The Journal of Immunology

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J Immunol 2005; 174:3087-3097; ;
doi: 10.4049/jimmunol.174.5.3087
http://www.jimmunol.org/content/174/5/3087

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Generation of an Optimized Polyvalent Monocyte-Derived Dendritic Cell Vaccine by Transfecting Defined RNAs after Rather Than before Maturation[1]

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Transfection with RNA is an attractive method of Ag delivery to dendritic cells (DCs), but has not yet been standardized. We describe in this study the methods to efficiently generate an optimized mature monocyte-derived DC vaccine at clinical scale based on the electroporation of several RNAs either into immature DC followed by maturation or, alternatively, directly into mature DCs, which has not been possible so far with such high efficiency. Electroporation of DCs resulted in high yield, high transfection efficiency (>90%), and high migration capacity. Intracellular staining allowed the study of the expression kinetics of Ags encoded by the transfected RNAs (MelanA, MAGE-3, and survivin) and a validation of the vaccine (≥90% transfection efficiency). Expression of all three Ags peaked 3–4 h after electroporation in DC transfected either before or after maturation, but decreased differently. The DC vaccine can also be cryopreserved and nevertheless retains its viability, stimulatory capacity as well as migratory activity. In addition, we uncover that DC transfected after rather than before maturation appear to be preferable vaccines not only from a production point of view but also because they appear to be immunologically superior for CTL induction in sharp contrast to common belief. DCs transfected after maturation not only more effectively generate and present the Mage-3.A1 and MelanA.A2.1 epitopes to T cell clones, but they even are superior in priming to the standard proteasome-dependent MelanA.A2.1 wild-type prototype tumor epitope, both in terms of T cell expansion and effector function on a per cell basis. *The Journal of Immunology,* **2005, 174: 3087–3097.**

Dendritic cell (DC)[4] based vaccination, i.e., the adoptive transfer of Ag-loaded DCs, is a new approach to induce immunity in humans, which is still in its infancy and so far has been exploited primarily in cancer treatment (1). To date over 1000 patients have been vaccinated with DC; largely the easily accessible and well-characterized monocyte-derived DC (Mo-DC) loaded with either tumor cell lysates or defined peptides, and more recently also with RNA (2). Encouraging clinical responses without any major side effects have also been observed. Only a small number of proof of concept studies used established immunomonitoring methods, yet it is encouraging that in these few studies the DC proved clearly immunogenic (reviewed in Refs. 3 and 4). Immature DC, notably Mo-DC, are now considered either weakly immunogenic or even tolerogenic based on experimental evidence (5) as well as clinical studies (6–8). Important obvious variables for DC-based vaccination (such as DC subsets and maturation stimulus; types of Ag and loading methods; dose, frequency, and route of DC delivery; and DC migration) have yet to be addressed in two-armed trials, and there are many upcoming opportunities to improve efficacy at the level of DC biology (9). There is, therefore, agreement that a systematic optimization of DC vaccination is necessary to exploit the potential of this approach and to finally perform meaningful direct comparisons to other vaccination strategies as well as phase III trials to test the clinical efficacy. There exists an even larger consensus that there is an urgent need to use well-characterized and standardized DC vaccines (9).

Our personal approach to develop the DC vaccination strategy was to first successively improve the generation of mature DC from monocytes following the initial observation made by our group and Sallusto and Lanzavecchia (10, 11) that (immature) DC can be generated under the aegis of GM-CSF plus IL-4. We always used a PGE_2 containing maturation stimulus (initially autologous monocyte-conditioned medium (12) and later a mixture composed of IL-1β plus IL-6 plus TNF-α plus PGE_2 as its synthetic mimic (13)), which might have been fortunate as PGE_2 appears critical to yield mature DC progeny exhibiting a high spontaneous and CCR7-directed migratory capacity (14). We learned to generate such mature Mo-DC in large numbers from apheresis products in semiclosed systems in a highly reproducible manner, and to prepare cryopreserved, ready-to-use standardized DC vaccine aliquots (15, 16). We have so far used peptide-loaded Mo-DC, which is the standard for loading with defined Ags and is useful as it facilitates detailed immunomonitoring of induced immune responses. In past and ongoing clinical trials, we have vaccinated almost 100 patients with such peptide-loaded DC addressing several variables. Tumor-specific CTL and Th1 responses have been demonstrated (17–20), and these initial immunomonitoring data together with upcoming responses will provide us with a solid database for further development of DC vaccination. Exogenous loading of DC by pulsing

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Received for publication August 4, 2004. Accepted for publication December 14, 2004.

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[1] This work was supported by Grants from the Deutsche Forschungsgemeinschaft (SCHU 1538), Bundesministerium für Bildung und Forschung GE0601, the Sonderforschungsbereich 643 Project C1, and Forimmune Project T5.

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[4] Abbreviations used in this paper: DC, dendritic cell; EGFP, enhanced GFP; EP, electroporation; EP→mat, DC electroporated at the immature state, then matured; mat→EP, DC electroporated at the mature state; MFI, mean fluorescence intensity; Mo-DC, monocyte-derived DC.

0022-1767/05/$02.00

with defined Ag-derived peptides that directly bind to the MHC molecules replacing endogenous peptides is obviously only applicable to patients carrying the appropriate MHC molecules/HLA haplotype (1, 21). In addition, the number of defined epitopes available for various Ags is still limited and it would also be very costly to generate a polyvalent vaccine using multiple peptides. Like others, we are therefore increasingly interested in using alternative strategies for Ag loading and favor the transfection of RNA as a particularly attractive novel method for Ag delivery, especially because RNA in contrast to DNA does not integrate into the genome, and therefore is much easier to work with from a regulatory point of view. In analogy to our previous approach we now set out to systematically investigate the production of a standardized and validated DC vaccine based on RNA-transfected rather than peptide-loaded Mo-DC.

RNA transfection as an alternative strategy for Ag loading of DC was first described in 1996 (22). Because the RNA-encoded Ag is expressed in the cytosol of the DC like a cellular protein, this method gives the Ag direct access to the classical MHC class I pathway. The expression of the whole polypeptide chain allows the cell's degradation machinery to potentially generate all possible MHC class I presented peptides, known or unknown, and is therefore not limited to certain HLA haplotypes. RNA-transfected Mo-DC have already been successfully explored in clinical trials but the respective vaccines were so far all based on transfection with naked RNA, which due to its low transfection efficiency, cannot readily be validated (23). In the meantime the method of electroporation was explored first for Mo-DC (24, 25) and recently for CD34-derived DC as well (26), and it has drastically increased the transfection efficiency over the use of naked RNA or lipofection. Both DC types if transfected at the immature stage with RNA coding for defined Ags were shown to induce or expand Ag-specific CTL responses (24–29). RNA transfection after maturation would be preferable for routine production of a DC vaccine as it is obviously more straightforward to transfect matured DC rather than to harvest DC at their immature stage, transfecting the DC and then putting them back into culture again for maturation and final harvest. In addition, immature DC upon transfection transiently get firmly adherent, which makes cell handling difficult. Unfortunately, electroporation of matured Mo-DC was found to be inferior with respect to transfection efficiency and viable DC yield (25).

Despite these reports that electroporation of mature Mo-DC is inferior and further concerns that transfecting DCs following maturation will only present epitopes generated by the immunoproteasome but not standard proteasome-dependent epitopes, we endeavored to optimize and carefully compare in parallel the transfection of RNA into mature DC with RNA transfection of immature DC that were matured after electroporation. We show that immature and mature DCs using appropriate methods can be electroporated with equally high yield and transfection efficiency. Migration capacity of electroporated DCs was in the same range as of nonelectroporated DCs. Ag expression was directly proportional to the RNA concentration used and could be validated and studied with respect to kinetics by intracellular Ab staining. Furthermore, we demonstrate that it is possible to simultaneously electroporate RNAs coding for three different tumor Ags (MelanA, Mage-3, survivin), without influencing each expression and presentation, and to cryopreserve transfected DC. Most interestingly and surprisingly, DCs transfected following maturation appeared immunologically superior to DCs transfected at the immature stage followed by maturation. Not only did DCs transfected after maturation more efficiently generate epitopes such as Mage-3.A1 and MelanA.A2.1, but they were also clearly superior in sensitizing IFN-γ-producing and lytic CTL specific for the HLA-A2.1-restricted MelanA peptide (aa 26–35), a classical standard proteasome-dependent CTL epitope.

Materials and Methods

Antibodies

The following mAbs were used for intracellular FACS staining of tumor Ags: mouse anti-human MelanA (A103; Novocastra), and mouse anti-human MAGE (clone 57b; kindly provided by Dr. G. C. Spagnoli, University Hospital, Basel, Switzerland). This Ab detects several members of the MAGE family, including MAGE-3 as described (30). In addition, rabbit anti-human survivin and rabbit IgG (Ab-105C; both of R&D Systems) were used. Secondary Abs used in intracellular staining were donkey anti-mouse PE (RDI) and goat anti-rabbit FITC (Loewe Biochemica). For determining phenotype of DC, the following Abs were used: PE-labeled anti-CD83 (HB15a; Immunotech), FITC-labeled anti-human CD86 (BU63), anti-human CD25 (Tu69; both of Cymbus Biotechnology), anti-HLA-DR (L243; BD Biosciences), and PE-conjugated anti-CD80 (L307.4). Isotype controls used were: IgG1-FITC and IgG1-PE (both of BD Biosciences), and IgG2b-PE, IgG2a-FITC, and IgG2a-PE (all of Cymbus Biotechnology).

DC generation from leukapheresis and whole blood

Mo-DC was generated essentially as described (15). In short, PBMC were prepared from leukapheresis products or whole blood of healthy donors (obtained following informed consent and approved by the institutional review board) by density centrifugation using Lymphoprep (Axis-Shield). PBMC were resuspended in autologous medium that consisted of RPMI 1640 (Cambrex) containing 1% heat-inactivated autologous plasma, 2 mM L-glutamine (BioWhittaker), 20 mg/L gentamicin (Sigma-Aldrich) and were transferred to cell factories (Nunc), or, for generation of DC in smaller scale, to tissue culture dishes (BD Falcon), at 1.2×10^9 cells/cell factory or 30×10^6 cells/dish. Cells were incubated for 1–2 h at 37°C to allow for adherence, and the nonadherent fraction was removed and cryopreserved while 200 ml (cell factory) or 10 ml (dish) of autologous medium was added to the adherent cells. On day 1, 20 ml of fresh medium containing GM-CSF (Leukine; Berlex Laboratories) and IL-4 (Strathmann Biotec) were added to cell factories, resulting in a final concentration of 800 and 250 IU/ml, respectively. GM-CSF and IL-4 were added to dishes directly, resulting in identical concentrations. On days 3 and 5, cells were fed with 40 ml (cell factory) or 4 ml (dish) of fresh medium containing GM-CSF and IL-4 to a final concentration of 800 and 250 IU/ml, respectively. On day 6 cells were used for electroporation as immature DC (see below). Following transfection the DCs were put back to culture in medium containing GM-CSF plus IL-4, and after 2 h, a maturation mixture consisting of IL-1β, 13.2 ng/ml; IL-6, 1000 U/ml (both of Strathmann, Biotec); TNF-α, 10 ng/ml (Bender); and PGE_2, 1 μg/ml (Minprostin; Pharmacia & Upjohn) (13) was added. After an additional 24 h of culture the transfected and matured DC were harvested. Alternatively, instead of harvesting the DC at their immature stage for RNA transfection, the maturation mixture was added to the cultures as described (15), and after 24 h of maturation the cells were used for electroporation as mature DC.

Production of in vitro transcribed RNA

For in vitro transcriptions, the pGEM4Z64A-enhanced GFP (EGFP), pGEM4Z64A-MAGE-3, pGEM4Z64A-MelanA, and pGEM4Z64A-survivin (survivin wild-type; Ref. 31) plasmids (kindly provided by Dr. I. Tcherepanova, Argos Therapeutics, Durham, NC) were linearized with *Spe*I enzyme, purified with phenol/chloroform extraction and ethanol precipitation, and used as DNA templates (32). The in vitro transcription was performed with T7 RNA polymerase (mMESSAGE mMACHINE kit; Ambion) according to the manufacturer's instructions. The transcribed RNA was recovered after DNaseI (Ambion) digestion on RNeasy columns (Qiagen) according to the manufacturer's instructions. RNA quality was verified by agarose gel electrophoresis, RNA concentration was measured spectrophotometrically, and RNA was stored at −80°C in small aliquots.

Electroporation of DCs

DCs were harvested from the cell factories and washed once with pure RPMI 1640 and once with PBS (all at room temperature). The cells were resuspended in OptiMEM without phenol red (Invitrogen Life Technologies) at a concentration of 4×10^7/ml. RNA was transferred to a 4-mm cuvette (50 μg/ml final concentration or as indicated; Equibio). A volume of 200–600 μl of cell suspension was added and incubated for 3 min before being pulsed in a Genepulser Xcell (Bio-Rad). Pulse conditions were square-wave pulse, 500 V, 0.5 ms. Immediately after electroporation,

the cells were transferred to autologous medium supplemented with the previously indicated concentrations of GM-CSF and IL-4.

Cryopreservation of cells

Cryopreservation was performed as described (16). In short, cells were taken up in 20% HSA (Pharmacia & Upjohn) at a concentration of 5–10 $\times$ 10^6 cells/ml for DC or 20–50 $\times$ 10^6 cells/ml for nonadherent fraction, and stored for 10 min on ice. An equal volume of cryopreservation medium, i.e., 55% HSA (20%), 20% DMSO (Sigma-Aldrich), and 25% glucose (Glucosteril 40; Fresenius), was added to the cell suspension. Cells were then frozen at -1°C/min in a cryofreezing container (Nalgene) to -80°C. Thawing was performed by holding cryotubes in a 37°C water-bath until detachment of the cells was visible. Cells were then poured into 10 ml of RPMI 1640, washed, and added to a cell culture dish containing prewarmed autologous medium of 250 IU IL-4/ml and 800 IU GM-CSF/ml. Cells were rested for 1–2 h in a 37°C incubator before additional experiments.

Flow cytometric analysis

For surface stainings, DCs were washed and thereafter suspended at 1 $\times$ 10^5 cells in 100 μl of cold FACS solution (Dulbecco's PBS; BioWhittaker) containing 0.1% sodium azide (Sigma-Aldrich) and 0.2% HSA (Octapharma) and incubated with mAb or appropriate isotype controls for 30 min. Cells were then washed twice and resuspended in 100 μl of cold FACS solution. For intracellular staining, electroporated cells were permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and stained with primary and secondary Abs according to the manufacturer's instructions. Stained cells were analyzed for two-color immunofluorescence with a FACStar cell analyzer (BD Biosciences). Cell debris was eliminated from the analysis using a gate on forward and side light scatter. A minimum of 10^4 cells was analyzed for each sample of surface stained cells and with intracellular staining at least 8 $\times$ 10^3 cells were analyzed. Results were analyzed using CellQuest software (BD Biosciences).

Transwell migration assay

DCs were counted and resuspended in migration medium (RPMI 1640 supplemented with 500 U/ml GM-CSF, 250 U/ml IL-4, 1% autologous serum and glutamine). Transwell inserts (Costar) with a pore size of 5 μm and 24-well plates (Nunc) were used as follows: inserts were preincubated with 100 μl of migration medium in 24-well plates, each well containing 600 μl of the same medium. A total of 2 $\times$ 10^5 cells were seeded in the upper compartment. To analyze migration toward the gradient, CCL19 (100 ng/ml; Tebu-Bio) was added to the lower wells. To analyze migration against a CCL19 gradient, the chemokine (100 ng/ml) was added to the upper well. Spontaneous migration was measured by incubation of the cells in a transwell without CCL19. DCs were allowed to migrate for 120 min. After this time period, DCs were harvested from the lower chamber and collected by brief centrifugation. Supernatant was removed and the cells were lysed by adding 25 μl of PBS (BioWhittaker) and 5 μl of 1% Triton X-100 (Roche Diagnostics). The β-glucuronidase activity in the lysates was determined photometrically using *p*-nitrophenyl-β-D-glucuronide (Sigma-Aldrich) as a substrate according to the manufacturer's instructions. The absorbance was measured at 405 nm using a Wallac Reader (Wallac) and the number of migrated cells was calculated using a separate standard curve for each cell population.

Induction and determination of IFN-γ production by MAGE-3- and MelanA-specific CTL

DCs electroporated with MAGE-3 RNA or MelanA RNA alone or with the three previously mentioned RNAs simultaneously were cocultivated with a CTL clone, specific for the MAGE-3-derived HLA-A1 binding peptide EVDPIGHLY (33) or a CTL clone or a cell line specific for the MelanA-derived HLA-A2 binding peptide EAAGIGILTV. DCs pulsed for 1 h at 37°C with 5 μg/ml of the peptide used in parallel as positive control. Four thousand T cells were cocultivated with 15,000 DCs in a volume of 100 μl of RPMI 1640 (Cambrex) supplemented with 10% pooled-plasma (heat-inactivated and sterile-filtered plasma from healthy donors), 10 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1% MEM nonessential amino acids 100$\times$ (Sigma-Aldrich), 2 mM L-glutamine (Cambrex), 20 mg/L gentamicin (Sigma-Aldrich), and 25 U/ml IL-2 (Sandoz). Supernatants were harvested after 16 h and IFN-γ production was determined using a commercially available ELISA kit according to the manufacturer's protocol (DPC Biermann).

Cytotoxic T cell induction assay

DCs were pretreated as indicated. DCs were pulsed for 1 h at 37°C with 10 μg/ml MelanA-derived HLA-A2-binding peptide EAAGIGILTV for comparison. Nonadherent fraction from the same healthy donor was used as source for generation $CD8^+$ T cell using MACS (Miltenyi Biotec) according to manufacturer's instructions. $CD8^+$ cells were then cocultivated with DC at final concentrations of 1 $\times$ 10^6/ml and 1 $\times$ 10^5/ml, respectively, in RPMI 1640 supplemented with 10% pooled-serum (Cambrex), 10 mM HEPES, 1 mM sodium pyruvate, 1% MEM nonessential amino acids (100$\times$), 2 mM L-glutamine, 20 mg/L gentamicin, and 20 U/ml IL-7. Twenty IU/ml IL-2 and 20 U/ml IL-7 were added on days 2 and 4. On day 7 the cells were harvested and used for readout or restimulation. Restimulations were performed under similar conditions, however IL-2 was added from day 0.

Tetramer staining and phenotyping of Ag-specific $CD8^+$ T cells

A total of 10^6 T cells were resuspended in 90 μl of RPMI 1640 supplemented with 5% pooled-serum, 10 mM HEPES, 1 mM sodium pyruvate, 1% MEM nonessential amino acids (100$\times$), 2 mM L-glutamine, and 20 mg/L gentamicin. Five hundred nanograms of HLA-A2-MelanA tetramer (peptide ELAGIGILTV) was added. Cells were incubated for 20 min at 37°C, 5% CO_2, and then cooled to 4°C. A mixture of the following Abs was added to determine the phenotype of the tetramer-positive cells: 5 μl of anti-CCR7 FITC, 5 μl of anti-CD45RA ECD (phycoerythrin-Texas Red), and 5 μl of anti-CD8 PC7 Ab. After 30 min on ice the cells were washed, and analyzed on a CYTOMICS FC500 from Beckman Coulter.

Cytotoxicity assay

Cytotoxicity was tested in a standard 4-h ^{51}Cr release assays. In short, target cells (T2 cells) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$/10^6 cells for 1 h at 37°C/5% CO_2, washed, loaded with peptides for 1 h at 37°C/5% CO_2, and washed again before cocultivation with effector T cells. Peptides (i.e., MelanA-derived HLA-A2 binding peptide EAAGIGILTV; MelanA-derived HLA-A2 binding analog peptide ELAGIGILTV; and irrelevant gp100-derived HLA-A2 binding analog peptide IMDQVPFSV) were loaded at a concentration of 10 μg/ml. Target cells were added to 96-well plates at 1000 cells/well. Effector cells, i.e., T cells generated in T cell induction assays, were added at an E:T ratio of 60:1, 20:1, 7:1, and 3:1. Percentage cytolysis, i.e., ^{51}Cr release, was calculated as follows: [(measured release $-$ background release)]/[(maximum release $-$ background release)] $\times$ 100%.

Results

Electroporation of RNA into immature as well as mature DC results in high migration capacity, transfection efficiency, and yields

Until now, almost all publications on RNA electroporation into DCs described transfection of DCs in the immature state, after which these cells were matured. As noted earlier, the transfection of DC following maturation would be more straightforward and thus preferable from a vaccine production point of view. However, previous data has shown low transfection efficiency and yield of mature DC using electroporation (25), and concerns were raised that immunoproteasomes predominantly expressed in mature DC do not generate the conventional epitopes for a number of tumor Ags, which are generated by the standard proteasomes in immature DC (34). Nevertheless, we investigated whether DC could also be electroporated in the mature state with high efficiency. Using EGFP expression as a readout, the electroporation protocol was optimized at several points: 1) previously described electroporation protocols (25) using an exponential decay pulse were compared with electroporation conditions using a square-wave pulse, and we found that for immature DC there was no difference in transfection efficiency using both methods (data not shown); 2) several electroporation settings for the square-wave pulse protocol were investigated, and we found that using 500 V, 0.5 ms with 40 $\times$ 10^6 cells/ml resulted in the best transfection efficiency with reasonable survival (Fig. 1 and data not shown); 3) moreover, we scaled-up the number of cells transfected with one pulse because it was necessary to generate large batches of transfected DC for clinical trials, and found that this could indeed easily be done when using the square-wave pulse protocol as implied by simple physics (data not shown). Considering these data, it was decided to use the square-wave protocol to carefully compare electroporation of DC

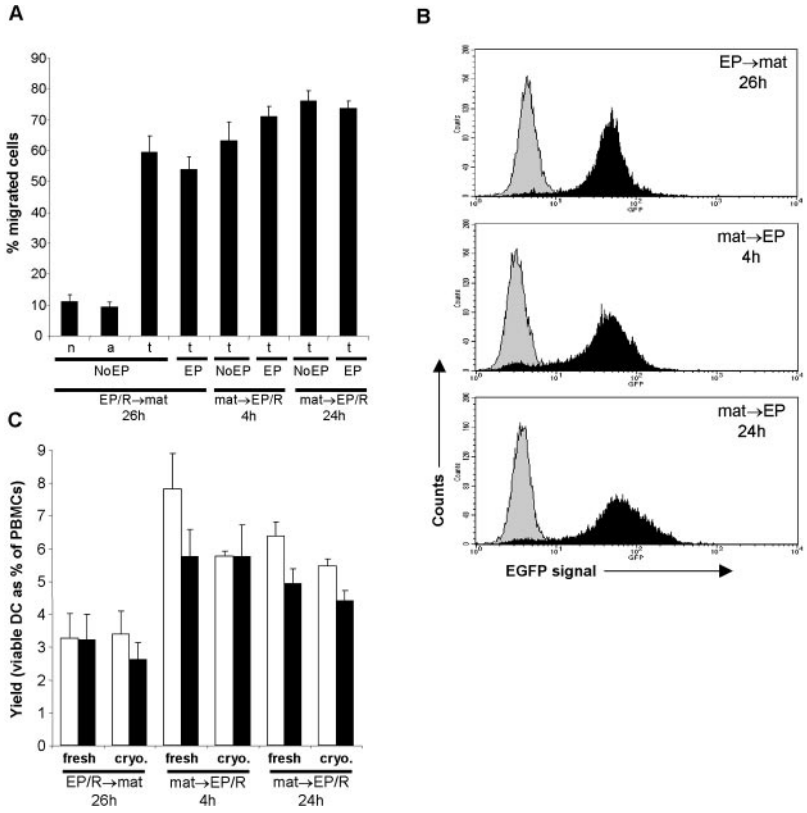

FIGURE 1. Migration capacity, transfection efficiency, and yields after electroporation of RNA into DC. *A*, Nonelectroporated DCs (NoEP) and DCs electroporated (EP) with MAGE-3, MelanA, and survivin RNAs, either in the immature state (EP/R→mat 26 h; which were given a maturation mixture 2 h after EP), or in the mature state (4 and 24 h after EP, respectively; mat→EP/R 4 h and mat→EP/R 24 h), were tested for their migration capacity toward (t) medium containing CCL19 in a standard transwell migration assay (see *Materials and Methods*). To measure spontaneous migration, cells were incubated in a transwell without CCL19 in the upper or lower compartment (n), or with CCL19 in the upper compartment (a). An average (± SEM) of three experiments is shown. EP/R→mat, DC electroporated or replated at the immature state, then matured; mat→EP/R, DC matured and then electroporated or replated. *B*, DCs were electroporated with EGFP RNA, and EGFP expression in these cells was determined by FACS analysis (black histogram). DC electroporated without RNA served as negative control (gray histogram). Data are representative for three independent and standardized experiments. *C*, DCs were electroporated with MAGE-3, MelanA, and survivin RNAs (■), either in the immature state (EP/R→mat 26 h), or in the mature state (mat→EP/R 4 h and mat→EP/R 24 h), and yield of living DC relative to the number of PBMC used to generate these DC was determined by trypan blue exclusion at the indicated time points before (fresh) and after (cryo.) cryopreservation. Nonelectroporated DCs served as controls (□). An average (± SEM) of three standardized experiments is shown. EP, Electroporation; NoEP, no electroporation; EP/R→mat, DC electroporated or replated at the immature state, then matured; mat→EP/R, DC matured and then electroporated or replated; cryo, frozen and thawed DC.

before and after maturation. Three different populations of DC were generated, including immature transfected DC that were matured 2 h after electroporation with maturation mixture for 24 h (i.e., EP→mat 26 h) and then used for analysis, and mature transfected DCs that were harvested 4 h (mat→EP 4 h) and 24 h (mat→EP 24 h) after electroporation and then used for analysis. It is important to note that electroporation neither significantly altered the characteristically high (15) surface expression of CD80, CD83, CD86, CD25, and HLA-DR (data not shown), nor the high stimulatory capacity in an alloreactive MLR (data not shown). Moreover, migration capacity of DCs is critical because they have to move from the skin into the lymph nodes. Therefore migration capacity of nonelectroporated and electroporated DCs were tested using established assays (35), and all three DC populations showed a clear CCL19-directed migration (Fig. 1*A*).

DC electroporated before or after maturation were efficiently transfected with EGFP RNA (Fig. 1*B*). Cell survival after transfection is critical to obtain enough DC for vaccination. Hence, we determined the yield of DC (i.e., percent living cells after electroporation relative to the number of PBMC used to generate these DC), of nonelectroporated DCs, and of DCs that were electroporated with MAGE-3, MelanA, and survivin RNAs. The DCs were electroporated and counted in trypan blue at the indicated time points (Fig. 1*C*). Yields of nonelectroporated DCs and RNA electroporated DCs were in the same range (i.e., 3.3 vs 3.4% for EP→mat 26 h, 7.8 vs 5.8% for mat→EP 4 h, and 6.4 vs 5.5% for mat→EP 24 h)(Fig. 1*C*). In addition, we determined the yield after freezing and thawing at the indicated time points. In general these yields were slightly lower than before cryopreservation (Fig. 1*C*). Furthermore, the yields of DCs electroporated in the mature state were both in fresh and cryopreserved populations higher than yields of DCs electroporated in the immature state (Fig. 1*C*). In summary these results show that there is no significant advantage or disadvantage in migration capacity, transfection efficiency, and yield when electroporating mature DC compared with immature DC. The yield of viable mature, electroporated, and cryopreserved DC is at least 50% (range from 50 to 90%) of the yield that is achieved for nonelectroporated DC (15).

Expression per cell is directly proportional to the RNA concentration used during electroporation

To determine transfection efficiency in immature and mature DC, and the relation of electroporated RNA to expressed protein, we used different concentrations of RNA coding for EGFP. As shown in Fig. 2, RNA concentration did not influence the percentage of

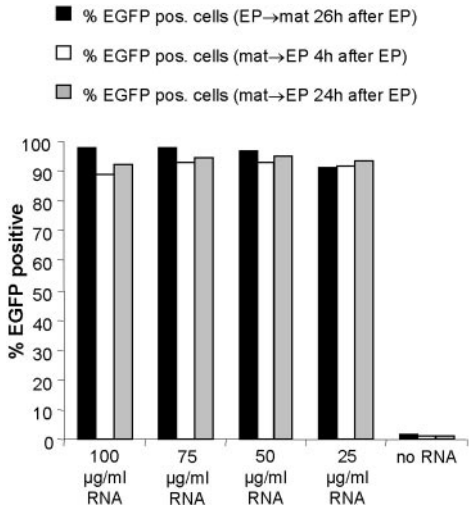

FIGURE 2. Electroporation of EGFP RNA into DC results in high transfection efficiency. DC were electroporated with different EGFP RNA concentrations (as indicated). EGFP expression in DC, either electroporated in the immature state (■), which were given a maturation mixture 2 h after EP, or in the mature state (□ and ▨, 4 and 24 h after EP, respectively), was determined by FACS at the indicated time points. Data are representative for three independent and standardized experiments.

EGFP-positive DC. Over 90% of the EP→mat 26 h, mat→EP 4 h, and mat→EP 24 h cells were EGFP-positive, even at the lowest RNA concentration used, i.e., 25 μg/ml (with 40 $\times$ 10^6 cells/ml) (Fig. 2). Furthermore, the EGFP expression per DC was determined by measuring the mean fluorescence intensity (MFI) of EGFP RNA-electroporated DC. As shown in Fig. 3, the MFI increased proportionally when RNA concentration was increased. This increase indicates that all RNA electroporated into the cell can be translated with equal efficiency, even in high concentrations. Moreover, these results show that mature DC can be electroporated with equally high efficiency as immature DC (Figs. 2 and 3). DCs electroporated without RNA were negative (Fig. 2). For additional experiments an intermediate concentration of 50 μg/ml RNA was chosen.

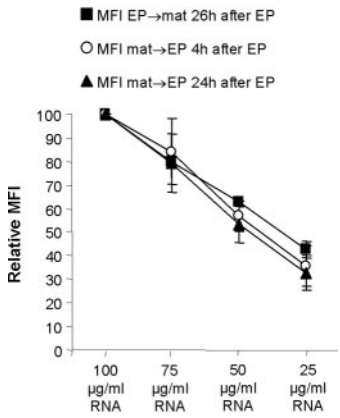

FIGURE 3. EGFP expression per cell is directly proportional to RNA concentration. DC were electroporated with different EGFP RNA concentrations (as described). EGFP expression in DC, either electroporated in the immature state (■), which were given a maturation mixture 2 h after EP, or in the mature state (○ and ▲, 4 and 24 h after EP, respectively), was determined by FACS at the indicated time points. MFI is expressed relative to the MFI of DC electroporated with the highest concentration of EGFP RNA (100 μg/ml = 100%). An average (± SD) of three separate standardized experiments is shown.

Different tumor Ags display different expression kinetics in electroporated DC

For clinical use of tumor Ag RNA-transfected DCs, it is essential to know the expression kinetics of the Ags to determine the optimal time to administer the DC to the patient. Therefore, we have investigated the expression kinetics of the three Ags (i.e., MAGE-3, MelanA, and survivin) planned for use in our clinical trial in RNA-electroporated DC by performing a time course intracellular FACS staining. Expression of these three Ags was determined at different time points in DCs electroporated in the immature state, which were matured 2 h after electroporation, and in DCs electroporated in the mature state. As shown in Fig. 4, there was hardly any difference in expression kinetics between immature and mature electroporated DC. Over 90% of the transfected DCs were positive 4 h after electroporation (Fig. 4, *right panels*). Expression of MAGE-3 and MelanA peaked at 4 h after electroporation and was stable over a longer period of time. After 24 h, expression of MAGE-3 was on background levels, whereas low MelanA expression was still detectable (Fig. 4). Survivin expression peaked already at 3 h after electroporation, and decreased rapidly within 6 h after electroporation, almost to background levels (Fig. 4), indicating a higher turnover of survivin RNA and/or protein. The limited persistence of expression of the three Ags is most likely relevant because DCs transfected before maturation are

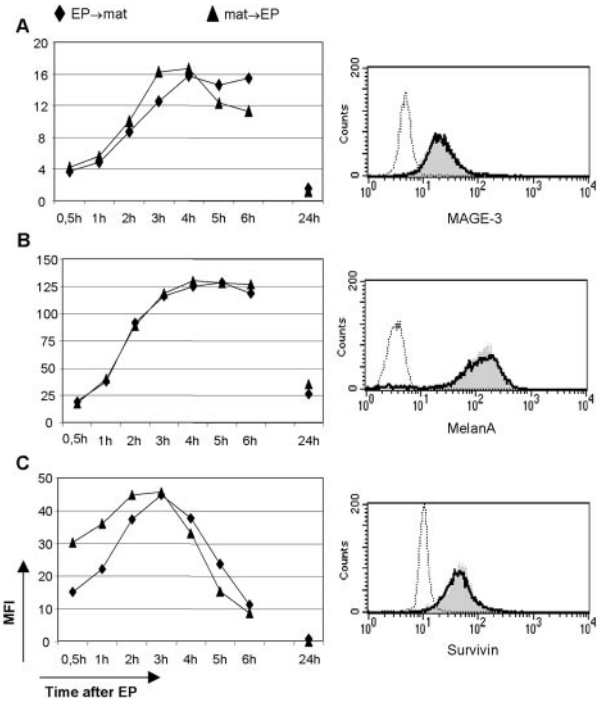

FIGURE 4. Ag-expression kinetics in electroporated DC. MAGE-3, MelanA, and survivin RNA were electroporated into DC. MAGE-3 (*A*), MelanA (*B*), and survivin (*C*) expression in DC, either electroporated (*left*) in the immature state (♦), which were given a maturation mixture 2 h after EP, or in the mature state (▲), was determined by intracellular FACS at the indicated time points. Expression levels were calculated as follows: [(MFI of DC electroporated with all three RNAs) − (MFI of DC electroporated without RNA)]. The *right panels* show intracellular Ag expression at the 4 h time point of DCs electroporated without RNA (dashed histogram), DCs electroporated in the immature state with Ag coding RNAs (gray histogram), and DCs electroporated in the mature state with Ag coding RNAs (thick histogram). Data are representative for three independent experiments.

usually harvested at least 24 h later, when intracellular Ag expression is hardly detectable, whereas mature electroporated DCs can be harvested 4 h after electroporation at the peak of Ag expression.

No differences in expression levels of tumor Ags in DC electroporated with one or three RNAs

To induce a broad antitumor response in vaccinated patients, and thereby prevent emergence of Ag-loss tumor cell variants, it would be advantageous to transfect different RNAs coding for several tumor Ags simultaneously into DC. Therefore we investigated whether expression levels of MAGE-3, MelanA, and survivin were influenced by each other when we electroporated all three RNAs simultaneously, compared with expression levels in DC electroporated with single Ags. We chose the time point of highest expression (Fig. 4) and analyzed Ag expression by intracellular FACS 4 h after electroporation. As shown in Fig. 5, there was no substantial difference in Ag expression in mature DC electroporated with RNA coding for a single Ag compared with DC electroporated with RNAs coding for all three Ags. Thus, DCs can be transfected with several RNAs simultaneously without effecting transfection and expression efficiency of the separate Ags.

Only mature electroporated DCs strongly induce production of IFN-γ by MAGE-3.A1-specific and MelanA.A2.1-specific CTL

To determine the Ag presentation capability of RNA-electroporated DC, MAGE-3.A1-specific and MelanA.A2.1-specific CTL were used. We directly compared DCs that were electroporated before maturation with DCs that were first matured, then electroporated and harvested after 4 and 24 h. To further determine whether the coexpression of three transgenes would influence their presentation, we compared DCs that were simultaneously electroporated with MAGE-3, MelanA, and survivin RNA with DCs electroporated with MAGE-3 or MelanA RNA alone.

The differently treated DCs were cocultivated with the CTL, and supernatants were harvested after 16 h. IFN-γ production by the CTL was analyzed in an ELISA. The EP→mat 26 h hardly induced IFN-γ production by the CTL (Fig. 6, *A* and *C*). However, the mat→EP 4 h and the mat→EP 24 h were able to induce IFN-γ production very efficiently (Fig. 6, *A* and *C*). Moreover, no difference in stimulatory capacity of DCs electroporated with RNA coding for a single Ag or for three Ags was observed (Fig. 6, *B* and *D*). As a positive control DCs transfected without RNA were loaded with the respective peptide. Also these cells induced a clear IFN-γ production by the CTL (data not shown). Background IFN-γ production of CTL alone was subtracted from the values, and CTL cocultivated with DC electroporated without RNA were not stimulated above background levels. From these data we conclude that mature-electroporated DC efficiently process the MAGE-3 and MelanA proteins, which results in efficient presentation of the respective HLA-restricted epitopes. Surprisingly, the long-lived MelanA Ag (Fig. 4) was not presented on the EP→mat 26 h DCs, although the standard proteasome, which is responsible for the generation of the MelanA.A2.1 epitope, is the predominate proteasome in immature DCs.

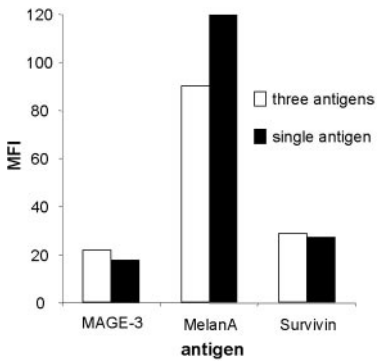

FIGURE 5. Electroporation of three RNAs simultaneously into DCs does not influence expression levels of each separately. MAGE-3, MelanA, and survivin expression in mature DC, either electroporated with RNA coding for a single Ag (■), or a mix of RNAs coding for all three Ags (□), was determined by intracellular FACS 4 h after electroporation. Expression levels were calculated as follows: [(MFI of DC electroporated with RNA) − (MFI of DC electroporated without RNA)]. Results are representative of three independent experiments.

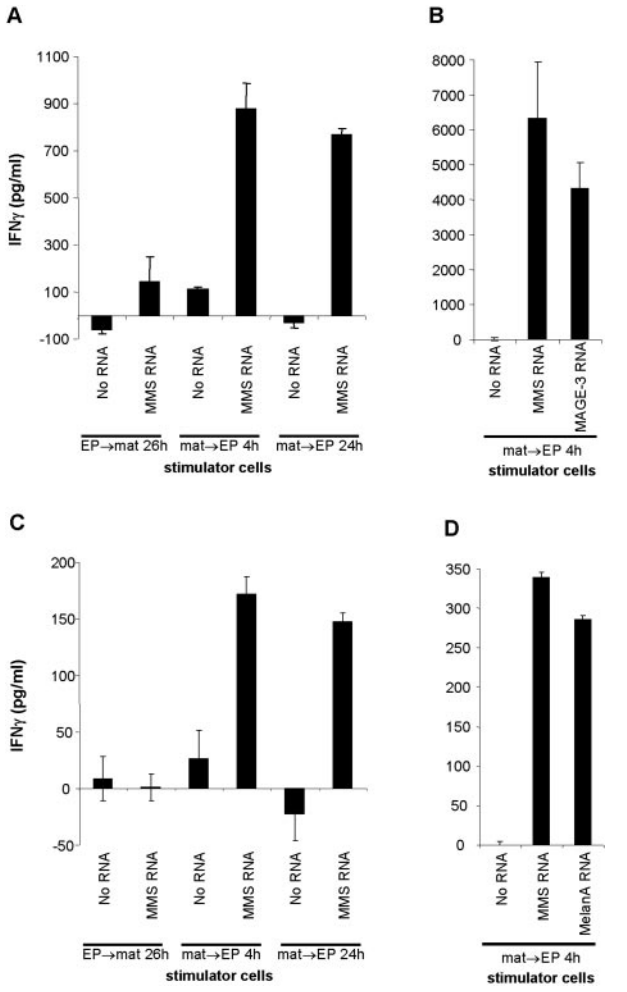

FIGURE 6. Superior presentation of MAGE-3 and MelanA epitopes by DC electroporated after maturation. A mixture of MAGE-3, MelanA, and survivin RNA (MMS RNA), or MAGE-3 RNA (MAGE-3 RNA) and MelanA RNA (MelanA RNA) alone was electroporated into DC, either in the immature state, which were given a maturation mixture 2 h after EP (EP→mat 26 h), or in the mature state (4 and 24 h after EP, respectively; mat→EP 4 h and mat→EP 24 h). MAGE-3/HLA-A1-specific CTL (*A* and *B*) and MelanA/HLA-A2.1-specific CTL (*C* and *D*) were stimulated with these DC for 16 h. Supernatants were harvested and IFN-γ production by the CTL was analyzed in a standard ELISA (shown as: [(average value of triplos ± SEM) − (average value of CTL alone)]). As a negative control, DCs electroporated without RNA were used. As a positive control, DCs electroporated without RNA but loaded with the MAGE-3.A1 peptide or MelanA.A2.1 peptide were used (data not shown).

Mature-electroporated DCs are more efficient in inducing MelanA-specific CD8$^+$ T cells

To compare the induction capacity of DC electroporated before or after maturation, we took advantage of the fact that sizeable frequencies of MelanA-reactive CD8$^+$ T cells exhibiting a naive phenotype are known to occur in the blood of healthy human adults (36). DC electroporated with MelanA RNA (either alone or together with MAGE-3 RNA and survivin RNA) were cocultivated with autologous CD8$^+$ T cells for 1 wk. Then the percentage and the phenotype of the MelanA-specific T cells were determined by tetramer and Ab staining. T cells were characterized as the four phenotypes: naive, central memory, effector memory, and lytic effectors by their CCR7 and CD45RA expression (37).

DC electroporated with MAGE-3, survivin, and MelanA RNA before maturation as well as after maturation were able to expand the pool of MelanA-specific T cells, but the mature-electroporated DC performed reproducibly better, resulting in higher expansion of MelanA-specific T cells and a stronger bias toward the effector population (Fig. 7*A*). Similar results were obtained with DC electroporated with MelanA RNA alone (data not shown). The lytic capacity of the generated T cells was determined by chromium release with peptide-pulsed T2 cells as targets, and was in line with the tetramer data, i.e., a stronger lysis was observed by T cell populations that were stimulated with mature electroporated DC (Fig. 7*B*). Interestingly, the higher lytic activity appeared not simply due to the higher percentage of MelanA tetramer-positive T cells in the T cell populations stimulated by DC electroporated after maturation: a lysis of ~30% (see Fig. 7*B*) was obtained in case of T cells expanded by mature electroporated DC at a 1:6 T:E ratio (equivalent to 200 MelanA tetramer$^+$ T cells in the coculture), while immature electroporated DC required a ratio of 1:60 (equivalent to 1000 MelanA tetramer$^+$ T cells) to achieve a comparable lysis (Fig. 7*B*). This suggests that MelanA-specific CD8$^+$ T cells induced by mature electroporated DC on a per cell basis exhibited about a five times higher activity. DC electroporated without RNA served as negative controls (Fig. 7*A*). For comparison, T cells were also stimulated with DCs that were sham-electroporated (either before or after maturation), and then loaded with MelanA peptide. These peptide-loaded DCs also lead to an expansion of lytic MelanA-specific T cells (Fig. 7) that was, however, less than that obtained by RNA-transfected DC. It is, however, noteworthy that there was no significant difference in expansion or lytic activity of MelanA-specific CD8$^+$ T cells (also not on a per cell basis) induced by peptide-loaded DC that were sham-electroporated before or after maturation. This is obviously an important control as it demonstrates (just like the equivalent stimulatory activity in the allo-MLR, data not shown) that the stimulatory capacity of the two mature DC populations is not different and is not the cause for the superiority of DC transfected with MelanA RNA at the mature stage.

Cryopreserved RNA-electroporated mature DCs retain their stimulatory capacity

These data collectively showed us that RNA transfection of DC following maturation was not only possible but likely immunologically superior apart from being preferable from a vaccine production point of view as discussed earlier. Because cryopreservation of RNA-electroporated DC is essential to be able to produce several large batches of cells for successive vaccinations of patients, we investigated whether cryopreservation influences the stimulatory capacity of such mature-electroporated DC by performing CTL induction experiments as aforementioned. Autologous CD8$^+$ T cells from the same batch were stimulated with freshly prepared or frozen and thawed electroporated DC. Their capability to expand MelanA-specific T cells was again determined by tetramer staining. Both the freshly prepared and the cryopreserved and thawed DC were able to expand the pool of MelanA-specific T cells to a similar level (Fig. 8*A*). For comparison, T cells were stimulated with MelanA peptide-loaded DC as aforementioned (Fig. 8*A*), and DC transfected without RNA served as negative control (Fig. 8*A*). In addition, an ELISPOT was performed with T cells that were stimulated with frozen and thawed electroporated DC (Fig. 8*B*). A clear increase in IFN-γ-producing CD8$^+$ T cells was observed when cells were stimulated with DC electroporated with the three RNAs and with peptide-loaded DC (Fig. 8*B*). Taken together, we conclude that mature-electroporated

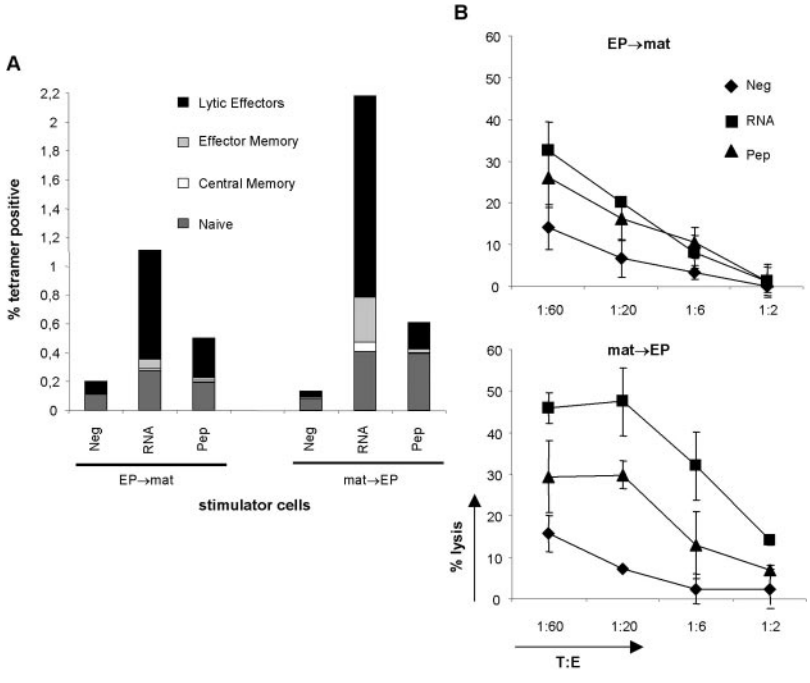

FIGURE 7. Superior induction of MelanA-specific lytic effector and effector memory T cells by DC electroporated after maturation. Immature (EP→mat) or mature (mat→EP 4 h) DCs were electroporated with a mixture of MAGE-3, MelanA, and survivin RNA (RNA) and were used to stimulate autologous CD8$^+$ cells for 1 wk. No RNA electroporated (Neg) and MelanA wild-type peptide-loaded (Pep) DC served as controls. After 1 wk the percentage of MelanA/A2 tetramer-binding CD8$^+$ cells and phenotype (*A*), and cytolytic capacity (*B*) was determined. Assignment of functional T cell phenotype was as follows: lytic effectors, CD45RA$^+$, CCR7$^-$; effector memory, CD45RA$^-$, CCR7$^-$; central memory, CD45RA$^-$, CCR7$^+$; naive, CD45RA$^+$, CCR7$^+$. As target cells in cytotoxicity assays T2 cells loaded with the MelanA analog peptide were used. As negative target T2 cells loaded with an irrelevant HLA-A2 binding gp100 peptide were used. Shown is the percentage lysis of target cells (± SD). Background lysis was always below 10% (data not shown). The target to effector ratio (T:E) is indicated. One representative experiment of three is shown.

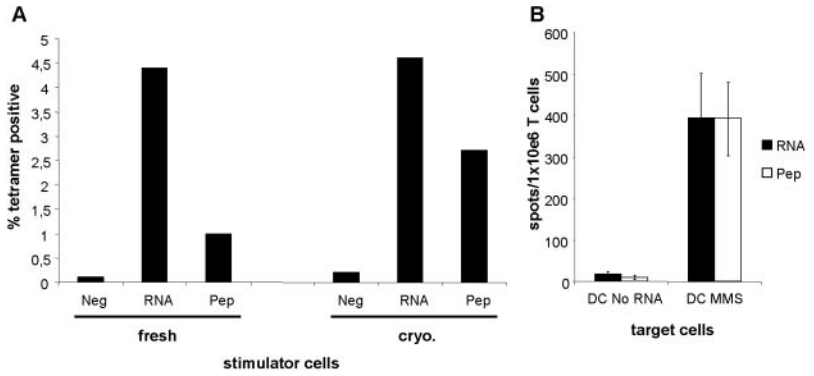

FIGURE 8. The stimulatory capacity of mature RNA-electroporated DC is retained after cryopreservation. DC electroporated in the mature state with MAGE-3, survivin, and MelanA RNA (RNA), either fresh (i.e., directly used 4–5 h after electroporation) or cryopreserved (cryo.) (i.e., frozen 4 h after electroporation and used 1 h after thawing) were used to stimulate autologous $CD8^+$ T cells for 2 wk. For comparison, T cells were expanded by stimulation with MelanA peptide-loaded DC (Pep). DCs electroporated without RNA served as negative controls (Neg). After 2 wk, $CD8^+$ T cells were harvested and analyzed by staining with MelanA/HLA-A2 tetramers (*A*). $CD8^+$ T cells stimulated by frozen and thawed RNA-electroporated (RNA) or MelanA peptide-loaded (Pep) DC were also tested in an IFN-γ ELISPOT (*B*). DC electroporated without RNA (DC No RNA) or electroporated with MAGE-3, survivin, and MelanA RNA (DC MMS) were used as target cells (see *Materials and Methods* for details). Number of spots per 1×10^6 T cells are indicated. One representative of two experiments is shown.

DC can be cryopreserved without loss of Ag presentation and induction capacity, and that specific tetramer binding correlates with a functional readout such as IFN-γ production. Mature-electroporated DCs therefore form a new vaccination tool that is potentially more active to induce resistance to cancer.

Discussion

DC vaccination is still at an early stage, and several parameters such as Ag loading need yet to be addressed systematically first by careful preclinical research and then in (preferably two-armed) clinical trials (reviewed in Refs. 1 and 9). The transfection of RNA is an attractive method of Ag delivery, particularly because of the recent development of effective electroporation protocols as a substitute for variable transfection with naked RNA, which although exclusively used so far in the initial clinical trials, would not readily allow to generate a standardized DC vaccine for widespread use. Electroporation protocols in contrast have so far not been systematically tested in DC vaccines with proven immunogenicity. In addition, electroporation of DC after maturation, although preferable from a vaccine production point of view per our earlier discussion, proved to be difficult. In this study we rigorously explored the electroporation of Mo-DC, which were generated by a well-standardized process and have proven immunogenic in human, if administered upon peptide loading as in earlier discussion. We demonstrate that large numbers of mature (>80% $CD83^+$) and migratory DCs, transfected simultaneously with one or several defined RNAs, can be generated with comparable high transfection efficiency of >90%, excellent viability, and sufficient yield in a reproducible standardized way both by transfecting DC at their immature stage followed by maturation, and, in contrast to previous reports, by directly transfecting matured DC (Fig. 1). Validation of RNA transfection is easily possible by intracellular FACS detection of proteins coded for by the transfected RNA (Fig. 4), and the RNA transfected DC can be cryopreserved without significant cell loss even upon 24 h further culture. Of particular relevance is the fact that we also found clear-cut evidence that DCs transfected after maturation were immunologically superior (Figs. 6 and 7).

Both immature and mature DC can be transfected with extraordinary efficiency and reproducible yield, without influencing migratory capacity

The mature DCs obtained from immature RNA-electroporated DC populations display a transfection and maturation status that is even higher than that reported by Van Tendeloo et al. (25) (60% transfection vs >90%). More remarkable is the fact that we could also electroporate mature DC with equal high yields and high transfection efficiencies, which is a clear improvement compared with the data from Van Tendeloo et al. (25). This discrepancy could be due to the different electroporation method used. Alternatively, their lower transfection efficiency might result from a different maturation stimulus given to the Mo-DC. Instead of the four-component mixture (IL-1β plus IL-6 plus TNF-α plus PGE_2; Ref. 13), Van Tendeloo et al. (25) used TNF-α plus LPS, possibly making the DC more resistant to RNA uptake. This could also explain the lower transfection efficiency in mature DC compared with immature DC when RNA was transfected by lipofection (25). DCs matured by TNF-α plus LPS might also be more vulnerable. We have found previously that the survival of DCs matured by the four-component mixture is excellent even after freeze and thaw (16), which might be due to the fact that this maturation stimulus appears to induce additional antiapoptotic molecules in the mature DC progeny (38, 39). Furthermore, we show that EGFP expression per cell is directly proportional to the EGFP RNA concentration used, i.e., the MFI increased 2-fold when RNA concentration was doubled (Fig. 3). Our observations with DC electroporated in the immature state are in line with data of Tuyaerts et al. (29) who showed that when immature DCs are electroporated with increasing concentrations of EGFP RNA, electroporation efficiency was not affected, whereas the MFI did increase. In this study we also show that for DC electroporated in the mature state, EGFP expression, measured 4 and 24 h after electroporation, is directly proportional to RNA concentration.

Transfection with several RNAs is possible and the expression of tumor Ags can be readily monitored by intracellular FACS staining over time. We sought to determine the expression of proteins encoded by the transfected RNAs both for validation of the vaccine but also to get information on the kinetics of expression because of its potential relevance for Ag presentation. In previous

electroporation studies only GFP or EGFP expression has been used to monitor intracellular protein expression (to determine transfection efficiency), but EGFP/GFP is an extremely stable protein (40) likely atypical of most Ags used in vaccination approaches. Intracellular detection of tumor Ags encoded by all three defined RNAs studied was readily and reproducibly possible by intracellular FACS staining. Using this approach we found that intracellular expression of MAGE-3, MelanA, and survivin (just like EGFP) all reached plateau already at 3–4 h after electroporation, indicating that rapid translation of transfected mRNA is a general phenomenon as one would expect. The kinetics of expression was, however, markedly different. Although EGFP levels stayed constant, both MAGE-3 and MelanA protein gradually decreased over time and had (almost completely) disappeared within 24 h. In sharp contrast, survivin decreased very rapidly and was no longer detectable after 6 h, which is in perfect accordance with its well-known short half-life (41) (Fig. 4). Of note, the kinetics were not different between DCs, which were transfected at their immature stage and then matured, and DCs, which were transfected after maturation. Because immature DCs need 24 h to mature after electroporation before they can be used for vaccination, the intracellular expression of the three Ags is very low or absent 26 h after electroporation when the DCs are harvested for use as a vaccine. This reduced expression could result in a lower and shorter Ag presentation on the DC surface following injection into the patient as immature transfected DC would no longer contain Ags available for prolonged internal supply of MHC class I binding peptides (which do not only derive from defective ribosomal subunits generated as a result of imperfect translation but also as a consequence of subsequent breakdown of unstable proteins). In contrast, mature DCs, 4 h after electroporation, still express large amounts of intracellular tumor Ags. In other words, most of the tumor Ag still can be processed and these cells could, therefore, be optimal for a clinical trial as a prolonged MHC-peptide presentation should enhance their immunogenicity (see below) (42).

Vaccination strategies using only one tumor Ag could impose selection pressure on the tumor, which could result in Ag-loss variants. The tumor cells that lost expression of that certain tumor Ag may then escape immune rejection (43–45). Therefore, vaccination with DC expressing several tumor Ags may be advantageous for immunotherapy, particularly for priming (46). Our report has shown that DC can be electroporated with RNAs coding for three different tumor Ags simultaneously without influencing the intracellular expression levels from each separately (Fig. 5). This is relevant as it had never been formally demonstrated in the context of RNA transfection, and is also reassuring for transfection of total tumor RNA. Furthermore, the capacity of the electroporated DC to generate the Mage-3.A1 or the MelanA.A2.1 epitope (Fig. 6) or to induce expansion of MelanA-specific T cells was not reduced when RNAs coding for all three Ags were coelectroporated.

DCs transfected with RNA at their mature stage appear superior in generating CTL epitopes and inducing CTL responses

There is evidence that the processing machinery is different in immature and mature DC, and there are well-grounded concerns that mature RNA-transfected DC due to their predominant expression of immunoproteasomes might not generate important tumor Ag-derived epitopes (34) We have, therefore, examined two well-characterized prototype tumor epitopes, namely the Mage-3.A1 and MelanA.A2.1 (wild-type) peptides. DC electroporated in the mature state with MAGE-3 RNA or MelanA RNA alone or in combination with the other RNAs, and assayed 4 or 24 h afterward, induced production of high amounts of IFN-γ by MAGE-3.A1- and MelanA.A2.1-specific T cells (Fig. 6), indicating a very efficient presentation of the MAGE-3 and MelanA epitope. Surprisingly, DC electroporated before maturation with either MAGE-3 RNA or MelanA RNA alone or with MAGE-3/MelanA/survivin RNA mixture, were far less efficient and sometimes totally incapable to stimulate IFN-γ production (although they worked perfectly well after exogenous peptide loading). This could be caused in part, as aforementioned, by the fact that MAGE-3 expression is no longer detectable, and MelanA expression is hardly detectable 24 h after electroporation (Fig. 4). Exogenous peptide-loading studies on human Mo-DC have demonstrated that MHC class I-peptide complexes display a much shorter half-life on the cell membrane of immature DC as compared with mature DC (47).[5] Furthermore, we have evidence from [^{35}S]methionine pulse/chase labeling studies that MHC class I molecules synthesized at the end of maturation have in general a much longer half-life (E. Kämpgen and N. Koch, unpublished observations) than the reportedly short-lived MHC class II molecules (48) synthesized earlier. Therefore, it is possible that after adding the maturation mixture, MAGE-3 and MelanA protein is processed and presented in MHC molecules, but because of the high turnover of the MHC-peptide complexes, the presentation rapidly decreases, and before the MHC-peptide complexes can become more stable due to maturation, the intracellular MAGE-3 and MelanA expression is already too low to serve as an efficient internal source of peptides for further MHC peptide loading. The low number of MHC-peptide complexes on the cell surface of immature electroporated DCs is not sufficient to stimulate IFN-γ production by CTL clones or lines (Fig. 6). However, this number is sufficient to expand T cells in a priming experiment, although the expanded T cells are not functional in a cytolysis assay (Fig. 7). It could, of course, also be that immature DC generate the epitope with only low efficiency, but this possibility awaits an exact quantification of MHC-peptide complexes (which is not possible by using CTL clones detecting less than a hundred complexes but is now feasible by using TCR-like Abs as a novel tool (49)), as well as investigation of the exact degradation pathway (currently it is unknown whether the Mage-3.A1 epitope is generated by the standard proteasome, the immunoproteasome, or alternatively, by nonproteasomal pathways). In this context it should also be considered what P. Pierre, E. Gatti, and colleagues (50) observed: namely that MHC class I-peptide complexes are rapidly and transiently down-regulated at the onset of maturation, and that the kinetics of this down-regulation correlate with the formation of DC aggresome-like inducible structures, in which newly formed ubiquitinated defective ribosomal initiation products accumulate (50, 51). One may speculate that transfection into mature DC is also superior because such defective ribosomal initiation products may no longer be retained in the DC aggresome-like inducible structures and get direct access to proteasomes.

In case of the well-characterized wild-type MelanA.A2.1 epitope EAAGIGILTV we could directly investigate the priming capacity of mature and immature RNA transfected DC, thanks to the presence of significant frequencies of primarily naive CTL precursors in adult human blood (36). The first surprise in this study was that in our system a single 7-day stimulation could reproducibly expand Ag-specific CD8$^+$ T cells and induce IFN-γ release and lytic effector function, even if DC loaded with the wild-type peptide were used, albeit these were clearly less efficient than immature or mature RNA transfected

[5] D. Dieckmann, E. S. Schultz, B. Ring, P. Chames, G. Held, H. H. Hoogenboom, and G. Schuler. Optimizing the antigen loading of monocyte-derived dendritic cells with exogenous peptides. *Submitted for publication*.

DC. The rapid and reproducible induction of MelanA.A2.1-specific responses from primarily naive precursors has usually only been observed with the highly immunogenic ELAGIGILTV analog peptide (52). Also, it has recently been reported that even with DC transfected at the immature stage with RNA and then matured, such responses were not observable despite multiple stimulations unless a mutated mRNA encoding full-length MelanA Ag with the A27L amino acid substitution to generate the analog peptide sequence was used (53). These observations certainly underscore the in vitro immunopotency of our DC vaccine. The second unanticipated observation was that mature transfected DC induced MelanA epitope-specific responses that were even more potent than those triggered by immature transfected DC, in that the extent of expansion was larger and the lytic activity on a per cell basis appeared several-fold higher. Based on published studies one might actually have even expected that immunoproteasome-rich mature DC could be unable to generate sufficient amounts of the standard proteasome-dependent MelanA epitope (34), although there is now more recent evidence that mature DCs still contain sizeable amounts of standard proteasomes (49, 54).

Of note, the standard proteasome-dependent MelanA epitope is generated from a long-lived protein (Fig. 4). Because DCs electroporated before maturation did not present the MelanA epitope, one can predict that short-lived proteins, such as survivin (Fig. 4), are also not effectively presented by these DCs. Actually, by optimizing the electroporation of already mature DC, we have likely created a possibility for the presentation of short-lived proteins. The more stable MHC class I expression on mature electroporated DCs will probably result in presentation of epitopes generated from short-lived proteins. Although we have evidence that mature transfected DCs present survivin epitopes (data not shown), we have yet to generate stable survivin-specific CTL clones to study in detail the kinetics of presentation of the survivin.A2-specific epitope. In addition we will for mechanistic studies include, of course, additional Ags, and we will also modify the Mage-3 and MelanA RNAs to artificially alter the half-life of the encoded proteins.

Unfortunately, there are no significant frequencies of primarily naive CTL precursors specific for the MAGE-3 and survivin epitopes present in human blood, and therefore we were not able to perform priming experiments with these Ags. However, we expect that the frequency of CTL precursors specific for these Ags is higher in melanoma patients, and that these specific CTL can be expanded in vivo, which will also facilitate further in vitro studies.

We are currently investigating the basis of the superiority of mature RNA-transfected DC, using novel tools like TCR-like Abs (49) and mutated tetramers detecting high avidity T cells (55). One possibility is certainly a stronger and prolonged Ag presentation (42, 52) as aforementioned. Recently Liao et al. (56) showed that mature DC, RNA transfected with 30–60% efficiency (as judged from EGFP expression) by a lipid-based reagent, are more potent than peptide-loaded DC in inducing MelanA-specific responses. This result is possibly due to a prolonged Ag presentation as shown by using a MelanA.A2.1-specific CTL clone as a readout. We are also considering other possibilities such as intracellular stimulation of TLRs (57) and induction of certain cytokines like IL-12 (58). Our data imply that mature RNA transfected DC might be superior immunogenes for CTL induction. Although priming to MHC class II epitopes has yet to be studied, the recent observation by Thielemans' group (59) that transfection into mature DC of endosomal targeting sequence-modified MAGE-3 mRNA is superior in generating the MAGE-3 DP.4 epitope suggests that mature RNA transfected DC might also be preferable over immature transfected DC for inducing Th cell responses.

Taken together, we have systematically developed a method to generate a standardized and validable RNA-electroporated, cryopreservable polyvalent Mo-DC vaccine. In addition, we have provided surprising evidence that DC transfected after maturation might be immunologically preferable to the use of DC transfected before maturation even in case of standard proteasome-dependent epitopes. This unexpected finding also leads to interesting questions regarding mechanisms responsible for their enhanced immunological potency, and respective hypotheses can now be experimentally addressed and may unravel interesting new biological aspects as well.

Acknowledgments

We thank Dr. Irina Tcherepanova for the pGEM4Z64A-EGFP, pGEM4Z64A-MAGE-3, pGEM4Z64A-MelanA, and pGEM4Z64A-survivin constructs, Dr. Giulio Spagnoli for the 57B anti-MAGE-3 Ab, Dr. Vincenzo Cerundolo and Dr. Pedro Romero for providing us with MelanA.A2.1-specific CTL clones, Dr. Alexander Prechtel for the DC migration assays, Waltraud Leisgang, Christiane Schwank, Elke Licha, Ester Müller, and Sonja Emmerling for their technical assistance, and Prof. Alexander Steinkasserer for fruitful discussions.

Disclosures

The authors have no financial conflict of interest.

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CORRECTIONS

Niels Schaft, Jan Dörrie, Peter Thumann, Verena E. Beck, Ina Müller, Erwin S. Schultz, Eckhart Kämpgen, Detlef Dieckmann, and Gerold Schuler. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. *The Journal of Immunology,* 2005, 174: 3087–3097.

An error was made in the grant information. The correct footnote is shown below.

[1] This work was supported by grants from the Deutsche Forschungsgemeinschaft (SCHU 1538), European Union (DCVACC, contract no.: 503037), the Sonderforschungsbereich 643 Project C1, and Forimmune Project T5.

Michelle A. Hurchla, John R. Sedy, Maya Gavrielli, Charles G. Drake, Theresa L. Murphy, and Kenneth M. Murphy. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly induced in anergic $CD4^+$ T cells. *The Journal of Immunology*, 2005, 174: 3377–3385.

The third author's last name is misspelled. The correct name is Maya Gavrieli.

Isabelle Béatrice Berkeredjian-Ding, Moritz Wagner, Veit Hornung, Thomas Giese, Max Schnurr, Stefan Endres, and Gunther Hartmann. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *The Journal of Immunology*, 2005, 174: 4043–4050.

The first author's last name is misspelled. The correct name is Isabelle Béatrice Bekeredjian-Ding. The error has been corrected in the online version, which now differs from the print version as originally published.

Gerard L. Bannenberg, Nan Chiang, Amiram Ariel, Makoto Arita, Eric Tjonahen, Katherine H. Gotlinger, Song Hong, and Charles N. Serhan. Molecular circuits of resolution: formation and actions of resolvins and protectins. *The Journal of Immunology,* 2005, 174: 4345–4355.

In *References*, an author's name was omitted from Reference 17. The correct citation is shown below.

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0022-1767/05/$02.00