cells persists in the lungs for several weeks [16]. Why these single viral antigen-positive cells are able to persist despite the presence of immunity is not known, but may be in part attributable to the apoptotic process in infected cells. It has been suggested that apoptosis of virus-infected cells may represent a very efficient mechanism for viruses to escape from humoral immunity. The progeny virus which is present in apoptotic bodies can be taken up by neighbouring macrophages while protected from antibodies. This type of immune-evasion has already been described for the human immunodeficiency virus type 1 [25], herpes simplex virus type 2 [2] and Epstein-Barr virus [13].

Taken together, apoptosis seems to have a homeostatic effect on the number of monocytes/macrophages during the massive influx of new monocytes in the lungs of PRRSV-infected pigs and may explain why a low number of single viral antigen-positive cells persists in the lungs for several weeks despite the presence of immunity.

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