

Classical swine fever virus induces activation of plasmacytoid and conventional dendritic cells in tonsil, blood, and spleen of infected pigs

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Abstract – Classical swine fever virus (CSFV) compromises the host immune system, causing indirect leucopenia and disruption of in vitro T cell stimulation capacity. In order to explore the potential role of dendritic cells (DC) in such phenomena, the activation of conventional DC (cDC) and plasmacytoid DC (pDC) in blood and secondary lymphoid organs of infected pigs was investigated in the early time course post-inoculation (pi), together with viral components dissemination and cytokine production in serum. Whereas CD11R1⁺CD172a⁺ cDC frequencies were markedly reduced in blood and spleen, analysis of CD4⁺CD172a⁺ pDC numbers revealed a rapid turn-over of this DC subset in tissues pi. Both subsets matured and were activated after infection, as demonstrated by down-regulation of CD1a, up-regulation of the co-stimulation molecule CD80/86 and expression of cytokines. cDC essentially expressed tumor necrosis factor alpha (TNF- α) and interleukin (IL)-10, whereas pDC produced alpha interferon (IFN- α) and IL-12. IFN- α and TNF- α productions revealed an enhancement of innate anti-viral immune responses. Detection of antigen activated B lymphocytes in tonsil T-cell areas at 72 h pi, subsequently to the transient translocation of the viral E2 protein within germinal centres at 48 h pi, indicates the initiation of humoral response. This response was also evidenced by an important IL-10 production in serum one week pi. IL-12 expression in organs, as well as transient detection of IL-18 and IFN- γ in serum, reflected the initiation of cellular immune responses. However, the uncommonly high levels of TNF- α and IFN- α produced by DC and measured in serum early post-infection, together with IL-10 expression in spleen, could play a role in the disruption of immune system cells, either inducing apoptosis or impairing DC functionalities themselves.

antigen presenting cells / secondary lymphoid organs / interferon / E2 antigen / pestivirus

1. INTRODUCTION

Classical swine fever virus (CSFV), a member of the genus *Pestivirus* within the Flaviviridae family, is a small, enveloped positive RNA virus [45]. Strains of high virulence cause a devastating disease in pigs, characterized by high fever, hemorrhagic lesions and high mortality [25]. CSFV can efficiently evade and compromise the host immune system, causing

lymphoid organ atrophy, thrombocytopenia, granulocytopenia, and severe lymphopenia, particularly in B cells, due to apoptosis and necrosis of uninfected lymphocytes [36, 40, 41, 44]. Additionally, in vitro studies showed that uninfected T cells isolated from CSFV-inoculated pigs lack their in vitro stimulation capacity [34].

Intending to explain immunosuppression mechanisms, recent studies investigated dendritic cell (DC) activation following an

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infection with CSFV. Indeed, DC are specialized antigen-presenting cells (APC) that bridge innate and adaptive immune systems with the unique capacity to initiate primary T-cell responses and efficiently stimulate memory responses [5]. Thus, their dysfunction may play a role in disruption of host immune responses. Previous reports concerned infection of monocyte-derived DC (mo-DC) and blood-isolated natural interferon (IFN) producing cells (NIPC), i.e. plasmacytoid DC (pDC). It was shown that CSFV replicates in mo-DC without cytopathological effect but prevents their activation and thereby the secretion of IFN- α , tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-10 and IL-12 [4, 13]. CSFV also blocks mo-DC maturation by inhibiting MHC-II up-regulation, although these cells remain able to present E2 viral envelop glycoprotein to cytotoxic T lymphocytes [14]. Similarly, CSFV replicates in NIPC [4]. However, unlike mo-DC, in vitro infected NIPC secrete large amounts of IFN- α . To date, these in vitro findings could not be correlated to observations made in infected animals. Interestingly, high serum levels of IFN- α were recently found during the acute phase of CSF [43]. They were statistically correlated to the lymphocyte depletion, thus a relationship between both events was hypothesized, supported by the known adverse haematological effects of high IFN- α doses in vivo [3]. The authors reported that the infection also induces a progressive depletion of NIPC in blood, and thus suggested that IFN- α expression does not occur in the peripheral blood but in the lymphoid tissue [43].

In a previous work, we showed that swine secondary lymphoid organs and blood contained two distinct DC subsets [23]. One subset is composed of CD172a⁺ CD11R1⁺ cells that correspond to conventional DC (cDC), whereas the other one is composed of CD172a⁺ CD4⁺ cells that correspond to pDC. In the steady state, cDC and pDC reside in T cell areas of secondary lymphoid organs and most of them are immature, as revealed by CD1a expression and lack of the co-stimulation molecule CD80/86. They express basal levels of TNF- α , IL-12, IL-10 and

IFN- α at the steady state, the rates depending on the nature and location of producing cells. In this study, we intended to further explore interactions between CSFV and DC in infected pigs. DC activation was investigated (i) in the tonsil, the primo-site of infection, (ii) in blood, that gives greater place to leukocyte migration than lymph in the pig, (iii) in the spleen, a secondary lymphoid organ of importance due to its ability to filter the leukocyte containing blood, especially after infections [8]. We report findings about variations in DC frequency, location, phenotype and cytokine expressions in the early time course post-inoculation. Together with virus dissemination in tissues and cytokine production in serum, these data lead to new hypotheses on the role of DC subsets in host systemic immune response after CSFV infection.

2. MATERIALS AND METHODS

2.1. Antibodies

mAb against pig cell surface molecules CD1a (clone # 76-7-4), CD4 (clone # 74-12-4), CD21 (clone # BB6-11C9), CD172a (porcine panmyeloid cell marker SWC3, clone # 74-22-15A) were obtained from Southern Biotechnology Associates (Birmingham, USA). Anti-human CD80/86 (hCTLA-4-mulg fusion protein) was from Ancell (Bayport, USA). All these mAb were conjugated with FITC or red-phycoerythrin (RPE) or biotin. Anti-porcine CD11R1 mAb (clone # MIL4) from AbDSerotec (Martinsried, Germany) was conjugated with FITC using the Fluoreporter FITC Protein Labeling Kit (Invitrogen, Carlsbad, USA). Two mAb against porcine IFN- α (clone # F17 and clone # K9), mAb against IL-12 (clone # 116219), biotinylated anti-porcine IL-12 polyclonal Ab (BAF912) and biotinylated anti-porcine TNF- α polyclonal Ab (BAF690) were from R&D Systems (Minneapolis, USA). Biotinylated anti-porcine IL-10 mAb (clone # 945A1A926C2) was from Biosource (Camarillo, USA). Anti-porcine IFN- α mAb (clone # F17) was biotinylated using the BiotinTag Micro Biotinylation Kit (Sigma-Aldrich, St. Louis, USA). FITC-conjugated mAb against CSFV E2 glycoprotein (clone # F34113) was from Euroclone (Pero, Italy) and mAb against pestivirus NS3 protein (clone # BVD-C16) was kindly provided by Dr I. Greiser-Wilke (School of Veterinary Medicine, Hannover, Germany). Streptavidin

coupled to RPE-cyanin-5, RPE-conjugated polyclonal goat F(ab')₂ anti-mouse Ig and negative substitution controls were obtained from Dako (Glostrup, Denmark).

2.2. Virus and animal infections

CSFV highly virulent Eystrup strain [30] was kindly provided by Dr A. Summerfield (Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland) and propagated on porcine kidney cell line PK15. Virus titre was determined as previously described [29]. Seven-week-old specific pathogen free Large White pigs of both sexes were housed in isolation units at the Section of Healthy Pig Production and Experimentation (AFSSA, Ploufragan). One group of 24 pigs was infected by the oronasal route with 10⁶ TCID₅₀ of the Eystrup strain and slaughtered in batches of 3 at 16, 72, 96 and 168 hours post-inoculation (hpi), and in batches of 6 at 24 and 48 hpi. A group of 8 pigs was similarly mock inoculated with culture supernatant of uninfected PK15 cells and slaughtered in batches of 2 at 0, 48, 96 and 168 hpi. Finally, a group of 10 control pigs was non-inoculated and slaughtered in a batch of 6 at 0 hpi and in batches of 2 at 24 and 72 hpi. Clinical signs such as anorexia, depression, fever, purple skin discoloration, diarrhoea and cough, were observed daily during the experiment and scored as previously described [30]. Accepted principles of animal care were followed and pigs were sacrificed by intravenous booster of sodium pentobarbital in compliance with the animal welfare experimentation agreement given by the Direction des Services Vétérinaires des Côtes d'Armor (AFSSA registration No. B-22-745-1, AJ and GKS authorisations No. 22-27 and 22-26, respectively).

2.3. Sample collection and treatments

Whole, EDTA and heparinized blood samples were taken before euthanasia. Immediately thereafter, palatine tonsils and spleens from each animal were entirely collected for laboratory investigations. Serum was obtained by centrifugation of coagulated blood at 2200 *g* for 5 min and stored at -20 °C until use. EDTA blood cell counts were performed with a MS9 Coulter Counter (Beckman Coulter, Fullerton, USA). EDTA blood samples and parts of tonsils and spleens were stored at -70 °C until viral genome detection. Other tonsil and spleen samples were quickly snap frozen in isopentane/liquid nitrogen and stored at -70 °C until cutting of serial 5 μm-thick frozen sections that

were fixed either on RNase free polylysine slides in 4% paraformaldehyde for 10 min for subsequent *in situ* hybridization, or on untreated slides in 80% cold acetone for 10 min for immunohistochemistry (IHC). After washing, both slide types were stored at -70 °C until use. Finally, the remaining tissue was weighed, cut, minced in RPMI 1640 (Invitrogen) and filtered through nylon membrane (100 μm). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll-Hypaque (Amersham Biosciences, Piscataway, USA) density centrifugation, at 2200 *g* for 15 min at 18 °C. After PBS washes, total tonsillar and splenic cell suspensions and PBMC were stored in fetal calf serum (Laboratoires Eurobio, Courtaboeuf, France) containing 10% DMSO (Sigma-Aldrich) at -70 °C until flow cytometry analyses.

2.4. Viral genome detection

Viral RNA was extracted from tissue samples using RNeasy kit (Qiagen, Courtaboeuf, France) and detected by closed-one tube RT-nested PCR. The same procedure as described by McGoldrick et al. [28] was followed except that A14 inner primer was replaced by PPC1 primer (5'-CAG GTC GTA CCC CAT CA-3') [27], giving rise to a 150 nucleotide-long CSFV specific amplification product from 5' NTR.

2.5. FITC-labeled probe synthesis

Primers from the Eystrup viral strain genome sequence (GenBank accession number NC002657) were used as 5'-ACCTACC-TGGCCACAGACAC-3' (nucleotides 1301-1320) and 5'-GGTGACCACGTTGATGTCAG-3' (nucleotides 1534-1553), corresponding to forward and reverse primers, respectively. The primer set resulted in an amplified fragment of 252 bp located in E^{rms} gene. After viral RNA extraction from virus stock, RT was performed with Moloney Murine Leukaemia Virus RT (M1701, Promega, Madison, USA), followed by PCR using Taq DNA polymerase (M1865, Promega) under these conditions: 94 °C for 10 min followed by 30 amplification cycles (denaturing at 94 °C for 45 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s) and final elongation at 72 °C for 7 min. The DNA product was purified using Qiaquick PCR purification kit (Qiagen), labeled with FITC-dUTP using Fluorescein High Prime kit (Roche Diagnostics, Meylan, France) and stored at -20 °C until use.

2.6. Fluorescence in situ hybridization

Tissue sections fixed on polylysine slides were treated in 0.2 N HCl at room temperature for 20 min and then incubated in PBS with 25 µg/mL of proteinase K (Roche Diagnostics) at 37 °C for 20 min. Reaction was stopped in PBS containing 0.2% glycine, and after successive washes in PBS and 2× SSC, slides were air-dried and mounted in a Gene Frame incubation chamber of 25 µL (Abgene, Epsom, UK). Tissues were pre-hybridized at 42 °C for 1 h in hybridization buffer consisting of 2× SSC with 50% formamide, 1× Denhart solution and 100 µg/mL fish sperm DNA. The hybridization mix consisted of 0.66 ng/µL of FITC-labeled probe diluted in hybridization buffer with addition of 10% dextran sulfate and 250 µg/mL fish sperm DNA. This reaction mix was denatured at 95 °C for 10 min and applied to tissue sections under cover slides. After overnight hybridization at 42 °C, sections were successively washed in 2× SSC, in 1× SSC, in 0.5× SSC and in 0.1× SSC, each for 30 min at 42 °C. Hoechst stain solution (Sigma-Aldrich) was added at 5 µg/mL on sections for 15 min. Finally, sections were air-dried before application of coverslip over fluorescence mounting medium (Dako). For each staining assay, three sequential sections per tissue for each pig were observed using an Olympus BX41 epifluorescence microscope. Images of representative areas for each tissue were recorded on a Spot Insight FireWire CCD camera using Spot Advanced software (Diagnostic Instruments, Sterling Heights, USA).

2.7. Fluorescence immunohistochemistry

Non-specific binding of Ab to tissue sections was blocked for 1 h in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X100. Then, all incubations were done in the dark at room temperature. Sections were incubated with 1 or 2 different conjugated-mAb at 20 ng/µL for 1 h in PBS containing 1% BSA, 0.1% Triton X100. When biotinylated-antibodies and anti-NS3 were used, sections were incubated for 1 h with streptavidin coupled to RPE-cyanin-5 and RPE-conjugated polyclonal goat F(ab')₂ anti-mouse Ig respectively. Finally, Hoechst stain solution was added on sections and microscopy observations were performed as described above.

2.8. Flow cytometry analyses

After thawing, PBMC, tonsillar and splenic cell viability was evaluated by the Trypan Blue dye ex-

clusion test. Each single-, double- or triple-color staining procedure was performed on 5×10^5 cells saturated with 0.5 µg FITC or/and RPE or/and biotin labeled primary mAb for 30 min at 4 °C. Cells stained with biotinylated mAb were incubated with streptavidin coupled to RPE-cyanin-5 for 30 min at 4 °C and fixed as other stained cells. After labeling of cell-surface proteins, cytokines were detected by intracellular staining using Cytofix/Cytoperm kit (BD Biosciences Pharmingen, San José, USA). Flow cytometry analyses were performed on a constant event number of 20 000 cells fixed with 2% paraformaldehyde, acquired on a FACSort flow cytometer using CellQuest Software (both from BD Biosciences) and analyzed using WinList 5.0 (Verity Software House, Topsham, USA). Due to the strong leucopenia and hemorrhagic disorders that resulted from the infection, low amounts of viable cells were available within PBMC, tonsil and spleen total cell suspensions, thus allowing only one repetition per immunostaining assay to be performed per pig. Each assay was repeated on 3 animals at each time point pi.

Cell subset frequencies obtained from flow cytometry analyses performed on PBMC were used to calculate absolute cell numbers per mL of blood, by reporting cell subset percentages to the number of leucocytes counted per mL of total blood reduced by the number of granulocytes.

Flow cytometry analyses on total cell suspension from the tonsil and spleen were realized on an electronic gate containing only mononuclear cells, by forwarding scatter versus side scatter (FSC/SSC) dot plot, in order to exclude granulocytes. Indeed, granulocytes are known to express CD172a as other myeloid cells such as monocytes, macrophages and DC, and to express CD11R1 as cDC [22]. CD11R1⁺ CD172a⁺ cells corresponding to cDC and CD4⁺ CD172a⁺ cells corresponding to pDC were detected in the mononuclear cell gate. Percentages of DC that expressed CD1a, CD80/86 or cytokine were subsequently obtained in another gate that included either CD11R1⁺ CD172a⁺ cells or CD4⁺ CD172a⁺ cells, respectively. Since the size of lymphoid organs and their different compartments were reported to be largely dependent on factors such as breed, age and antigenic stimulation, absolute numbers of cells per gram of tissue and per whole organ should be given in any experiment dealing with alterations of the immune system [9]. However, we observed that the size and weight of the tonsil and spleen were constant between individuals at a given time point post-infection but that the

cell amounts, obtained after isolation and freezing-thawing, varied from one experiment to another (data not shown). Since flow cytometry analyses were performed on a constant event number, after immunostaining of a constant cell number, all results concerning tonsillar and splenic cell subsets were given as relative numbers.

2.9. Detection of cytokines in serum

IFN- α and IL-12 were detected by sandwich ELISA as previously described [23]. TNF- α and IL-10 were detected using commercial ELISA kits from Biosource, IL-6 using a kit from R&D Systems (Minneapolis, USA), IL-18 and IFN- γ using kits from Tebu-Bio (Le Perray-en-Yvelines, France).

2.10. Statistical analysis

Due to the small size of the data sets, all the statistical tests used were non-parametric. For each data set, variations in the time course post-inoculation were analysed by comparing data obtained at t ($h = 16, 24, 48, 72, 96$ or 168) with data obtained at $t(0)$. When presented as numbers or concentrations, data were analysed using the Mann & Whitney test. In case of results expressed as percentages, analyses were performed on total strengths using the χ^2 test. All statistical analyses were performed using Systat 9 software¹. Comparisons were performed with a limit of significance of $p < 0.05$ (indicated by *) or of $p < 0.01$ (indicated by **).

3. RESULTS

3.1. Clinical signs and blood cell counts

Control and mock-infected animals remained healthy with normal body temperature (39.5°C) and normal blood cell count values throughout the experiment (Fig. 1). Both groups were reassembled for laboratory investigations.

All CSFV-inoculated animals had high increases in body temperature from 48 hpi, reaching a mean peak of 41.1°C at 72 hpi and remaining at an average of 40.3°C until 168 hpi. Growth was affected from 24 hpi and weight loss was observed from 96 hpi (data not shown). Clinical scores rose from 0 level

at 24 hpi to 8 level at 168 hpi. Tonsils were congested and haemorrhagic in 1/6 pig killed at 48 hpi, 1/3 at 72 hpi, and in all animals from 96 hpi. Spleens of all infected pigs were congested and haemorrhagic from 72 hpi.

In the infected group, EDTA-blood cell counts revealed a thrombocytopenia from 24 hpi, by comparison to normal platelet values in control pigs (data not shown). They also showed a significant leucopenia from 16 hpi (Fig. 1a), particularly due to a severe decrease in lymphocyte number (Fig. 1b). At 96 hpi, the mean lymphocyte number represented only 15% of its value in control pigs. By contrast, monocyte numbers were not significantly modified after infection (Fig. 1c) but statistical analyses revealed an obvious granulocytopenia one week pi (Fig. 1d).

3.2. Dissemination of CSFV in blood and organs

The presence of the CSFV genome was investigated by RT-nested PCR in blood, tonsil and spleen at different times pi (Tab. I). No CSFV RNA was detected in tissues of control and mock-inoculated animals. Viral RNA was detected in all infected pigs from 16 hpi. It was detected in one tissue out of three, the tissue identity depending on the individual. At 24 hpi, CSFV RNA was detected in blood of 4/6 animals, in the tonsils of 6/6 animals and in the spleen of 3/6 animals. At 48 hpi, all the tissues of infected pigs contained viral genome.

Fluorescence in situ hybridization analyses revealed that the CSFV genome was located in the tonsil crypt epithelium (CE) at 24 hpi and then in interfollicular areas (IFA) at 48 hpi (Fig. 2a). Detection of the non-structural protein NS3 by IHC confirmed the virus location in CE and IFA at 24 hpi and its multiplication in IFA, around the crypts, at 48 hpi (Fig. 2b).

NS3 was also detected in periarteriolar lymphoid sheaths (PALS) of the spleen, as soon as 24 hpi for some pigs or at 48 h in other cases (data not shown). When already detected at 24 hpi, the labeling intensity increased at 48 hpi (data not shown). CSFV RNA and NS3 protein were not detected neither in follicular germinal centres, nor in organs from control pigs.

¹ SPSS Inc., 1998.

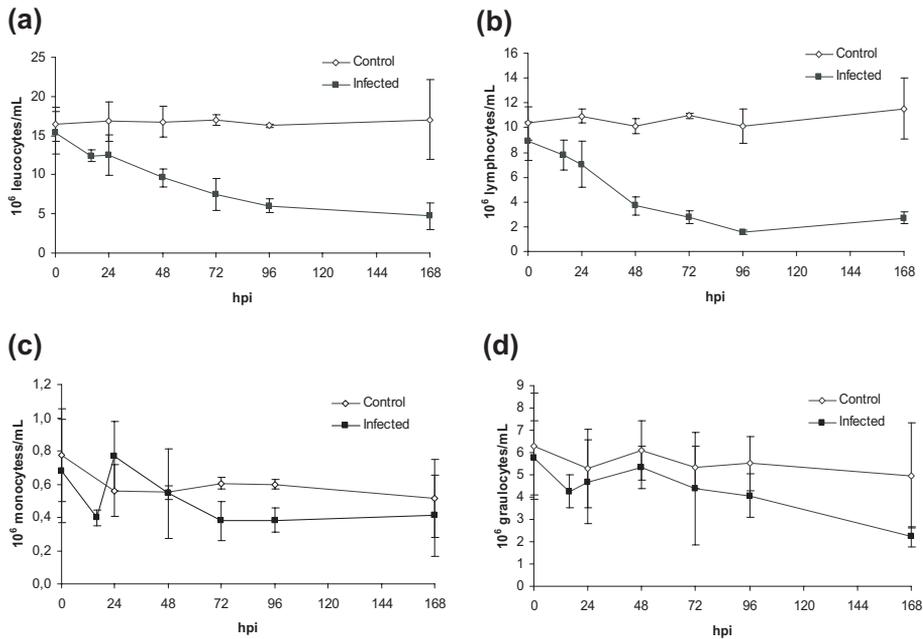


Figure 1. Numbers of white cells per mL of peripheral blood in the time course post-infection (hpi). (a) Leucocytes. (b) Lymphocytes. (c) Monocytes. (d) Granulocytes. The results are presented as mean ± SD of samples from 14 (Control group) and 23 animals (Infected group) at 0 hpi, and then from 6 animals at 24 and 48 hpi and from 3 animals at other time points pi.

Table I. Detection of CSFV RNA in control, mock-inoculated and infected pigs in the time course post-inoculation (hpi). The presence of virus genome was assessed by RT-PCR in blood (B), tonsil (T) and spleen (S). Results are expressed as negative (–) and positive (+) samples. *n* = number of analysed pigs.

Animals	hpi	0			16			24			48			72			96			168			
		Tissues	B	T	S	B	T	S	B	T	S	B	T	S	B	T	S	B	T	S			
Control	<i>n</i> = 10		–	–	–																		
Mock-inoculated	<i>n</i> = 8		–																				
Infected No. 1	<i>n</i> = 1		–			+	–	–															
Infected No. 2	<i>n</i> = 1		–			–	+	–															
Infected No. 3	<i>n</i> = 1		–			–	–	+															
Infected No. 4	<i>n</i> = 1		–						+	+	+												
Infected No. 5	<i>n</i> = 1		–						–	+	+												
Infected No. 6	<i>n</i> = 1		–						+	+	+												
Infected No. 7	<i>n</i> = 1		–						+	+	–												
Infected No. 8	<i>n</i> = 1		–						+	+	–												
Infected No. 9	<i>n</i> = 1		–						–	+	–												
Infected No. 10–15	<i>n</i> = 6		–									+	+	+									
Infected No. 16–18	<i>n</i> = 3		–											+	+	+							
Infected No. 19–21	<i>n</i> = 3		–														+	+	+				
Infected No. 22–24	<i>n</i> = 3		–																		+	+	+

3.3. Location of viral E2 antigen with respect to APC in secondary lymphoid organs

Intending to analyse interactions between CSFV and DC in secondary lymphoid organs, we investigated the location of the viral envelop glycoprotein E2, the most immunogenic viral protein, together with APC in the tonsil and spleen. Double fluorescence IHC analyses first focused on E2 location with respect to myeloid CD172a⁺ cells, i.e. monocytes, macrophages or DC. E2 protein was not detected in tonsil from non-infected pigs (Fig. 3). Anti-E2/anti-CD172a double staining assays revealed numerous double positive cells in IFA as early as 24 hpi, demonstrating that most of E2 were located within myeloid cells despite some green fluorescence spots due to the presence of viral particles taken up by non-myeloid cells (Fig. 3a). Interestingly, only rare CD172a⁺ E2⁺ cells were still detected in IFA at 48 hpi, while the number of CD172a⁺ cells seemed to increase in that area. Surprisingly, at that time point, quite all E2 proteins were transiently located into follicle centres. At 72 hpi, CD172a⁺ E2⁺ cells were observed again in IFA, around CE, where E2 free of CD172a⁺ cells was strongly detected.

IHC analyses also investigated E2 location with respect to IFN- α ⁺ cells, i.e. pDC. Rare IFN- α positive cells were detected in IFA of tonsil in non infected pigs (Fig. 3b). Their number markedly increased in IFA from 24 hpi and several IFN- α ⁺E2⁺ cells were easily detected. At 48 hpi, only few IFN- α ⁺E2⁺ cells were observed in T cell areas, at the periphery of follicles. E2 was mostly condensed in germinal centres as already observed above from IHC analyses with anti-CD172a. At 72 hpi, the number of IFN- α ⁺E2⁺ cells increased again. Double positive cells were detected around and in CE, together with E2 antigen located in non IFN- α secreting cells.

Intrigued by the transient translocation of E2 in germinal centres at 48 hpi, we analysed sections after anti-CD21/E2 double staining (Fig. 3c). CD21⁺ cells include B cells and constitute primary follicles of tonsil from non-infected pigs [23]. At 24 hpi, E2 was clearly observed in IFA and the CD21⁺ cells did not present the viral antigen. At 48 hpi, E2

was stained in germinal centres where some CD21⁺E2⁺ cells were also noticed. Rare double positive cells were still found in follicles at 72 hpi, most of them being located in IFA, between follicles and CE, while CD21⁺ cell-free E2 already reached CE.

IHC analyses of E2 location in the spleen gave equivalent results to studies of tonsil sections (data not shown). In spleens that were invaded by the virus very early post-inoculation, a large number of IFN- α ⁺ cells contained E2 protein at 24 hpi. This was revealed by the presence of double positive E2/IFN- α cells in IFA. No E2⁺IFN- α ⁺ cell was detected at 48 hpi in these areas, whereas double positive cells were visualized again in PALS at 72 hpi, surrounding other E2⁺. IFN- α ⁻ cells were located close to blood vessels.

3.4. Variations in DC frequencies

In order to further analyse the effect of CSFV on DC subsets, we investigated the variations in cDC and pDC frequencies in organs and blood.

IHC assays aimed to detect CD4⁺IFN- α ⁺ cells in tonsil (Fig. 4a) and spleen (Fig. 4b), revealed an increase in the pDC number in T cell areas after infection. Double labeling assays with anti-CD172a together with anti-IFN- α or anti-CD4 antibodies provided similar pictures (data not shown).

DC frequencies were also analysed in blood, tonsil, and spleen in the time course pi, by flow cytometry analyses. Around 0.3×10^6 CD11R1⁺CD172a⁺ cDC and 0.25×10^6 CD4⁺CD172a⁺ pDC were measured in mean per mL of blood in control pigs (Fig. 5a). After CSFV infection, the cDC number immediately decreased markedly in blood, reaching at 48 and 96 hpi frequencies significantly different from frequencies at 0 hpi. By contrast, the number of pDC was not found significantly different. In tonsil, CD172a⁺CD11R1⁺ cDC and CD172a⁺CD4⁺ pDC frequencies slightly decreased at 24 and 16 hpi, respectively, but these modifications were punctual and the frequencies remained globally constant, representing 0.7% and 0.9% of total cell suspension, respectively (Fig. 5b). In the spleen, both CD11R1⁺CD172a⁺ cDC and

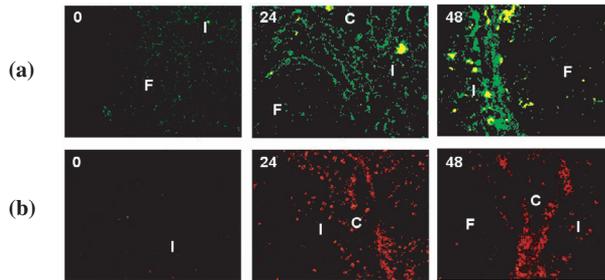


Figure 2. Location of CSFV in the tonsil in the time course post-infection. (a) CSFV RNA detection by green fluorescence in situ hybridization; magnification $\times 630$. (b) NS3 protein detection by red fluorescence IHC; magnification $\times 200$. Numbers correspond to hpi; C, crypt; F, follicle; I, interfollicular area. At each time point, a representative section out of three per one animal out of three is shown.

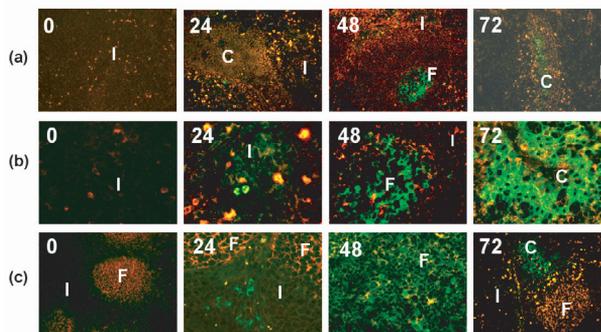


Figure 3. Co-location of E2 viral antigen and APC in tonsil in the time course after CSFV infection. (a) Anti-E2 (green) and anti-CD172a (red) double staining identifying $E2^+CD172a^+$ cells (yellow); magnification $\times 200$. (b) Anti-E2 (green) and anti-IFN- α (red) double staining identifying $E2^+IFN-\alpha^+$ cells (yellow); magnification $\times 630$. (c) Anti-E2 (green) and anti-CD21 (red) double staining identifying $E2^+CD21^+$ cells (yellow); magnification $\times 200$ at 0 and 72 hpi and $\times 630$ at 24 and 48 hpi. Numbers correspond to hpi; C, crypt; F, follicle; I, interfollicular area. At each time point, a representative section out of three per one animal out of three is shown.

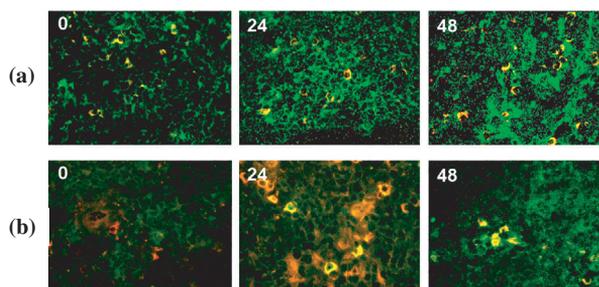


Figure 4. Location of IFN- α expressing cells in secondary lymphoid organs after CSFV infection. Anti-CD4 (green) and anti-IFN- α (red) double staining identifying $CD4^+IFN-\alpha^+$ pDC (yellow) in T cell areas of (a) tonsil and (b) spleen. Magnification $\times 630$. Numbers correspond to hpi. At each time point, a representative section out of three per one animal out of three is shown.

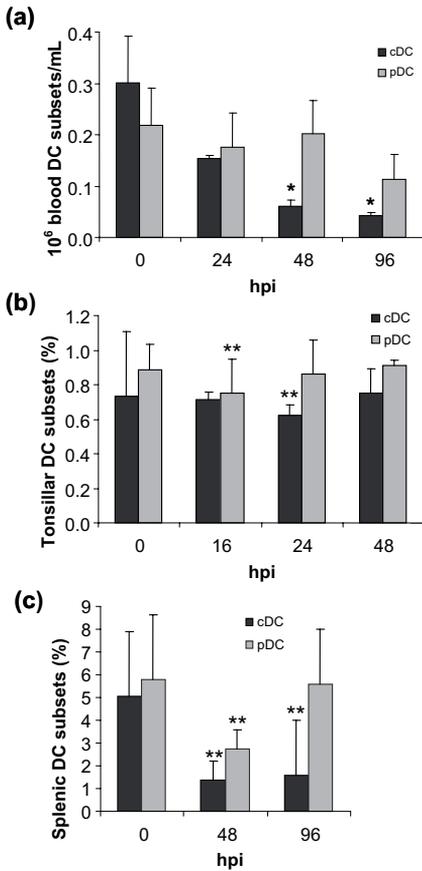


Figure 5. Variations in CD11R1+CD172a+ cDC and CD4+CD172a+ pDC numbers after CSFV infection. (a) DC numbers per mL of blood. (b) DC percentages within total cell suspensions from the tonsil. (c) DC percentages within total cell suspensions from the spleen. Results are presented as mean ± SD of samples from 6 to 9 animals at 0 hpi and from 2 to 3 individuals at various times pi. * $p < 0.05$ compared with corresponding values at 0 hpi using Mann & Withney test. ** $p < 0.01$ compared with corresponding values at 0 hpi using χ^2 test.

CD4+CD172a+pDC subsets, that represented each one around 5–6% of total splenic cell suspension in non infected pigs, markedly decreased post-infection. Thus, cDC only represented 1.6% at 96 hpi. pDC were reduced to 2.7% at 48 hpi but increased again to their normal value at 96 hpi (Fig. 5c).

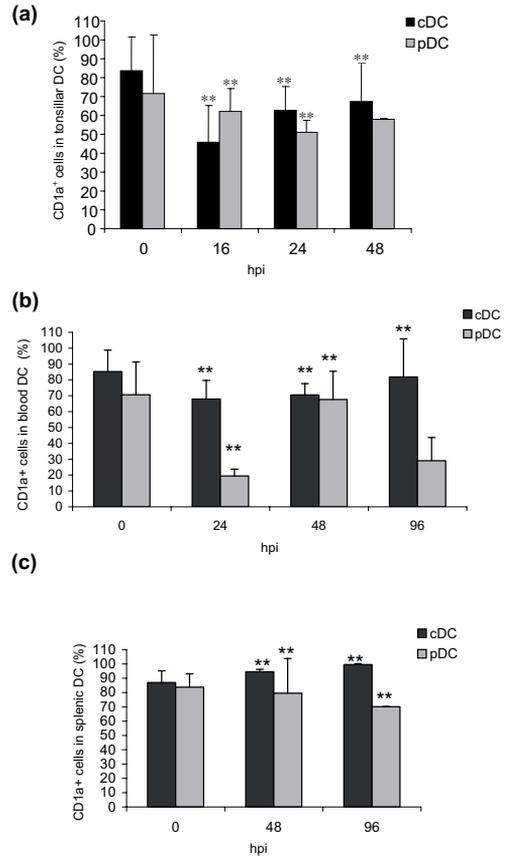


Figure 6. Variation in CD1a expression by DC subsets after CSFV infection. Percentages of CD1a+ cells within CD11R1+CD172a+ cDC and CD4+CD172a+ pDC in (a) tonsil, (b) PBMC and (c) spleen were measured by triple-color flow cytometry. Results are presented as mean ± SD from analyses conducted on samples from 6 to 9 individual animals at 0 hpi and 2 to 3 animals per time point pi. ** $p < 0.01$ compared with corresponding values at 0 hpi using the χ^2 test.

3.5. Maturation status of DC subsets

To evaluate the maturation status of DC subsets after CSFV infection, we intended to measure the expression of CD1a, implicated in lipid antigen binding, as well as the expression of CD80/86, a co-stimulation molecule, by CD172a+CD11R1+ cDC and CD172a+CD4+ pDC from the three studied cell suspensions, using a three-color flow cytometry analyses.

In the tonsils of control pigs, most of DC, i.e. around 80% of cDC and 70% of pDC, expressed CD1a (Fig. 6a). After infection, less than 50% of cDC were still CD1a⁺ as early as 16 hpi. Then, cDC expressed again CD1a at 48 hpi without reaching a normal level. These decreases were found significant as compared to the CD1a expression level before infection. Similarly to cDC but to a lesser extent, CD1a⁺ pDC frequency decreased after challenge, with around 60% of pDC expressing this marker at 24 hpi. Within PBMC, 85% of cDC expressed CD1a at 0 hpi. After infection, this percentage decreased and was maintained at 70% between 24 and 48 hpi. It raised back up to the initial value at 96 hpi (Fig. 6b). In pDC, CD1a appeared to be expressed with a particular kinetic during the first days of infection. Indeed, this marker was detected in 70% of pDC at 0 hpi, whereas only 20% of these cells were positive at 24 hpi. At 48 hpi, CD1a expression concerned again 70% of pDC but was limited to 40% of the subset at 96 hpi. In spleen, around 90% of cDC expressed CD1a at 0 hpi, as in blood. Conversely to the phenomenon observed in blood, CD1a expression increased from 48 hpi and concerned about 100% of the subset at 96 hpi (Fig. 6c). By contrast, CD1a expression by pDC was significantly reduced from 85% to 70% between 0 and 48 hpi.

Interestingly, CD80/86 appeared to be expressed by DC with an opposite kinetic to CD1a. In blood, co-stimulation molecule expression increased both in cDC and pDC between 0 and 24 hpi (Fig. 7a). Thus, the frequency of CD80/86⁺ cDC varied on average from 7% to 24% and CD80/86⁺ pDC from 9 to 14%. Then, a strong decrease in CD80/86 expression was observed. At 48 hpi it only concerned 2% and 4% of cDC and pDC, respectively. This lack of expression was slightly reversed in pDC at 96 hpi. In spleen, CD80/86 was expressed by only an average of 5% of both DC subsets at 0 hpi but increased post-infection up to 20% of pDC at 96 hpi (Fig. 7b). Because of low numbers of viable cells within tonsil cell suspensions, CD80/86 expression by DC could not be investigated in this tissue.

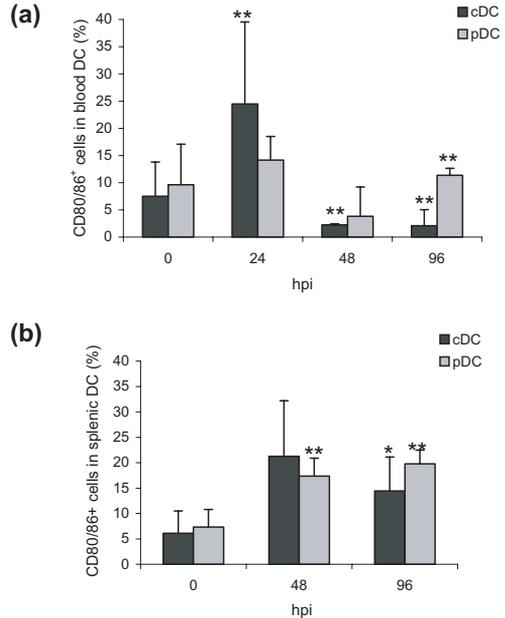


Figure 7. Variation in CD80/86 expression by DC subsets after CSFV infection. Percentages of CD80/86⁺ cells within CD11R1⁺CD172a⁺ cDC and CD4⁺CD172a⁺ pDC in (a) PBMC and (b) spleen were measured by triple-color flow cytometry. Results are presented as mean ± SD from analyses conducted on samples from 6 to 9 individual animals at 0 hpi and 2 to 3 animals per time point pi. ** *p* < 0.01 compared with corresponding values at 0 hpi using χ^2 test.

3.6. Cytokine expression by DC subsets

In order to evaluate DC activation status after CSFV infection, we measured cytokine expression by CD172a⁺CD11R1⁺ cDC and CD172a⁺CD4⁺ pDC in the tonsil, blood and spleen in the time course pi.

Three-color flow cytometry analyses conducted on tonsil cell suspensions showed that numbers of TNF- α and IFN- α positive cells significantly increased in both DC subsets in the time course post-infection, but with different kinetics and frequencies (Fig. 8). Thus, 18% of cDC expressed TNF- α in the tonsils of control pigs, whereas only 8.5% of pDC expressed this cytokine (Fig. 8a). After infection, TNF- α ⁺ cDC frequency immediately raised up, reaching 70% at 48 hpi. pDC were also

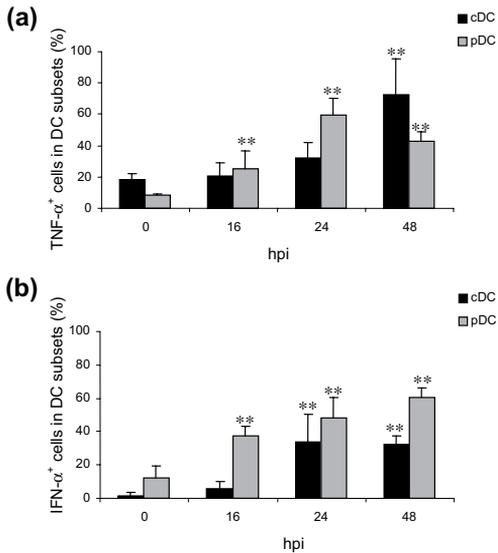


Figure 8. Activation status of DC subsets in tonsil after CSFV infection. Percentages of (a) TNF- α^+ cells and (b) IFN- α^+ cells within CD11R1 $^+$ CD172a $^+$ cDC and CD4 $^+$ CD172a $^+$ pDC, measured by triple-color flow cytometry. Results are presented as mean \pm SD from analyses conducted on samples from 6 to 9 individual animals at 0 hpi and 2 to 3 animals per time point pi. ** $p < 0.01$ compared with corresponding values at 0 hpi using χ^2 test.

activated to express TNF- α after challenge. TNF- α^+ pDC represented 60% of the subset at 24 hpi but decreased to 42% at 48 hpi. 12% of pDC expressed a basal level of IFN- α in the steady state, whereas very few cDC expressed this cytokine (Fig. 8b). After infection, cDC were activated with respect to IFN- α expression, as 5% of them were IFN- α positive at 16 hpi and 32% at 24 hpi. A more important effect was observed in pDC which rapidly enhanced their IFN- α expression after challenge. Thus, around 60% of them were found IFN- α^+ at 48 hpi. Regarding the low number of viable cells obtained from tonsils to perform flow cytometry analyses, no other cytokine expression in DC subsets was investigated in this tissue.

Analyses performed on PBMC showed that 10–20% of both CD172a $^+$ CD11R1 $^+$ cDC and CD172a $^+$ CD4 $^+$ pDC expressed TNF- α , IL-12 and IL-10 in blood at 0 hpi (Figs. 9a–9b). In

non-infected pigs, IFN- α was also expressed by 15% of pDC, but less than 2% of cDC. After infection, around 20–25% of cDC and 70% of pDC were detected as TNF- α^+ and IL-12 $^+$ cells at 24 hpi. Expression of IL-10 by cDC slightly decreased pi, while IL-10 expression was up-regulated in pDC early pi. Expression of IFN- α was stimulated in pDC and in cDC up to 48 hpi, before being reduced.

As observed in blood, 5–10% of cDC from spleen expressed basal levels of TNF- α , IL-12, IL-10 but no IFN- α at the steady-state (Fig. 9c). Less than 5% of splenic pDC expressed these cytokines before inoculation (Fig. 9d). After infection, cDC expressed TNF- α and IL-10 in an amount of 65% and 85%, respectively. IL-12 expression by cDC also increased in the time course post-infection but to a lower extent, and 15% of cDC were able to secrete IFN- α (Fig. 9c). Expression of these four cytokines by splenic pDC also significantly increased after infection (Fig. 9d), although numbers of cytokine expressing pDC were lower than cDC except for IFN- α .

3.7. Release of cytokines in serum after infection

Since expression of IFN- α , TNF- α , IL-12, IL-10 was stimulated in DC early post-infection, we measured their productions in serum in the time course pi. IFN- α level increased as early as 16 hpi, reaching a mean peak of 33 800 IU/mL at 96 hpi, i.e. 200 times more than in serum of non infected animals (Fig. 10a). Similarly, TNF- α level increased in the first hpi and reached a maximum of 320 pg/mL at 96 hpi, i.e. 8 times more than the basal level, before decreasing (Fig. 10b). On the contrary, IL-12 secretion did not significantly vary during the first week pi, remaining quite constant at 30–40 ng/mL (Fig. 10c). For IL-10, two peaks of release were observed, the first one at 24 hpi, and the second one, more important, 6 days later, i.e. 1-week pi (Fig. 10d). IL-6 was transiently detected at 96 hpi in 2/3 infected animals, with levels around 200 pg/mL (Fig. 10e). Finally, IFN- γ and more rarely IL-18, were also transiently detected at 72 hpi in some animals (Figs. 10f and 10g).

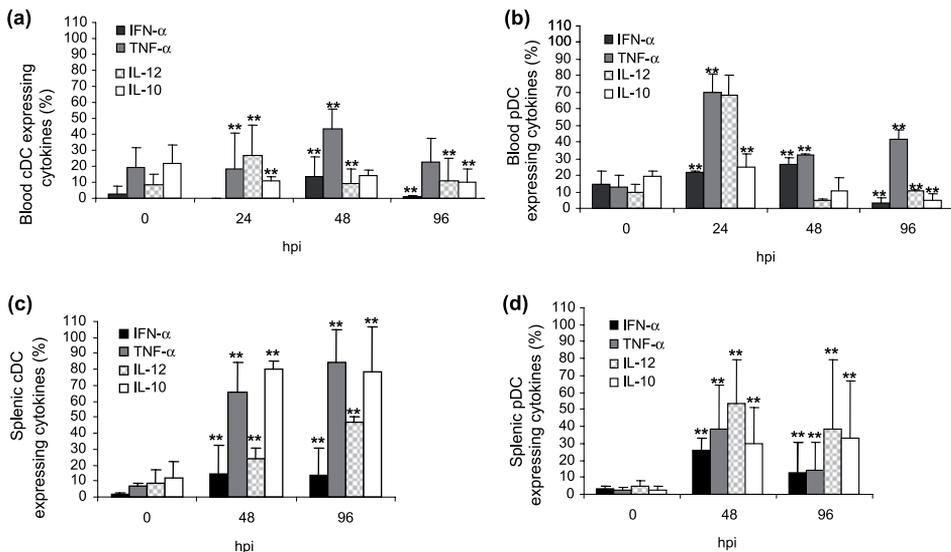


Figure 9. Expression of IFN- α , TNF- α , IL-12 and IL-10 by CD11R1⁺CD172a⁺ cDC and CD4⁺CD172a⁺ pDC. Percentages of cDC (a) and pDC (b) that express cytokines in blood. Percentages of cDC (c) and pDC (d) that express cytokines in spleen. Results from triple-color flow cytometry analyses are presented as mean \pm SD from experiments conducted on samples from 6 to 9 individual animals at 0 hpi and 2 to 3 animals per time point pi. ** $p < 0.01$ compared with corresponding values at 0 hpi using χ^2 test.

4. DISCUSSION

In this study, we first described the activation of cDC and pDC in secondary lymphoid organs and blood in the early time course after CSFV inoculation, together with viral components dissemination and cytokine production in serum.

Clinical signs and lesions observed in infected pigs were in accordance with previous data describing the Eyrstrup strain as a highly virulent CSFV strain [26, 30]. As revealed by viral RNA and NS3 protein detections, CSFV first invaded the tonsil, its entry site, and rapidly disseminated in the blood system, reaching the spleen between 24 and 48 hpi. In the tonsil, the virus was detected in crypts at 24 hpi, in accordance with previous reports describing mucosal epithelial cells as virus target cells [25]. At that time, numerous particles had already reached the IFA where they immediately multiplied. At 72 hpi, an increasing amount of viral particles was located in the crypt epithelium, supporting virus dissemination. CSFV also actively replicated in

PALS from spleen early post-infection, confirming hypotheses drawn by others [38]. The viral genome was never detected in tonsil and spleen germinal centers, unlike observations made in porcine lymph nodes [44]. These differences were in agreement with the fact that follicles constitute the virus entry site in lymph nodes, due to the inverted structure of these organs in the pig.

Looking for viral antigen uptake by APC in the tonsil and spleen, we showed that CSFV E2 protein was located in T cell areas within CD172a⁺ myeloid cells and IFN- α ⁺ cells. Cells expressing CD172a antigen and/or IFN- α included two DC subsets we previously characterized in swine secondary lymphoid organs [23]. Further investigations would be necessary to ascertain that the viral immunogenic protein was processed in cDC and pDC, and/or to know if CSFV replicated in these cells. Uptake of viral antigen by APC is also supported by the recruitment of CD172a⁺ and IFN- α ⁺ cells in both tonsil and spleen T cell areas and by the intriguing disappearance of

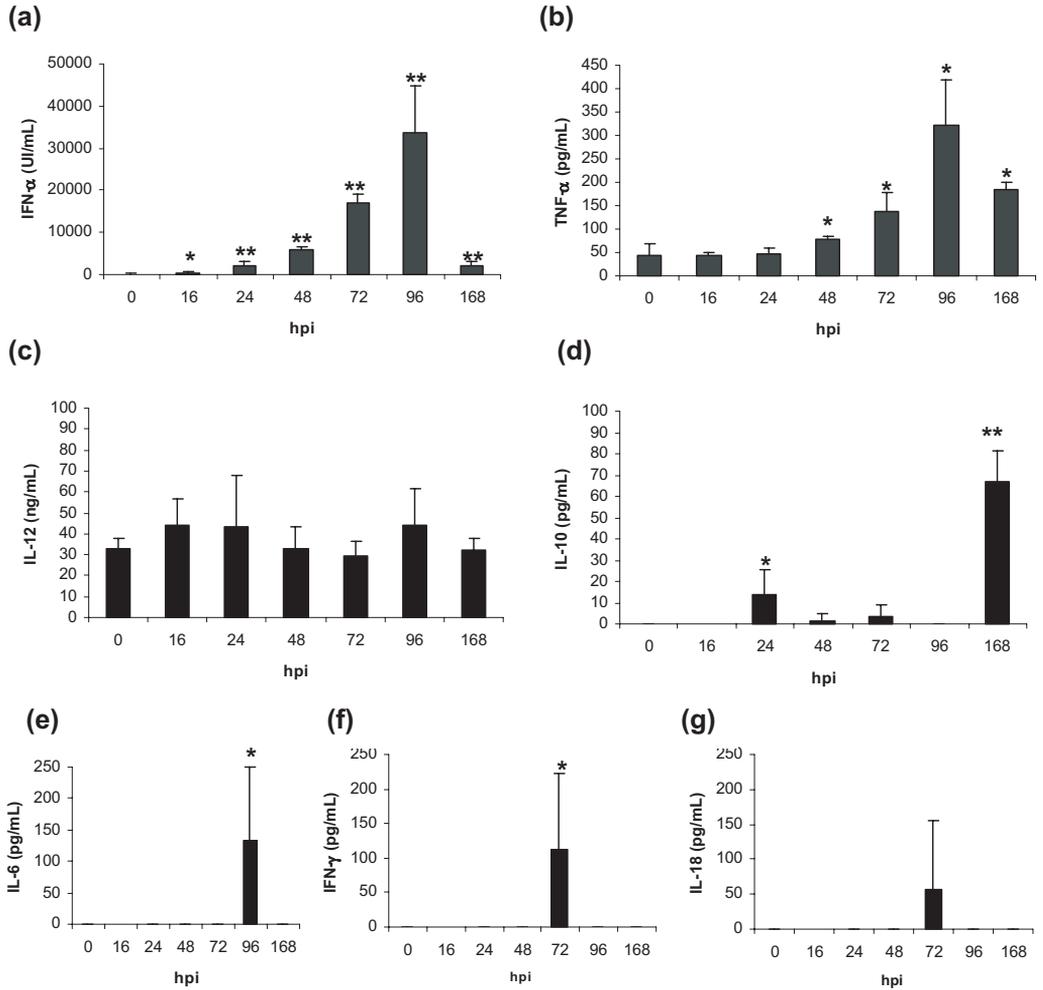


Figure 10. Cytokine levels in serum in the time course post-infection. (a) IFN- α , (b) TNF- α , (c) IL-12, (d) IL-10, (e) IL-6, (f) IFN- γ and (g) IL-18. Results are presented as mean \pm SD of samples from 6 to 7 individual animals at 0 hpi and from 3 individual animals at other hpi. * $p < 0.05$ and ** $p < 0.01$ compared with values at 0 hpi using Mann & Whitney test.

E2 from these areas at 48 hpi. Investigations on tonsil sections clearly showed the transient location of the viral antigen into follicles at that time point. Such a translocation of the viral immunogenic protein, but not of viral particles, into germinal centers could indicate that the virus was captured and degraded by APC, then transported by complement components from T-cell areas to follicles. Double labeling with anti-E2 and anti-CD21 antibodies revealed only sparse CD21⁺E2⁺ cells into

follicles at 48 hpi, and confirmed that E2 was not located at the follicle periphery but in the germinal center, within cells that were not B lymphocytes. Further investigations would be necessary to identify the cells that loaded E2, which could be follicular DC. Indeed, follicular DC are stromal cells located in germinal centers that present Ag-Ab complexes to B cells through Fc receptor or complement receptors such as CD21 [33]. Such a mechanism is supported by the fact that numerous

CD21⁺E2⁺ cells, i.e. antigen-activated B cells, migrated to IFA at 72 hpi. It is also in accordance with a recent report that revealed co-localization of B cells and IgM in spleen early after CSFV infection [39], supporting the demonstration of humoral immune response induction. Altogether, these data complete previous reports about E2 antigen processing by APC such as macrophages and B cells in the spleen [19, 38, 39].

Intending to know more about DC subset frequencies, maturation and activation status in CSFV infected pig, further analyses were performed on cell tissue suspensions. It appeared that cDC and pDC frequencies slightly and transiently decreased in tonsil between 16 and 24 hpi, while the cDC number markedly diminished in blood and spleen in the time course pi. We did not find any significant variation in blood pDC number, despite a jagged curve, but a strong transient decrease in spleen at 48 hpi. Altogether, these results revealed a rapid turn-over of pDC in the spleen, implying a migration of stimulated NIPC between blood and spleen, as previously demonstrated in mice [1] and hypothesized in pigs [43]. These data also showed that cDC, unlike pDC, disappeared permanently from blood after infection, preventing an efficient turn-over of these DC subset in the spleen. This observation was in agreement with in vitro studies showing that mo-DC functionalities were more affected than NIPC by CSFV infection [4, 13].

DC maturation after viral stimulation, demonstrated by down-regulation of CD1a expression and up-regulation of CD80/86 expression [12, 15, 31], was observed in both DC types. Less cDC expressed CD1a in tonsil and blood early after inoculation, and cDC maturation was clearly evidenced in blood through CD80/86 up-regulation. pDC matured in all tissues, with clear opposite CD1a and CD80/86 expression kinetics that also reflected, with two-stage variations, the turn-over we described above about pDC circulation between blood and spleen.

cDC and pDC activation was demonstrated through cytokine expression. Panels of various cytokines significantly increased in both

subsets in the tonsil, blood and spleen post-inoculation. Generally, the frequency of cDC expressing TNF- α markedly increased in the tonsil and spleen, and to a lesser extent in blood. Some cDC also started to express IFN- α after infection, especially in the tonsil. They increased their IL-12 expression in blood, then in the spleen. The number of cDC expressing IL-10 was remarkably high in the spleen. These results differed from previous in vitro studies that indicated that CSFV-infected mo-DC do not express any cytokine [13]. This discrepancy is discussed below. It has to be noticed that expression of IFN- α by porcine cDC was never demonstrated before but is in line with the constitutive expression of IFN- α by other porcine DC of myeloid origin, the Langerhans cells [6]. IFN- α expression by CD172a⁺CD11b⁺CD4⁻ cDC was also demonstrated in bovine lymph nodes after calf infection with bovine viral diarrhoea virus, another pestivirus [10].

Moreover, increasing percentages of pDC expressed IFN- α , TNF- α and IL-12 in all studied tissues after infection. pDC preferentially expressed IFN- α in the tonsil, TNF- α in blood and IL-12 in blood and spleen, respectively. pDC also increased their IL-10 expression, but only in the spleen. In blood, jagged variations in frequencies of pDC expressing cytokines illustrated the pDC turn-over presented above. These results were in agreement with cytokine production by in vitro CSFV-infected NIPC [4, 42]. Moreover, porcine blood-isolated NIPC secreted IFN- α , TNF- α and IL-12, but not IL-10, after CpG-oligonucleotide stimulation [20].

The correlation between increasing frequencies of DC expressing cytokines and cytokine production levels in serum was interesting. As a consequence of the huge amounts of DC expressing IFN- α and TNF- α in secondary lymphoid organs and blood after infection, IFN- α and TNF- α productions in serum were uncommonly high, in accordance with previous reports [36, 43]. By contrast, IL-12 level remained constant in serum after infection, probably because of the high IFN- α concentration known to inhibit IL-12 production [2]. IL-6 was also transiently detected at 96 hpi.

It proved impairment in this proinflammatory cytokine induction, known to be a better marker of bacterial infection in pigs [18].

As previously noticed, the discrepancy between our data that revealed maturation and activation of cDC, and results from previous *in vitro* studies indicating that CSFV-infected mo-DC did neither mature nor express any cytokine [13] was intriguing, whereas these *in vitro*-derived cells were able to produce TNF- α or IFN- α after mRNA transfection [15]. Other *in vitro* investigations showed that CSFV also disrupted secretion of IFN- α and proinflammatory cytokines in porcine aortic and kidney endothelial cell lines [7, 11]. It is to emphasize that CSFV-infected mo-DC were able to mature only when TNF- α and IFN- α were added into culture medium [13]. A recent *in vitro* study showed that TNF- α and IFN- α produced by porcine NIPC were closely involved in inducing maturation and activation of cDC [21]. Altogether, these data support our findings and lead to think about a close cooperation between DC subsets after CSFV infection. Thus, IFN- α and TNF- α expressions by pDC would be involved in induction of innate antiviral responses as well as in cDC stimulation. Subsequently, mature and activated DC subsets would then be involved in induction of adaptive immune responses, illustrated by punctual IFN- γ and IL-18 secretions in serum. This was in agreement with CTL stimulation by CSFV-infected mature mo-DC [14] and with the initiation of cellular immune responses described in pigs surviving infection [35]. Additionally, the fact that high proportions of both DC subsets expressed IL-10 in the spleen could reflect the starting of humoral immune response, in accordance with the detection of secreting IgM E2⁺ B cells the first days post-infection [39].

However, regarding their production of large amounts of TNF- α and IFN- α , DC could be involved in induction of the lymphocyte apoptosis described in the thymus, spleen, lymph nodes and blood [41, 44]. Indeed, TNF- α and IFN- α can have adverse effects when produced in huge amounts after viral infection [32]. Thus, it was suggested that TNF- α secreted by CD172a⁺ cells, together

with IL-1, IL-6 and prostaglandin, were responsible for apoptosis in non-infected immune system cells in lymphoid organs [16, 24, 38]. These authors implicated macrophages as TNF- α producing cells, but our data showed that DC could also play a role in this phenomenon. Similarly, the high IFN- α level in serum was recently correlated to leucopenia [43]. As described in other viral infections, IFN- α and TNF- α expressions by pDC could have an inhibitory effect in IL-12 expression by cDC becoming unable to induce efficient cellular immune responses [2]. IL-10 expression in cDC was also shown to stimulate the inhibition of both DC subset functions [17, 46].

In conclusion, CSFV infection induced DC recruitment and activation in secondary lymphoid organ T-cell areas during the first hours post-infection, leading to the initiation of innate immune responses, via IFN- α and TNF- α productions. IL-10 and IL-12 expressed by splenic DC participated in the induction of the known adaptive immune responses to CSFV [37, 38, 39]. However, the uncommonly high levels of cytokines could also play a role in disrupting immune system cells, either inducing apoptosis and/or impairing DC maturation and T cell priming.

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