

Dendritic cells pulsed with hepatitis C virus NS3 protein induce immune responses and protection from infection with recombinant vaccinia virus expressing NS3

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Infections with *Hepatitis C virus* (HCV) pose a serious health problem worldwide. In this study, the hypothesis that adoptive transfer of dendritic cells (DCs) pulsed with HCV NS3 protein and matured with an oligodeoxynucleotide (ODN) containing CpG motifs (CpG) *ex vivo* would initiate potent HCV-specific protective immune responses *in vivo* was tested. NS3 protein was efficiently transduced into DCs and treatment of DCs with CpG ODN induced phenotypic maturation and specifically increased the expression of CD40. DCs matured with CpG ODN produced higher interleukin 12 levels and a stronger allogeneic T-cell response compared with untreated DCs. Notably, there were no differences between NS3-pulsed DCs and DCs pulsed with a control protein with respect to phenotype, cytokine production or mixed lymphocyte reaction, indicating that transduction with NS3 protein did not impair DC functions. Compared with the untreated NS3-pulsed DCs, the NS3-pulsed DCs matured with CpG ODN induced stronger cellular immune responses including enhanced cytotoxicity, higher interferon- γ production and stronger lymphocyte proliferation. Upon challenge with a recombinant vaccinia virus expressing NS3, all mice immunized with NS3-pulsed DCs showed a significant reduction in vaccinia virus titres when compared with mock-immunized mice. However, the NS3-pulsed DCs matured with CpG ODN induced higher levels of protection compared with the untreated NS3-pulsed DCs. These data are the first to show that NS3-pulsed DCs induce specific immune responses and provide protection from viral challenge, and also demonstrate that CpG ODNs, which have a proven safety profile, would be useful in the development of DC vaccines.

INTRODUCTION

Infections with *Hepatitis C virus* (HCV) pose a serious health problem worldwide. An estimated 170 million people are currently infected, which amounts to 3 % of the world population. About 80 % of infected people remain chronic carriers and are at high risk of developing severe liver disease including cirrhosis and hepatocellular carcinoma (WHO, 1999). Treatment with interferon- α (IFN- α) and ribavirin is the only effective therapy against HCV infection, but the overall response rate is only 40–50 % (Fried *et al.*, 2002; Manns *et al.*, 2001). Currently, there is no licensed vaccine against HCV; therefore, developing strategies for vaccination as well as for treatment of HCV infection is of great importance.

Dendritic cells (DCs) are the most potent type of antigen presenting cells (APCs) and are responsible for the initiation and maintenance of immune responses. Situated in peripheral tissues and in lymphoid organs, DCs are uniquely suited to detect and capture pathogens. They express members of the recently identified Toll-like receptor (TLR) family, which bind common chemical moieties associated with microbial organisms. TLR ligands include bacterial lipopolysaccharide, lipopeptides, hypomethylated CpG DNA motifs, dsRNA and flagellin (Akira *et al.*, 2001; Iwasaki & Medzhitov, 2004). TLR signalling triggers a maturation programme in DCs that leads to the upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules and the expression of pro-inflammatory cytokines. As a result, DCs acquire the unique ability to prime naive T cells (Kaisho & Akira, 2003; Pulendran, 2004). Because of their pivotal functions, DCs have begun to be appreciated as a mandatory target in the creation of new adjuvants. Most importantly, DCs can be used as exogenous adjuvants by loading them with the antigen of interest *ex vivo* and injecting them back into animals or humans to manipulate the immune response (Ardavin *et al.*, 2004; Berger & Schultz, 2003; Ludewig, 2003; Nieda *et al.*, 2003; Walsh *et al.*, 2003).

The therapeutic potential of DC-based vaccines has been demonstrated for numerous murine tumour models and some human clinical trials (Banchereau *et al.*, 2001; Cerundolo *et al.*, 2004). Recently, DCs have been examined for their capacity to serve as adjuvants and vaccine carriers mediating protection against bacterial, viral, parasitic or fungal pathogens (Moll & Berberich, 2001a, b). For example, DC-based vaccination improved immunity to malaria (Pouniotis *et al.*, 2004), human immunodeficiency virus (Brown *et al.*, 2003), *Candida albicans* (d'Ostiani *et al.*, 2000) and *Leishmania major* (Berberich *et al.*, 2003; Ramirez-Pineda *et al.*, 2004).

An effective vaccine against HCV infection should be capable of inducing strong, cross-reactive, helper T-cell (Th) and cytotoxic T-lymphocyte (CTL) responses (Esser *et al.*, 2003; Neumann-Haefelin *et al.*, 2005). A major inherent problem in the design of an effective vaccine against HCV infection is the heterogeneity of its genome (Pawlotsky, 2003; Simmonds, 1999). Thus, the antigens included in HCV vaccines should not only be immunogenic but also conserved between HCV genotypes. HCV NS3 protein has serine protease and helicase activity and is one of the most conserved proteins of HCV (Grakoui *et al.*, 1993). It contains an immunodominant CD4⁺ T-helper epitope and several CTL epitopes, which have been associated with control of HCV in patients with self-limiting infection (Diepolder *et al.*, 1997; Jiao

et al., 2003; Takaki *et al.*, 2000). These characteristics make NS3 protein an appropriate vaccine candidate for HCV.

In this study, we tested the hypothesis that adoptive transfer of DCs transduced *ex vivo* with HCV NS3 protein can initiate potent HCV-specific protective immune responses *in vivo*. DCs were generated from murine bone marrow, pulsed with NS3 protein and stimulated with a CpG oligodeoxynucleotide (ODN). Mice vaccinated with these NS3-pulsed, CpG ODN stimulated DCs developed stronger cellular immune responses and were better protected from a challenge with vaccinia virus expressing NS3 protein than animals immunized with unstimulated NS3-pulsed DCs or DCs pulsed with a control protein.

METHODS

HCV NS3 protein and CpG ODN. The production and purification of HCV NS3 recombinant protein has been described previously (Yu *et al.*, 2004). Briefly, the NS3 gene (HCV 1b strain) consisting of aa 1027–1657 of the polyprotein was cloned into the expression vector pRSETA (Invitrogen). The recombinant clone was transformed into *Escherichia coli* BL21 (DE3) and the NS3 protein plus a six histidine amino-terminal linker from the vector sequence was expressed as inclusion bodies. Recombinant NS3 protein (rNS3) was purified by nickel-chelate affinity resin under denaturing condition according to the recommendations of the supplier (Invitrogen). The NS3-containing fraction was renatured by reduction of the urea concentration by dialysis (Jin & Peterson, 1995). The purity of the NS3 protein was analysed by SDS-PAGE and determined to be about 90 %. The endotoxin level, determined using the QCL-1000 Chromogenic *Limulus* amoebocyte lysate test (BioWhittaker), was 51 ng NS3 protein mg⁻¹. ODN 1826 (5'-CCATGACGTTCTGACGTT-3') (Qiagen), which is a strong immune cell mitogen known to stimulate mouse splenocytes *in vitro* (Davis *et al.*, 1998; Rankin *et al.*, 2001), was used in this study. The CpG ODN was phosphorothioate modified to increase resistance to nuclease degradation (Samani *et al.*, 2001).

DC generation from bone marrow. Murine DCs were generated following the protocol described by Lutz *et al.* (1999). Briefly, bone marrow cells prepared from the femora and tibiae of normal BALB/c mice (H-2^d) were depleted of red blood cells with ammonium chloride (17 mM Tris, 144 mM NH₄Cl, pH 7.2) and cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % fetal bovine serum (JRH Biosciences), 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 5×10⁻⁵ M 2-mercaptoethanol and 10 mM HEPES (complete RPMI) containing 20 ng GM-CSF (PeproTech) ml⁻¹ at 37 °C and 5 % CO₂. On day 3, the non-adherent granulocytes and T and B cells were gently removed and fresh medium was added. On days 5 and 7, 50 % of the medium was replaced with fresh culture medium containing 20 ng GM-CSF ml⁻¹. On day 9, non-adherent cells were harvested for protein pulsing.

DC pulsing. DCs harvested on day 9 were washed twice in RPMI 1640. One hundred microlitres of the liposomal transfection reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP; Roche) and 20 µg rNS3 or control protein, human serum albumin (HSA; Sigma), were mixed with 500 µl RPMI 1640 at room temperature in polystyrene tubes for 20 min (Liu *et al.*, 2001; Nonn *et al.*, 2003; Santin *et al.*, 1999). DCs (2×10⁷) in 2 ml RPMI 1640 were added to the DOTAP/protein mixtures. The DCs were incubated for 3 h at 37 °C, washed three times, resuspended in complete RPMI 1640 containing 20 ng GM-CSF ml⁻¹ and then cultured for 18 h in the absence or presence of 25 µg ODN 1826 ml⁻¹. These pulsed DCs were used for *in vitro* phenotypic analysis, immunohistochemical analysis, mixed lymphocyte reaction (MLR) assay, cytokine measurements and *in vivo* immunization.

Phenotypic analysis of DCs. After the DCs were subjected to different treatments they were collected on day 10 and incubated with FITC-labelled monoclonal antibodies (anti-mouse CD11c, I-A^d, CD86, CD54 and CD40; BD PharMingen) for 30 min at 4 °C in PBS (0.137 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M NaH₂PO₄, pH 7.3). After three washes, the cells were resuspended in PBS. Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

Immunohistochemistry. The NS3- and HSA-pulsed DCs were plated in four-well LAB-TEK chamber slides (Nalge Nunc International). After 6 h, the DCs were fixed for 20 min in 4 % paraformaldehyde in PBS. Subsequently, they were permeabilized for 10 min in 0.5 % Triton X-100 in PBS. The cells were blocked for 20 min with PBS containing 1 % goat serum, and incubated with NS3-specific polyclonal rabbit serum (1:200) for 1.5 h. Biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:5000) (Zymed Laboratories) was added and then the cells were incubated again for 1.5 h. Finally, the cells were incubated for 45 min with ABC Reagent (Vector Laboratories) and incubated with peroxidase substrate solution (DAB substrate kit SK-4100; Vector Laboratories) until the desired stain intensity developed. The slides were rinsed in H₂O, counterstained with 0.1 % toluidine blue, and again rinsed in H₂O. All incubations were performed at room temperature and the slides were washed in PBS three times between incubations. The slides were observed and the images were captured with a Zeiss Axiovert 200M microscope (Carl Zeiss).

MLR assay. Splenocytes from C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (Charles River Laboratories) were passed over nylon wool fibre columns (Polyscience) and T cells were separated and used as responder cells at 2×10⁵ cells per well in U-bottom 96-well plates. The DCs from BALB/c mice were collected on day 10, irradiated at 50 Gy and added to the responder cells in varying cell numbers as stimulator cells. Cells were cultured for 5 days in complete RPMI 1640 at 37 °C and 5 % CO₂. The cell cultures were pulsed with 0.4 μCi (14.8 kBq) [*methyl*-³H]thymidine (Amersham Pharmacia Biotech) per well during the last 18 h. The cells were harvested and radioactivity was determined by scintillation counting. Data are expressed as mean c.p.m. of triplicate wells.

Cytokine measurements. The culture supernatants of the treated DCs were collected on day 10 and analysed with respect to interleukin 12 (IL12) and IL10 production with a sandwich ELISA using corresponding specific capture and detection antibodies. Cytokine levels were calculated using standard curves constructed by recombinant murine cytokines (BD PharMingen).

Immunization of mice. Eight groups of 12 eight-week-old female BALB/c mice (Charles River Laboratories) were immunized twice with a 2 week interval, subcutaneously in the base of the tail with 5×10⁶ DCs or 1 μg rNS3 formulated with 25 μg alum (2 % Alhydrogel; Superfos

Biosector) or PBS in a 100 μl volume. The DCs were transduced either with rNS3 or HSA and then left either untreated or incubated with ODN 1826. Ten days after the last immunization, half of the mice of each group were sacrificed to isolate splenocytes for lymphocyte proliferation, enzyme-linked immunospot (ELISPOT) and CTL assays. The other half of the mice were used for viral challenge. The experiments were carried out according to the guidelines provided by the Canadian Council for Animal Care.

ELISPOT assay. A cytokine-specific ELISPOT assay was performed as described previously (Ioannou *et al.*, 2002; Lewis *et al.*, 1999). Briefly, 96-well MultiScreen-HA filtration plates (Millipore) were coated overnight at 4 °C with 0.1 μg murine IFN- γ or IL4-specific monoclonal antibodies (BD PharMingen) per well. Splenocytes were isolated from the mice as described previously (Baca-Estrada *et al.*, 1996), resuspended in AIM-V medium (Gibco-BRL) and added to the coated plates at 10^6 cells per well in the absence or presence of rNS3 at a final concentration of 1 $\mu\text{g ml}^{-1}$. After a 20 h incubation at 37 °C and 5 % CO_2 , the plates were washed extensively and incubated with biotinylated anti-murine IFN- γ or IL4 monoclonal antibodies (BD PharMingen) at 2 $\mu\text{g ml}^{-1}$. This was followed by incubation with streptavidin–alkaline phosphatase (Gibco-BRL) at a 1:1000 dilution. The spots were visualized with a substrate consisting of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The number of cytokine-secreting cells was expressed as the difference between the number of spots per 10^6 cells in rNS3-stimulated wells and the number of spots per 10^6 cells in non-stimulated wells.

Proliferation assay. The splenocytes isolated from the immunized mice were dispensed at 3.5×10^6 cells ml^{-1} in AIM-V medium and cultured in 96-well tissue culture plates at 3.5×10^5 cells per well in the absence or presence of 1 $\mu\text{g rNS3 ml}^{-1}$. After 72 h in culture, the cells were pulsed with 0.4 μCi (14.8 kBq) [*methyl*- ^3H]thymidine (Amersham Pharmacia Biotech) per well. The cells were harvested 18 h later and radioactivity was determined by scintillation counting (TopCount NXT Microplate Scintillation & Luminescence Counters; Packard Instrument Company). The $\Delta\text{c.p.m.}$ was determined by subtracting background activity of cells incubated with medium only without antigen.

CTL assay. To prepare effector cells, splenocytes were isolated from each group of mice. Syngeneic splenocyte stimulators were prepared by infection for 1 h at 37 °C with a recombinant vaccinia virus VP1461, which encodes NS3/NS4/NS5 from HCV-1b strain BK (kindly provided by Sanofi Pasteur MSD, Toronto, Canada), at an m.o.i. of 10. After infection, the stimulators were suspended at a concentration of 10^6 cells ml^{-1} and irradiated with 30 Gy. The splenocytes from each group were cultured with the stimulators at 37 °C and 5 % CO_2 for 5 days in AIM-V medium. Mouse IL2 (Roche) was added to a final concentration of 5 U ml^{-1} after 2 days. To generate target cells, P815 cells (H-2^d) (ATCC) were stably transformed with NS3. NS3-transformed and control P815 cells were labelled for 1 h with 100 μCi (3.7 MBq) $\text{Na}_2^{51}\text{CrO}_4$ per 10^6 cells. Cells were washed four times and used as targets at 5×10^4 cells ml^{-1} . One

hundred microlitres of labelled target cells was added to each well of a U-bottom 96-well plate and 100 μ l of effector cells were added to the target cells in triplicate wells at various effector-to-target (E:T) ratios. Plates were incubated for 4 h at 37 °C and 5 % CO₂. The supernatant from each well was counted in a 1470 Wizard gamma counter (Perkin Elmer). The percentage specific cytotoxicity was calculated as [(experimental ⁵¹Cr release–spontaneous release)/(total ⁵¹Cr release–spontaneous release)] \times 100.

Recombinant vaccinia virus challenge and plaque assay. Ten days after the last immunization, mice were challenged intraperitoneally with 5×10^6 p.f.u. of recombinant vaccinia virus encoding NS3/NS4/NS5, designated VP1461. Five days after challenge, mice were scarified and the ovaries were harvested, homogenized and sonicated. Recombinant vaccinia virus titres were determined by plating 10-fold dilutions of the homogenized ovaries onto BSC-1 cells. The BSC-1 cells were stained with 0.075 % (w/v) crystal violet (Murata *et al.*, 2003; Pancholi *et al.*, 2003) to identify the vaccinia virus-infected cells.

Statistical analysis. All data were analysed with the aid of a software program (GraphPad Prism 3.0). Differences between the means of experimental groups were analysed using an independent two-tailed *t*-test.

RESULTS

Efficient uptake of recombinant NS3 protein by DCs using DOTAP

The cationic lipid DOTAP was used to deliver rNS3 or HSA into DCs after 9 days of culture. The uptake of rNS3 protein by DCs was analysed by immunohistochemistry 6 h after pulsing. Strong expression of rNS3 was detected in the cytoplasm of the rNS3-pulsed DCs (Fig. 1a), whereas no signal was detected in the HSA-pulsed DCs as expected (Fig. 1b).

Induction of phenotypic maturation of pulsed DCs exposed to CpG ODN 1826 *in vitro*

After 9 days of culture, DCs were pulsed with rNS3 or HSA and stimulated with 25 μ g CpG ODN ml⁻¹ for 18 h or left untreated. Subsequently, the cell surface antigen expression of the DCs was evaluated by flow cytometry. CD11c-positive cells ranged from 86.78 to 94.16 %, indicating that about 90 % of the cells were DCs in all cultures. No phenotypic differences were detected between DCs pulsed with rNS3 and DCs pulsed with HSA. However, DCs stimulated with CpG ODN expressed enhanced levels of CD40, MHC class II and CD86 compared with unstimulated DCs. The CpG ODN treatment, in particular, dramatically increased the expression of CD40, which functions in the adaptive immune response as a trigger for the expression of co-stimulatory molecules for efficient T-cell activation (Fig. 2).

Production of IL12 and induction of allogeneic T-cell responses by pulsed DCs exposed to CpG ODN 1826 *in vitro*

To functionally evaluate the DCs, we measured IL12 and IL10 secretion and evaluated primary allogeneic and syngeneic MLRs. DCs stimulated with CpG ODN produced high levels of IL12, whereas unstimulated DCs did not generate IL12 (Fig. 3a). No IL10 production was detected (data not shown) by any DCs. The production of high levels of IL12, but no IL10 by DCs stimulated with CpG ODN suggests the development of a Th1-biased response, which is likely to clear HCV most efficiently. The results also showed that DCs matured with CpG ODN induced a stronger allogeneic T-cell response compared with untreated DCs, indicating a more efficient antigen presenting capacity of the CpG ODN-treated DCs (Fig. 3b). There were no differences between rNS3-pulsed DCs and HSA-pulsed DCs with respect to cytokine production and MLR (Fig. 3).

Induction of HCV NS3-specific cellular immune responses

To assess the potential of the rNS3-transduced DCs to induce specific cellular immune responses, mice were immunized with the differently treated DCs and sacrificed 10 days after the last immunization. Splenocytes were isolated to measure lymphocyte proliferation, cytokine production and CTL activity. All groups of mice developed a significant NS3-specific lymphocyte proliferative response when compared with the animals immunized with PBS. Furthermore, the groups vaccinated with rNS3-pulsed DCs developed significantly ($P<0.001$) stronger NS3-specific lymphocyte proliferative responses than the groups vaccinated with HSA-pulsed DCs. The mice vaccinated with CpG ODN-matured, rNS3-pulsed DCs showed significantly ($P<0.001$) stronger NS3-specific lymphocyte proliferation than the animals vaccinated with unstimulated rNS3-pulsed DCs. Both groups of mice immunized with rNS3-pulsed DCs developed significantly ($P<0.001$) stronger NS3-specific lymphocyte proliferation than the group vaccinated with rNS3 formulated with alum, whereas the mice immunized with rNS3 formulated with alum had a stronger NS3-specific proliferative response than the animals immunized with HSA-pulsed DCs ($P<0.01$) (Fig. 4).

The mice vaccinated with rNS3-pulsed DCs developed high numbers of IFN- γ and low numbers of IL4-secreting cells, indicating that a Th1-biased immune response was induced. Furthermore, the animals immunized with CpG ODN-matured, rNS3-pulsed DCs produced significantly ($P<0.05$) higher numbers of IFN- γ -secreting cells than the animals vaccinated with untreated rNS3-pulsed DCs. In contrast, the group of mice immunized with rNS3 formulated with alum developed relatively high numbers of IL4-secreting cells and low numbers of IFN- γ -secreting cells, indicating that a Th2-biased immune response was induced (Fig. 5).

Since CTL responses are essential to eliminate virus from infected cells, we studied the ability of splenocytes derived from the mice subjected to the different vaccination regimens to lyse P815 target cells stably expressing NS3 protein. As shown in Fig. 6, both groups of mice immunized with rNS3-pulsed DCs developed a CTL response. However, the CTL response obtained by immunization with rNS3-pulsed DCs stimulated with CpG ODN was significantly stronger than that observed after immunization with unstimulated rNS3-pulsed DCs ($P<0.05$). In contrast, as expected, the mice immunized with HSA-pulsed DCs or rNS3 protein formulated with alum did not develop a NS3-specific CTL response. These results were confirmed in a

second trial and demonstrate the induction of a NS3-specific CTL response in mice immunized with rNS3-pulsed DCs. In addition, the fact that P815 is an Ia-negative cell line (Mozdzanowska *et al.*, 2000) indicates that these cytotoxic T-cell responses are mediated by NS3-specific CD8 cells.

Protection against challenge with recombinant vaccinia virus expressing NS3/NS4/NS5

Since HCV does not infect mice, we used a previously described surrogate challenge model (Pancholi *et al.*, 2003) to assess the efficacy of adoptive transfer of rNS3-pulsed DCs in the protection of mice against virus infection. Ten days after the last immunization, all mice were challenged with 5×10^6 p.f.u. of recombinant vaccinia virus expressing NS3/NS4/NS5. Five days after challenge, the mice were sacrificed and the presence of recombinant vaccinia virus in the ovaries was determined. As shown in Fig. 7, the mean vaccinia virus titres decreased at least five orders of magnitude in mice vaccinated with rNS3-pulsed DCs stimulated with CpG ODN ($P < 0.001$) and decreased four orders of magnitude in mice vaccinated with unstimulated rNS3-pulsed DCs ($P < 0.01$) compared with the mice immunized with PBS. The two groups of animals immunized with HSA-pulsed DCs also shed less virus than the PBS group ($P < 0.05$), but the reduction in virus titre was at most 1 log. This was most likely due to the development of natural killer cell activity (van den Broeke *et al.*, 2003). No reduction in virus titre was seen in the group immunized with NS3 and alum.

DISCUSSION

Several reports have shown that DC function and maturation are impaired during persistent HCV infection (Auffermann-Gretzinger *et al.*, 2001; Larsson *et al.*, 2004; Sarobe *et al.*, 2002, 2003). If we assume that this results in impaired antigen presentation, which in turn fails to result in an expansion of the Th cell population, then the result will be a limited expansion of the humoral and cell-mediated immune response. Indeed, characteristics of HCV infection include low HCV-specific antibody (Chen *et al.*, 1999) and a low frequency of HCV-specific CTLs (Rehermann & Chisari, 2000), both of which are consistent with a failure of CD4⁺ Th cells to induce proliferation of the B- and CTL-effector cells. IFN treatment of HCV carriers indicates that the frequency of HCV core-specific Th cell precursors is significantly higher in sustained responders than in transient or non-responders (Lasarte *et al.*, 1998), suggesting that the expansion of Th cell precursors correlates with and is critical for HCV elimination. There is therefore a high probability that DCs from HCV carriers that are loaded and matured *ex vivo* with HCV proteins followed by autologous transfusion will be able to prime naive T-cells and/or stimulate existing HCV-specific cellular immunity. The aim then is to change the equilibrium between the virus and the immune response in patients that will result in viral clearance. In addition, it has been suggested that the number of impaired DCs is small (Sarobe *et al.*, 2002), so autologous transfusion of a large number of HCV antigen-loaded matured DCs may overcome the impairment.

The therapeutic potential of DC-based immune interventions has been reported for a variety of murine tumour models and more recently in human clinical trials. However, only two studies have explored the *in vivo* efficacy of DC-mediated vaccination against HCV. One study showed that core-specific CTL are effectively primed in mice by injecting DCs treated *in vitro* with an anthrax toxin fusion protein composed of a lethal factor (LF) and the HCV-core 133–142 epitope (Moriya *et al.*, 2001). However, epitope-based DC immunotherapies induce limited CTL responses and are only applicable in patients with the appropriate HLA haplotype. In the other study, DCs transfected with recombinant adenovirus expressing HCV core (Adex1SR3ST) more efficiently prime core-specific CTLs than Adex1SR3ST-transfected macrophages or DCs treated with an anthrax toxin fusion protein mentioned above. Upon challenge with recombinant HCV-core-expressing vaccinia virus, vaccinia virus titres were significantly reduced in mice immunized with Adex1SR3ST-transfected DCs (Matsui *et al.*, 2002). Although the strategy of vaccination with DCs transfected with recombinant virus containing HCV genes might result in efficient transfection and high levels of transgene expression in DCs, it may also negatively impact DC functions. In addition, immunodominant viral products could suppress an immune response against the transgene in an unpredictable manner. Since a variety of practical and theoretical concerns may limit the utility of these methods in patients, DCs pulsed with HCV proteins as an alternative strategy would be more efficient, safer and more feasible for human immunotherapy.

DCs are capable of internalizing macromolecules by macropinocytosis and receptor-mediated endocytosis (Sallusto *et al.*, 1995). Thereby, exogenous proteins are usually processed and presented via the MHC class II pathway. However, DCs are able to channel

antigenic peptides efficiently towards MHC class I, a process termed 'cross priming' (Rodriguez *et al.*, 1999). In this study, we used the cationic liposomal transfection reagent DOTAP to deliver HCV NS3 protein to DCs. The rNS3-pulsed DCs induced strong cellular immune responses and protection against recombinant vaccinia virus containing the HCV NS3 gene. Several studies reported that if proteins were precomplexed with the DOTAP, cytoplasmic uptake of proteins was increased, and processing and presentation of incorporated protein by the MHC class I pathway was further enhanced (Nonn *et al.*, 2003; Santin *et al.*, 1999).

Recently, CpG ODNs have attracted a great deal of attention as a novel and safe adjuvant. Indeed, CpG DNA induces stronger immune responses with less toxicity than other adjuvants (Lonsdorf *et al.*, 2003; Oumouna *et al.*, 2005; Weeratna *et al.*, 2000). CpG ODNs bind to TLR9 and preferentially induce Th1-biased immune responses with the production of cytokines such as IL12 and IFN- γ (Harandi *et al.*, 2003; Rao *et al.*, 2004). In this study, we used ODN 1826, a mouse-specific CpG ODN, to mature protein-pulsed DCs. The results show that the CpG ODN caused upregulation of MHC class II, CD86 and CD40 and triggered cytokine release such as IL12. CpG ODN 1826, in particular, dramatically increased the expression of CD40 on DCs. CD40 has emerged as a key signalling molecule for the function of DCs in the immune system. CD40 is expressed by DCs and is upregulated when DCs migrate from the periphery to draining lymph nodes in response to microbial challenge. CD40 functions in the adaptive immune response as a trigger for expression of co-stimulatory molecules, and for potent T-cell activation (O'Sullivan & Thomas, 2003). DC stimulation via CD40 also has the capacity to induce high levels of the cytokine IL12, which can activate natural killer cells to enhance proliferation of CD8⁺ T cells, and polarize CD4⁺ T cells toward a Th1 type (O'Sullivan & Thomas, 2003), which plays a major role in clearing HCV. In this study, DCs matured with CpG ODN produced a greater allogeneic T-cell response compared with DCs not treated with CpG ODN, indicating that CpG ODN-mediated DC maturation/activation is associated with a more efficient functional transition to professional APC. Notably, there were no differences between rNS3-pulsed DCs and HSA-pulsed DCs with respect to phenotype, cytokine production and MLR implying that transduction with rNS3 protein does not impair DC functions.

Our study also demonstrates that compared with the unstimulated rNS3-pulsed DCs, the rNS3-pulsed DCs matured with CpG ODN 1826 induced more robust cellular immune responses including enhanced cytotoxicity, higher IFN- γ production and stronger lymphocyte proliferation in mice. Upon challenge with a recombinant vaccinia virus expressing HCV NS3, the two groups of mice vaccinated with rNS3-pulsed DCs showed a remarkable reduction in vaccinia virus titre compared with mock-immunized controls, and the mice immunized with the rNS3-pulsed DCs matured with CpG ODN showed stronger immune protection than those not matured with CpG ODN.

These data have important clinical implications and are the first to show that NS3-pulsed DCs can induce specific immune responses and provide antiviral protection. CpG ODNs as non-toxic, safe agent would be useful in the development of DC vaccines. Thus, this study provides a novel vaccination strategy against hepatitis C that ultimately should be transferable to humans.

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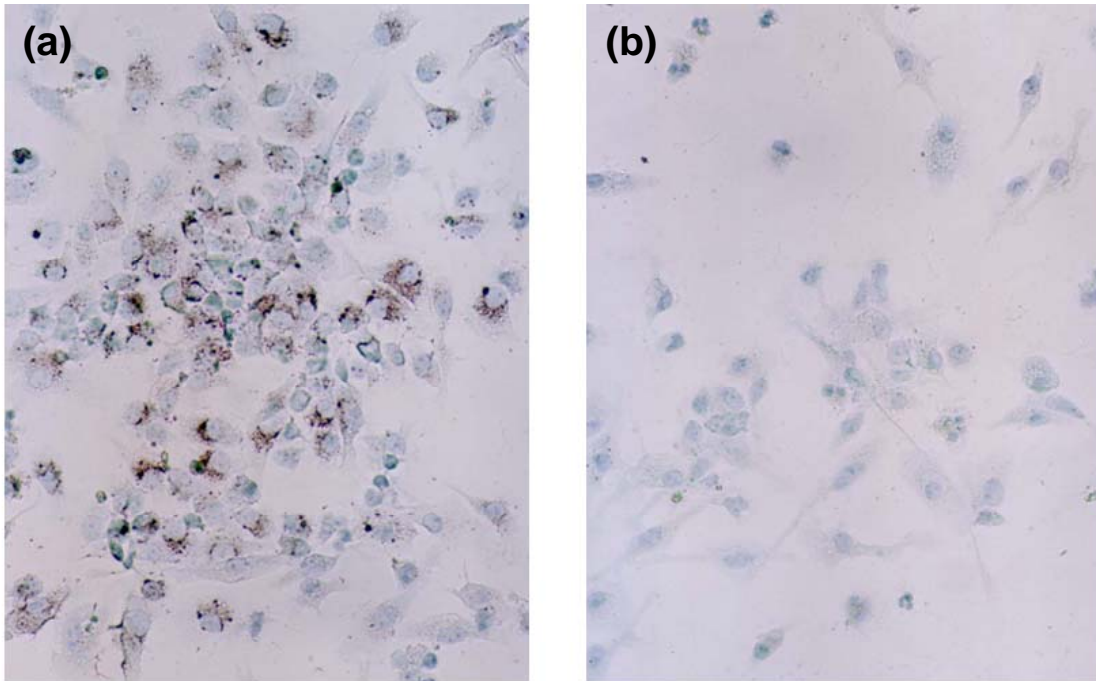


Fig. 1. Protein uptake identified by immunohistochemistry in DCs pulsed with rNS3 protein (a) or a control protein, HSA (b). The cationic lipid DOTAP was used to deliver rNS3 or HSA into the DCs. The presence of rNS3 in the DCs 6 h after pulsing was visualized with an NS3-specific polyclonal rabbit antibody followed by a biotinylated goat anti-rabbit IgG (Zymed) and a DAB substrate (Vector laboratories).

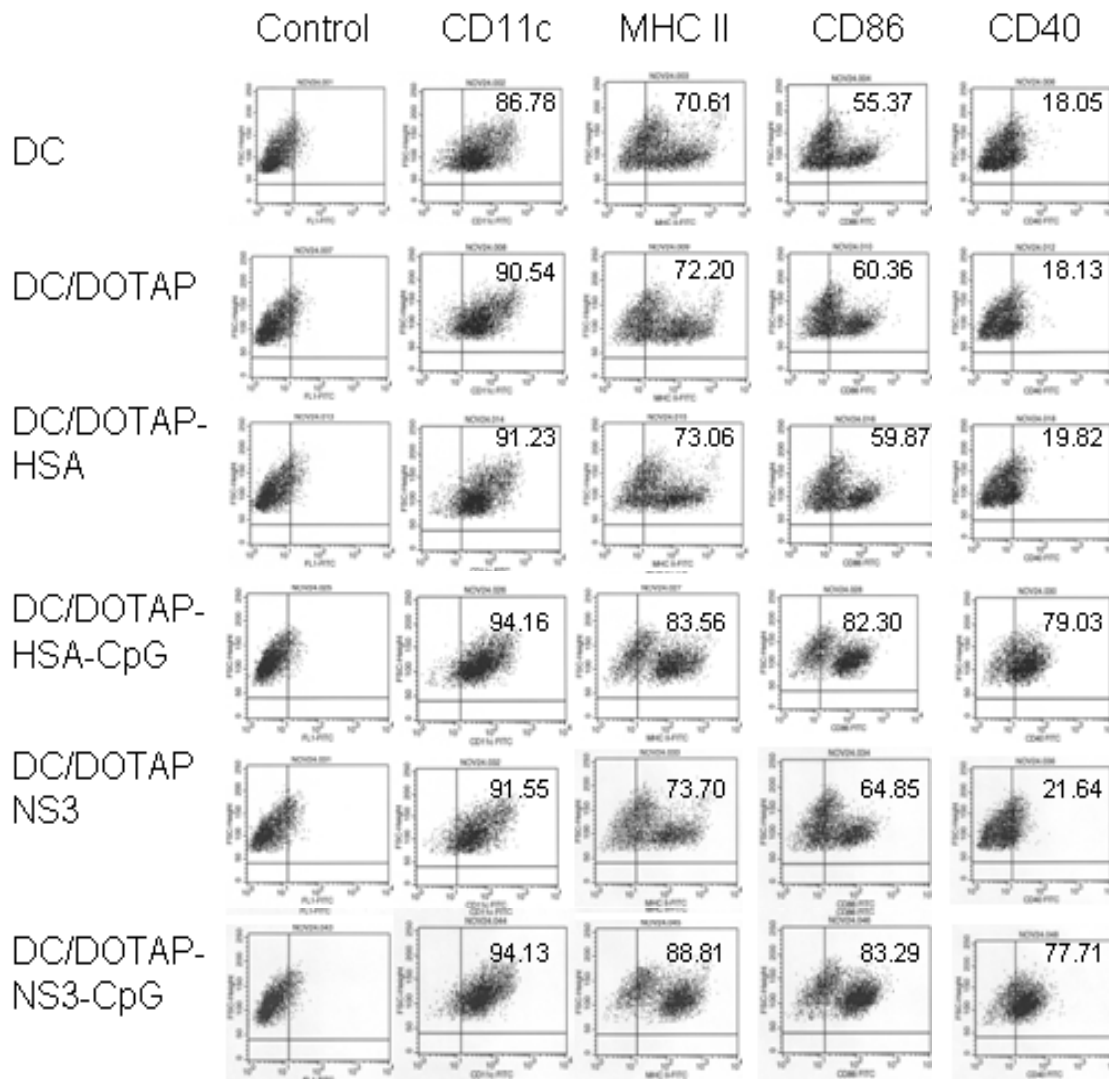


Fig. 2. Enhanced phenotypic maturation of DCs stimulated with CpG ODN 1826 *in vitro*. After 18 h incubation of the rNS3- or HSA-pulsed DCs stimulated with or without 20 ng CpG ODN 1826 ml⁻¹, surface phenotypes of the DCs were gated and analysed by flow cytometry. DCs treated with DOTAP (DC/DOTAP), DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3) and DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG). Each number indicates the percentage of positive cells for a representative experiment. These experiments were repeated twice with similar results.

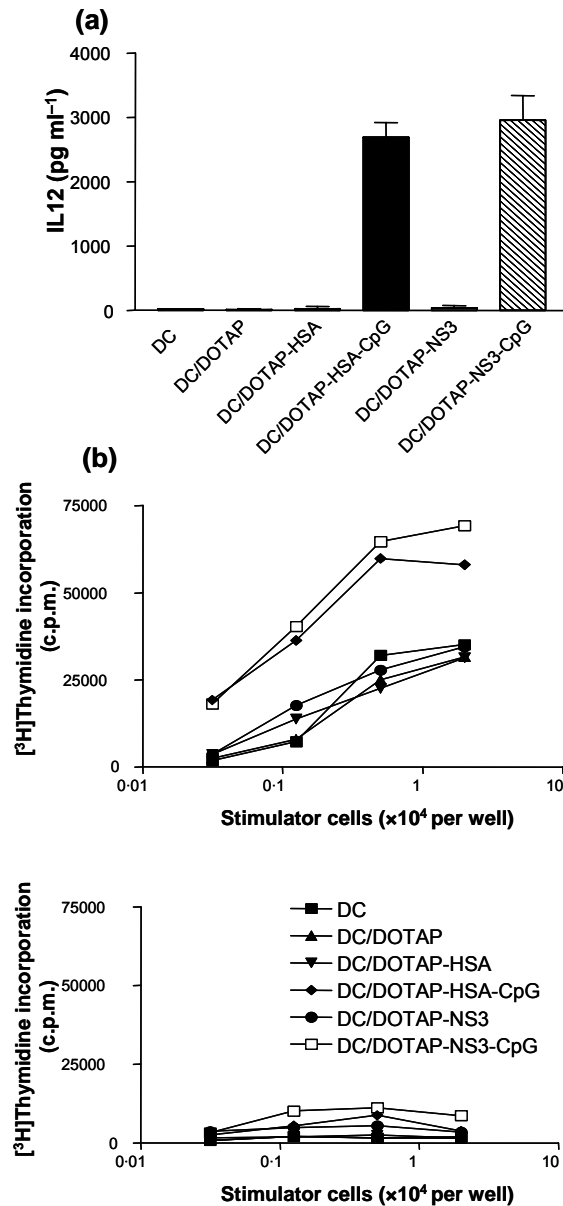


Fig. 3. Functional evaluation of DCs subjected to different treatments. (a) IL12 production by DCs. Supernatants of cultures of DCs that had been treated as shown were assayed for IL12p70 levels by ELISA. The results are representative of three independent experiments. (b) Primary allogeneic and syngeneic MLR of DCs generated from the bone marrow of BALB/c mice. Different numbers of irradiated (50 Gy) DCs (stimulator cells) were incubated in U-bottom 96-well plates with 2×10^5 cells allogeneic (C57BL/6) or syngeneic (BALB/c) T cells (responder cells) per well. Uptake of [³H]thymidine (c.p.m.) was determined on day 5. (b, upper panel) Allogeneic (C57BL/6) T cells as responder cells. (b, lower panel) Syngeneic (BALB/c) T cells as responder cells. DCs treated with DOTAP (DC/DOTAP), DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3) and DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG). Similar results were obtained in three separate experiments.

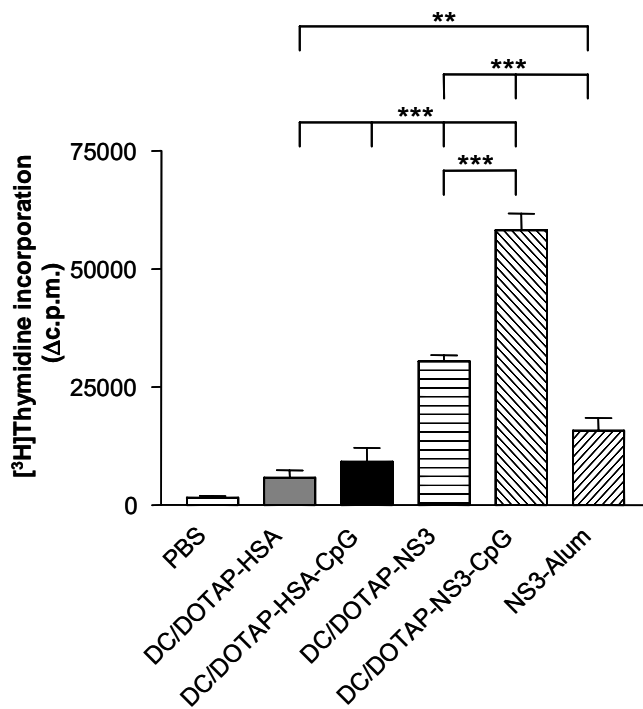


Fig. 4. Antigen-specific lymphocyte proliferative responses. Mice were vaccinated with PBS, DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3), DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG) or rNS3 and alum (NS3-Alum). Splenocytes were cultured at 3.5×10^6 cells ml^{-1} in medium or with $1 \mu\text{g}$ rNS3 ml^{-1} . After 72 h in culture, the cells were pulsed with [*methyl*-³H]thymidine. The cells were harvested 18 h later and radioactivity was determined. The $\Delta\text{c.p.m.}$ was calculated by subtracting background activity (incubation without rNS3). $n=6$. **, $P<0.01$; and ***, $P<0.001$.

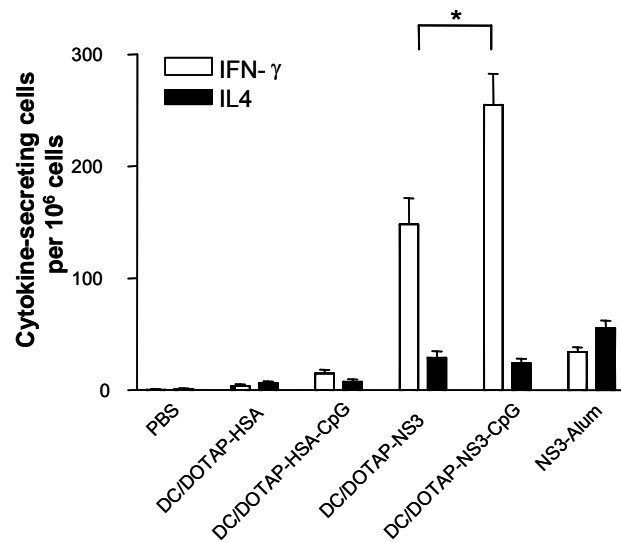


Fig. 5. IFN- γ and IL4 secretion detected by ELISPOT assay. Mice were vaccinated with PBS, DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3), DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG) or rNS3 and alum (NS3-Alum). Splenocytes were restimulated *in vitro* for 20 h with 1 $\mu\text{g rNS3 ml}^{-1}$ or left untreated. Differences in the number of cytokine-secreting cells per 10⁶ cells in rNS3-stimulated wells and the number of cytokine-secreting cells per 10⁶ cells in non-stimulated wells are shown. The results represent the mean of triplicate wells and are expressed as the mean \pm SEM of NS3-induced cytokine-secreting cells per 10⁶ cells for groups of six mice. *, $P < 0.05$.

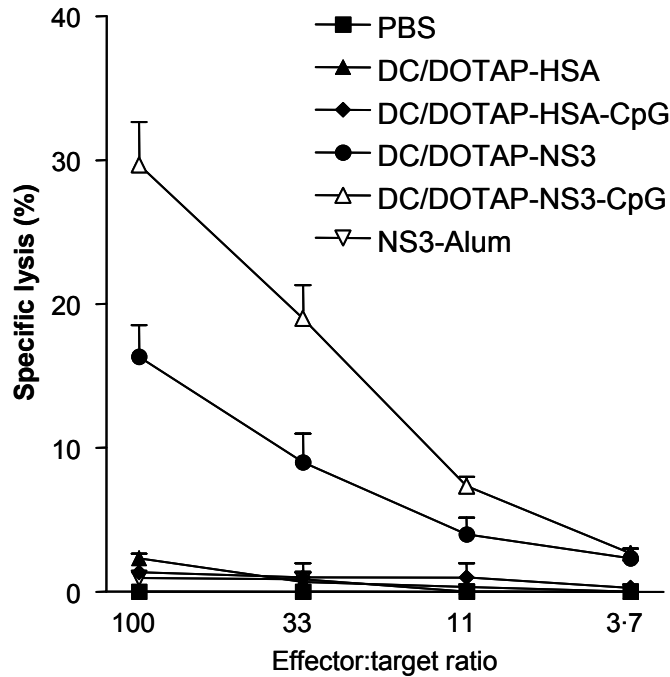


Fig. 6. CTL responses to NS3 at different E:T ratios. Mice were vaccinated with PBS, DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3), DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG) or rNS3 and alum (NS3-Alum). Ten days after the last immunization, splenocytes were prepared and incubated *in vitro* for 5 days with irradiated syngeneic spleen cell stimulators infected with recombinant vaccinia virus – NS3/NS4/NS5. CTL activity was determined in a 4 h ⁵¹Cr release assay against an NS3-transformed P815 cell line. Background activity against P815 cells was subtracted to obtain specific lysis. Data are shown as the mean±SEM of three mice. Similar results were obtained in another independent experiment.

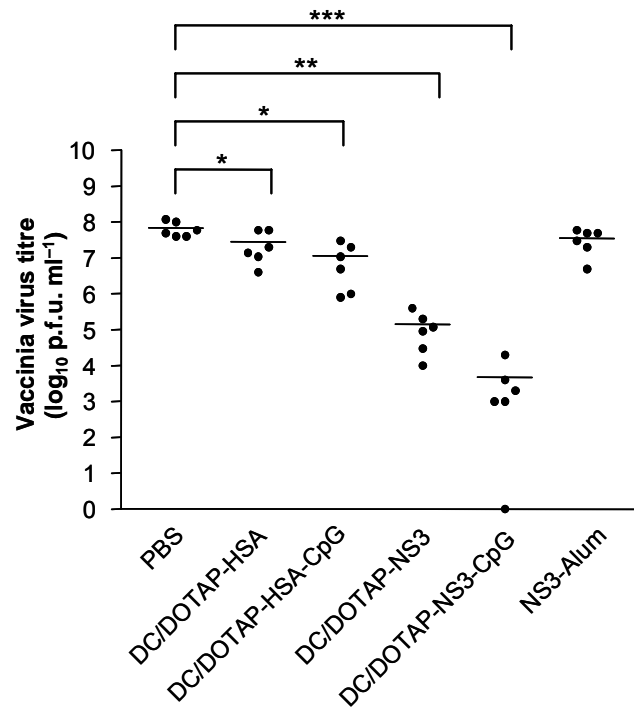


Fig. 7. Vaccinia virus titres in the ovaries of mice challenged with recombinant vaccinia virus expressing NS3/NS4/NS5, VP1461. Mice were vaccinated with PBS, DCs pulsed with HSA (DC/DOTAP-HSA), DCs pulsed with HSA and stimulated with CpG ODN (DC/DOTAP-HSA-CpG), DCs pulsed with rNS3 (DC/DOTAP-NS3), DCs pulsed with rNS3 and stimulated with CpG ODN (DC/DOTAP-NS3-CpG) or rNS3 and alum (NS3-Alum). Ten days after the last immunization, mice were challenged intraperitoneally with 5×10^6 p.f.u. of HCV NS3-encoding recombinant vaccinia virus. Five days after challenge, the ovaries were removed and vaccinia virus titres were measured in BSC-1 cells. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.