NONVIRAL TRANSFER TECHNOLOGY

RESEARCH ARTICLE

Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes

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The use of tumor antigen loaded dendritic cells (DC) is one of the most promising approaches to induce a tumor specific immune response in vivo. Several strategies have been designed to load DC with tumor antigens. In this study, we investigated the delivery of in vitro transcribed RNA and plasmid DNA into monocyte-derived, ie non-proliferating human DC, using several nonviral transfection methods including electroporation and lipofection. Green fluorescent protein (GFP) was used as a reporter gene and influenza matrix protein 1 (M1) as a model antigen for HLA class I restricted antigen presentation. Using electroporation in combination with DNA or with RNA, up to 11% of DC were GFP-positive. Using liposomes as a vehicle for DNA transport up to 10% of the DC were GFP-positive. A significant

increase in transfection efficacy, of up to 20%, was observed when GFP RNA was used in combination with liposomes. Importantly, the RNA transfected DC retained their typical morphological and immunophenotypical characteristics. In addition, DC transfected with M1 RNA were able to stimulate autologous peripheral M1-specific memory cytotoxic T lymphocytes (CTL), as well as M1-specific CTL clones. Furthermore, comparison of DNA-transfected DC with RNA-transfected DC revealed the latter to be far better stimulators of antigen-specific T cells. This RNA transfection technique consequently represents a very promising tool for future immunotherapy strategies. Gene Therapy (2000) 7, 2028–2035.

Keywords: dendritic cells; RNA transfection; CTL; immunotherapy

Introduction

Recent studies using tetrameric MHC/peptide complexes have unequivocally demonstrated the existence of tumor-specific CD8⁺ cytotoxic T lymphocytes (CTL) in blood and metastatic tissue.^{1–3} However, these tumor-specific CTL are quantitatively and/or qualitatively unable to eradicate the tumor for several reasons. One major criterion for the weak CTL induction is that tumor cells are weak antigen presenting cells (APC) due to the lack of adhesion-, MHC class II- and co-stimulatory molecules.⁴ One way to improve tumor antigen presentation is the use of cancer therapies based on professional APC modified with tumor-associated antigens to induce tumor-specific CTL. In this respect, the use of dendritic cells (DC) represents a particularly promising approach as DC are the most potent professional APC of the immune system and are the only cells able to stimulate naive T lymphocytes.^{5,6}

Recently, several strategies have been developed in order to use modified DC as a tumor vaccine.⁷ Encouraging results have been reported for both murine models and human clinical trials using tumor peptide-pulsed DC.^{8–14} Nevertheless, the use of peptides has considerable

limitations, including MHC restrictions and the fact that only a limited number of tumor-specific epitopes are known.

In this respect, the transfection of DC with DNA or RNA has several advantages in comparison to peptide loading. First, transfection of DNA/RNA can be performed without prior knowledge of the patient's MHC haplotype. Second, processing of the endogenously expressed protein leads to multiple MHC class I epitopes.¹⁵

In this study, we investigated the transfection of human monocyte-derived DC with *in vitro* transcribed RNA (RNA) and plasmid DNA (DNA) using different transfection methods. We demonstrate that DC can efficiently be transfected with RNA using electroporation or lipofection and that RNA transfected DC are far superior inducers of antigen-specific CD8⁺ T cell responses when compared with DNA transfected DC.

Results

Transfection efficacy of GFP in monocyte-derived DC using different methods

In order to establish an efficient method to transfer genes into monocyte-derived DC, we investigated the transfection efficacy of *in vitro* transcribed RNA and plasmid DNA using different physical and chemical transfection techniques. A human codon-optimized mutant of the

green fluorescent protein (GFP) was used as the reporter gene in order to test the different transfection methods. Successful GFP expression was monitored by fluorescence microscopy and/or FACS analyses. DC generated from CD14⁺ monocytic cells were cultured in RPMI supplemented with 1% AB-plasma, GM-CSF and IL-4. These immature DC were transfected on day 5 or 6 and after a culture of 12–16 h, to allow for protein expression and processing, a cytokine cocktail consisting of IL-1 β , IL-6, PGE₂ and TNF- α was added to induce the final DC maturation. After an additional 24 h, the transgene expression was monitored.

In a first series of experiments, the electroporation technique was tested. To determine efficient electroporation conditions for monocyte-derived DC, the following parameters determining the electric field were tested: (1) the cell density was tested in the range from 2×10^6 – 4×10^7 cells/ml while capacitance and voltage were kept constant at 300 μ F and 250 V, respectively, showing that an increased cell density resulted in a decreased mortality; (2) the impact of the voltage was investigated in the range from 250 to 350 V, while capacitance and cell density were kept constant at 300 μ F and 4×10^7 cells/ml, respectively, demonstrating that an increasing voltage resulted in a higher mortality; (3) the capacitance was evaluated in the range from 300 to 1500 μ F while cell density and voltage were kept constant at 4×10^7 cells/ml and 250 V, respectively. Increasing capacitance yielded in an increased mortality. Although pulse times below 22 ms increased cell viability, only a very low heterologous gene expression was detectable. In contrast pulse times above 28 ms increased transient gene expression but resulted in high cell loss. In conclusion, the optimal electroporation conditions for immature monocyte-derived DC were as follows: (1) cell density of 4×10^7 cells/ml; (2) voltage of 250 V; (3) capacitance of 300–500 μ F; and (4) pulse times between 22 and 28 ms.

Using these optimized electroporation conditions up to 11% of DC were GFP⁺ after 48 h, when GFP RNA was transfected. A similar transfection efficacy was obtained using GFP DNA (see Table 1).

Next we tested lipofection using eight commercially available liposomal reagents (Lipofectin, Lipofectamine, GenePorter, DOTAP, Tfx-50, DAC-30, DMRIE-C, TransFast). Four of them (Tfx-50, Lipofectin, GenePorter, TransFast) are composed of cationic lipid and the neutral lipid DOPE which facilitates endosomal disruption, thereby allowing the release of the complexes into the cytoplasm. Interestingly, using liposomal reagents transgene expression was higher with GFP RNA when compared with GFP DNA (see Table 1).

Using our experimental settings, the best results were obtained with the liposome TransFast. Up to 20% of the DC were GFP⁺ when RNA was used. In contrast, only up to 10% were positive when GFP DNA was used (see Table 1). Importantly, incubation with this liposome showed no DC toxicity at the concentration used. A lower transfection efficacy was observed using the cholesterol-derivative DAC-30 (up to 14% GFP⁺ with RNA, up to 10% GFP⁺ with DNA). Interestingly, DOPE had no influence on the transfection efficacy of DC. In addition, we also tested the transfection efficacy of the non-lipid reagents FuGene6 and SuperFect. Both resulted in a transgene expression of less than 1%.

In summary, transfection of RNA in combination with

Table 1 Transfection efficacy in human DC transfected with GFP RNA (a) or GFP plasmid DNA (b) using different methods

	Transfection efficacy (% of GFP ⁺ cells)	
	Maximum	Mean $\pm$ s.e.m.
(a) GFP RNA		
Liposomal reagents		
TransFast	20.7	16.2 $\pm$ 2.8
DAC-30	14.5	9.5 $\pm$ 2.5
DMRIE-C	<1	<1
Electroporation	11.0	7.6 $\pm$ 1.9
(b) GFP DNA		
Liposomal reagents		
TransFast	10.2	4.6 $\pm$ 2.8
DAC-30	10.5	4.2 $\pm$ 3.1
DMRIE-C	<1	<1
DOTAP	<1	<1
LipoFectAmine Plus	<1	<1
Lipofectin ^a	<1	<1
Tfx-50 ^a	<1	<1
Gene Porter ^a	<1	<1
Non-liposomal reagents		
SuperFect	<1	<1
FuGene6	<1	<1
Electroporation	10.2	6.2 $\pm$ 2.1

The mean transfection efficiency data of each set of parameters are derived from at least three independent experiments.

^aIndicates two independent experiments.

cationic lipids like TransFast represents an efficient way to introduce foreign genes into DC.

Maturation potential of DC is retained after RNA lipofection and electroporation

The potential of transfected DC to mature was evaluated by FACS analysis. Counterstaining of the transfected DC was performed using monoclonal antibodies specific for typical DC markers including CD83, CD86 and CD25. Phenotypical analysis of DC incubated with the liposomal reagent TransFast alone showed that this liposome has only little effect on the surface expression of CD83 and CD86, and no effect on the expression of CD25 (Figure 1a). Addition of RNA or DNA increased the surface expression of CD83 and CD86. Furthermore, addition of the cytokine cocktail further increased the surface expression of CD83, CD86 and also CD25. Finally, all GFP⁺ DC transfected with RNA expressed CD83, CD86 and CD25 simultaneously, typical for fully mature and stable DC.

The majority of GFP⁺ DC electroporated with GFP RNA simultaneously expressed CD83 and CD86 (Figure 1b). Furthermore, these GFP⁺ cells expressed CD80, CD40, CD54, HLA class I and HLA-DR (data not shown). CD25, a late-appearing DC marker, was expressed on 50% of the GFP⁺ cells. A similar antigen expression pattern was observed when GFP DNA transfected DC were analyzed. Cell pulsing, without DNA or RNA did not influence the expression of the above-mentioned antigens.

Allostimulatory capacity of RNA transfected DC is maintained

In order to evaluate whether or not gene transfer into DC affected their allo-stimulatory capacity, transfected DC

Figure 1 Phenotypal analysis of transfected DC. Immature DC were transfected with either RNA or DNA encoding GFP using (a) lipofection (TransFast) or (b) electroporation (capacitance, 300 μ F; voltage, 250 V). A cytokine cocktail was added 12 h after transfection to induce final maturation of DC. After an additional 24 h, DC were counterstained with monoclonal antibodies specific for CD25, CD83, CD86 and PE-labeled goat anti-mouse IgG F(ab')₂. Results are representative of three independent experiments. CC, cytokine cocktail.

were cocultured with purified allogeneic T cells at different DC:T cell ratios. Electroporation of DC with GFP RNA did not alter their ability to stimulate allogeneic T cells (Figure 2a). Even low numbers of electroporated DC (DC:T cell ratio of 1:900) were able to induce a strong T cell proliferation. Electroporation of DC with GFP DNA showed only a slightly reduced stimulatory capacity at a DC:T cell ratio of 1:33 and 1:100. The electroporation procedure *per se* had no effect on the allo-stimulatory capacity of DC when compared with non-electroporated DC. A similar stimulatory capacity was observed when DC, electroporated with RNA or DNA encoding M1 were analyzed (Figure 2b).

GFP RNA transfected DC, using liposomes as vehicle, also showed a very potent allo-stimulatory capacity (Figure 2c). The same was observed for DC transfected with RNA or DNA encoding M1 (Figure 2d).

Transfected DC stimulate antigen-specific CD8⁺ CTL *in vitro*

The antigen-presenting capacity of transfected DC was assessed by the stimulation of antigen-specific memory CTL. Therefore, M1-transfected DC were cocultured for 7 days with autologous purified CD8⁺ T cells without addition of exogenous cytokines. M1-specific CTL were identified by FACS analysis using CD8-specific monoclonal antibodies and tetrameric HLA-A2/M1 peptide complexes. As shown in Figure 3, DC transfected with M1 RNA/liposomes efficiently stimulate and expand an M1-specific CTL response. In contrast, using M1 DNA-transfected DC this antigen-specific CTL response was not observed. M1 peptide-loaded DC were used as positive control and induced a specific CTL response, comparable to RNA transfected DC.

The antigen-presenting capacity of transfected DC was

Figure 2 Allo-stimulatory capacity of transfected DC. Immature DC were transfected either with RNA or DNA encoding GFP or M1 using electroporation (a and b) or lipofection (c and d). A cytokine cocktail was added 12 h after transfection, and after an additional 24 h, MLR analyses were performed by culturing graded numbers of DC with purified allogenic T cells (2×10^5). At day 4, cells were pulsed with ^3H -thymidine and incorporation was determined 16 h later. The mean $\pm$ s.d. of triplicates are shown. Counts of T cells or DC alone were always below 500 c.p.m. Results are representative of three independent experiments.

Figure 3 Detection of M1-specific memory CTL using M1-specific tetramers. DC were left untreated or transfected with either RNA or DNA encoding M1 using TransFast or were pulsed with M1 peptide and subsequently cocultured for 7 days with purified autologous CD8⁺ T cells. M1-specific CD8⁺ T cells were stained with PE-labeled HLA-A2/M1 peptide tetramers following counterstaining with Tricolor-labeled CD8 specific monoclonal antibodies. Results are representative of two independent experiments.

further confirmed by stimulation of M1-specific CTL clones. Activation of CTL clones was immunochemically visualized by IFN- γ secretion at a single T cell level using the ELISPOT technique. As shown in Figure 4, DC transfected with M1 RNA/liposomes (TransFast) stimulated IFN- γ secreting CTL in high numbers. Comparable results were obtained when M1 peptide-loaded DC were used. In contrast, DC transfected with M1 DNA/liposomes stimulated only a very small number of IFN- γ producing CTL, indicating the presence of lower numbers of MHC class I/M1 peptide complexes. DC treated with irrelevant GFP RNA/liposomes, GFP DNA/liposomes or liposomes alone, as well as DC or CTL alone showed no activation. In summary, these data clearly demonstrate that M1 RNA transfected DC are efficient antigen presenting cells able to induce antigen-specific CD8⁺ CTL responses.

Discussion

Modified DC represent a very promising tool for the development of vaccines for the treatment of tumors and viral diseases. Antigen-pulsed DC have been shown to elicit tumor specific CTL *in vitro*^{16,17} and induce therapeutic anti-tumor responses in animal models.^{18,19} In addition, recent reports on several human vaccination studies using peptide- and/or tumor lysate-pulsed DC as a vaccine describe promising anti-tumor immunity.⁹⁻¹⁴

In order to induce an effective immune response against tumors and virus-infected cells, antigen-specific CD8⁺ T cells, which are also able to kill the target cells, are indispensable. In this regard the antigen-specific peptides are presented in an MHC-class I restricted manner.²⁰ To be presented on MHC class I molecules, the antigens have to be expressed endogenously or introduced into the cytoplasm. Therefore, genetic modification of DC with genes encoding tumor- or viral antigens represents

Figure 4 DC transfected with M1 RNA stimulate M1-specific CTL. The IFN- γ secretion of M1-specific CTL was determined using the ELISPOT-technique. DC (1.5×10^5) were transfected with either DNA or RNA encoding GFP or M1 using TransFast. After 18 h of coculture with 5×10^3 M1-specific CTL, IFN- γ production of single T cells was detected using horseradish-peroxidase/ACE-substrate. The mean $\pm$ s.d. of triplicates are shown. Results are representative of seven independent experiments.

a potentially very powerful method to induce an MHC class I-specific antigen presentation. Several systems have been investigated in order to deliver various genes into murine and human DC. These include viral vectors such as retroviruses,^{21–23} adenoviruses,^{24–28} vaccinia virus^{29–31} and influenza virus.⁴⁴ Although high levels of heterologous gene expression could be obtained with some of these viral vectors, the immunogenicity of viral vectors might be an obstacle for long-term genetic modifications. Cellular immunity directed against viral proteins might lead to the destruction of genetically modified DC and may also lead to major toxic side-effects.

Therefore, nonviral transfection systems such as gene gun,³² electroporation^{26,28} and lipofection^{26,28,33,34} have been investigated to transfect monocyte-derived DC using plasmid DNA. A major drawback of these reported studies was the fact that only very low transfection levels were obtained.

In this study, we investigated the transfection efficacy of differentiated DC with DNA and RNA using several different nonviral vehicles. The transfection efficacy was determined by the GFP expression using fluorescence microscopy and FACS analyses. Using electroporation we observed a comparable transfection rate of up to 10% and 11% for DNA and RNA, respectively. A comparable efficacy for DNA transfection has been reported for DC derived from hematopoietic progenitors.^{28,34} However, in contrast to our data monocyte-derived DC were not transfected efficiently.^{28,34} To our knowledge, this is the first published report on electroporation of RNA.

Regarding transfection of DC using liposomes, the commercially available product TransFast gave the best results in our experimental settings. Although the efficacy with DNA was comparable to that obtained by means of electroporation, the transfection of DC with GFP RNA was significantly increased up to 20% with the use of liposomes. Also the influenza matrix protein was successfully introduced and expressed in DC using M1 RNA. These M1 RNA transfected DC expressed the heterologous protein, generated and expressed MHC-peptide complexes as assessed using M1-specific CTL clones in combination with the IFN- γ ELISPOT technique. Furthermore, these DC were able to expand M1-specific memory T cells, in the absence of exogenous cytokines, as analyzed using M1-specific tetramers. Of note, comparison of DNA-transfected DC with RNA-transfected DC revealed the latter to be far better stimulators of antigen-specific T cells (see Figures 3 and 4). This might be due to the fact that RNA can directly be translated into proteins, whereas DNA has first to be transcribed and only then can it be translated into proteins and subsequently peptides derived from these proteins can be presented via MHC class I molecules.

As previously shown, antigens encoded by RNA have the advantage of presenting multiple epitopes for different MHC haplotypes and therefore permit the CTL induction independent from the patients genetic background.^{15,35–38} In addition, in the mouse system it has been shown that using RNA it was possible to induce a CTL specific immune response.^{15,39} Nevertheless, in contrast to other reports,^{35–38,40} in our hands DC could not be transfected efficiently with naked RNA only.

In summary, the transfection of DC with RNA presents a unique opportunity to induce an anti-tumor or anti-viral vaccine. It combines efficient gene expression

together with full retention of DC phenotype and function. In addition, it has the advantage that no non-human gene products are expressed and it does not integrate into the host genome.

Materials and methods

Synthetic peptides

The influenza virus peptide 1 (M1) (GILGFVFTL; amino acids 58–66) was purchased from Clinalfa (Läufelingen, Switzerland), dissolved in DMSO (10 mg/ml) and stored at -20°C .

Plasmids

The plasmid pEGFP-C1, expressing GFP under the control of a cytomegalovirus (CMV) promoter was purchased from Clontech (Palo Alto, CA, USA). The plasmid pUC19 M1 (CMV promoter) was kindly provided by Dr Klenk, University of Marburg, Germany. For *in vitro* transcription, cDNA encoding GFP or M1 were digested with *NheI/HindIII* and *Asp718/BglII*, respectively. The expression vector pGEM4Z/A64 (a kind gift from E Gilboa, Duke University, Durham, NC, USA) was digested using *HincII* for the GFP cloning and with *Asp718/EcoRI* for M1 cloning. The *EcoRI* site was blunt-ended before subcloning the *Asp718/BglII*-M1 fragment. The vectors were denominated pGEM4Z/GFP and pGEM4Z/M1, respectively. All plasmids were transformed in *E. coli* XL-1 blue and purified using Qiagen Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany).

In vitro transcription of RNA

The plasmids pGEM4Z/GFP or pGEM4Z/M1 were linearized with *SpeI* and *in vitro* transcription was performed with the Ribomax large-scale RNA production system T7 (Promega, Mannheim, Germany) according to the manufacturer's instructions. Briefly, linearized DNA templates were incubated for 4 h at 37°C in a reaction mixture containing T7 1 $\times$ transcription buffer, ATP, CTP, UTP (1.8 mM each), 0.6 mM GTP, 3 mM $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ cap-analogons (Amersham Pharmacia Biotech, Freiburg, Germany) and T7 RNA polymerase. RNA was resuspended in water and stored at -80°C . The functional activity of the *in vitro* transcribed RNA was tested with a rabbit reticulocyte lysate system (Promega).

Cell lines and clones

The CTL clone C9, which recognizes the M1 peptide in context of HLA-A2.1 was kindly provided by PR Dunbar (Institute of Molecular Medicine, University of Oxford, Oxford, UK) and cultured in Iscove's medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% human pooled serum, L-arginine (116 $\mu\text{g}/\text{ml}$), L-asparagine (36 $\mu\text{g}/\text{ml}$), 10 mM sodium pyruvate and 0.1 M Hepes. Cells were stimulated every 3 weeks using irradiated (30 Gy), PHA-treated PBMC as stimulator cells and irradiated (100 Gy) LG-2-EBV as feeder cells. The EBV-transformed B cell line LG-2-EBV was cultured in Iscove's (Life Technologies) supplemented with 10% FCS (PAA, Cölbe, Germany). Both LG-2-EBV-, as well as M1-CTL C9 cells were maintained at 37°C in an 8% CO_2 atmosphere. Cell lines and clones were checked to be free of mycoplasma contamination using a mycoplasma detection Kit (Roche Diagnostics, Mannheim, Germany).

Generation of dendritic cells from CD14⁺ monocytic cells

As source for peripheral blood mononuclear cells (PBMC), buffy coats and leukapheresis products from healthy donors were obtained from the Bavarian Red Cross (Nuremberg, Germany) and the local Department of Transfusion Medicine (University of Erlangen). The donors were HLA-typed by flow cytometry using monoclonal antibodies specific for HLA-A1 and HLA-A2.1 (One Lambda, Canoga Park, USA, and American Tissue Culture Collection, Rockville, USA, respectively). PBMC were isolated by density gradient centrifugation (460 g, 25 min, room temperature) on a Ficoll-Hypaque cushion ($d = 1077$ g/ml) (Nycomed, Oslo, Norway). PBMC were collected at the interface and washed four times with phosphate-buffered saline (PBS) (BioWhittaker, Walkerville, USA) at 4°C and decreasing g forces (10 min at 250 g, 10 min at 175 g and twice 12 min at 110 g) to remove platelets. Resulting cells were stored overnight at 4°C. CD14⁺ cells were positively selected using anti-CD14 antibody conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. MACS-buffer consisted of PBS supplemented with 2% human serum albumin. As assessed by FACS analyses, the cells were 93–98% pure and expressed high levels of HLA class I, HLA-DR, CD86, CD95, CD45RO, moderate levels of CD40, CD45 RA. No CD83, CD80 and CD25 expression was observed. The isolated CD14⁺ cells were cultured at a cell density of 7×10^6 in 12 ml culture medium in 10 cm dishes (Falcon 3003 from Becton Dickinson, Heidelberg, Germany). Culture medium consisted of RPMI 1640 (BioWhittaker) supplemented with 1% heat-inactivated AB-plasma or 1% heat-inactivated, autologous plasma in the case of buffy coats or leukapheresis, respectively. On setting up cultures (day 0) 800 U/ml rh GM-CSF (Leukomax; Novartis, Basel, Switzerland) and 1000 U/ml rhIL-4 (kindly provided by Dr E Liehl, Novartis Research Institute, Vienna, Austria) were added. On day 3, one third of the culture medium was replaced with fresh complete medium supplemented with 400 U/ml rhGM-CSF and 500 U/ml rhIL-4. On day 5 or 6, immature DC were used for the transfection experiments. These immature DC expressed high levels of HLA class I, HLA-DR, CD45RO, CD40, CD11b, CD13, CD54, CD50, mannose-receptor, CD1a, moderate levels of CD80, CD51, CD11a, low levels of CD4, CD95, CD71 and no expression of CD14, CD83, CD25 was observed. Terminal differentiation to mature DC was induced by the addition of IL-1 β (2 ng/ml) (Sigma, Deisenhofen, Germany), IL-6 (1000 U/ml) (kindly provided by Novartis), PGE₂ (1 μ g/ml) (Sigma), TNF- α (10 ng/ml) (kindly provided by Dr Adolf, Boehringer Ingelheim, Vienna, Austria) according to the protocol of Jonuleit *et al*⁴¹. These mature DC are characterized by their high level expression of CD83, CD25, CD86, HLA class I, HLA-DR, CD80, CD40, CD11b, CD13, CD54, CD58, CD71, CD50, CD1a, CD45RO, CD11a and Fascin.

Flow cytometric analysis

Cell surface antigens were detected with monoclonal antibodies specific for CD4 (SK4), CD25 (2A3), CD122 (TU27) (all from Becton Dickinson), CD11a, CD11b, CD13 (WM15), CD14 (UCHM-1), CD40 (B-B20), CD51 (13C2), CD54 (15.2), CD58 (BRIC5) (all from Cymbus Biotechnology, Chandlers Ford, UK), CD80 (MAB104), CD83

(HB15a) (both from Immunotech, Marseille, France), CD95 (PharMingen, San Diego, CA, USA), CD1a (OKT6; Ortho, Neckargemund, Germany), CD71 (VIP-1; Dianova, Hamburg, Germany) and mannose-receptor (3.29 B1 γ) (a kind gift of A Lanzavecchia, Basel Institute of Immunology, Basel, Switzerland). FITC- or PE-conjugated goat anti-mouse IgG F(ab')₂ fragments (Jackson Immuno Research, West Grove, PA, USA) were used as secondary antibodies. For the staining of surface antigens of freshly MACS-isolated CD14⁺ cells FITC-conjugates of the above-mentioned monoclonal antibodies were used. In order to stain intracellular antigens, cells were permeabilized using the Fix & Perm kit (An der Grub, Kaumberg, Austria) according to the manufacturer's instructions. The Fascin (p55k) specific antibody was a kind gift of E Langhoff, Massachusetts General Hospital, Boston, MA, USA. For the detection FITC-conjugated goat anti-mouse IgG F(ab')₂ fragments (Jackson Immuno Research) were used.

M1-specific CTL were stained for 15 min with PE-labeled tetrameric HLA-A2.1/M1 peptide complexes (tetramers) at 37°C.⁴² Subsequently, cells were counterstained with Tricolor-labeled-anti-CD8 (Caltag, Burlingame, CA, USA) for 15 min on ice. After several washes 0.5×10^6 cells were analyzed by flow cytometry. FACS analysis was performed using a FACScan (Becton Dickinson) using the CellQuest software.

Transfection methods

DC were transfected on day 5 or 6 in their immature stage. Immediately after transfection, DC were resuspended at a density of 1×10^6 cells per well in six-well plates in a final volume of 3 ml culture medium supplemented with 400 U/ml GM-CSF and 500 U/ml IL-4 and incubated for 12–16 h. Thereafter, the cytokine cocktail composed of IL-1 β , IL-6, PGE₂ and TNF- α was added to induce the final DC maturation.⁴¹ After an additional 24 h incubation the transfection efficacy was determined and morphological, phenotypical and functional assays were performed.

Electroporation

DC were adjusted to a final cell density of 4×10^7 cells/ml in PBS (BioWittaker). The cell suspension (250 μ l) was preincubated in a 0.4-cm gap electroporation cuvette for 5 min on ice. Twenty μ g of DNA or RNA were added and cells were pulsed at different settings using the Gene Pulser II apparatus (BioRad, Munich, Germany). Afterwards, cells were incubated for 5 min on ice and then reseeded in culture medium.

Lipofection

Transfection using liposomes (Lipofectin, LipofectAmine Plus, DMRIE-C (all from Life Technologies), TransFast, Tfx-50 (both from Promega), DAC-30 (Eurogentec, Seraing, Belgium), DOTAP (Roche Diagnostics), Gene Porter (Gene Therapy Systems, San Diego, CA, USA) and non-liposomal reagents SuperFect (Qiagen) and FuGene6 (Roche Diagnostics)) was performed and optimised according to the manufacturer's instructions. Briefly, using the TransFast reagent (Promega) 4 μ g DNA or RNA were added to 400 μ l Opti-MEM medium (Life Technologies) and 12 μ l TransFast reagent was added to the same tube and incubated for 15 min at room temperature. Cell suspension (600 μ l) containing 1×10^6 DC was

added to DNA/liposome or RNA/liposome complexes and incubated at 37°C for 1 h. Then the cells were washed once and reseeded in culture medium.

Allogeneic T cell proliferation

For the lymphocyte proliferation assays allogeneic T cells were isolated by positive MACS-sorting of CD4⁺ cells according to the manufacturer's instructions (Miltenyi). Freshly isolated CD4⁺ T cells (2×10^5) were cocultured with graded numbers of transfected DC in 96-well flat-bottomed plates in 200 μ l RPMI1640 containing 5% heat-inactivated human serum. On day 4 of culture, cells were pulsed with 1 μ Ci ³H-methyl-thymidine (Amersham, Buckingham, UK) and 16 h later harvested on to glass fiber filters and measured using a microplate 1450 counter (Wallac, Turku, Finland).

Stimulation of memory CTL

DC transfected with M1 DNA or M1 RNA were cocultured with autologous CD8⁺ T cells (DC:T cell ratio = 1:20) in 24-well plates in RPMI supplemented with 10% human serum. After 7 days of culture without addition of exogenous cytokines, the cells were harvested and specificity of CTL was evaluated by staining with tetramers.⁴²

Enzyme-linked immunospot assay (ELISPOT)

The ability of the transfected DC to stimulate antigen-specific CTL was determined on a single T cell basis using the ELISPOT technique.⁴³ Microtiter plates (96 wells, MAHA S4510, Millipore, Eschborn, Germany) were coated with 0.1 μ g/ml anti-IFN- γ antibody 1-D1k (Mabtech, Stockholm, Sweden). After blocking with 5% human serum, 5×10^3 antigen-specific T cells were added per well. Then, 1.5×10^5 of non-transfected or transfected DC were added. After 18 h of culture at 37°C the plates were washed six times with PBS/0.05% Tween and then incubated with biotinylated anti-IFN- γ detection antibody (7-B6-1, Mabtech). Plates were washed six times with PBS/0.05% Tween and stained with streptavidin/peroxidase (Vectastain Elite Kit, Vector Laboratories) and the substrate 3-amino-9-ethyl carbazole (ACE from Sigma). Spots were counted with a special assisted video imaging analysis system (Carl Zeiss Vision, Eching, Germany), using the KS ELISPOT software version 4.1.143.

Acknowledgements

We thank Waltraud Leisgang for technical assistance. We gratefully acknowledge Armin Bender for setting up the ELISPOT technique in the laboratory, for many helpful comments and fruitful discussions. We thank Manfred B Lutz and Heidi C Joao for critical reading of the manuscript. This work was supported by the Bundesministerium für Bildung und Forschung, grant No. 01GE9601, Forschungsverbünde zur somatischen Gentherapie.

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