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Carcinoembryonic antigen (CEA) presentation and specific T cell-priming by human dendritic cells transfected with *CEA*-mRNA

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Abstract

The feasibility of dendritic cells (DC) for cancer immunotherapy after transfection by electroporation with mRNA encoding the human carcinoembryonic antigen (CEA) was investigated. Both, total RNA from the CEA^+ colon cancer cell line SW480 and mRNA transcribed *in vitro* from cDNA3.1-plasmids (pcDNA3.1+/-HisC) with a *CEA*-insert (ivt-*CEA*-mRNA, ivt-*CEA*/HisC-mRNA) were used. Labelled ivt-*CEA*-mRNA was detectable in DC by light and electron microscopy and by fluorescence-activated cell-sorting (FACS) even 15 min after electroporation. Four hours after transfection with ivt-*CEA*/HisC-mRNA, we detected specific expression of CEA and the histidine-tag by immunofluorescence microscopy and by FACS. CEA-specific T lymphocytes were successfully primed by transfected DC and were able to lyse CEA-expressing target cells, even from the CEA-expressing human colon adenocarcinoma cell line SW480. Thus, DC transfected by electroporation with *CEA*-mRNA are valuable tools for the immunotherapy of CEA^+ tumour entities.

Keywords: Antigen presentation; Carcinoembryonic antigen; Dendritic cells; Human; Immunotherapy; mRNA

1. Introduction

Immunotherapy targeted against cancer aims to mobilise the immune system for destroying tumour cells [1]. Tumour-associated antigens (TAA) as targets for immunotherapy have been found to be expressed by diverse tumours [2]. Many TAA-expressing malignancies are only weakly recognised by the immune system which may be one mechanism by which tumours escape recognition [3]. Since antigen presentation by the tumour cells themselves appears insufficient to induce an adequate immune response, professional antigen-presenting cells (APC) are required [4,5]. Dendritic cells (DC) are the most potent APC, crucial for the initiation of T cell responses *in vitro* and *in vivo*. They are capable of activating naïve $CD4^+$ and $CD8^+$ T lymphocytes by antigen presentation on their surface major histocompatibility complex (MHC) class I and II molecules [6]. Both, $CD4^+$ and $CD8^+$ T cells are required for an efficient anti-tumour immune response [7].

The first clinical applications of the use of DC for cancer immunotherapy have recently been published using blood-derived DC loaded in *vitro* with TAA [8,9]. The results of this kind of adoptive immunotherapy are very promising. Nevertheless, there are still difficulties concerning antigen delivery to the DC. TAA delivered as whole protein are usually processed towards MHC II presentation, which activates $CD4^+$, but not $CD8^+$ cytotoxic T lymphocytes (CTL) [10]. For CTL activation, the MHC I complex on DC can be loaded with immunogenic peptides that have to fit to the polymorphic and patient-specific human leucocyte antigen (HLA) haplotype [11]. Therefore, patients must be classified by their HLA type, and binding properties of the immunogenic peptides for the specific HLA have to be characterised. One way to avoid this problem is the use of TAA encoded by DNA [12]. For this purpose, the patient-specific TAA gene has to be cloned into a vector before transfer into the DC, a labour-intensive and

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time-consuming process. Additionally, there is no optimal expression system for human DC.

Another approach is the introduction of mRNA encoding TAA into DC [13]. This idea is very appealing as RNA can be extracted from small, even embedded tumour samples and amplified *in vitro* [14]. The mRNA extracted from the tumour is patient-specific and encodes all TAA expressed by the tumour sample.

Few studies have used DC transfected with mRNA [13–17]. Among these studies, some used mRNA encoding the human carcinoembryonic antigen (CEA) [14,16,17]. CEA, a 180 000 kDa oncofetal glycoprotein, is highly expressed by adenocarcinomas of colon, breast and lung, by the fetal colon, and, at a lower level, by normal colonic epithelium [18,19]. As CEA-expressing malignancies are only weakly recognised by the immune system [3,19], various immunotherapeutical approaches were tested to enhance immune surveillance against these widespread tumour entities [19].

Transfection of DC with *CEA*-mRNA performed by lipofection successfully induced CEA-specific $CD4^+$ and $CD8^+$ T lymphocytes *in vitro* [14,16,17]. Moreover, in mice specific *in vivo* CTL response and regression of lung metastases were achieved [14]. These promising results using DC transfected with TAA-encoding RNA justify further investigations to obtain more experience with this important immunotherapeutical approach. Therefore, a variety of experimental settings are necessary to improve safety and efficacy before these results can be transferred into clinical use [20].

As none of the studies so far used electroporation for DC transfection or further characterised the CEA-specific T cells generated by the mRNA-transfected DC, we focused on these aspects. Moreover, CEA expression by transfected DC has not yet been detected. Therefore, we intended in this *in vitro* study to characterise CEA expression by human DC transfected via electroporation with *CEA*-mRNA. First, we tested whether electroporation might be suitable for transfection as regards the efficiency and influence on DC function. Second, we investigated CEA protein expression. Finally, we generated and characterised *in vitro* CEA-specific CD4+ and $CD8^+$ T cells using autologous DC treated with *CEA*-mRNA.

2. Materials and methods

2.1. Generation of CEA-mRNA

Total tumour RNA was extracted from the human HLA-$A2.1^+$ CEA-expressing colon adenocarcinoma cell line SW480 (ATCC, Rockville, MD, USA) using Ultraspec II RNA isolation system (Biotecx, Houston, TX, USA), *in vitro*-transcribed (ivt)-*CEA*-mRNA generated from the plasmid pcDNA3.1-*CEA* (kindly provided by Judy Kantor, NIH, Bethesda, MD, USA), and ivt-*CEA*/HisC-mRNA from pcDNA3.1/HisC-*CEA* expressing the full-length 2.1 kb *CEA* sequence. Plasmid DNA was linearised with *Sal 1* (Amersham Pharmacia Biotech, Dübendorf, Switzerland) for subsequent *in vitro* transcription with the Ribomax Large Scale RNA Production System T7 (Promega, Madison, WI, USA). Fluorochrome-labelled ivt-*CEA*-mRNA was produced by *in vitro* transcription using Alexa Fluor 488-5-uridine triphosphate (UTP) and gold-labelled ivt-*CEA*-mRNA using biotin-11-deoxyuridine triphosphate (dUTP) (both Molecular Probes, Leiden, The Netherlands) by combining the manufacturers' *in vitro* transcription protocols. Gold-labelling of the biotin-coupled ivt-*CEA*-mRNA was performed with nanogold-coupled streptavidin (Nanoprobes Inc., Stony Brook, NY, USA). RNA purity and concentration were evaluated by photospectrometric analysis of adsorbance at A_{260} and A_{280} and by 1% agarose gel electrophoresis stained with ethidium bromide (Fig. 1).

2.2. Generation of DC

Human DC were generated *in vitro* from blood-derived precursors as already described in Ref. [21]. Briefly, human peripheral blood mononuclear cells (PBMC) obtained from venous blood of healthy donors (Blood Bank SRK, Zurich, Switzerland) were isolated by Ficoll-Paque (Pharmacia Biotech) density centrifugation. PBMC were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with penicillin/streptomycin (all Life Technologies, Paisley, UK) and 10% heat-inactivated pooled human A serum (Blood Bank SRK) for 2 h. The adherent cells were then cultured in RPMI 1640 containing 5% heat-inactivated pooled human A serum in the presence of recombinant human (rh) granulocyte monocyte colony-stimulating factor (GM-CSF, 50 ng/ml, Novartis, Basel, Switzerland) and rh interleukin (IL)-4 (100 U/ml, R&D Systems, Abingdon, UK) for 1–5 days to obtain *in vitro*-generated DC.

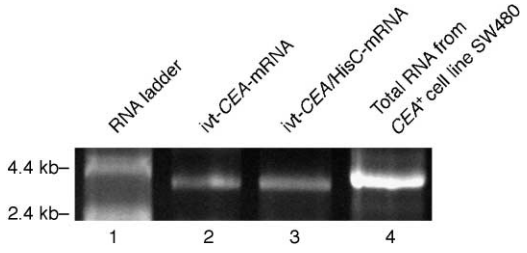

Fig. 1. Carcinoembryonic antigen (*CEA*)-mRNA preparations used for transfection. Quality of the RNA was analysed by electrophoresis with an ethidium bromide-stained 1% agarose gel (1). RNA molecular weight marker 9.5–0.24 kb; (2) 3.800 kb band containing 2,3ivt-*CEA*-mRNA from pcDNA3.1-*CEA*; and (3) ivt-*CEA*/HisC-mRNA from pcDNA3.1/HisC-*CEA*, respectively; (4) Total RNA extracted from colon cancer cell line SW480.

2.3. Transfection of DC

DC were electroporated with 5–10 μg of the various *CEA*-mRNA preparations per 1–5×10^6 DC in 0.8 ml RPMI 1640 in a 0.4 cm gap sterile cuvette (voltage: 0.2 kV, capacitance: 960 μFD) using the Gene Pulser System (all Biorad, Hercules, CA, USA).

To evaluate the influence of the mRNA and electroporation on DC, cells were tested by fluorescence-activated cell-sorting (FACS) with a FACScan instrument and the Cell Quest software (all Becton-Dickinson, San José, CA, USA) using antibodies (Abs, all at a dilution of 1:50) directed against CD83 (PharMingen, Becton-Dickinson), CD26, CD80 and CD86 (all from Serotec Ltd., Kidlington, Oxford, UK) and a mouse monoclonal (m)Ab directed against HLA-DR, DP and DQ (PharMingen, Becton-Dickinson). For a control, we used anti-γ_1-γ_1 Ab (Becton-Dickinson) and as secondary Ab, fluorescein isothiocyanate (FITC)-labelled polyclonal goat anti-mouse Ab (Kirkegard & Perry Lab., Gaithersburg, MD, USA).

Viability of the cells was tested using the fluorescein diacetate (FDA) viability test [22]. Furthermore, DC function was investigated in mixed lymphocyte reaction (MLR) assays. Briefly, DC electroporated with ivt-*CEA*-mRNA were co-incubated with allogeneic T lymphocytes derived *in vitro* from non-adherent PBMC from healthy donors in a 5 days assay (in quadruplets, 10^5 T cells and 10^4 DC per well, 96-well round-bottomed plates, Nunc, Roskilde, Denmark). Untreated DC and DC electroporated without RNA served as controls. After 5 days in culture, a 6-[^{3}H]-thymidine pulse (0.5 mCi/well, specific activity 20 Ci/mmol, Amersham Pharmacia Biotech) was administered. Thereafter, cells were harvested with the LKB harvesting system using glass fibre filtermats, and the incorporated 6-[^{3}H]-thymidine was detected by liquid scintillation using the Beta-plate liquid scintillation counting system (all components LKB, Wallac Oy, Turku, Finland).

Fluorescence of the DC treated with FDA or fluorescent-labelled ivt-*CEA*-mRNA was detected with confocal laser scanning microscopy (CLSM, Leica, Heidelberg, Germany) and FACS, respectively. DC transfected with nanogold-labelled ivt-*CEA*-mRNA were processed for transmission electron microscopy (TEM) as already described in Ref. [23]. The gold-labelling was intensified using silver enhancement (Nanoprobes Inc.).

2.4. CEA protein expression

CEA was detected with a polyclonal anti-CEA rabbit Ab (DAKO A/S, Glastrup, Denmark, diluted 1:200) followed by a biotinylated secondary goat anti-rabbit Ab and FITC-conjugated streptavidin (both BioScience, Emmenbrücke, Switzerland). The histidine-tag was stained with a mouse anti-HisC mAb (Invitrogen, Groningen, The Netherlands) followed by a biotin-coupled sheep anti-mouse Ab (Amersham Pharmacia Biotech, each diluted 1:100) and FITC-coated streptavidin. Nuclei were stained using 4′, 6-diamidine-2-phenyl-indole-dihydrochloride (DAPI, Boehringer Mannheim, Mannheim, Germany). For quantification of transfected cells with FACS, the anti-CEA rabbit Ab was used followed by FITC-labelled secondary goat anti-rabbit Ab (Kirkegard & Perry Lab., both diluted 1:50).

2.5. Generation of CEA-specific T lymphocytes

CEA-specific T cells were generated *in vitro* by weekly stimulation of non-adherent PBMC (5×10^6/ml) from HLA-A2$^+$ healthy donors with irradiated (1×10^4 rad, ^{60}Co source) autologous DC (10^5/ml, six-well-plates, Costar, NY, USA) either incubated with native CEA glycoprotein (2 μg/ml per 10^6 DC, Sigma, Buchs, Switzerland) or transfected with ivt-*CEA*-mRNA (5 μg/ml per 10^6 DC). The T cells were cultured in RPMI 1640 containing 5% human A serum and antibiotics. No additional cytokines were used for the first 3 weeks. After 3 weeks, the antigen-specific T cells were cultured in the presence of 100 U/ml rhIL-2 (R&D Systems). After 4 weeks, T cells were re-stimulated with solid phase-bound mitogenic anti-human CD3 mAb (clone CB3G) at the maximal stimulatory concentration [24]. FACS analysis was performed using FITC-labelled anti-human CD4, phycoerythrin (PE)-coated anti-human CD8 (both Serotec Ltd.), FITC-labelled anti-human CD3, PE-coated anti-human CD16 and anti-human CD56 (all Becton-Dickinson) Abs (dilution 1:50) on day 0 and after 3, 4, 5, 6 and 8 weeks in culture. Analysis was not performed after 1 and 2 weeks in cultivation so as to obtain a sufficient amount of cells for the various experiments.

2.6. CEA-specificity assays

The specificity of the T lymphocytes generated either by use of DC loaded with the native CEA glycoprotein (CEA protein-primed T cells) or by use of DC transfected with ivt-*CEA*-mRNA (ivt-*CEA*-mRNA-primed T cells) was tested after 4 weeks of stimulation in proliferation assays as already described in Ref. [21]. Briefly, the proliferative response of the T cells was measured in a 3 day assay by application of a 12-h pulse with 6-[^{3}]H-thymidine (0.5 mCi/well). Autologous DC either loaded with the native *CEA* glycoprotein or transfected with ivt-*CEA*-mRNA were washed and co-cultured with the CEA-specific T cells. Untreated DC served as negative controls. Anti-CD3 mAb [24] was used as a non-specific mitogenic stimulus for the positive controls. For blocking of MHC class II antigen presentation, we used the mouse mAb directed against HLA-DR, DP and DQ antigens. Cells were harvested and the incorporated 6-[^{3}H]-thymidine detected.

CEA-specific HLA-A2$^+$ T lymphocytes generated with DC transfected with ivt-*CEA*-mRNA were investigated in cytotoxicity tests after 8 weeks of stimulation. Their cytotoxic potential towards CEA-expressing tumours was investigated using the CEA$^+$ tumour cell line SW480 in a JAM test [25]. In brief, SW480 cells were labelled with 6-[^{3}H]-thymidine (0.5 mCi/ml) and exposed overnight to the HLA-A2$^+$ CTL in round-bottomed plates. Thereafter, cells were harvested and the incorporated 6-[^{3}H]-thymidine detected. As we did not have a CEA$^-$ colon cancer cell line as an adequate control, we further analysed the specificity of lysis in a fluorescence release assay [26] using 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) (5 μg/ml, Nova Biochem, Lucerne, Switzerland) as a marker. T2-A2 cells (HLA-A2$^+$, transporter associated with antigen-processing deficient T-B cell hybrid, kindly provided by F. Nestlé, Department of Dermatology, University of Zurich) were pulsed for 2 h with the HLA-A2.1-restricted CEA-associated peptide-1 (CAP-1, 1μg/ml) [27] and subsequently labelled with BCECF/AM. Unpulsed T2-A2 cells served as negative controls. HLA-A2$^+$ ivt-*CEA*-mRNA-specific CTL were co-incubated with T2-A2 cells at various ratios of effector to target cells (E:T ratio) for 90 min in 96-well round-bottomed plates. Thereafter, plates were centrifuged and the pellets resuspended in 2% Nonidet (N)P-40/5 mM borate lysis buffer. After transfer of the cell suspensions into flat-bottomed plates (Nunc), fluorochrome release was detected with Cytofluor 2300 (Millipore, Volketswil, Switzerland). Specific lysis was calculated as: (maximal release−specific release)/(maximal release−spontaneous release)×100.

3. Results

3.1. Transfection of human DC with CEA-mRNA by electroporation

Various *CEA*-mRNA preparations were used for transfection: ivt-*CEA*-mRNA, ivt-*CEA*/HisC-mRNA and total tumour RNA containing *CEA*-mRNA (Fig. 1).

Transfection efficiency of human DC by electroporation with *CEA*-mRNA was analysed using fluorochrome-

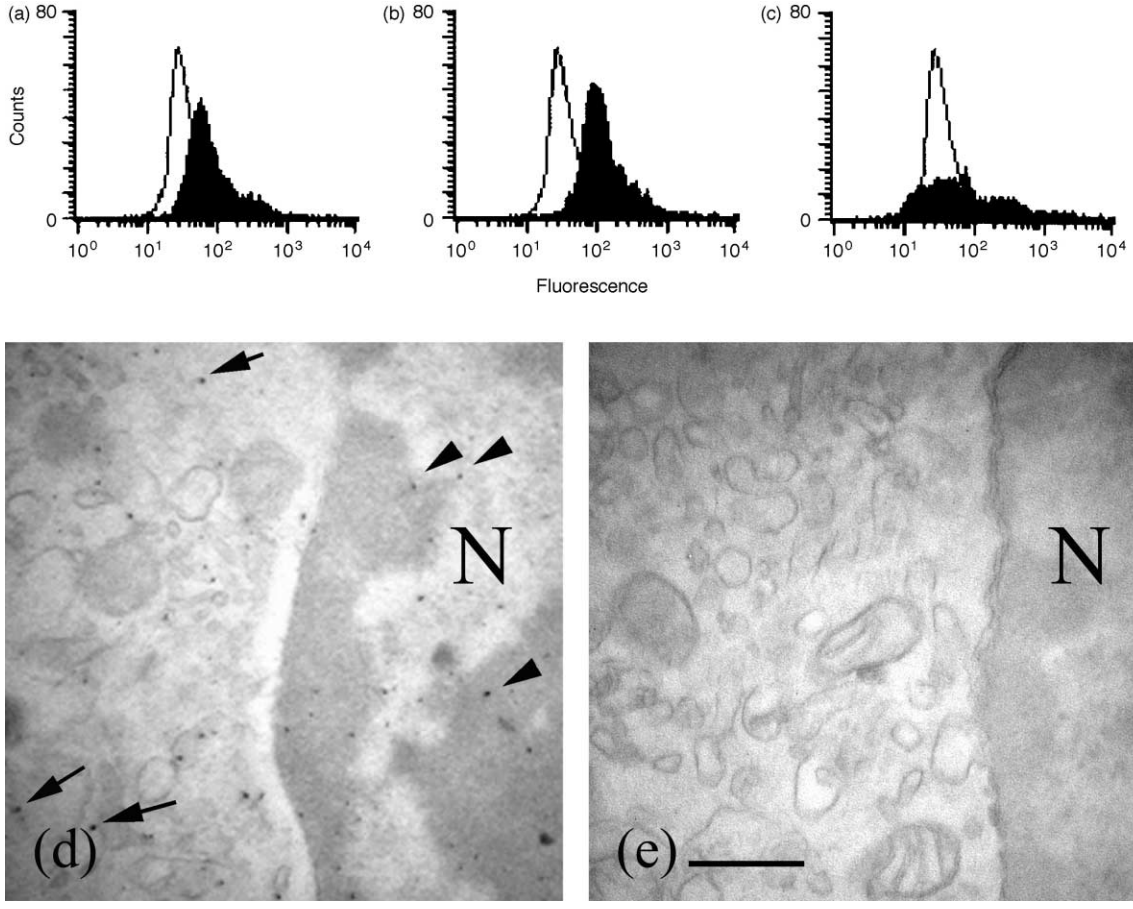

Fig. 2. Transfection of dendritic cells (DC) by electroporation with labelled *CEA*-mRNA. Fluorescence-activated cell-sorting (FACS): (a) DC co-incubated with fluorochrome-labelled ivt-*CEA*-mRNA (solid area) before electroporation, (b) 15 min after electroporation, (c) 4 h after electroporation. Control (a–c): DC electroporated without RNA (empty area). Transmission electron microscopy (TEM): (d) Localisation of nanogold-labelled ivt-*CEA*-mRNA 15 min after transfection in the cytosol of the DC (arrows) and in the nucleus (N, arrowheads). (e) Control DC electroporated without RNA. Bar 0.5 μm.

and gold-labelled ivt-*CEA*-mRNA preparations. CLSM revealed fluorochrome-labelled ivt-*CEA*-mRNA at the cell surface of the DC before electroporation and in the cytoplasm 15 min after electroporation (data not shown). FACS analysis showed a slight increase (half a log) of the fluorescence of the DC before electroporation due to fluorochrome-labelled ivt-*CEA*-mRNA bound to the cell surface (Fig. 2a). 15 min after electroporation, a pronounced increase (1 log) of the fluorescence of the DC transfected with fluorochrome-labelled ivt-*CEA*-mRNA was detected compared with DC electroporated without RNA (Fig. 2b). 4 h after electroporation with fluorochrome-labelled ivt-*CEA*-mRNA, fluorescence in the transfected cells decreased to the control level, probably due to RNA degradation (Fig. 2c). To localise the transfected *CEA*-mRNA at the ultrastructural level, nanogold-labelled ivt-*CEA*-mRNA was visualised by silver enhancement. 15 min after transfection, nanogold particles were found in large amounts in the cytosol of DC and in small amounts in the nucleus (Fig. 2d). DC electroporated without RNA showed no labelling (Fig. 2e). After 4 h, nanogold-labelled ivt-*CEA*-mRNA was no longer detectable (data not shown) which is consistent with the results obtained with fluorochrome-labelled ivt-*CEA*-mRNA.

To determine the impact of electroporation on DC function, viability, maturation and immunogenic potential were tested in DC transfected with ivt-*CEA*-mRNA. Viability was not influenced by electroporation, with or without mRNA, as examined by FDA assays. Over 95% of the DC were viable, independent of whether they were electroporated or not, with or without mRNA (data not shown). The influence of transfection on DC maturation was assessed by analysis of relevant surface markers, including CD26, CD80, CD83, CD86 and MHC II. FACS analysis after 24 and 48 h revealed no difference concerning the phenotypical appearance between DC transfected by electroporation with ivt-*CEA*-mRNA, DC electroporated in the absence of mRNA and untreated DC (Fig. 3a–c). When tested for their immunogenic potential in MLR, DC displayed sufficient induction of allogeneic lymphocyte proliferation after either treatment (electroporation ±ivt-*CEA*-mRNA and untreated DC; Fig. 3d).

3.2. *Specific CEA protein expression after transfection with* CEA-*mRNA*

CEA-expression in ivt-*CEA*/HisC-mRNA-transfected DC was visualised by CLSM using polyclonal anti-CEA and monoclonal anti-HisC antibodies. 4 h after transfection, the majority of the transfected DC expressed both the CEA protein (Fig. 4a) and the histidine-tag (Fig. 4c). Autologous DC treated by electroporation without RNA used as negative controls displayed only low unspecific background fluorescence signal (Fig. 4b and d). FACS analysis of CEA surface expression after transfection with a high amount of ivt-*CEA*/HisC-mRNA (up to 400 μg) revealed a distinct increase of fluorescence (Fig. 4e). We used this high amount of ivt-*CEA*/HisC-mRNA since the usual amount of 5–10 μg did not provoke a pronounced increase of fluorescence (data not shown).

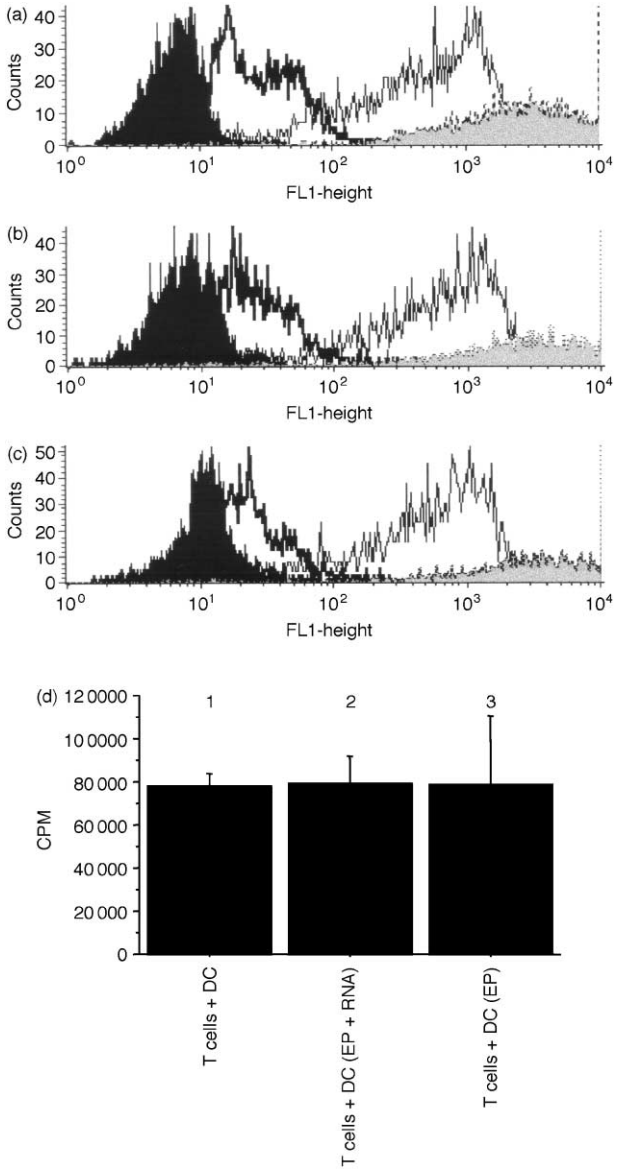

Fig. 3. Influence of transfection on dendritic cell (DC) function. Fluorescent-activated cell-sorting (FACS) analysis of (a) untreated DC, (b) DC electroporated without RNA and (c) DC transfected by electroporation with ivt-*CEA*-mRNA. The following Abs directed against relevant surface maturation markers were used: anti-CD83 (thick line), anti-CD86 (thin line), anti-MHC II (grey area). Control: anti-γ_1–γ_1 Ab (black area). (d) Mixed lymphocyte reaction (MLR): T cells co-incubated for 5 days with allogeneic DC either untreated (1) or electroporated with ivt-*CEA*-mRNA (2) or without RNA (3) at a T cell:DC ratio of 100:1. Standard 6-[^{3}H]-thymidine incorporation assay (counts per minute, CPM). Means (±standard deviation, bars) calculated from quadruplets. Representative of four separate experiments from three healthy donors.

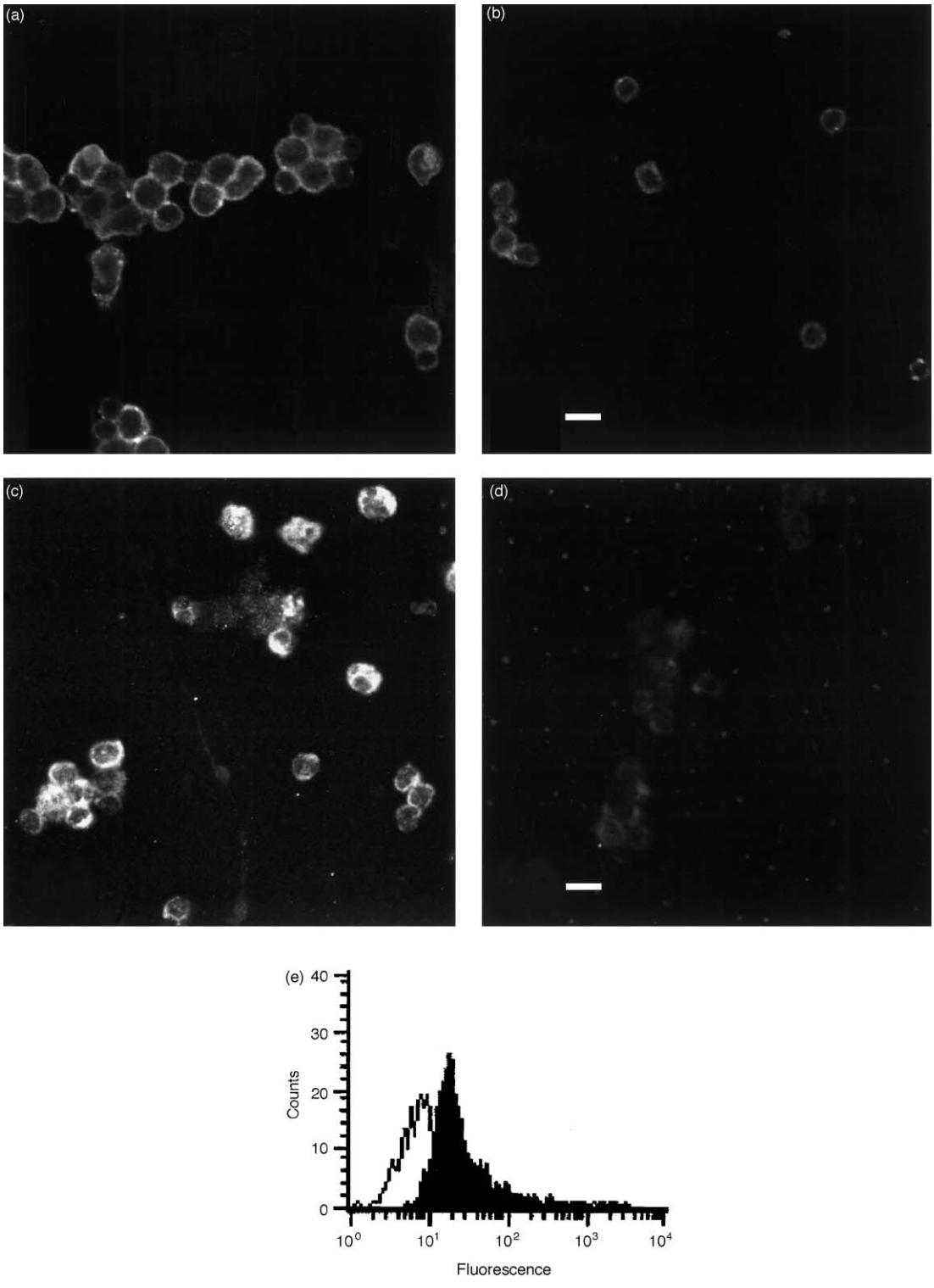

Fig. 4. Carcinoembryonic antigen (CEA)-expression of dendritic cells (DC) 4 h after transfection. Confocal laser scanning microscopy (CLSM): DC after electroporation with ivt-*CEA*/HisC-mRNA (a and c) or without RNA (b and d) examined with a polyclonal rabbit anti-CEA Ab (a and b) or a mouse anti-HisC mAb (c and d). Bar 13 μm. (e) Fluorescent-activated cell-sorting (FACS): CEA surface expression of DC electroporated with ivt-*CEA*/HisC-mRNA (solid area) or without RNA (empty area).

3.3. CEA-specific T lymphocytes

Distinct CEA-specific T cell populations were generated from freshly isolated human PBMC obtained from HLA-A2$^+$ healthy donors. Specificity of the T lymphocytes and the stimulatory capacity of the CEA-presenting DC were tested by standard 6-[^{3}H]-thymidine incorporation assays (Fig. 5). Proliferative activity of the T lymphocytes was tested after 4 weeks of stimulation when CD4$^+$ T cells were the dominating T cell subpopulation in culture.

First, we tested if CEA protein-primed T cells were able to proliferate in the presence of DC loaded with CEA protein or transfected with total RNA derived from CEA$^+$ SW480 tumour cells. Both DC populations induced a specific proliferative response in CEA protein-primed T cells comparable to that induced by a non-specific mitogenic anti-CD3 mAb at maximal stimulatory concentration (Fig. 5a), whereas untreated DC did not.

Second, we tested ivt-*CEA*-mRNA-primed T lymphocytes. Phenotypic characterisation of T cells by FACS (Fig. 6) showed that after 4 weeks of stimulation by DC pulsed with ivt-*CEA*-mRNA the PBMC-derived population consisted of 89% CD3$^+$ T lymphocytes (CD4$^+$: 63%, CD8$^+$: 26%) and 11% natural killer (NK) cells (CD3$^-$, CD56$^+$, CD16$^+$). After 5 weeks, NK cells had completely disappeared, and 100% CD3$^+$ T lymphocytes (CD4$^+$: 14%, CD8$^+$: 86%) were obtained. After 8 weeks, 23% of the T lymphocytes showed a CD4$^+$ phenotype and 77% a CD8$^+$ phenotype. These data indicate that, following our culture protocol, CD4$^+$ T cells predominate up to 4 weeks in culture, and afterwards selection towards CD8$^+$ T cells occurs.

Ivt-*CEA*-mRNA-primed T cells obtained after 4 weeks of cultivation were tested to see whether they were able to proliferate in the presence of DC transfected with ivt-*CEA*-mRNA (Fig. 5b). DC transfected with ivt-*CEA*-mRNA induced a potent proliferative response of the ivt-*CEA*-mRNA-primed T lymphocytes compared with non-transfected DC. The ivt-*CEA*-mRNA specific proliferative response could be blocked with a mouse anti-human MHC II mAb.

In a further experiment, the ivt-*CEA*-mRNA-primed T cells were tested concerning their cytotoxic activity after 8 weeks in culture when they were predominantly CD8$^+$ T-cells. Their cytolytic capacity was, on the one hand, examined using the HLA-A2$^+$ CEA$^+$ colonic cancer cell line SW480 which was efficiently lysed (58% at an E:T ratio of 8:1) by the ivt-*CEA*-mRNA-primed CTL (Fig. 7a). As we did not possess an adequate control to test CEA-specificity, i.e. a colon cancer cell line that does not express CEA and, furthermore, is suitable for the HLA status of our CTL, we used as tumour-analogue model TAP-deficient HLA-A2$^+$ T2-A2 cells

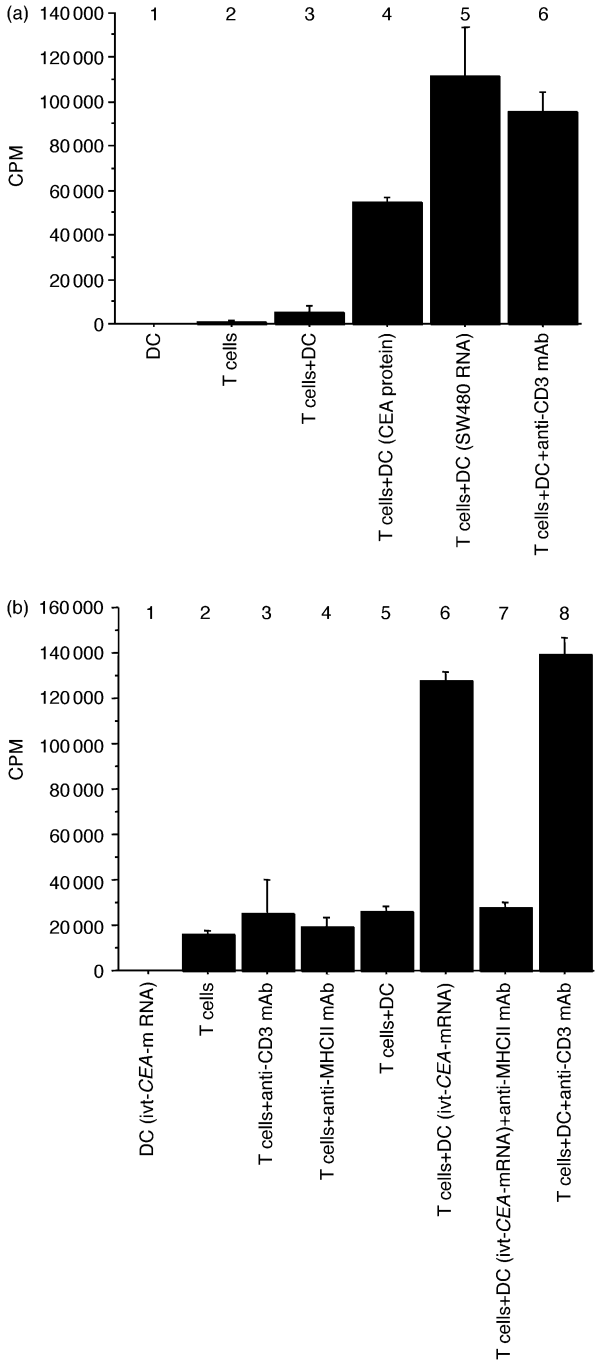

Fig. 5. Proliferative response of the CEA-specific T cells (predominantly CD4$^+$) after 4 weeks of stimulation. (a) CEA protein-primed T cells incubated without DC (2), with untreated DC (3), with DC loaded with CEA protein (4) or with total RNA from CEA$^+$ SW480 cells (5). Co-culture with untreated DC in the presence of mitogenic anti-CD3 mAb served as a positive control (6). Untreated DC alone (1). (b) Ivt-*CEA*-mRNA-primed T cells alone (2) treated with anti-CD3 mAb (3) or with anti-MHC II mAb (4), and co-incubated with untreated DC (5) plus anti-CD3 mAb as an unspecific mitogenic stimulus (8). T cells incubated with ivt-*CEA*-mRNA-transfected DC (6) with anti-MHC II mAb to block MHC II-restricted antigen presentation (7). DC transfected with ivt-*CEA*-mRNA alone (1). 6-[^{3}H]-thymidine incorporation assay. Means (±standard deviation, bars) were calculated from quadruplets. Representative of three separate experiments. CPM, counts per minute.

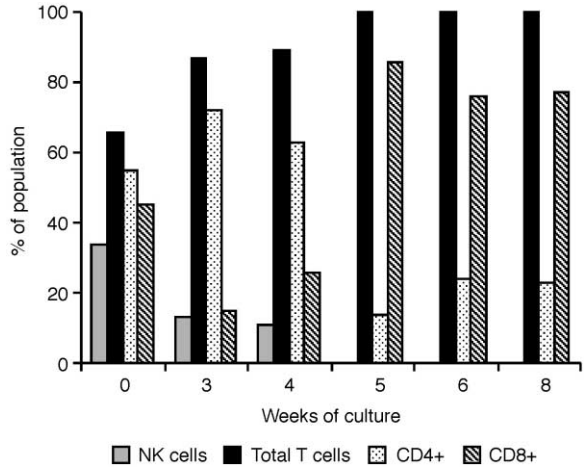

Fig. 6. Phenotype of the ivt-*CEA*-mRNA-primed T lymphocytes analysed by Fluorescent-activated cell-sorting (FACS) on day 0 and after 3, 4, 5, 6 and 8 weeks in culture (*X*-axis) using fluorochrome-labelled mAbs directed against the surface markers CD3, CD4, CD8, CD16 and CD56. Natural killer (NK) cells (grey columns), total T cells (black columns), CD4+ T cells (dotted columns) and CD8+ T cells (striped columns) as a percentage of the population (*Y*-axis). Representative of 3 independent experiments.

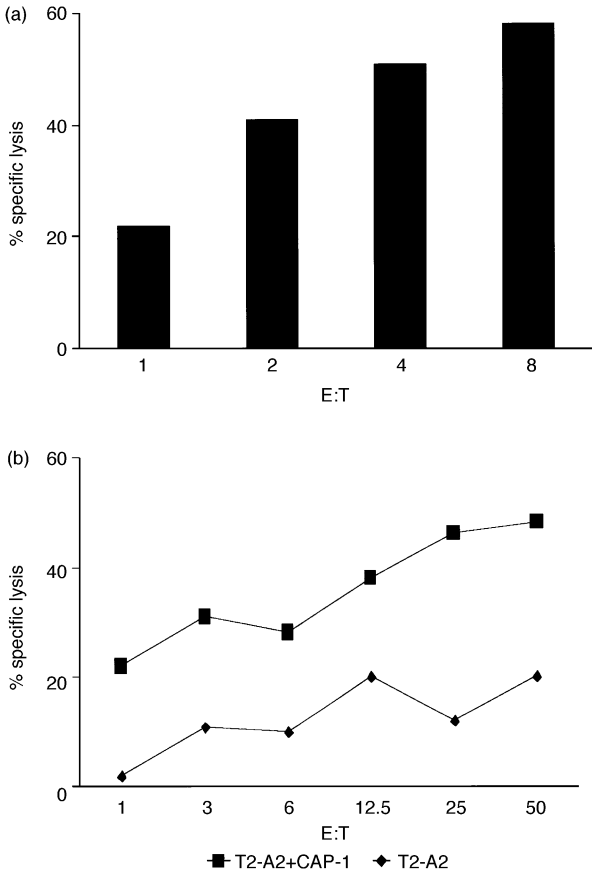

Fig. 7. Cytolytic capacity of the ivt-*CEA*-mRNA-specific T lymphocytes after 8 weeks in culture (predominantly CD8+). T-cells were used as effectors and co-cultured at different effector to target (E:T) ratios (X-axis) with (a) CEA+ tumour cells from colon cancer cell line SW480 in a JAM test and (b) T2-A2 cells loaded with antigen CEA-associated peptide-1 (CAP-1) (squares) or un-loaded (diamonds) in a fluorescence release assay. Lysis shown in % (*Y*-axis). Means were calculated from quadruplets. Representative of three separate experiments (standard deviation of the single values used for the cytotoxicity formula did not exceed 15%).

pulsed with the immunogenic HLA-A2.1-restricted CEA peptide CAP-1 (Fig. 7b). T2-A2 target cells pulsed with CAP-1 were killed to 49% at an E:T ratio of 50:1 by the ivt-*CEA*-mRNA-primed CTL.

4. Discussion

There is increasing evidence that mRNA encoding TAA might be useful for adoptive immunotherapy using DC in the treatment of cancer [28]. However, basic understanding about mRNA uptake by DC leading to TAA presentation and T cell priming is still lacking [20]. The aim of this study was to investigate the transfection efficiency of human DC by electroporation with *CEA*-mRNA, to localise the transfected mRNA in the DC, to detect specific protein expression, and to characterise CEA-specific T cells generated *in vitro* using *CEA*-mRNA transfected DC.

Transfection by electroporation was found to be a very efficient method. Light and electron microscopy revealed that ivt-*CEA*-mRNA was spread in the cytosol of the DC and even in the nucleus. However, it has to be considered that the mRNA is degraded within few hours after transfection indicating that specific protein synthesis is performed just during the first hours after transfection.

In a next step, specific CEA protein expression was analysed with immunolabelling for CLSM and FACS. This procedure implies some specificity difficulties, as all anti-CEA Abs not only recognise CEA (CD66e, CEACAM5), but also other gene products from the CEA family expressed constitutively by leucocytes [29]. In order to overcome this problem, we used histidine-tagged *CEA*-mRNA for transfection. By this procedure, we were able to demonstrate both, the specific CEA protein and the histidine-tag indicating active *CEA*-mRNA-encoded synthesis after electroporation. The translated protein was detectable already 4 h after electroporation in the majority of cells. Similar results were recently published (using luciferase as the reporter protein) 4 h after lipofection with *luciferase*-mRNA [30]. Detection of CEA expression on the surface of the DC using FACS analysis was only possible using high amounts of ivt-*CEA*-mRNA, which is in agreement with recently published data indicating a correlation between the amount of transfected RNA and subsequent protein expression [30]. Such high amounts of mRNA can only

be transfected via electroporation, as lipofection may be toxic in this range.

An important issue is the influence of the transfection method on the maturation of DC [30]. There was no such influence detectable in experiments measuring surface markers for DC maturation like CD83, CD80 and CD86. This is in contrast to lipofection where maturation of DC may be influenced [30].

To ensure that the translated CEA is presented by the DC, we generated CEA-specific T cells *in vitro*. Functional tests confirmed that DC transfected with *CEA*-mRNA by electroporation activate CEA specific $CD4^+$ and $CD8^+$ T cells. The CTL were even capable of lysing cells from the human CEA-expressing colon adenocarcinoma cell line SW480. These observations correlate with the published data using lipofection for transfection [13–16].

In summary, transfection via electroporation with mRNA is a good option to introduce antigens into DC for adoptive immunotherapies. Electroporation is an easy and very efficient method for transfection and might be employed even in protocols for the treatment of cancer patients. As translation of the protein and degradation of the transfected mRNA occur within few hours, the adoptive transfer can also be envisaged within few hours after electroporation.

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