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Interaction of classical swine fever virus with dendritic cells

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Functional disruption of dendritic cells (DCs) is an important strategy for viral pathogens to evade host defences. Monocytotropic viruses such as classical swine fever virus (CSFV) could employ such a mechanism, since the virus can suppress immune responses and induce apoptosis without infecting lymphocytes. Here, CSFV was shown to infect and efficiently replicate in monocyte- and in bone marrow-derived DCs. Interestingly, the infected DCs displayed neither modulated MHC nor CD80/86 expression. Stimulation of DCs with IFN-α/TNF-α or polyinosinic–polycytidylic acid (pIC) induced phenotypic maturation with increased MHC and CD80/86 expression, both with mock-treated and infected DCs. In addition, the T cell stimulatory capacity of CSFV-infected DCs was maintained both in a polyclonal T cell stimulation and in specific antigen-presentation assays, requiring antigen uptake and processing. Interestingly, similar to macrophages, CSFV did not induce IFN-α responses in these DCs and even suppressed pIC-induced IFN-α induction. Other cytokines including interleukin (IL)-6, IL-10, IL-12 and TNF-α were not modulated. Taken together, these results demonstrated that CSFV can replicate in DCs and control IFN type I responses, without interfering with the immune reactivity. These results are interesting considering that DC infection with RNA viruses usually results in DC activation.

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease of pigs caused by CSF virus (CSFV) and leads to important economic losses worldwide. CSFV together with bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) form the genus *Pestivirus* within the family *Flaviviridae*.

CSFV is a monocytotropic viral pathogen, which can efficiently evade and compromise the host's immune system. The virus has a high affinity for reticulo-endothelial cells (Cheville & Mengeling, 1969; Ressang, 1973; Susa *et al.*, 1992) causing lymphopenia, thrombocytopenia, coagulation disorders and atrophy of the thymus and bone marrow (Gomez-Villamandos *et al.*, 2003; Pauly *et al.*, 1998; Sanchez-Cordon *et al.*, 2002; Summerfield *et al.*, 2000, 2001). Lymphopenia is caused, at least in part, by apoptosis detectable in uninfected lymphocytes (Sanchez-Cordon *et al.*, 2003; Summerfield *et al.*, 1998b). In addition, viable lymphocytes isolated from CSFV-infected pigs do not respond to mitogen stimulation (Pauly *et al.*, 1998; Summerfield *et al.*, 1998b; Van Oirschot *et al.*, 1983). These modulated cells are not infected. Instead, it is the myeloid population, particularly monocytes (Mo) and macrophages (Mϕ), that contains the early target cell for infection and replication, both *in vivo* (Ressang, 1973; Gomez-Villamandos *et al.*, 2001; Sanchez-Cordon *et al.*, 2003; Summerfield *et al.*, 2000; Trautwein, 1988) and *in vitro* (Knoetig *et al.*, 1999). Despite this clear targeting and tropism, no direct evidence has been found of a role for infected Mo and Mϕ in the observed immunosuppression and death of T lymphocytes (Knoetig *et al.*, 1999).

Dendritic cells (DCs) are one of the primary immunological sentinels of the immune system (Banchereau *et al.*, 2000; Steinman, 1991). They can efficiently sense invading pathogens by a set of pattern recognition receptors. Their strategic localization at the site of pathogen entry – mucosal surfaces and dermal layers – makes them a particularly early target for virus contact (MacPherson & Liu, 1999). After detection, uptake and degradative processing of the virus, DCs should mature and subsequently promote effective immune responses by migrating into lymphoid tissue to present the processed viral antigens to T lymphocytes (Pulendran *et al.*, 2001).

Interestingly, certain viruses are tropic for DCs. Human immunodeficiency virus, measles virus (MV),

cytomegalovirus and herpes simplex virus type 1 infect, survive and even replicate in these potent antigen-presenting cells, inducing immunosuppression, mediated in part through the infected DCs (Klagge & Schneider-Schaulies, 1999). A problem for the virus could arise if the infection results in activation of receptors for double-stranded (ds)RNA such as toll-like receptor 3 and protein kinase R, which would activate and mature the DCs. Such an observation has been made particularly with certain DC-tropic RNA viruses including MV (Fugier-Vivier *et al.*, 1997), influenza virus (Cella *et al.*, 1999), Semliki Forest virus (Johnston *et al.*, 1996), dengue virus (Ho *et al.*, 2001) and Sindbis virus adapted to human DCs (Gardner *et al.*, 2000). As a consequence, type I IFN production is induced and the virus-infected DCs mature to acquire the capacity to induce potent MHC class I- and II-restricted antiviral immune responses. In the context of CSFV, the results with dengue virus, also a member of the *Flaviviridae*, are interesting, yet nothing is known about the interaction of CSFV with DCs.

Considering these fundamental elements in virus–host interactions, particularly of monocytotropic viruses, the objective of the present study was to determine the affinity of CSFV for DCs and the functional consequences of that infection.

METHODS

Media and reagents. DCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 2 mM glutamine, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 50 μM 2-ME (all from Invitrogen). Serum supplements for DC cultures employed 10 % (v/v) porcine serum (Sigma Chemicals). Recombinant porcine (rp) cytokines utilized for DC generation were as follows: rpGM-CSF (kindly provided by Dr S. Inumaru, Institute for Animal Health, Ibaraki, Japan; Inumaru *et al.*, 1998) and rpTNF-α (kindly provided by Dr G. Bertoni, Institute of Veterinary Virology, Berne, Switzerland; Von Niederhausern *et al.*, 1993). Recombinant porcine IL-4 was prepared in our laboratory, as described previously (Carrasco *et al.*, 2001). MHC class I was detected with mAb 74-11-10 (Pescovitz *et al.*, 1984) (kindly donated by Dr J. K. Lunney, USDA, Beltsville, USA) and MHC class II with mAb MSA3 (Hammerberg & Schurig, 1986) (from VMRD, Pullman, WA, USA). CD80/86 expression was measured using a huCTLA4–mouse Ig fusion protein (Alexis, Lausen, Switzerland).

Generation of porcine DCs. Bone marrow (BM)-derived and blood Mo-derived DCs were generated as described previously (Carrasco *et al.*, 2001). Briefly, low-density bone marrow haematopoietic cells (BMHCs) and PBMCs were obtained by density-gradient centrifugation over Ficoll-Paque (1·077 $g\,l^{-1}$; Amersham Pharmacia Biotech). BM-DCs were generated from BMHCs using rpGM-CSF (25 ng ml^{-1}) and rpTNF-α (30 U ml^{-1}), cultured for 8 days at 39 °C. At days 3 and 6, the BM-DC culture was fed with the cytokines. Mo-DCs were generated from blood Mo isolated by plastic adherence, using rpGM-CSF (150 ng ml^{-1}) and rpIL-4 (100 U ml^{-1}), cultured for 7 days at 39 °C. At days 2, 4 and 6, the Mo-DC culture was fed with the cytokines. Maturation of DCs was induced by treatment with a cocktail of recombinant porcine IFN-α (1000 U ml^{-1}; R&D Systems) and TNF-α (10 ng ml^{-1}; Endogen) for 48 h. Alternatively, maturation was induced with polyinosinic–polycytidylic acid (pIC; Sigma) transfection to mimic a virus infection. DCs were washed twice with serum-free DMEM at 37 °C and resuspended at a concentration of 4×10^6 cells ml^{-1} in 500 μl Opti-MEM (Invitrogen). pIC was first incubated for 30 min with 2 μl of a single-stranded RNA-specific RNase cocktail composed of 500 U RNase A ml^{-1} and 20 000 U RNase T1 ml^{-1} (both from Ambion) in 100 μl at 37 °C. This digestion improved the stimulatory activity of pIC for DCs, whereas treatment with dsRNA-specific RNase abolished the capacity of pIC to induce DC maturation (M. Ceppi, unpublished results). This mixture was allowed to form complexes with 24 μl TransFast (Promega) in 500 μl Opti-MEM for 15 min at room temperature, before adding to the DCs and incubating for 1 h at 39 °C. After lipofection, cells were washed twice with serum-free DMEM at 37 °C before use in T cell stimulation assays.

CSFV infection. The virulent Brescia strain (Summerfield *et al.*, 1998b) was used if not otherwise indicated. For certain other experiments, the virulent Eystrup strain (Mayer *et al.*, 2003), the moderately virulent Alfort/187 strain (Ruggli *et al.*, 1996) and the avirulent C-strain (vflc2 corresponding to Flc 133 described by Moormann *et al.*, 1996) were employed. These viruses and mock controls were prepared in swine kidney SK-6 cells as described (Knoetig *et al.*, 1999). In some experiments, UV-inactivated virus and preparations (Knoetig *et al.*, 1999) were used as additional controls. DCs (BMHCs or Mo) were mock-treated or infected with CSFV at the m.o.i.s specified in the results. The presence of viral antigen in the DCs was quantified by immunofluorescent analysis using microscopic or flow cytometric detection of the viral E2 structural glycoprotein with mAb HC/TC26 (kindly provided by Dr Bommeli, Diagnostics AG, Berne, Switzerland; Greiser-Wilke *et al.*, 1990). The viral non-structural NS3 protein was detected with mAb C16 (kindly provided by Dr I. Greiser-Wilke, Hannover Veterinary School, Germany; Greiser-Wilke *et al.*, 1992). Due to the internal expression of E2 and NS3, the DCs were fixed and permeabilized (Cell Permeabilization Kit; Harlan Sera-Lab) before labelling with the mAbs.

Analysis of CSFV replication in BM- and Mo-DCs was assessed by incubating the non-adherent DCs with CSFV at an m.o.i. of 10 $TCID_{50}$ per cell for 1 h at 39 °C. The cells were then washed a total of ten times by centrifugation. Cell-associated virus (CAV) from these DCs was obtained by two cycles of freeze–thawing of the cell pellets, which were then clarified by centrifugation at 10 000 ***g*** for 20 min and resuspended to the original culture volume. The measurement of virus titres from extracellular virus (ECV) and CAV were quantified by end-point titration on PK-15 cells and immunofluorescent detection.

DC-dependent T lymphocyte stimulation assays. T cell purification for all assays (SEB-, FMDV- and CSFV-dependent) used MACS (magnetic activated cell sorting), wherein either $CD6^+$ T cell enrichment or $SWC3^+$ Mo depletion was used. T cells were cultured at 2×10^5 cells per well. The microbial superantigen staphylococcal enterotoxin B (SEB) (Toxin Technology) was used to measure the potency of DCs at inducing T cell responses dependent on MHC class II–T cell receptor cross-linking (Bhardwaj *et al.*, 1992). BM-DCs were incubated simultaneously with mitomycin C (10 μg ml^{-1}) and SEB (100 ng ml^{-1}) for 1 h at 39 °C, washed four times and titrated in a 96-well flat bottom microtitre plate as described. After 2 days, 1 μCi (37 kBq) [^{3}H]thymidine was added for another 18 h to quantify proliferation.

The processing and presentation of antigen was assessed in two antigen-specific assays. For the CSFV-specific antigen-presentation assays, PBMCs for both Mo-DC generation and lymphocyte isolation were obtained from pigs 4–8 months after infection with CSFV Alfort187 (Ruggli *et al.*, 1996). CSFV was added to the DC/lymphocyte co-cultures at an m.o.i. of 0·01 $TCID_{50}$ per cell. The foot-and-mouth disease virus (FMDV)-specific restimulation assay employed T cells and Mo-DCs isolated from pigs vaccinated against FMDV (three

booster injections, serotype C_1 Oberbayern; vaccine kindly provided by Dr P. Barnett, Institute for Animal Health, Pirbright, UK). The DCs and T cells were co-cultured in the presence of β-propiolactone-inactivated FMDV antigen as described previously (Carrasco *et al.*, 2001). After 4 days for the FMDV and 5 days for the CSFV restimulation, 1 μCi [^{3}H]thymidine was added for another 18 h to quantify proliferation. T cell activation was only observed with immune animals.

Cell viability and apoptosis analysis. For quantification of apoptotic cells expressing phosphatidylserine on their surface and dead cells permeable to propidium iodide (PI; Sigma), dual parameter analysis of AnnexinV-FITC (Bender Med Systems) and PI were performed (Vermes *et al.*, 1995). To this end, 5×10^5 cells were labelled with 2 μg AnnexinV-FITC ml^{-1} in 140 mM NaCl, 2·5 mM $CaCl_2$, 10 mM HEPES (pH 7·4) buffer for 10 min. After FL1/FL2 compensation, PI (100 ng ml^{-1}) was added, to discriminate between apoptotic and dead cells and the sample analysed by flow cytometry.

Cytokine responses. Cytokines produced by DCs were induced with either pIC or lipopolysaccharide (LPS, *Escherichia coli*-derived; Sigma). The concentration of secreted IFN type I in the supernatants was quantified using a bioassay based on the antiviral effect of IFN type I against vesicular stomatitis virus (VSV). Reduction of VSV-induced cytopathic effect in PK-15 cells induced by the test samples or by recombinant porcine IFN-α (R&D Systems) as a standard was quantified as described previously (Ruggli *et al.*, 2003). The presence of IFN-α was controlled by addition of a neutralizing anti-porcine IFN-α polyclonal antiserum (R&D Systems).

Other cytokines produced by DCs were measured using commercially available ELISAs designed for porcine IL-6, IL-10, IFN-γ (Biosource) and TNF-α (Perbio Science).

For quantification of IL-10 and IL-12 mRNA levels, RNA was isolated from DCs using the RNeasy Kit (Qiagen). This RNA was reverse-transcribed to cDNA using the Omniscript RT Kit (Qiagen). The cDNA levels of cyclophilin (housekeeping gene), IL-10 and IL-12 (subunits p35 and p40) were quantified on the Icycler IQ Multicolour Real-Time PCR Detection System (Bio-Rad) using the Lightcycler Faststart DNA Master SYBR Green kit (Roche Molecular Diagnostics). Duplicate samples as well as serial dilutions of each target amplicon were analysed using the following optimized protocol. A 10 min incubation at 95 °C was employed to activate the polymerase and denature the targets. This was followed by 50 cycles of 30 s at 95 °C, 30 s at 55 °C (cyclophilin), 59 °C (IL-10, IL-12p40) or 61 °C (IL-12p35), and 30 s at 72 °C. After a final extension for 10 min at 72 °C, the programme was ended with a melt curve programme. All reactions were performed in 20 μl (1 μl sample added), the $MgCl_2$ concentration was 3 mM in all cases and the primer concentrations were 0·25 μM. The following forward and reverse primers, respectively, were used for PCR: cyclophilin: 5′-TAACCCCACCGTCTTCTT-3′ and 5′-TGCCATCCAACCACTCAG-3′; IL-10: 5′-GCATCCACTTCCCAACCA-3′ and 5′-CTTCCTCATCTTCATCGTCAT-3′; IL-12p35: 5′-GCCTCAACCACTCCCAAAATC-3′ and 5′-CTTCCAGGGAGGGCTTCTGT-3′; and IL-12p40: 5′-GCAAAACCCTGACCATCCAC-3′ and 5′-TGCCCTCCTGACACTCCACT-3′. Serial dilutions were used to generate standard curves from which the concentration of each sample was deduced. All cytokine data were normalized to the expression of cyclophilin.

RESULTS

CSFV infection of DCs

It is known that CSFV has an affinity for myeloid cells (Knoetig *et al.*, 1999; Ressang, 1973; Summerfield *et al.*, 1998a, b, 2000, 2001). However, there is no information on the susceptibility of DCs to CSFV infection. Considering the central role that DCs play in the stimulation and control of immune defence development, this is a subject of particular importance. As a cell culture model, Mo- and BM-DCs, both representing myeloid DCs, were used. Similar to other species, these cells are characterized as DCs based on their dendritic morphology, non-adherence, their potent T cell stimulatory capacity and their expression of CD1, CD80/86 and MHC class II (Carrasco *et al.*, 2001). Consequently, BM- or Mo-DCs were infected with CSFV at an m.o.i. of 1 $TCID_{50}$ per cell. At 48 h post-infection, the majority of the infected BM- and Mo-DCs were seen to express viral E2 and NS3 proteins (Fig. 1). The detection method for these viral proteins did not permit the detection of virus entry, but only *de novo* protein synthesis (Knoetig *et al.*, 1999). These results suggested a productive infection of the DCs, which was confirmed by the observation of a 3–4 $\log_{10}$-fold increase in the virus titres of ECV and CAV between 24 and 72 h post-infection (Fig. 2). Interestingly, the CAV titres were invariably lower than the ECV titres, contrasting with infected SK-6 cells, where the majority of *de novo*-synthesized virus was cell-associated (data not shown). Similar elevated ECV titres compared with CAV titres have been observed with CSFV-infected Mo (S. M. Knoetig and A. Summerfield, unpublished data).

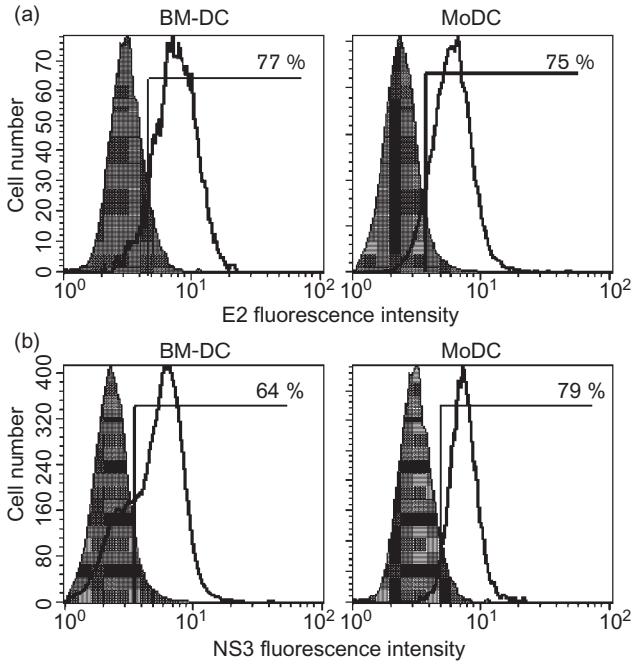


Fig. 1. Viral protein expression in BM-DCs and Mo-DCs at 48 h after CSFV infection at an m.o.i. of 1 $TCID_{50}$ per cell. (a) Structural glycoprotein E2 expression. (b) Non-structural protein NS3 expression. The filled histograms represent the staining of mock-treated cells used to determine the non-specific staining. Data shown in (a) and (b) are representative of 11 independent experiments.

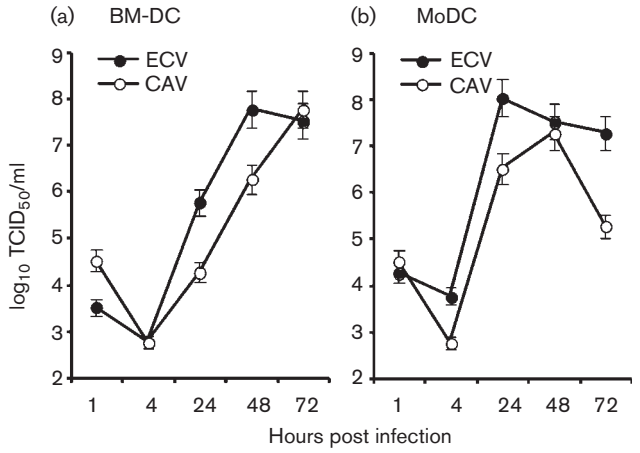


Fig. 2. Replication kinetics of CSFV in BM-DCs (a) and Mo-DCs (b), infected at an m.o.i. of 10 $TCID_{50}$ per cell for 1 h at 39 °C. At the indicated time points, the culture supernatant medium was harvested for ECV and the cells for CAV, as described in Methods. Data shown in (a) and (b) are representative of three independent experiments.

DC morphology and maturation after CSFV infection

No morphological difference between CSFV-infected and mock-treated DCs was noted (data not shown). This correlated with the observation that CSFV is non-cytopathic for Mo and Mϕ (Knoetig *et al.*, 1999). In addition, CSFV infection of DCs had no apparent influence on their 'maturation status' with respect to the expression of MHC class I, II and CD80/86 (Fig. 3). CD80/86 was already expressed without addition of maturation signals, but could be induced to be further upregulated by *in vitro* stimulation. This is characteristic of porcine Mo-DCs (Carrasco *et al.*, 2001) and also porcine blood DCs (Summerfield *et al.*, 2003). Consequently, it was investigated whether CSFV-infected DCs were altered in their response to maturation signals. When CSFV-infected or mock-treated DCs were stimulated either with a TNF-α/IFN-α cocktail or with pIC, upregulation of molecules involved in antigen presentation, typical of DC maturation, was observed in both mock-treated and CSFV-infected DCs (Fig. 3 shows the results for the IFN-α/TNF-α-induced maturation). The observation that CSFV did not influence the expression of maturation markers such as CD80/86 was also observed with other strains of CSFV, including the avirulent C-strain, the moderately virulent Alfort strain and the virulent Eystrup and Brescia strains. In addition, no modulation was observed using UV-inactivated virus as an additional control (Fig. 3b).

Functional activity of DCs infected with CSFV

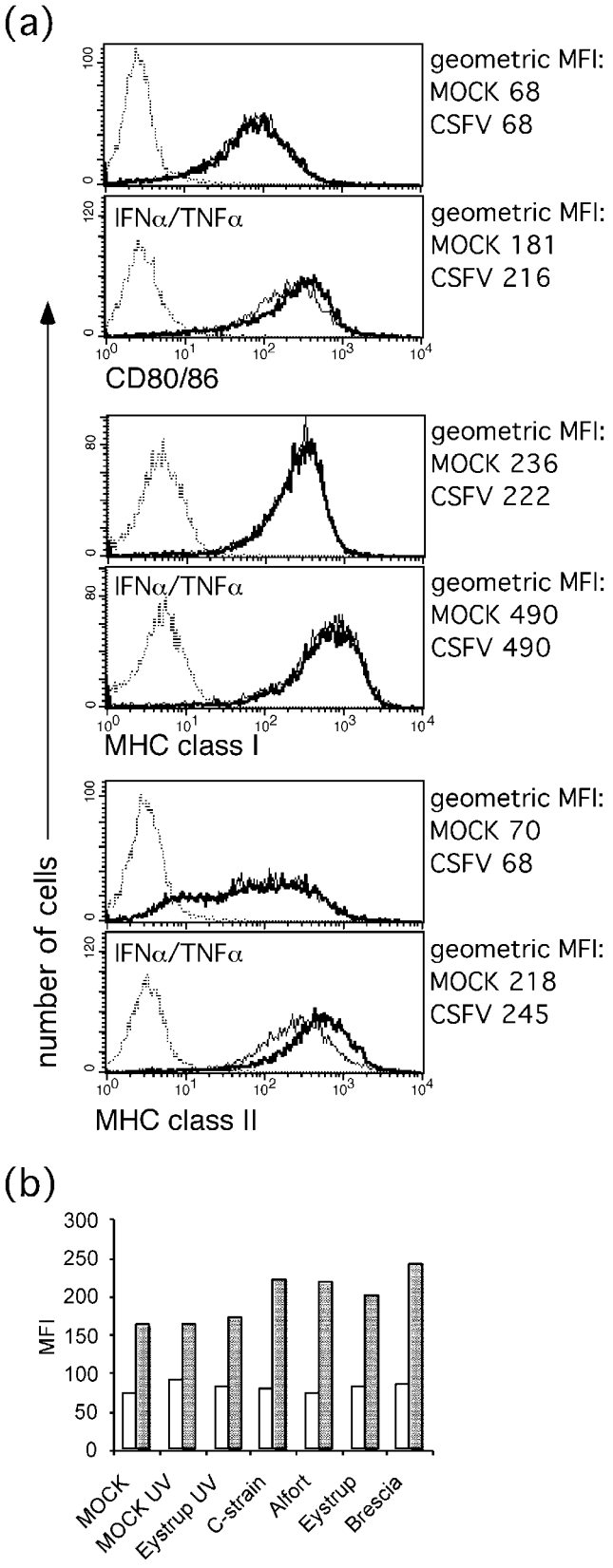
Having observed efficient CSFV replication in DCs without detectable alteration in cell morphology or consistent modulation of MHC and CD80/86 expression, the influence of infection on the functional activity of the DCs was assessed. To this end, DCs were pulsed either with the microbial superantigen SEB or with viral antigens, and their capacity to stimulate proliferative T cell responses was quantified. Although CSFV-infected DCs were seen to be more stimulatory for SEB-induced T cell proliferation compared with mock-treated DCs (Fig. 4), the difference was not always reproducible. A statistical analysis (Student's *t*-test) of 11 SEB-dependent proliferation assays demonstrated no significant differences between mock-treated and CSFV-infected DCs in this functional test ($P=0{\cdot}2$).

When the same experiments were performed with IFN-α/TNF-α-stimulated infected or mock-treated DCs, it was often observed that the CSFV-infected cells promoted a higher lymphoproliferation compared with the mock controls (Fig. 4a). Nevertheless, this observation was also not seen in all experiments and a statistical analysis of the results indicated no significant difference ($P=0{\cdot}23$). When the DCs were stimulated with lipofected pIC as the maturation signal, both the mock-treated and the CSFV-infected DCs upregulated their capacity to stimulate T cell proliferation to a similar degree (Fig. 4b).

To investigate further any possible functional modulation of CSFV-infected DCs, the influence of virus infection on an MHC-restricted antigen-specific T cell response, which requires antigen uptake, processing and presentation, was tested. CSFV-infected DCs were able to stimulate T cell proliferation in an FMDV-specific antigen-presentation assay and this was of a similar intensity to that obtained with mock-treated Mo-DCs (Fig. 5a). Another important observation was that IFN-γ protein levels were not altered when supernatants from T lymphocytes activated by mock-treated or CSFV-infected DCs presenting FMDV antigen were analysed (Fig. 5b). This indicated that CSFV did not shift the T cell response towards a Th1 or a Th2 pattern. Finally, CSFV-infected DCs could also restimulate CSFV-specific T cell proliferation (Fig. 5c). Taken together, these results demonstrated that CSFV-infected DCs were certainly functionally intact with respect to their capacity to induce T cell activation.

Cell viability following CSFV infection

In the acute phase of severe CSF, death of uninfected lymphocytes can be detected in the animals. Consequently, even though CSFV was not apparently cytopathic for DCs in terms of morphological changes, it was necessary to determine whether an augmented rate of cell death was present in the infected DC/lymphocyte co-cultures. Using lymphocyte co-cultures with DCs at a ratio of 10 : 1, viability analysis at 24, 48 and 72 h showed no significant difference in the number of AnnexinV-positive and PI-positive cells between mock controls and CSFV-infected cultures (data not shown).



Cytokine responses

In a final series of analyses, the capacity of CSFV to modulate the cytokine response of virus-infected DCs was characterized. Similar to infection of Mo and Mϕ (Knoetig *et al.*, 1999; Ruggli *et al.*, 2003), CSFV did not induce type I IFN responses in Mo-DCs. On the contrary, CSFV-infected DCs had a defective type I IFN response to pIC stimulation (Table 1). As for other cytokines, the protein levels of IL-6, IL-10 and TNF-α measured by ELISA, as well as the mRNA levels of IL-10 and IL-12 (p35 and p40), were not modulated by CSFV infection (Table 1). No modulation of spontaneously induced TNF-α, IL-6 or IL-10 protein was detectable. The same was found for IL-10 and IL-12 p40 mRNA levels (Table 1). IL-12 p35 mRNA was not detectable in mock- or in CSFV-infected DCs (data not shown). Furthermore, the induction of TNF-α by LPS and of IL-6 by LPS and pIC were not modified by CSFV (Table 1).

DISCUSSION

CSFV is a haemorrhagic and immunosuppressive viral pathogen that has been seen in histological sections associated with dendritic-like cells (Susa *et al.*, 1992). Non-infected T lymphocytes from animals with CSF do not respond to antigen or mitogen stimulation and rapidly undergo apoptosis *in vivo* and after *ex vivo* culture. Yet, CSFV is well documented as being non-cytopathogenic. Furthermore, *in vitro* infection of Mo or Mϕ with this monocytotropic virus did not induce detectable lymphocyte immunosuppression nor was apoptosis discernible (Knoetig *et al.*, 1999). This would indicate that infected Mϕ are not directly responsible for the reported lymphocyte death and anergy in CSF (Pauly *et al.*, 1998; Van Oirschot *et al.*, 1983). Consequently, the present work sought to determine whether DCs, the major myeloid cell type interacting with lymphocytes, are involved in CSFV immunopathogenesis.

CSFV was seen to be highly efficient at infecting and productively replicating in DCs, both Mo-DCs and BM-DCs. These observations that CSFV is tropic for DCs indicated that these critically important promoters and

Fig. 3. CD80/86, MHC class I and MHC class II expression on Mo-DCs after infection with CSFV. (a) The cells were mock-treated (light-line histograms) or infected with CSFV (dark-line histograms) at an m.o.i. of 1 $TCID_{50}$ per cell and cultured for 48 h. DC maturation was induced with IFN-α/TNF-α for another 48 h in some cultures as indicated. The dotted-line histograms represent the conjugate controls. For each histogram plot, the geometric mean fluorescence intensity (MFI) for the mock controls and the virus-infected DCs is indicated. The data shown are representative of five independent experiments. (b) Influence of various CSFV strains and UV-inactivated virus on CD80/86 expression. The geometric means determined as described in (a) for unstimulated DCs (white bars) and for IFN-α/TNF-α-stimulated DCs (grey bars) are shown. Experimental conditions were as described in (a).

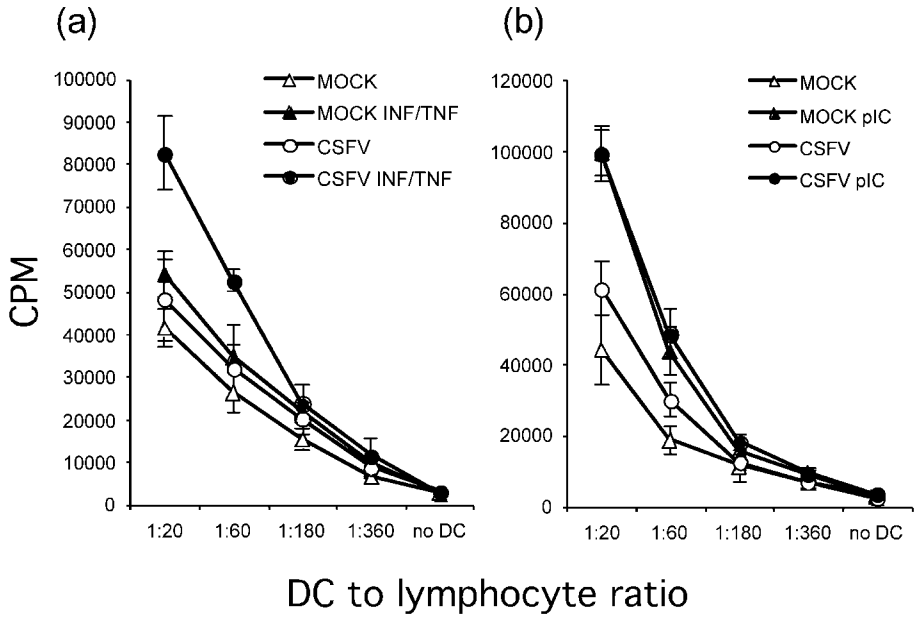


Fig. 4. Antigen-presentation capacity of CSFV-infected DCs, influenced by maturation signals. Mo-DCs were mock-treated (▲, △) or CSFV-infected (m.o.i. of 1 $TCID_{50}$ per cell; ●, ○) for 48 h. Some cultures were further stimulated (filled symbols) with either IFN-α/TNF-α for 48 h (a) or pIC (transfection) for 24 h (b). The capacity of the Mo-DCs to present cell-bound SEB to T lymphocytes and to induce T cell proliferation at different DC : lymphocyte ratios is shown. Data shown are representative of 11 experiments, displaying the mean ± SD of triplicate values.

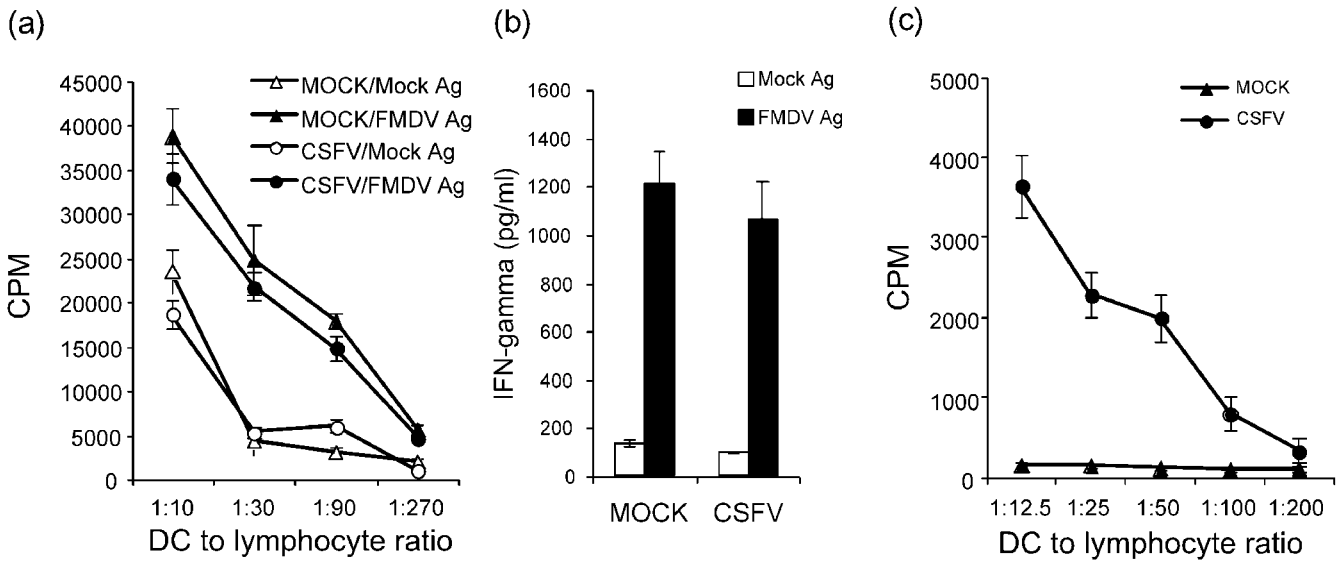


Fig. 5. Capacity of CSFV-infected DCs to process and present antigen. Mo-DCs were exposed to mock (▲, △) or CSFV (m.o.i. of 1 $TCID_{50}$ per cell; ●, ○) for 48 h. For (a) and (b), these DCs were then incubated with FMDV antigen (filled symbols) or mock BHK-cell antigen (open symbols) for another 24 h, before co-culturing with autologous T lymphocytes from an FMDV-immune animal. The T cell responses were measured in a proliferation test (a) and by quantification of IFN-γ protein in supernatants after 48 h co-culture (b). The data shown are representative of four (a) or two (b) independent experiments. (c) The CSFV-infected DCs (●) or mock-treated controls (▲) were co-cultured with autologous T lymphocytes from a CSFV-immune animal. The antigen specificity of these responses was controlled by performing the same experiments with non-immune animals (data not shown). DCs were titrated to obtain different DC : T lymphocyte ratios and the mean ± SD of triplicate c.p.m. values are displayed.

Table 1. Cytokine responses of CSFV-infected DCs

	IFN I (U ml^{-1})	TNF-α (pg ml^{-1})	IL-6 (pg ml^{-1})	IL-10		IL-12 p40
				pg ml^{-1}	mRNA*	mRNA*
Mock	−	11 ± 1	166 ± 4	24 ± 1·2	0·5 ± 0·1	0·1 ± 0·01
CSFV	−	19 ± 2	220 ± 5	43 ± 2·3	0·9 ± 0·3	0·2 ± 0·01
Mock LPS	−	431 ± 23	2365 ± 59	ND	ND	ND
CSFV LPS	−	480 ± 13	1720 ± 31	ND	ND	ND
Mock pIC	14 ± 2	ND	2373 ± 57	ND	ND	ND
CSFV pIC	0·5 ± 0·2	ND	2237 ± 43	ND	ND	ND

*mRNA molecules, cytokine/cyclophilin.
−, Not detectable; ND, not determined.

controllers of immune response development would serve as a main target cell for CSFV replication. Considering the non-cytopathogenic nature of CSFV and the migratory characteristics of DCs, infected DCs would present a reservoir for the efficient dissemination of the virus. The latter aspect is particularly important due to the trafficking of DCs from peripheral sites, such as the mucosal surfaces at which CSFV would invade the host, to the secondary lymphoid tissue where close contact to lymphocytes could have detrimental consequences.

Despite this tropism of CSFV for DCs, there was no clear morphological, phenotypic or functional modulation. This contrasts with dengue virus, another flavivirus, which induced DC maturation (Ho *et al.*, 2001; Libraty *et al.*, 2001; Wu *et al.*, 2000). It has been reported that DCs can be activated through dsRNA-dependent stimulation (Cella *et al.*, 1999). Considering the fact that many RNA viruses forming dsRNA intermediates during their replicative cycle activate DCs (for example, see Introduction), it would appear that CSFV has developed mechanisms to control or limit DC activation. Both the non-cytopathogenic and the cytopathogenic biotypes of another pestivirus, BVDV, do not activate DCs (Glew *et al.*, 2003), suggesting that such common means for evading immune recognition may be a characteristic of all pestiviruses. This would relate to their capacity to interfere with pIC-induced IFN responses in Mϕ (Ruggli *et al.*, 2003; Schweizer & Peterhans, 2001). Indeed, CSFV impairs pIC-induction of type I IFN in DCs and the recent report of Glew *et al.* (2003) has shown a similar effect of BVDV in bovine DCs. However, the current analyses demonstrating that the response of CSFV-infected DCs to maturation signals for DCs – including pIC – was not impaired contrasted with this effect on type I IFN inducibility. It would therefore appear that the DC maturation induced by pIC was IFN-independent. In fact, the independence of virus-induced DC maturation from secreted IFN-α has been reported recently (Lopez *et al.*, 2003).

Considering this inability of CSFV to interfere with DC maturation, the question arose concerning the functionality of the DCs as antigen-presenting cells. Using antigen-presentation assays for polyclonal SEB stimulation, as well as FMDV- and CSFV-specific restimulation assays, it was observed that CSFV did not impair the capacity of DCs to process and present antigens. The recent report of Glew *et al.* (2003) has also shown that BVDV-infected bovine DCs retained their capacity to function as antigen-presenting cells. With the CSFV infections in the present report, it was particularly interesting to note that the infected DCs could still present CSFV antigen and stimulate CSFV-specific lymphocyte responses. Furthermore, apart from suppressed IFN type I responses, the cytokine profile of the DCs was not modified. For the interpretation of these results and particularly the cytokine responses, it is important to note that *in vitro*-derived DCs represent a cell culture model for myeloid DCs, which does not reflect the complexity of the DC system. Consequently, further studies investigating the interaction of CSFV with, for instance, plasmacytoid DCs are required in the future.

The interaction of CSFV with DCs can be seen as a double-edged sword. On one side, CSFV has developed a mechanism to prevent antiviral responses, permitting the virus to replicate in DCs and to use these highly migrating cells as a 'taxi' for transport to different sites in the host body. This would enable virus 'delivery' to lymphoid tissue, where it is know that considerable lymphocyte destruction occurs during CSF. The simple interaction between CSFV-infected DCs and lymphocytes is inadequate for the induction of such cell death. It would therefore appear that the environment particular to lymphoid follicles has a major role to play. On the other side, the present report demonstrates that CSFV-infected DCs do remain functional and are able to induce T cell responses against the virus.

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