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Identification of classical swine fever virus protein E2 as a target for cytotoxic T cells by using mRNA-transfected antigen-presenting cells

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Vaccination of pigs against *Classical swine fever virus* (CSFV) by using live-virus vaccines induces early protection before detectable humoral immune responses. Immunological analyses indicate that this is associated with T-cell activation, underlining the importance of targeting cytotoxic T-lymphocyte (CTL) responses for vaccine improvement. Antigen-presenting cells (APCs) transfected with mRNA encoding structural protein E2 or non-structural viral proteins NS3–NS4A were used to identify viral genes encoding CTL epitopes. Monocyte-derived dendritic cells (DCs) and fibrocytes served as the APCs. *In vitro* translation of the mRNA and microscopic analysis of transfected cells demonstrated that E2 and NS3–NS4A could be identified. APCs transfected with either of the mRNA molecules restimulated CSFV-specific T cells to produce gamma interferon and specific cytotoxic activity against CSFV-infected target cells. The presence of CTL epitopes on E2 was confirmed by using d/d-haplotype MAX cells expressing E2 constitutively as target cells in d/d-haplotype CTL assays. A potent CTL activity against E2 was detected early (1–3 weeks) after CSFV challenge. This work corroborates the existence of CTL epitopes within the non-structural protein domain NS3–NS4A of CSFV. Furthermore, epitopes on the E2 protein can also now be classified as targets for CTLs, having important implications for vaccine design, especially subunit vaccines. As for the use of mRNA-transfected APCs, this represents a simple and efficient method to identify viral genes encoding CTL epitopes in outbred populations.

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INTRODUCTION

The development of new vaccines against members of the family *Flaviviridae* is an issue of growing importance, because these pathogens continue to present a medical and economical problem in many areas of the world. The family *Flaviviridae* comprises small, enveloped, single-stranded RNA viruses, including the flaviviruses, the hepaciviruses and the pestiviruses. *Classical swine fever virus* (CSFV) is a highly contagious pestivirus that causes a severe and often lethal disease of pigs, leading to important economic losses worldwide. The CSFV genome is a positive-stranded RNA molecule of about 12·3 kb, encoding a single open reading frame (ORF) (Fig. 1a). This ORF is translated into a 3898 aa polyprotein, which gives rise to the different CSFV proteins after co- and post-translational processing (Meyers *et al.*, 1989). An autoprotease (N^{pro}) located at the N terminus of the polyprotein is followed by the structural proteins – core (C), E^{rns}, E1 and E2 – and the non-structural proteins – p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Meyers & Thiel, 1996).

Vaccination against CSFV with a C-strain vaccine induces protection at least as early as 1 week post-vaccination. This is interesting, because detectable neutralizing antibodies usually appear at 2–4 weeks after vaccination (Terpstra *et al.*, 1990; Dahle & Liess, 1995; van Oirschot, 2003). An earlier indication of immunological activity is the appearance of CSFV-specific gamma interferon (IFN-γ)-secreting cells (SFCs) in the peripheral blood as early as 6 days after vaccination (Suradhat *et al.*, 2001). Indeed, this was associated

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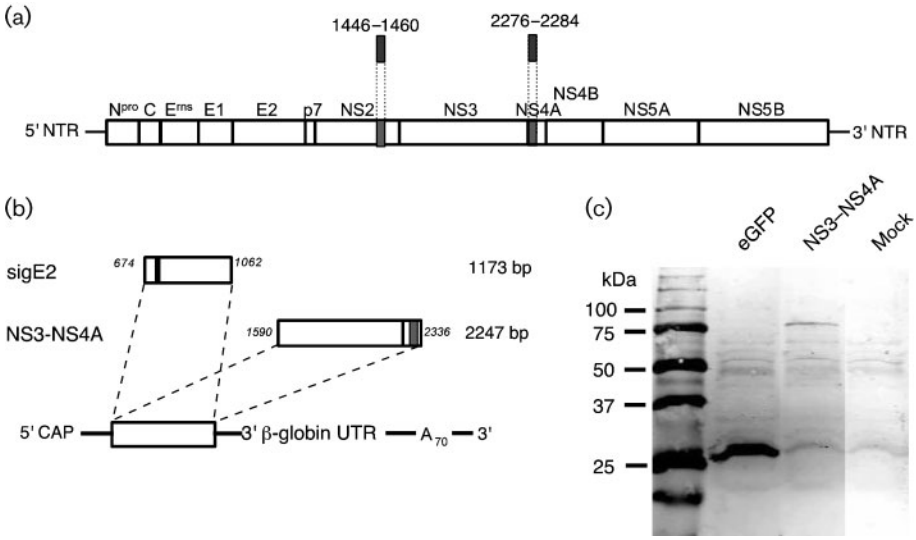

Fig. 1. Overview of CSFV-derived mRNA molecules constructed. NTR, Non-translated region; N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, non-structural proteins; C, E^{rns}, E1 and E2, structural proteins; 5′ CAP, 5′-terminal capping; 3′ *β*-globin UTR, *Xenopus laevis β*-globin untranslated region; A_{70}, 3′-terminal poly(A) nucleotide stretch. (a) Linear map of the recombinant CSFV ORF. The localization of the CSFV-specific CTL epitopes known for d/d-haplotype miniature pigs is indicated (shaded boxes). (b) Constructed mRNA molecules with their respective lengths in bp. Amino acid positions are indicated in italics. (c) Detection of eGFP and NS3 translated *in vitro* by Western blotting. After translation with the rabbit reticulocyte lysate system and SDS-PAGE separation, the proteins were immunodetected by using an eGFP-specific mAb and an NS3-specific polyclonal rabbit serum at the expected molecular masses (27 and 80 kDa, respectively).

with protective immunity, indicating the importance of cellular immunity during the early phases of immunity against CSFV.

Consequently, the localization of T-cell epitopes – in particular, epitopes recognized by cytotoxic T lymphocytes (CTLs) – is important for new vaccine development and improvement of existing vaccines, with respect to inducing early protective immunity. Viral proteins capable of inducing virus-specific T-cell responses have been examined previously, and a nonapeptide CTL epitope was identified on NS4A (Pauly *et al.*, 1995). More recently, 26 different pentadecapeptides capable of inducing virus-specific T-cell responses have been found, distributed over different viral proteins (Armengol *et al.*, 2002). One of these peptides – present on the NS2 protein – was shown to carry a CTL epitope (Armengol *et al.*, 2002). In contrast, no CTL epitopes have been identified on the CSFV structural proteins.

T-lymphocyte epitopes have to be recognized in the context of major histocompatibility complex (MHC) molecules, which imparts an influence of the MHC haplotypes on epitope recognition. Considering that the above CTL epitopes were detected in d/d-haplotype inbred animals, it was necessary to verify that CTL epitopes on CSFV proteins would be recognized in outbred animals. To this end, we employed a novel methodology based on mRNA-transfected antigen-presenting cells (APCs) – both dendritic cells (DCs) and fibrocytes were employed. DCs can be loaded efficiently with mRNA molecules, leading to induction of antigen-specific T-cell responses (Ponsaerts *et al.*, 2003). DCs transfected with mRNA encoding viral antigens can induce strong, virus-specific CTL responses (Strobel *et al.*, 2000; Weissman *et al.*, 2000; Zarei *et al.*, 2003; Ueno *et al.*, 2004). Unlike the peptide-antigen approach, this methodology permits the identification of T-cell epitopes arising naturally from the antigen processing by DCs and is therefore not influenced by the MHC haplotype. The procedure is therefore particularly applicable to use with outbred animals, and does not require the establishment of target-cell lines for CTL assays. By using this approach of mRNA-transfected DCs, the existence of T-cell epitopes recognized by T lymphocytes from outbred animals was determined. The mRNA-transfected DCs induced CSFV-specific responses efficiently, showing that CTL epitopes were present on both non-structural proteins and the E2 structural protein.

METHODS

Virus preparation. CSFV was prepared from SK-6 cells that were infected at an m.o.i. of 0·01 $TCID_{50}$ per cell and incubated for 72 h at 39 °C, or from SK-6 cells expressing E^{rns} for propagation of Flc23, an E^{rns}-deletion mutant (Widjojoatmodjo *et al.*, 2000). Lysates from these cultures and uninfected control cultures (mock) were prepared as described previously (Knoetig *et al.*, 1999).

Immunization of pigs against CSFV. Specific pathogen-free, 3-month-old pigs (Swiss Land race), bred at the IVI, were infected intranasally with 10^3 $TCID_{50}$ vA187-1, a recombinant CSFV derived from the Alfort/187 strain (Ruggli *et al.*, 1996). Peripheral blood

mononuclear cells (PBMCs) from these outbred animals were used 3–6 months after infection for the antigen-presentation assays using transfected APCs.

For the CTL analyses using the MAX.b2 cells (see below), three miniature pigs inbred for swine leukocyte-antigen complex (haplotype d/d) were used (kindly obtained from Dr Henry Salmon, INRA, Tours-Nouzilly, France). Three pigs were vaccinated intramuscularly with 10^4 immunofluorescence-stained 'foci' of vFlc23 complemented on SK-6 cells expressing E^{rns} (Widjojoatmodjo *et al.*, 2000) at 2 months of age. After 4 weeks, the pigs were challenged intranasally with $10^{3\cdot2}$ $TCID_{50}$ CSFV strain Brescia 456610, corresponding to 100 LD_{50} (Terpstra & Wensvoort, 1988), followed by a second challenge after 8 weeks. This protocol was selected to induce a strong cellular immune response with sufficient virus-specific T lymphocytes in the circulation. Unvaccinated pigs were used as controls to determine the specifity of the antigen-presentation assays.

Plasmids and mRNA molecules encoding CSFV-derived genes. The E2 gene, including its signal sequence (sigE2), and the sequences encoding NS3–NS4A of the CSFV genome (strain Alfort/187; Fig. 1) were cloned into the pCR-XL-TOPO cloning vector (Invitrogen). The sigE2 sequence, including the E2 gene and the 3′-terminal 48 nt of the E1 gene, encoding the E2 signal sequence, was amplified with primers sigE2_*Bam*HI_L3 and E2_*Xba*I_R2; the NS3–NS4A sequence was amplified with primers NS3_*Bgl*II_L and NS4A_*Xba*I_R3. Subsequent cloning in the T7TSbetaglobinA70 vector (Hoerr *et al.*, 2000; Carralot *et al.*, 2004) (CureVac) was via the *Bgl*II and the *Spe*I restriction sites, and the resulting vectors were linearized by *Pst*I followed by *in vitro* transcription using a T7-Opti-mRNA transcription kit (CureVac). The 5′-capped and polyadenylated (A_{70}), *in vitro*-transcribed mRNA molecules were purified by LiCl precipitation, quantified, extracted with phenol/chloroform, precipitated with ethanol/NaCl, washed with 75 % (v/v) ethanol and air-dried. All constructs were verified by sequencing on a LI-COR 4200 sequencer (LI-COR Biosciences). The sequences of the oligonucleotide primers used were as follows [polarity of the primers: (+), sense orientation; (−), antisense orientation]: sigE2_*Bam*HI_L3(+), 5′-CC*GGATCC*GCCACCATGATCGTGCAAGGTGTGATATG-3′; E2_*Xba*I_R2(−), 5′-GG*TCTAGA*TTAACCAGCGGCGAGTTGTTC-TGT-3′; NS3_*Bgl*II_L(+), 5′-GAAACGCAT*AGATCT*GCCGCCA-CCGCCATGGGGCCTGCCGTTTGC-3′; NS4A_*Xba*I_R3(−), 5′-GACGGC*TCTAGA*TTATAGCTCTTTCAACTCTCT-3′ (restriction sites are shown in italics and start and stop codons are underlined). Stabilized, capped and polyadenylated, *in vitro*-transcribed enhanced green fluorescent protein (eGFP) mRNA molecules (produced from the T7TSbetaglobinA70 vector) were purchased from CureVac.

For the construction of the pcDNA-E2/NS3 plasmid, viral RNA isolated from CSFV strain Glentorf (GenBank accession no. U45478)-infected cells was amplified by using primers with a *Bgl*II restriction site at the 5′ end (5′-TTTTTT**AGATCT**ATCGTGCAAGGTGTGATA-TGGCTG-3′) and a *Not*I restriction site at the 3′ end (5′-AAA-AAA**GCGGCCGC**TCATAGACCAACTACTTGTTTTAGTGC-3′) (restriction sites are shown in bold). To fuse the E2 and NS3 sequences, overlapping primers were designed (forward primer E2–NS3, 5′-ACAGAACAACTCGCCGCTGGTGGTGGTATGCCAAGGGGCACC-3′; reverse primer E2–NS3, 5′-GGTGCCCCTTGGCATAACACCA-CCAGCGGCGAGTTGTTCTGT-3′). After cDNA synthesis with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), the E2–NS3 gene was amplified by PCR using the TripleMaster PCR system (Eppendorf). The PCR product was purified, digested with *Bgl*II and *Not*I and cloned into the pcDNA4 vector (Invitrogen), and the inserted sequence was verified by sequencing.

***In vitro* translation of CSFV-specific mRNA and protein detection by Western blotting.** Proteins were expressed *in vitro* by using the rabbit reticulocyte lysate system, using 1 μg RNA translated in a 25 μl reaction without radioactive labelling, following the manufacturer's instructions (Promega). Proteins were separated by SDS-PAGE under reducing conditions according to standard protocols and analysed by Western blotting. Gels were equilibrated for 30 min in SDS blotting buffer (12·5 mM Tris/HCl, 125 mM glycine, 0·05 % SDS, 20 % methanol, pH 8·3) prior to transfer to a Trans-Blot nitrocellulose membrane (Bio-Rad) at 15 V for 30 min using a Trans-Blot SemiDry transfer device (Bio-Rad). The membranes were blocked for 1 h at room temperature with Odyssey blocking reagent (LI-COR Biosciences) diluted 1 : 1 with PBS. Immunodetection was performed for 30 min at room temperature with a mixture of the eGFP-specific mAb JL8 (BD Biosciences) and a rabbit antiserum against NS3 (J. D. Tratschin, unpublished data), followed by Alexa Fluor 680 goat anti-mouse and anti-rabbit IgG (Molecular Probes) secondary antibody, using standard protocols. Image acquisition used the Odyssey Infrared Imaging system (LI-COR).

PBMC, DC and fibrocyte preparation. PBMCs were purified from citrated blood by density-gradient centrifugation over Ficoll-Paque (1·077 g l^{-1}; Amersham Biosciences) (McCullough *et al.*, 1993). For generation of DCs, monocytes were enriched from the PBMCs by magnetic cell sorting using the magnetic-activated cell-sorting (MACS) system (Miltenyi Biotec) and an anti-CD172a mAb (74-22-15, kindly provided by Dr J. K. Lunney, USDA, Beltsville, MD, USA) (Alvarez *et al.*, 2000). DCs were cultured for 5 days at 39 °C, 6 % (v/v) CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10 % (v/v) porcine serum (Sigma-Aldrich) and recombinant porcine (rp) granulocyte–macrophage colony-stimulating factor (150 ng ml^{-1}, kindly provided by Dr S. Inumaru, Institute for Animal Health, Ibaraki, Japan) and rp interleukin 4 (100 U ml^{-1}) (Carrasco *et al.*, 2001).

Porcine fibrocytes were derived and cultured as described previously (Balmelli *et al.*, 2005). Briefly, PBMCs were plated at 4×10^6 cells ml^{-1} in fibrocyte medium [DMEM, 20 % (v/v) fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin (Invitrogen)]. After 24 h culture at 39 °C, 6 % (v/v) CO_2, non-adherent cells were removed by a single, gentle aspiration. After 10–12 days further culture, the loosely adherent, growing fibrocytes were further passaged or used as antigen-presenting cells (Balmelli *et al.*, 2005).

Transfection of SK-6 cells, DCs and fibrocytes. At 16 h before transfection, SK-6 cells were detached by using trypsin/EDTA solution and plated in six-well plates with 10^5 cells per well. Prior to transfection, the cells were rinsed twice with PBS. For the transfection, 2 μg mRNA (or 4 μg plasmid DNA) diluted in 1 ml Opti-MEM was mixed with 8 μl TransFast lipofection reagent (Promega) at a lipid : mRNA ratio of 4 : 1 (or 2 : 1 lipid : DNA ratio). After 15 min incubation at room temperature, the transfection mix was added to the cells and incubated for 1 h at 37 °C. Following transfection, 4 ml pre-warmed Earl's medium containing 7 % (v/v) horse serum was added to the cells. At 24 h post-transfection, the cells were analysed for protein expression.

DCs were transfected by mRNA electroporation as described previously (Ceppi *et al.*, 2005). Briefly, DCs were washed twice and adjusted to 5×10^6 cells ml^{-1} in Opti-MEM (Invitrogen) in a 0·2 cm gap electroporation cuvette (Bio-Rad). After 5 min on ice, 5 μg mRNA was added and the cells were pulsed for 2–4 ms by using a Gene Pulser II apparatus (300V, 125 μF; Bio-Rad) and cultured for another 48 h. Fibrocytes were transfected by mRNA lipofection. At 4 h before transfection, fibrocytes were plated in six-well plates with $0{\cdot}5 \times 10^5$–$1{\cdot}0 \times 10^5$ cells per well. Then, the cells were rinsed twice with PBS, and 3 μg mRNA diluted in 1 ml Opti-MEM was mixed with 6 μl TransFast (Promega) at a lipid : mRNA ratio of 2 : 1. After 15 min at room temperature, the transfection mix was added to the cells and incubated for 1 h at 39 °C. Following transfection, 4 ml fibrocyte medium was added and, 24 h later, the cells were employed.

Establishment of MAX cell lines expressing CSFV E2. MAX cells, an SV40-immortalized swine kidney-cell line of MHC class I haplotype d/d (Pauly *et al.*, 1995), were grown in DMEM containing 5 % (v/v) FBS, glutamine (0·3 mg ml^{-1}), penicillin (200 U ml^{-1}), streptomycin (0·2 mg ml^{-1}) and mycostatin (100 U ml^{-1}). The MAX cells were transfected with the pPRb2 plasmid containing the E2 gene of CSFV strain Brescia (Widjojoatmodjo *et al.*, 2000) under control of the transcription and translation signals of the pEVhisD12 vector (Peeters *et al.*, 1992). For transfection, 2 μg lipofectamine (Invitrogen) was diluted in 50 μl Opti-MEM I (Invitrogen), mixed with plasmid DNA (1 μg) in 50 μl medium and the mixture was incubated for 15 min at room temperature. MAX cells grown in 10 cm^2 tissue-culture plates were washed with Opti-MEM I and cultured for 20 h in 0·5 ml medium. Then, the cells were harvested for limiting-dilution cultures in microtitre plates in medium containing 7·5 mM histidinol, which was replaced every 3–4 days until single colonies were visible. After 2 weeks, surviving colonies were recloned by limiting dilution and screened for E2 expression by immunostaining with mAbs b3 and b6, directed respectively against the conserved domain A and non-conserved domain B of E2 from CSFV strain Brescia (Wensvoort *et al.*, 1989). The two domains were recognized by the mAbs in 70–80 % of the cells, referred to as MAX.b2.

Confocal-microscopy analysis. For CSFV-specific protein expression, transfected or infected cells were washed twice in PBS, fixed in 4 % (w/v) paraformaldehyde for 15 min and washed again. Then, the cells were stained for 20 min at 4 °C with the anti-E2 mAb HC26 (Greiser-Wilke *et al.*, 1990) (kindly provided by Bommeli AG) or the anti-NS3 mAb C16 (Greiser-Wilke *et al.*, 1992) (kindly provided by Dr Greiser-Wilke, Hannover, Germany), diluted in 0·3 % (w/v) saponin (S4521; Sigma-Aldrich) permeabilization solution. After washing with PBS containing 0·1 % (w/v) saponin, Alexa-488 or Alexa-546 fluorochrome-labelled anti-mouse secondary antibody (Molecular Probes) – diluted in the permeabilizing solution – was added. Following incubation for 20 min at 4 °C, washing and mounting in Mowiol mounting solution, the cells were analysed by using a Leica TCS-SL spectral confocal microscope (CFM) and Leica LCS software (Leica Microsystems AG).

Antigen-presentation assays. Purified T lymphocytes were sorted by MACS using an anti-CD6 mAb, as described previously (Carrasco *et al.*, 2001). Purity of the positive fraction was >95 %. Either autologous DCs or fibrocytes (transfected or infected) were employed as APCs at a ratio of 1 : 20 or 1 : 400, respectively. Where indicated, CSFV strain Eystrup was added to the co-cultures at an m.o.i. of 0·01 $TCID_{50}$ per cell. These cultures were incubated for 5 days and then tested for the presence of activated T cells.

IFN-γ ELISPOT. The ELISPOT assay was performed as described previously (Armengol *et al.*, 2002) with minor modifications. Stimulated lymphocytes were added to duplicate wells on nitrocellulose plates (Nunc) coated with mouse anti-pig IFN-γ mAb (MP700, 5 μg ml^{-1}; Pierce Biotechnology). After 48 h incubation, IFN-γ spots were visualized with rabbit anti-pig IFN-γ polyclonal antibody (PP700, 2·5 μg ml^{-1}; Pierce Biotechnology) followed by peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) and developed with 3,3′-diaminobenzidine (Sigma) for 20 min at room temperature in the dark. The number of spots was determined with a computer-assisted video-image analyser (AID ELISPOT reader; AID GmbH).

Cytotoxic assay. Autologous fibrocytes infected with CSFV were used as target cells in a flow-cytometry assay for cytotoxicity (Balmelli *et al.*, 2005). Briefly, fibrocytes were detached, stained with PKH26 (Sigma) and plated at 10 000 cells per well in 96-well flat-bottomed plates. The fibrocytes were infected with CSFV or treated with mock antigen for 24 h at an m.o.i. of 2 $TCID_{50}$ per cell. Restimulated lymphocytes (see above) were then added to the target fibrocytes at various effector : target-cell (E : T) ratios for 16 h at 39 °C. Then, the percentage of dead cells was quantified by using propidium iodide (Sigma).

For the experiments using d/d-haplotype MAX cells as target cells, miniature pig-derived PBMCs were restimulated with CSFV strain C1.1.1 (5×10^6 p.f.u. ml^{-1}) for 7 days and CTL activity was tested in a ^{51}Cr-release assay, as described previously (de Bruin *et al.*, 2000). Briefly, MAX.b2 cells, MAX cells transfected with an empty control plasmid or infected MAX cells were labelled with ^{51}Cr [400 μCi (14·8 MBq); Amersham Biosciences, catalogue no. CJS4] and cultured with restimulated PBMCs in 96-well V-bottomed microtitre plates (Nunc) at E : T ratios decreasing from 50 to 6·25 for 5 h. The supernatant was then tested for radioactivity in a Wallac Microbeta^{plus} 1450 scintillation counter (EG&G Instruments). Maximal release of ^{51}Cr was induced by adding 50 μl 20 % Triton X-100. Spontaneous release was determined in wells that did not contain effector cells. The percentage of specific lysis was calculated as (c.p.m. experimental release − c.p.m. spontaneous release)/(c.p.m. maximal release − c.p.m. spontaneous release) × 100.

Statistical analysis of data. Statistical evaluation of the experimental data was performed by using Student's *t*-test. A value of $P < 0·05$ was considered statistically significant.

RESULTS

mRNA molecules encoding CSFV-specific T-cell epitopes

Two different mRNA molecules encoding CSFV-specific T-cell epitopes were designed (Fig. 1b). The sigE2 mRNA encodes the E2 structural protein and the C-terminal 16 aa of the E1 protein with the E2 signal sequence. The NS3–NS4A mRNA encodes the NS3 and NS4A non-structural proteins, and should contain at least one CTL epitope on the basis of peptide-antigen studies (Pauly *et al.*, 1995). All mRNA molecules have a common structure, characterized by a 5′-terminal cap, a 3′ *Xenopus laevis* β-globin untranslated region for mRNA stabilization and an A_{70} stretch at the 3′ end (Fig. 1b).

As a first step, it was considered important to verify the translation of the mRNA molecules. To this end, *in vitro* translation in the rabbit reticulocyte lysate system followed by Western blotting was used. Both eGFP and NS3 were detectable with the expected molecular masses, indicating that both proteins are translated, although only NS3 at lower levels (Fig. 1c). This technique was not used for the E2 mRNA, due to the lack of a suitable antibody.

Expression of the E2 and NS3 proteins in transfected cells

The protein-expression capacity of the CSFV-derived mRNAs was tested in transfected cells and compared with the expression obtained with plasmid DNA transfection or CSFV infection. Protein expression was first tested in SK-6 cells, an established porcine cell line that is permissive for CSFV infection and replication. At 24 h after lipofection of pcDNA-E2/NS3 (for construct, see Fig. 1d), both E2 and

NS3 were detectable (Fig. 2a, top panels). The proteins were not detectable in all cells due to the limited transfection efficiency of SK-6 cells (25 %, relative to an eGFP reporter plasmid). Moreover, in some cells, E2 and NS3 could not be detected simultaneously, probably because of differences in the sensitivity of their detection. Lipofection of SK-6 cells with the sigE2 mRNA also resulted in E2 protein expression (Fig. 2a, bottom panels). No signal for NS3 was detected with the sigE2-transfected cells, as expected.

Having demonstrated the potential for transfection with these constructs, translation of the mRNA molecules was analysed in the immunological target for this work – DCs. At 24 h post-infection with CSFV, both E2 and NS3 were detectable in DCs (Fig. 2b, top panels). When DCs were electroporated with sigE2 mRNA, the E2 protein could again be detected; this was noted in the majority of cells as a focal inclusion. This appeared as a perinuclear inclusion somewhat weaker than that obtained with transfected SK-6 cells (Fig. 2b, bottom panels). The high number of mRNA-transfected DCs expressing E2 was expected, as electroporation of porcine DCs with eGFP mRNA results in 80 % transfection efficiency (Ceppi *et al.*, 2005). In contrast, no NS3 could be detected by immunofluorescence in DCs electroporated with NS3 mRNA (data not shown).

mRNA-transfected DCs induce CSFV-specific CTL responses

Although only the E2 mRNA produced detectable protein in the transfected DCs, it was necessary to determine whether this had any immunological relevance. Consequently, DCs transfected with the mRNA molecules encoding E2 and NS3–NS4 were tested for their capacity to induce virus-specific T-cell responses *in vitro*. The mRNA-transfected DCs were co-cultured with CSFV-immune lymphocytes at a ratio of 1 : 20, whilst CSFV-infected DCs (m.o.i. of 0·01 $TCID_{50}$ per cell) were employed as a positive control. Mock-treated DCs were the negative control. After 5 days DC/lymphocyte co-culture, the presence of activated T cells was determined by using an IFN-γ ELISPOT assay. As expected, the co-cultures using virus-infected DCs yielded significantly higher numbers of IFN-γ SFCs than the mock-treated controls (Fig. 3a, 'CSFV' compared with 'Mock'; $*P<0{\cdot}05$). The co-cultures using DCs transfected with the different CSFV protein-encoding sigE2

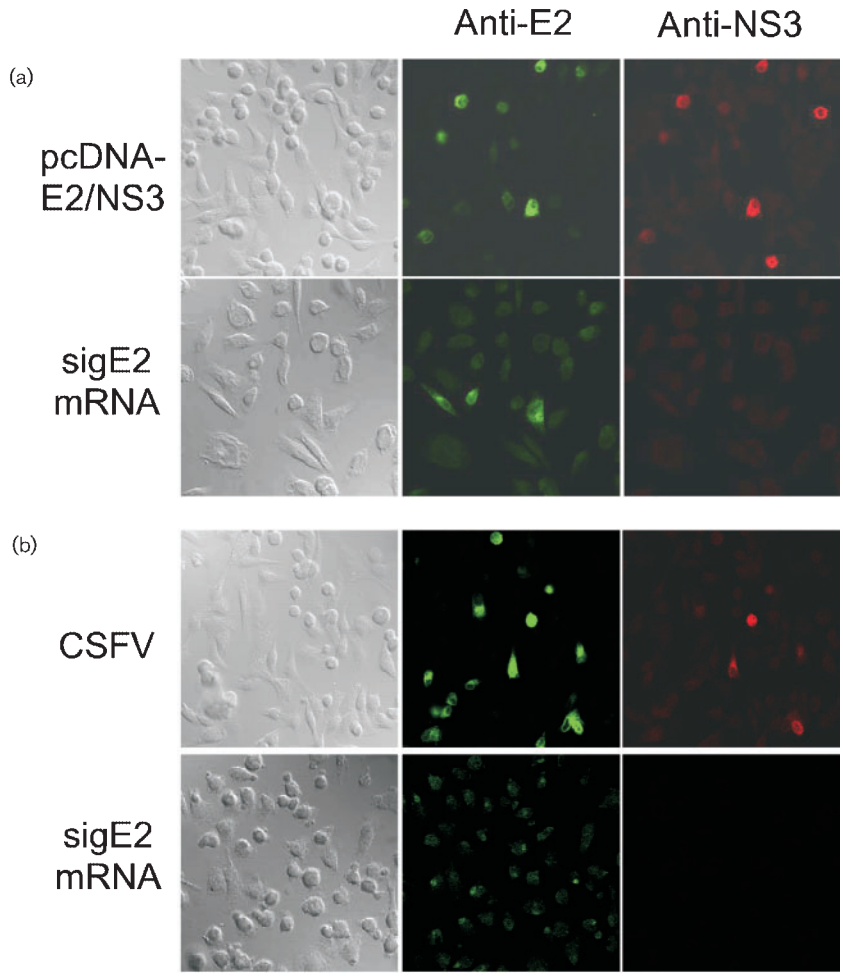

Fig. 2. Detection of E2 and NS3 proteins in transfected cells. (a) SK-6 cells were lipofected with either a plasmid DNA encoding the E2–NS3 fusion protein (pcDNA-E2/NS3, top panels) or with the sigE2 mRNA (bottom panels). (b) DCs were either infected with CSFV (top panels) or electroporated with sigE2 mRNA (bottom panels). At 24 h after treatment, the cells were stained for intracytoplasmic E2 and NS3 proteins, using specific mAbs, and imaged by confocal microscopy.

and NS3–NS4A mRNAs resulted in significantly higher numbers of IFN-γ SFCs compared with co-cultures employing eGFP-transfected DCs (Fig. 3a, 'sigE2' or 'NS3–NS4A' compared with 'eGFP'; $*P<0{\cdot}05$).

The presence of IFN-γ SFCs indicates the induction of a virus-specific T-lymphocyte response and can often relate to the presence of CTL activity. In order to test this, lymphocytes from a CSFV-immune animal were stimulated with the mRNA-transfected DCs and then incubated at various E : T ratios by using virus-infected target cells of the same haplotype as the lymphocytes. A statistically significant three- to fourfold increase in specific lysis of the target cells was observed for the lymphocytes stimulated by sigE2-transfected DCs, when compared with lymphocytes stimulated by eGFP-transfected DCs (Fig. 3b, compare 'sigE2' and 'eGFP' in the graph and in Table 1; $P<0{\cdot}05$). A statistically significant two- to threefold increase in specific lysis was observed for lymphocytes stimulated by NS3–NS4A-transfected DCs (Fig. 3b, compare 'NS3–NS4A' and 'eGFP' in the graph and in Table 1; $P<0{\cdot}05$). The results demonstrate consistently that CSFV-specific CTL responses

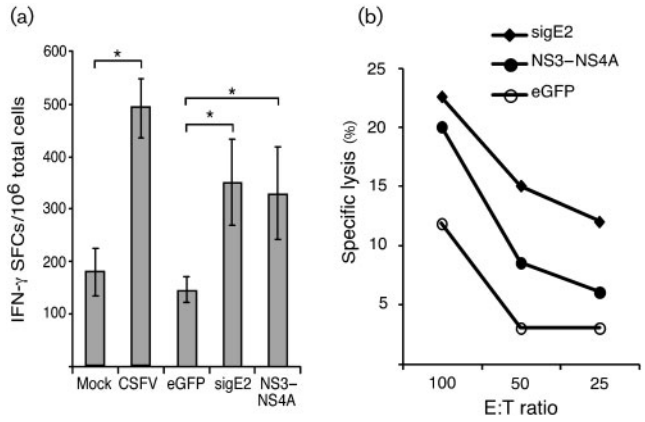

Fig. 3. Stimulation of antiviral CTL activity by mRNA-transfected DCs. DCs were generated from CSFV-immunized animals 3–6 months post-infection. At 48 h after mRNA transfection, DCs were incubated with autologous lymphocytes at a ratio of 1 : 20. As a positive control, DCs were infected with CSFV (m.o.i. of 0·01 $TCID_{50}$ per cell); the negative control was mock-treated DCs. (a) After 5 days DC/lymphocyte co-culture, the lymphocytes were analysed in an IFN-γ ELISPOT assay. Errors bars indicate the SD between replicate wells. The result is representative of three independent experiments performed with two different animals. $*P<0{\cdot}05$ (paired *t*-test). (b) The cytotoxic activity of the lymphocytes following 5 days co-culture with transfected DCs was tested by using additional cultures of CSFV-infected (m.o.i. of 2 $TCID_{50}$ per cell) autologous fibrocytes as target cells. The target cells were prepared 24 h prior to adding the stimulated lymphocytes (effector cells). The result reported in the graph relates to experiment 1 in Table 1 and is representative of three independent cytotoxic assays performed with one animal.

Table 1. CTL responses induced by transfected DCs

Experiment	sigE2 mRNA	NS3–NS4A mRNA
1	3·6*	2·2
2	3·2	2·6
3	3·4	2·5
Mean ± SD	3·4 ± 0·2	2·4 ± 0·2

*Fold increase compared with eGFP mRNA-transfected DCs at an E : T ratio of 50.

were induced by sigE2 and NS3–NS4A mRNA-transfected DCs. This demonstrated that CTL epitopes were encoded within the structural E2 region.

Transfected fibrocytes are stimulators of CSFV-specific CTL activity

Fibrocytes represent a population of blood-borne cells, described recently as potent stimulators of CSFV-specific CTLs (Balmelli *et al.*, 2005). Consequently, fibrocytes transfected with the above mRNA constructs were tested for their capacity to activate CSFV-specific T cells. mRNA lipofection of fibrocytes using an eGFP mRNA reporter molecule resulted in 30 % transfection efficiency and 92 % cell viability (data not shown). At 24 h after lipofection of the fibrocytes, they were co-cultured with CSFV-immune lymphocytes at a ratio of 1 : 400 (based on the known characteristics of these cells; Balmelli *et al.*, 2005). As a positive control, CSFV-infected fibrocytes (m.o.i. of 0·01 $TCID_{50}$ per cell) were employed and mock-treated fibrocytes were the negative control. After 5 days fibrocyte/lymphocyte co-culture, the lymphocytes were tested for the presence of activated T cells by the IFN-γ ELISPOT assay. The co-cultures of immune lymphocytes with virus-infected fibrocytes yielded significantly higher numbers of IFN-γ SFCs compared with the mock-treated controls (Fig. 4, 'CSFV', 'Mock'; $*P<0{\cdot}05$). Similar to the DCs, co-cultures of lymphocytes with fibrocytes transfected by using sigE2 or NS3–NS4A mRNA resulted in significantly higher numbers of IFN-γ SFCs when compared with co-cultures of lymphocytes with eGFP-transfected fibrocytes (Fig. 4; $*P<0{\cdot}05$).

MAX cells expressing E2 are killed efficiently by CSFV-restimulated PBMCs

Due to the above results presented in Figs 3 and 4, it was considered important to confirm the enhanced CTL activity in an independent manner. To this end, MAX cells stably expressing CSFV E2 protein were generated (MAX.b2 cells) and used as target cells in CTL assays. MAX.b2 cells, but not mock-transfected MAX cells (MAXtf ctrl), were lysed by PBMCs restimulated with CSFV (Fig. 5a). Although the cytotoxicity did not reach the same levels as CSFV-infected MAX cells (MAX infected), the presence of E2 clearly permitted a viral-specific CTL response. The cytotoxic

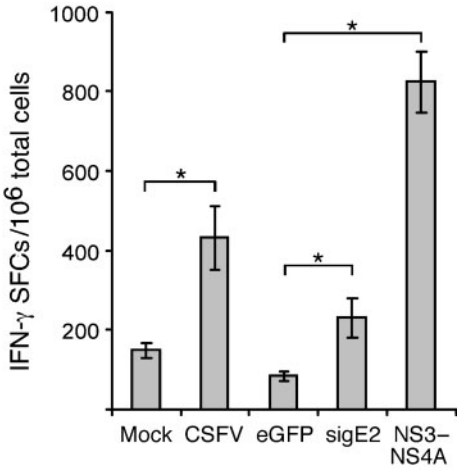

Fig. 4. Stimulation of antiviral CTL activity by mRNA-transfected fibrocytes. Fibrocytes were generated from CSFV-immunized animals 3–6 months post-infection. At 24 h after mRNA transfection, fibrocytes were incubated at a ratio of 1 : 400 with autologous lymphocytes. Positive controls were fibrocytes infected with CSFV (m.o.i. of 0·01 $TCID_{50}$ per cell), whilst mock-treated fibrocytes served as negative controls. After 5 days fibrocyte/lymphocyte co-culture, the lymphocytes were analysed in an IFN-γ ELISPOT assay. Errors bars indicate the SD between replicate wells. The result is representative of three independent experiments performed with two different animals. *$P < 0{\cdot}05$ (paired *t*-test).

response that could be induced in PBMCs was then analysed with cells isolated at different time points after vaccination. High levels of CTL activity were detected at 1–3 weeks after challenge (Fig. 5a, b), with a significant reduction 4 weeks after challenge (Fig. 5c). The levels of cytotoxicity obtained at this latter time point related to those seen when the mRNA-transfected DCs or fibrocytes were employed to stimulate lymphocytes obtained 3–6 months post-infection (see Figs 3 and 4).

DISCUSSION

The present study presents a rationalized approach to identifying viral proteins containing CTL epitopes. T-cell epitopes are often identified by loading APCs with peptides encoding epitopes or with proteins encoding whole antigens. However, the production of peptides or sufficiently pure proteins is both laborious and expensive. In contrast, *in vitro*-transcribed mRNAs and the direct use of these molecules to target APCs avoids such drawbacks, representing an alternative method for the identification of T-cell epitopes (Strobel *et al.*, 2000; Thornburg *et al.*, 2000; Weissman *et al.*, 2000; Tuyaerts *et al.*, 2003; Zarei *et al.*, 2003; Ueno *et al.*, 2004). Importantly, APCs such as DCs can be transfected efficiently with mRNA molecules, whereas gene expression after plasmid DNA transfection is not detectable (Van Tendeloo *et al.*, 2001; Ponsaerts *et al.*, 2003; Ceppi *et al.*, 2005). Moreover, mRNA-transfected APCs potentially display the complete spectrum of epitopes relevant to a particular protein, rather than those present on the synthesized peptides. This has the advantage of uncovering previously unknown epitopes, but is particularly valuable in not suffering from restrictions imposed by MHC haplotype – the APC will select the epitopes from the translated proteins that fit best to their MHC haplotype.

Whilst DCs represent a well-established and potent APC, a relatively new cell type has been identified with a propensity for inducing CTL activity. This is the fibrocyte, an APC derived from blood (Balmelli *et al.*, 2005). Fibrocytes have the additional advantage that they can be cultured easily in the absence of cytokines and frozen as primary cell lines

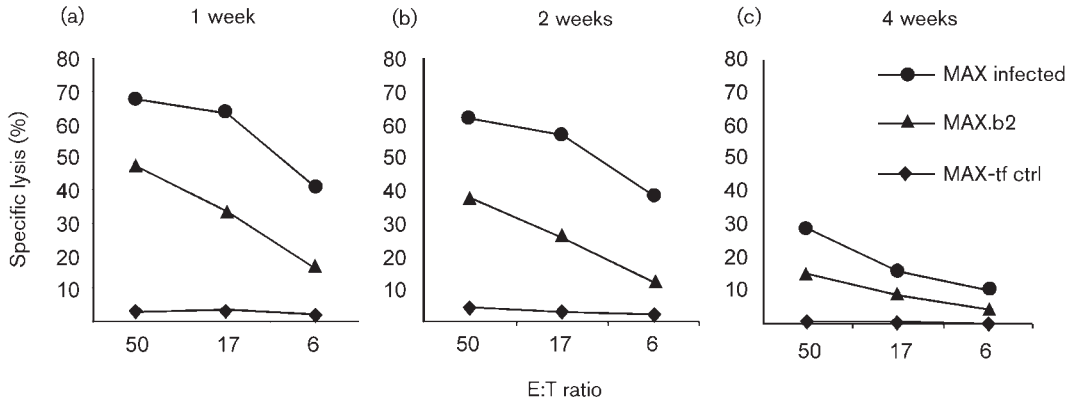

Fig. 5. CTL activity against E2-expressing cells demonstrable in PBMCs of a challenge-infected pig. PBMCs were isolated from pigs at 1 (a), 3 (b) or 4 (c) weeks after challenge with CSFV. These cells were restimulated *in vitro* with virus and tested for their cytolytic activity in a ^{51}Cr-release assay, using MAX cells infected with CSFV (MAX infected), MAX cells expressing E2 (MAX.b2) or mock-transfected MAX cells (MAX-tf ctrl) as target cells. The specific cytotoxic activity at different E : T ratios is shown for a experiment representative of three performed with three different animals.

for future use. This makes them a particularly flexible alternative to monocyte-derived DCs for antigen-processing/presentation studies.

Both DCs and fibrocytes were employed for the transfection studies. The mRNA used encoded the structural E2 protein or non-structural proteins NS3–NS4A of CSFV. Transfection of APCs with the mRNA encoding E2 was revealed easily, in terms of detecting the E2 protein by immunofluorescent staining. In contrast, the NS3 protein could not be detected by immunofluorescence in transfected cells, but was identified in immunoblots after *in vitro* translation, albeit in low quantities. Furthermore, APCs transfected with the mRNA encoding the non-structural proteins were capable of restimulating T lymphocytes from immune animals, although any quantitative comparison between the different mRNA-transfected APCs is difficult. The difficulty in detecting the viral protein *in situ* could be the result of viral or cellular protease digestion. Furthermore, the formation of defective ribosomal products (DRiPs) – resulting from translational and post-translational errors – could have been involved (Schubert *et al.*, 2000). There is evidence showing that DRiPs are a major source of peptides presented in the context of MHC class I (Yewdell *et al.*, 2001) and could therefore play a role in the antigen processing of mRNA-loaded APCs.

This is the first report demonstrating CTL epitopes on CSFV E2. Flavivirus-specific CTL epitopes have mainly been identified on the viral non-structural proteins (Roehrig, 2003); nevertheless, reports demonstrating the presence of CTL epitopes on the structural proteins of *Hepatitis C virus*, including E2 (Koziel *et al.*, 1993; Sarobe *et al.*, 2001), are related to our study, considering that both viruses are in the family *Flaviviridae*.

These findings could be of importance for future vaccine design, considering the immunology of CSFV. Although the presence of neutralizing anti-E2 antibodies can be related to protective immunity against CSFV, pigs can be protected in the absence of such antibodies, such as 1–3 weeks after vaccination (Terpstra & Wensvoort, 1988; Dewulf *et al.*, 2004). Cellular immunity has been identified as early as 1 week post-vaccination, in the form of IFN-γ SFCs (Suradhat *et al.*, 2001). The present work demonstrates that E2-specific CTL activity is particularly evident 1–3 weeks after challenge. With CSFV being non-cytopathogenic and the inaccessibility of the cell-associated virus to neutralizing antibodies, CTL-based immune defences have an important role to play.

Both the CTL epitopes and major epitopes for inducing neutralizing antibodies should be targeted in novel CSFV vaccines. The application of mRNA vaccines or self-replicating pestivirus replicons are known to induce potent CTL responses (Hoerr *et al.*, 2000; Racanelli *et al.*, 2004). Such replicon-based vaccines have been described for classical swine fever (Widjojoatmodjo *et al.*, 2000; van Gennip *et al.*, 2002; Maurer *et al.*, 2005) and represent promising vaccine candidates. However, such vaccines could possibly lose immunogenicity if E2 is deleted or replaced by a heterologous E2 from another pestivirus. On the other hand, for subunit protein vaccines based on E2, the present study would indicate that protein-delivery systems suitable for the induction of both neutralizing antibodies and CTL response have to be investigated. Therein, those systems known to mediate cross-presentation, such as liposomes, bacterial toxins, heat-shock proteins and particles, are particularly interesting (Bungener *et al.*, 2002; Chikh & Schutze-Redelmeier, 2002; Morón *et al.*, 2004).

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