Single-Step Antigen Loading and Activation of Dendritic Cells by mRNA Electroporation for the Purpose of Therapeutic Vaccination in Melanoma Patients

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Abstract

**Purpose:** A critical factor determining the effectiveness of currently used dendritic cell (DC)-based vaccines is the DC activation or maturation status. We have recently shown that the T-cell stimulatory capacity of DCs pulsed with tumor-antigen-derived peptides can be considerably increased by activating the DCs through electroporation with mRNA encoding CD40 ligand, CD70, and a constitutively active Toll-like receptor 4 (Tri-Mix DCs). Here, we investigate whether TriMix DCs can be coelectroporated with whole tumor-antigen–encoding mRNA.

**Experimental Design:** The T-cell stimulatory capacity of TriMix DCs pulsed with the immunodominant MelanA-A2 peptide and that of TriMix DCs coelectroporated with MelanA mRNA were compared *in vitro*. TriMix DCs were also coelectroporated with mRNA encoding Mage-A3, Mage-C2, tyrosinase, or gp100. The capacity of these DCs to stimulate tumor-antigen–specific T cells in melanoma patients was investigated both *in vitro* before vaccination and after DC vaccination.

**Results:** Like peptide-pulsed TriMix DCs, TriMix DCs coelectroporated with MelanA mRNA are very potent in inducing MelanA-specific CD8+ T cells *in vitro*. These T cells have an activated phenotype, show cytolytic capacity, and produce inflammatory cytokines in response to specific stimulation. TriMix DCs coelectroporated with tyrosinase are able to stimulate tyrosinase-specific CD8+ T cells *in vitro* from the blood of nonvaccinated melanoma patients. Furthermore, TriMix DCs coelectroporated with Mage-A3, Mage-C2, or tyrosinase are able to induce antigen-specific CD8+ T cells through therapeutic DC vaccination.

**Conclusions:** TriMix DCs coelectroporated with whole tumor-antigen mRNA stimulate antigen-specific T cells *in vitro* and induce antigen-specific T-cell responses in melanoma patients through vaccination. Therefore, they represent a promising new approach for antitumor immunotherapy.

The past five decades have witnessed a steady increase in the incidence of malignant melanoma. Whereas early detection and appropriate surgery have improved outcomes, at least one third of patients with early-stage melanoma will develop metastases. The prognosis for patients with malignant metastatic melanoma remains poor. These patients have a median survival of approximately 6 to 8 months, and <5% will generally survive for 5 years or more (1). There is universal agreement that further research to address this problem is critically warranted.

Many strategies to enhance specific or nonspecific immunity in melanoma patients have been explored in clinical studies (2). Although the field is relatively new and many clinical variables remain to be investigated, vaccination with tumor-associated antigen (TAA)–expressing dendritic cells (DC) might provide a therapeutic benefit (3). Roughly, the DC life cycle can be divided into two stages: the immature and the mature stage. Immature DCs reside in the periphery and are specialized...
Different kines and chemokines. Different maturation stimuli lead to HLA and costimulatory molecules, changes in chemokine re-maturation is highly complex and involves up-regulation of characteristics, mature DCs seem to be the ideal cellular tools for CD40 and TLR4 ligation through addition of soluble mature, cytokine/chemokine-secreting DCs, as has been shown TLR4 electroporation would mimic CD40 ligation (7) and TLR4 (caTLR4; ref. 6). Here, the combination of CD40L and gand (CD40L), CD70, and a constitutively active form of poration with mRNA encoding a so-called TriMix of CD40 li-

them with three different molecular adjuvants through electro-

nation protocols could be improved by providing the DCs with tumor immune response through vaccination, and there is mining whether these cells will be potent inducers of an anti-(5). Thus, the activation state of DCs is a critical factor deter-

phologic, phenotypic, and functional changes and transform in antigen recognition and uptake. On receiving a maturation signal such as a Toll-like receptor (TLR) ligand or other pathogen-associated molecular pattern, DCs undergo several morphologic, phenotypic, and functional changes and transform into efficient antigen-processing/presenting cells capable of stimulating both CD4+ and CD8+ T cells (4). Based on these characteristics, mature DCs seem to be the ideal cellular tools for use in cancer immunotherapy. Nonetheless, the process of maturation is highly complex and involves up-regulation of HLA and costimulatory molecules, changes in chemokine receptor repertoire, and enhanced secretion of inflammatory cyto-

kines and chemokines. Different maturation stimuli lead to different "mature DC" that possess different effector functions (5). Thus, the activation state of DCs is a critical factor deter-

ming whether these cells will be potent inducers of an anti-
tumor immune response through vaccination, and there is general belief that the effectiveness of currently used DC vacci-
nation protocols could be improved by providing the DCs with a more potent activation signal.

We have recently shown that the T-cell stimulatory capacity of peptide-pulsed DCs can be greatly enhanced by providing them with three different molecular adjuvants through electroporation with mRNA encoding a so-called TriMix of CD40 li-
gand (CD40L), CD70, and a constitutively active form of TLR4 (caTLR4; ref. 6). Here, the combination of CD40L and caTLR4 electroporation would mimic CD40 ligation (7) and TLR4 signaling (8) of the DCs and generates phenotypically mature, cytokine/chemokine-secreting DCs, as has been shown for CD40 and TLR4 ligation through addition of soluble CD40L and lipopolysaccharide (9). On the other hand, the intro-
duction of CD70 into the DCs would provide a costimulatory signal to CD27+ naive T cells by inhibiting activated T-cell apoptosis and by supporting T-cell proliferation (10).

Providing the DCs with a maturation signal through mRNA electroporation offers several advantages. There is no need to preincubate the DCs for up to 48 hours with soluble matura-
signals like proinflammatory cytokines or TLR ligands to achieve DC activation, which can render the cells "exhausted" and inferior for vaccination purposes (11). As a result, TriMix-electroporated DCs, which can be injected into the patient with-
in a few hours after electroporation, will mature and secrete most of their immunostimulatory cytokines and chemokines in situ. Furthermore, it has been postulated that maturation of DCs in situ resembles more closely the physiologic process in-
volved in response to pathogen infection and may therefore lead to enhanced T-cell immunity (12).

Here, we investigate whether TriMix DCs can be coelectropo-

rated with TAA-encoding mRNA instead of being pulsed with antigenic peptides. This approach offers several additional ad-
vantages. First, the maturation and TAA loading of the DCs can be combined in one simple electroporation step. Obviating the peptide pulsing step in the vaccine production thus results in less manipulation of the cells and less cell loss and contamination risk. Second, by using full-length TAA-encoding mRNA, all possible antigenic epitopes of the TAA will be presented instead of some selected epitopes. Consequently, this strategy could in-
duce a broader TAA-specific T-cell response, and it is not depend-
ent on the knowledge of each patient's HLA haplotype or on the prior identification of TAA-derived epitopes (13). Third, the TAA-encoding plasmid can be genetically modified by adding an HLA class II targeting sequence. This not only routes the TAA to the HLA class II compartments for processing and pre-

sentation of HLA class II–restricted TAA-derived peptides but also enhances processing and presentation in the context of HLA class I molecules (14, 15).

We show that TriMix DCs can stimulate specific T cells when coelectroporated with whole MelanA-encoding mRNA instead of being pulsed with MelanA-derived peptide. We also show that TriMix DCs are able, both in vitro and in vivo, to induce T cells specific for other TAA with a lower precursor frequency.

Translational Relevance

Therapeutic vaccination with activated, tumor-
antigen–expressing autologous dendritic cells (DC) might provide a clinical benefit for patients with me-
tastatic malignant melanoma. We have recently shown that DCs pulsed with a MelanA-derived pep-
tide and activated by electroporation with a combina-
tion of CD40 ligand, CD70, and constitutively active Toll-like receptor 4 mRNA (TriMix) are far more supe-

in inducing MelanA-specific T cells than DCs ac-

tivated with the conventional methods. We now report that TriMix electroporation can be combined with whole tumor-antigen mRNA electroporation. For the MelanA antigen, mRNA coelectroporated Tri-

Mix DCs had comparable immunostimulatory capac-

ity as peptide-pulsed TriMix DCs in vitro. For melanoma-associated antigens with lower precursor frequencies (such as Mage-A3, Mage-C2, and tyrosi-
nase), we show that TriMix DCs coelectroporated with tumor-antigen–encoding mRNA can also induce specific T cells, both in vitro and following therapeu-
tic vaccination of patients with advanced melanoma. We therefore believe that this work is relevant for the further clinical development of an improved DC-based vaccine for the treatment of melanoma and potentially also other types of cancer, whereby in one simple and effective step both the tumor-antigen information and an activation signal are given to the DCs.

Materials and Methods

Genetic constructs. The pGEM-C40L, pGEM-C70, and pGEM-caTLR4 plasmids encoding CD40L, CD70, and caTLR4 (containing the intracellular and transmembrane fragments of TLR4, as described in ref. 8); the pGEM-NFGR plasmid encoding a truncated form of the nerve growth factor receptor (NGFR, containing the extracellular and transmembrane fragments); the pGEM-sig-MageA3-DCLamp plasmid encoding the full-length Mage-A3 antigen linked to the HLA class II tar-
getting sequence of DC-Lamp (transmembrane/luminal region); and the pGEM-sig-MelanA-DCLamp plasmid encoding the full-length MelanA antigen, containing the optimized immunodominant MelanA-A2 epitope and linked to the DC-Lamp targeting signal, have previously been described (6, 14).

The pGEM-sig-MageC2-DCLamp plasmid contains the full-length Mage-C2 gene, flanked by the signal sequence and the HLA class II targeting sequence of DC-Lamp. The pGEM-sig-gp100-Lamp and pGEM-sig-tyrosinase-Lamp plasmids contain the gp100 and tyrosinase genes, respectively, with their own signal sequence and with their transmembrane and luminal regions replaced by the HLA class II targeting sequence of Lamp-1 (14).
In vitro generation of human monocye–derived DCs, in vitro transcrip- 
tion of capped mRNA, and mRNA electroporation of DCs. Generation, 
maturaton, and cryopreservation of immature and cytokine- 
cocktail–matured DCs, capped mRNA production, and mRNA electropo-
ration of TriMix DCs, either pulsed with MelanA peptide or coelectro-
porated with MelanA-DCLamp mRNA, have been described elsewhere 6). For coelectroporation with MageA3-DCLamp, MageC2-DCLamp, tyrosinase-Lamp, or gp100-Lamp mRNA, DCs were electroporated with a 
slightly different protocol. Here, 50 × 10^6 DCs were electroporated with 
20 μg of CD40L, CD70, and caTLR4 mRNA together with 60 μg of TAAs-
encoding mRNA. The following conditions were used for electropo-
ration: voltage, 300 V; capacitance, 450 μF; and resistance, 99 Ω, in a final 
volume of 600 μL.

Patients, vaccine preparation, and vaccination schedule. Three HLA-A2+ 
patients (two male, one female) with recurrent stage III or stage IV me-
anoma were recruited in an ongoing institutional (UZ Brussels) pilot 
trial with autologous TriMix-DC vaccine for patients with advanced 
melanoma. Patients were studied after written informed consent was 
obtained and with approval of the study protocol by the institutional 
ethical commission and national competent authorities.

For vaccination purposes, DCs were electroporated with mRNA en-
coding one of four TAA (Mage-A3, Mage-C2, tyrosinase, and gp100) 
and the TriMix mRNA. After a rest period of 1 h, the cells were mixed at 
equal ratios. The first vaccine was administered before cryopreservation 
of the DC vaccine, and subsequent vaccines were done with cells that 
were thawed at the day of vaccination. Vaccines consist of 2 ± 10^6 TriMix DC per antigen and are administered by four biweekly in-
tradermal injections at four different injection sites (axillary and/or in-
guinal region).

Synthetic peptides and peptide pulsing. The HLA-A*0201–restricted 
Mage-A3 (aa 112-120; KVAELVHFL), Mage-C2 (aa 336-344; ALK-
DVEERV), tyrosinase (aa 369-377; YMDGTMSQV), gp100 (aa 209-
217; ITDQVPFSV), and MelanA/MART-1 (optimized immunodominant 
epitope, aa 26-35; ELAGIGILTV) derived peptides were purchased from 
Thermo Electron. The HLA-A2–restricted gag peptide (gag-A2 peptide, 
HXB2 gag peptide–complete set, NIH, AIDS Research & Reference 
Reagent Program, McKesson BioServices Corporation) was used as a 
negative control. The HLA-DRB1*0401–restricted Mage-A3 peptide 
(aa 243-258; KKLITQHFVQYNELEY) was purchased from Henogen. 
For peptide pulsing, DCs or T2 cells were diluted to a final density of 
2 × 10^7/mL in Iscove’s modified Dulbecco’s medium (Invitrogen) 
containing 10 μg/mL peptide and were incubated for 2 h at 37°C.

Flow cytometry. DCs were stained using the following monoclonal 
antibodies (mAbs): CD40-allophycocyanin (APC), CD70-phycocerythrin 
(PE), CD80-PE, CD83-PE, CD86-PE, HLA-ABC-APC, and CD83-FITC (all 
from BD Pharmingen), and HLA-DR (purified from clone I243). The anti-
HLA-DR antibody was biotin labeled and detected through streptavi-
din-APC (BD Pharmingen). T cells were phenotyped with the following 
mAbs: CD8-FITC, CD8-APC-Cy7, CD27-APC, CD28-APC, CD45RA-
biotin, CD45RO-APC, CD62L-FITC (all from BD Pharmingen), and 
CCR7-APC (R&D Systems). Biotinylated CD45RA was detected with 
mAbs: CD8-FITC, CD8-APC-Cy7, CD27-APC, CD28-APC, CD45RA-
HLA-DR antibody was biotin labeled and detected through streptavi-
dine-phycocyanin (PE), CD80-PE, CD83-PE, CD86-PE, HLA-ABC-FITC (all from BD 
Pharmingen). For coelectroporation with MageA3-DCLamp, MageC2-DCLamp, tyrosinase-Lamp, or gp100-
derived peptide (sequences KVAELVHFL, ALKDVDEERV, YMDGTMSQV, 
and ITDQVPFSV, respectively; mixed at equal ratios) or TriMix DCs as 
prepared for vaccination were used. CD8+ T cells were restimulated weekly with the same stimulator DCs as used in the primary stimulation. 
After two rounds of stimulation, CD8+ T cells were harvested and their 
antigen specificity and function were determined.

Tetramer staining. T cells were stained with a FITC–labeled anti-CD8 (BD 
Pharmingen) and with 10 nmol/L PE–labeled HLA-A2 tetramers (prepared 
in-house). The tetramers contained one of the following HLA-A2–restricted, TAA–derived peptides: ELAGIGILTV (MelanA 
derived); FLWGPRLAV or KVAELVHFL (Mage-A3 derived); ALKDVDEERV 
(Mage-C2 derived); YMDGTMSQV (tyrosinase derived); ITDQVPFSV, 
YESLCPVTAY, or KTWWGYYQVQ (gp100 derived); or SLLMWTIQC 
(NY-ESO-1 derived, negative control). Cells were analyzed by flow 
cytometry.

Intracellular cytokine staining and CD107a/CD137 assay. For intracellu-
lar cytokine staining, 2 × 10^6 primed CD8+ T cells were restimulated with 2 × 10^6 stimulator cells in the presence of Golgi-plug [brefeldina, Becton Dick-
inson (BD)]. After 12 h of incubation at 37°C, CD8+ T cells were then 
stimulated with IFN-γ–APC–conjugated anti-CD8 mAb, washed, per-
meabilized, and stained intracellularly using the BD Cytofix/Cytoperm plus 
kit with IFN-γ–PE/TFN-α–APC or IFN-γ–PE/TFN-α–FITC, respectively. For the 
CD107a/CD137 assay, 1 × 10^5 primed CD8+ T cells were re- 
stimulated with 2 × 10^6 stimulator cells in the presence of Golgi-stop (monesin, BD) 
and PE-Cy5–labeled anti-CD107a mAb (BD Pharmingen). After 12 h of incuba-
tion at 37°C, the cells were harvested and stained with FITC–labeled anti-
CD8 mAb and PE-labeled CD137 mAb (both from BD Pharmingen). As stimu-
lator cells, TAM-deficient, HLA-A2+ T cells pulsed with peptide or cytokine 
cocktail–matured DCs electroporated with TAA mRNA were used. Cells 
were analyzed by flow cytometry using a FACS Canto flow cytometer and 
FACSDiva software. Cells were electronically gated according to light scatter 
properties to exclude dead and contaminating cells.

Antigen presentation assays for HLA class II–restricted epitopes. Four 
hours after electroporation, 2 × 10^6 HLA-DR4* or control HLA-DR4* 
TriMix DCs pulsed with specific peptide or coelectroporated with MageA3-
DCLamp mRNA were cocultured with 5,000 T cells from clone R12-C9. 
The specific HLA restriction of these T cells have been described elsewhere 
(16, 17). Clone R12-C9 was kept in culture as described previ-
ously (17), is HLA-DR4 (HLA-DRB1*0401) restricted, and is specific for 
the Mage-A3 epitope aa 243-258 with sequence KKLITQHFVQYNELEY. 
Each coculture was done in triplicate in round-bottomed microwells in 
200 μL stimulation medium. After 20 h of coculture, the supernatant 
was assessed for the presence of IFN-γ by ELISA using commercially available 
antibodies (Thermo Scientific).

Results and Discussion

TriMix DCs can be coelectroporated with TAA mRNA without 
affecting their electroporation efficiency, mature phenotype, and 
cytokine secretion. DCs electroporated with a TriMix of 
CD40L, CD70, and caTLR4 mRNA are typically very efficiently 
electroporated: on average, ~80% of the DCs express the CD70 
molecule on their surface 24 h after electroporation. Because we 
observed that the electroporation efficiency slightly decreased 
when a combination of three different mRNAs was electropo-
ration in comparison with a single mRNA, we investigated

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whether adding a fourth mRNA would affect electroporation efficiency. We found that, when TriMix DCs are coelectroporated with TAA mRNA, electroporation efficiency does not alter notably as shown by CD70 expression 24 h after electroporation (Fig. 1A).

After electroporation with TriMix mRNA, immature DCs acquire a mature phenotype and enhance their cytokine secretion as shown by up-regulation of costimulatory molecules (CD40, CD80, CD83, and CD86) and HLA molecules and IL-12p70 secretion, respectively. Here also, when TriMix DCs are coelectroporated with TAA mRNA, the mature phenotype (Fig. 1B) and cytokine secretion (Fig. 1C) are not markedly altered. Thus, there were no differences in electroporation efficiency, maturation potential, and cytokine secretion when TriMix DCs were prepared as such or coelectroporated with TAA mRNA.

**Induction of MelanA-specific CD8+ T cells by TriMix DCs pulsed with peptide or coelectroporated with whole TAA mRNA.** We investigated whether TriMix DCs coelectroporated with full-length MelanA-encoding mRNA could prime naïve MelanA-specific CD8+ T cells. Therefore, DCs from HLA-A2+ healthy donors were
electroporated with TriMix mRNA and either pulsed with the immunodominant MelanA peptide or coelectroporated with MelanA-DCLamp mRNA. The DCs were then cocultured with autologous CD8+ T cells without the addition of exogenous cytokines. Immature and cytokine cocktail–matured DCs, electroporated with irrelevant NGFR mRNA and pulsed with MelanA peptide, were used as controls. Cells were stimulated thrice with a weekly interval. After each stimulation round, the number of remaining cells and the percentage of tetramer-positive, MelanA-specific CD8+ T cells were determined. From these data, the absolute number of tetramer-positive, MelanA-specific CD8+ T cells was calculated (Table 1). Furthermore, the relative percentage of MelanA-specific T cells obtained after each stimulation was compared with the absolute number of MelanA-specific CD8+ T cells obtained after three weekly stimulations with peptide-pulsed TriMix DCs (set at 100%; Fig. 2A). We observed that, after one or two stimulations, TriMix DCs coelectroporated with TAA mRNA were slightly less potent in inducing MelanA-specific CD8+ T cells than, after one or two stimulations, TriMix DCs coelectroporated with MelanA-DCLamp mRNA. The DCs were then cocultured with an immunodominant MelanA peptide or coelectroporated with MelanA-DCLamp mRNA. The DCs were then identified by flow cytometry. Background staining with MAGE-A3–specific HLA-A2 tetramers, which never reached higher than 0.5%, was subtracted. The number of living cells was determined by trypan blue exclusion.

Table 1. Induction of HLA-A2–restricted MelanA-specific CD8+ T cells by TriMix DCs pulsed with MelanA-A2 peptide or coelectroporated with MelanA-DCLamp mRNA

<table>
<thead>
<tr>
<th></th>
<th>%CD8+ MelanA tetramer+ T cells/no. of CD8+ T cells (10^6)</th>
<th>Absolute no. of CD8+ MelanA tetramer+ T cells (10^3)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>Imm + MelanA peptide</td>
<td>0.3/1.2</td>
<td>0.1/1.35</td>
</tr>
<tr>
<td>TriMix + MelanA peptide</td>
<td>72.4/6.8</td>
<td>63.2/1.1</td>
</tr>
<tr>
<td>TriMix + MelanA mRNA</td>
<td>72.5/6.3</td>
<td>25.9/1.6</td>
</tr>
<tr>
<td>Mat + MelanA peptide</td>
<td>ND</td>
<td>0.1/1.5</td>
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NOTE: Results are shown for four individual experiments from different healthy donors.

Abbreviations: Imm, immature DCs electroporated with irrelevant NGFR mRNA; Mat, cytokine cocktail–matured DCs electroporated with NGFR mRNA; ND, not done.

*The T-cell population generated after three weekly stimulations with the different DCs was stained with MelanA peptide–loaded HLA-A2 tetramers and anti-CD8 antibody. MelanA-specific CD8+ T cells were then identified by flow cytometry. Background staining with TAA mRNA, which never reached higher than 0.5%, was subtracted. The number of living cells was determined by trypan blue exclusion.

†Absolute number of MelanA-specific CD8+ T cells was calculated with the following formula: (number of CD8+ T cells/100) × % of CD8+ MelanA tetramer+ T cells.

For all donors tested, we observed that the percentage of MelanA-specific T cells correlated with the percentage of Lyt/activated T cells and with the percentage of IFN-γ/TNF-α–producing T cells. Overall, no major differences were observed between T cells stimulated with peptide-pulsed or TAA-coelectroporated DCs, except a slight but reproducible increase in mean fluorescence intensity of IFN-γ staining and also in percentage of IFN-γ/TNF-α double-positive cells. We also analyzed the phenotype of the induced MelanA-specific CD8+ T cells. The primed CD8+ MelanA-specific T cells were all CD45RA−CD45RO+CD27−CD28−, together with a variable expression of CD62L and CCR7 (data not shown), suggesting that both central memory T cells (CD62L+ and CCR7+) and early effecter memory T cells (CD62L- and CCR7-) had been induced (20). Overall, there were no significant differences in the phenotype of the MelanA-specific CD8+ T cells of the different donors regardless of whether peptide-pulsed or TAA-coelectroporated DCs were used for stimulation.

Although coelectroporated TriMix DCs seem to induce a lower number of epitope-specific T cells than their peptide-pulsed counterparts in this setting, this does not necessarily mean that they will be less efficient when used for vaccination purposes, and this is for a number of reasons. First, when investigating the qualitative functionality of the induced T cells, we consistently observed that the T cells stimulated with coelectroporated TriMix DCs induced more cells secreting both IFN-γ and TNF-α. Moreover, the mean fluorescence intensity of the intracellular IFN-γ staining was increased, indicating that more cytokine per cell had been produced. These data suggest that these T cells are multifunctional, which has been correlated with a better effecter function (21). Second, by electroporating full-length TAA mRNA linked to an HLA class II targeting signal into the DCs, all antigenic epitopes are introduced, including unidentified epitopes and epitopes restricted to all possible HLA haplotypes, being HLA class I as well as class II. Therefore, this approach is prone to induce a broader TAA-specific T-cell response. Third, data from the literature suggest that TriMix DCs in general and TriMix DCs coelectroporated with TAA mRNA in particular will induce T cells with a higher TCR avidity. Indeed, it has been shown that functional avidity is dependent on IL-12p70 (22) and that DCs coelectroporated with IL-12 mRNA induce T cells with an enhanced functional avidity (23). Because TriMix DCs secrete more IL-12p70 than do classic DCs, it is conceivable that they will also induce T cells with a higher TCR avidity. On the
other hand, it has been shown that DCs electroporated with TAA-encoding mRNA exhibit a prolonged duration of presentation of immunogenic epitopes compared with peptide-pulsed DC and, as a result, generate a CTL population that exhibits higher target avidity (24). Therefore, TriMix DCs coelectroporated with TAA mRNA might be even more potent in increasing TCR avidity.

**Stimulation of Mage-A3–specific CD4+ T cells by TriMix DCs pulsed with peptide or coelectroporated with whole TAA mRNA.**

We have shown before that TriMix DCs preferentially induce Th1 CD4+ T cells (6). Because all TAA constructs used contain an HLA class II targeting signal, we now investigated whether TriMix DCs coelectroporated with TAA mRNA could process and present HLA class II–restricted epitopes from electroporated TAA mRNA to established CD4+ T cells. Therefore, TriMix DCs were either pulsed with Mage-A3- DP4 peptide or coelectroporated with MageA3-DCLamp mRNA. Four hours later, the cells were cocultured with Mage-A3–specific, HLA-DP4–restricted T cells for 20 hours. Immature DCs electroporated with irrelevant NGFR mRNA were used as a negative control. IFN-γ released in the supernatant during the coculture was measured by ELISA (Fig. 3). We observed that TriMix DCs are indeed capable of presenting antigenic epitopes in the context of HLA class II molecules. Moreover, their capacity to do so is similar to the CD4+ T-cell stimulatory capacity of peptide-pulsed cells. Of note, TriMix DCs derived from an HLA-DP4+ donor and loaded with Mage-A3-DP4 peptide or coelectroporated with MageA3-DCLamp mRNA were not able to induce IFN-γ secretion from the T-cell clone (results not shown), confirming the specificity and restriction of the clone.

**In vitro induction of CD8+ T cells specific for other antigens than MelanA in the blood of unvaccinated melanoma patients.**

Unlike most TAA-derived epitopes, the HLA-A2–restricted immunodominant peptide of MelanA, which was used in our CD8+ T-cell stimulations up to now, is an epitope for which a very high precursor frequency in the blood exists. We therefore evaluated whether TriMix DCs coelectroporated with other TAA would also be able to induce antigen-specific CD8+ T-cell responses. Because this work is part of the preclinical assessment of a vaccination study where TriMix DCs coelectroporated with...
Mage-A3, Mage-C2, tyrosinase, or gp100 mRNA will be injected into melanoma patients, we wanted to investigate whether these DCs are able to induce CD8+ T cells specific for these antigens in vitro in the peripheral blood mononuclear cells of unvaccinated melanoma patients. Therefore, CD8+ T cells from HLA-A2+ melanoma patients were cocultured with autologous DCs as prepared for vaccination (i.e., electroporated with TriMix mRNA together with one of four TAA mRNAs and mixed afterward at equal amounts). Cytokine cocktail–matured DCs pulsed with an HLA-A2–restricted Mage-A3–, Mage-C2–, tyrosinase–, or gp100–derived peptide (also mixed at equal amounts) were used as control. During the whole stimulation period, no exogenous cytokines like IL-2 or IL-7 to support T-cell proliferation and survival were added. After three weekly stimulations, the T cells were stained with a panel of tetramers recognizing seven different HLA-A2–restricted Mage-A3–, Mage-C2–, tyrosinase–, or gp100–derived epitopes. For all three patients tested, we observed that TriMix DCs coelectroporated with TAA mRNA were able to induce HLA-A2–restricted tyrosinase-specific T cells, whereas cytokine cocktail–matured DCs pulsed with the tyrosinase-A2 peptide failed to do so (Fig. 4A). We did not observe T cells recognizing the other Mage-A3–, Mage-C2–, or gp100–specific tetramers, neither when TriMix DCs nor cytokine cocktail–matured DCs were used for in vitro stimulation (data not shown). Although TriMix DCs were coelectroporated with full-length TAA mRNA encoding all possible TAA–derived epitopes, we observed no induction of other Mage-A3–, Mage-C2–, tyrosinase–, or gp100–specific T cells, as assessed by CD137/CD107a and intracellular cytokine staining assays (Fig. 4B and C, and data not shown), although low frequencies of specific T cells might have been concealed by the aspecific T-cell activation induced by TriMix DCs.

This aspecific T-cell activation seems to be inherent to TriMix DCs and occurs both in vitro and in vivo. The reason for this, however, remains unclear at this point. On the one hand, it might be due to the fact that DCs electroporated with CD40L and cTLR4 secrete quite high amounts of cytokines and chemokines (6), which might attract and activate T cells in an aspecific manner. On the other hand, it has been shown that chronic stimulation of naïve T cells by antigen-presenting cells continuously expressing CD70 leads to activation of the T-cell pool and conversion into effector memory cells (25). In this CD70 transgenic mouse model, the T-cell activation eventually led to exhaustion of the naïve T-cell pool and lethal immunodeficiency. Although we also use antigen-presenting cells continuously expressing CD70, we do not expect this in our vaccination study because the T-cell pool is not continuously stimulated with CD70 because the DCs are injected biweekly and have a limited life span in vivo. Another reason for this aspecific T-cell activation might be found in the natural killer T-cell population. Indeed, natural killer T cells are known to be reactive to DCs, and they are particularly activated by mature, IL-12–secreting DCs (26). Thus, it is conceivable that stimulation with TriMix DCs may expand invariant natural killer T cells. This might be beneficial for the outcome of cancer immunotherapy (27) because natural killer T cells can, in turn, positively modulate DCs, and their activation in the presence of antigenic proteins can enhance antigen-specific T-cell responses (28).

When compared with the massive induction of MelanA–specific T cells by TriMix DCs, the induction of T cells specific for other tumor-antigens in vitro seems rather poor. This is most probably due to the low precursor frequency of the latter. It is difficult, however, to compare our results with other reports on the induction of MelanA–specific CD8+ T cells because they are scarce and because exogenous IL-2 and/or IL-7 are commonly added during these stimulations, which support T-cell activation and proliferation and thus create an artificial T-cell stimulatory environment.

Induction of CD8+ T cells specific for other antigens than MelanA in the blood of melanoma patients after vaccination with TriMix DCs coelectroporated with TAA mRNA. Finally, we investigated whether TriMix DCs coelectroporated with Mage-A3, Mage-C2, tyrosinase, or gp100 mRNA would be able to induce an antigen-specific CD8+ T-cell response in vivo. Therefore, two HLA-A2+ melanoma patients (patients 2 and 3) were vaccinated four times at biweekly intervals with a mixture of the four different TriMix DCs (i.e., expressing one of the four vaccinal antigens). Two weeks after the last vaccination, CD8+ T cells isolated from the blood of these patients were restimulated in vitro with autologous DCs, either with TriMix DCs as prepared for vaccination or with cytokine cocktail–matured DCs coelectroporated with TAA mRNA. Again, during the whole stimulation period, no exogenous cytokines were added. After two weekly stimulations, the antigen specificity and functionality of the T cells were investigated by staining with the HLA-A2 tetramer panel and by the CD137/CD107a and intracellular cytokine staining assays; this was compared with the response induced in the CD8+ T cells of the same patients, but before vaccination. For both patients, we observed no T cells specific for the known HLA-A2–restricted Mage-A3–, Mage-C2–, tyrosinase–, or gp100–derived epitopes in tetramer staining (data not shown), although we were able to detect tyrosinase-A2–specific T cells in the CD8+ T cells of these same patients before vaccination (Fig. 4A). This was still the case after the T cells had received an extra stimulation round in vitro (data not shown). Because the patients were vaccinated...
Fig. 4. Induction of CD8+ T cells specific for other antigens than MelanA in melanoma patients both in vitro and in vivo. A, TriMix DCs as prepared for vaccination were used to stimulate CD8+ T cells isolated from the blood of unvaccinated HLA-A2+ melanoma patients. Cytokine cocktail–matured DCs pulsed with HLA-A2–restricted Mage-A3–, Mage-C2–, tyrosinase–, or gp100–specific peptide were used as control. After three weekly stimulations, the cells were stained with anti-CD8 antibody and a panel of HLA-A2 tetramers loaded with different Mage-A3–, Mage-C2–, tyrosinase–, or gp100–specific peptides. TAA-specific CD8+ T cells were then identified by flow cytometry. Background staining with NY-ESO-1–specific HLA-A2 tetramers, which never reached higher than 0.1%, was subtracted. B, the activation status and cytolytic activity of CD8+ T cells from melanoma patients before or after vaccination with TriMix DCs were determined by a CD107a/137 assay. CD8+ T cells isolated from the blood of HLA-A2+ melanoma patients before or after vaccination with TriMix DCs were stimulated twice in vitro with the same DCs as used for vaccination. One week after the last stimulation, cells were restimulated overnight with mature DCs electroporated with TAA mRNA or NGFR as irrelevant control in the presence of anti-CD107-PE-Cy5 mAb and Golgi-stop. Cells were harvested, stained with anti-CD8-FITC and anti-CD137-PE, and analyzed by flow cytometry. T cells were gated on forward scatter/side scatter characteristics and CD8 positivity. The percentage of CD137/CD107a double-positive cells is given. C, cytokine production of CD8+ T cells from melanoma patients before or after vaccination with TriMix DCs was determined by intracellular cytokine staining. CD8+ T cells isolated from the blood of HLA-A2+ melanoma patients before or after vaccination with TriMix DCs were stimulated twice in vitro with the same DCs as used for vaccination. One week after the last stimulation, cells were restimulated overnight with mature DCs electroporated with TAA mRNA or NGFR as irrelevant control in the presence of Golgi-plug. Then, T cells were stained for CD8, IFN-γ, and TNF-α positivity. T cells were gated on forward scatter/side scatter characteristics and CD8 positivity. The percentage of IFN-γ– and/or TNF-α–secreting cells is given.
with DCs coelectroporated with full-length TAA mRNA encoding all possible TAA-derived epitopes, we investigated whether a T-cell response specific for other epitopes than the known HLA-A2-restricted epitopes had been induced. Therefore, 1 week after the second restimulation in vitro, T cells were restimulated overnight with mature DCs electroporated with TAA mRNA or NGRF as irrelevant control, after which a CD137/CD107a assay (Fig. 4B) and an intracellular cytokine staining assay (Fig. 4C) were done. We observed strong, vaccine-induced responses against other Mage-A3 (patient 2), Mage-C2 (patients 2 and 3), and tyrosinase epitopes (patient 2), which were not present before vaccination. Overall, similar results were obtained when TriMix or cytokine-cocktail-matured DCs were used for restimulation in vitro, except for the fact that the latter induced less aspecific T cells (data not shown).

Remarkably, although the responses induced in CD8+ T cells of unvaccinated patients were quite poor, we observed that TriMix DCs are able to induce robust responses for the Mage-A3, Mage-C2, and tyrosinase antigens through vaccination. Tetramer staining showed that these responses were not directed toward the known HLA-A2–restricted epitopes tested, clearly evidencing the advantage of using full-length TAA mRNA. Of note, the HLA-A2–restricted tyrosinase-specific responses that could be found in patients 2 and 3 before vaccination were no longer detected after vaccination. Several reasons can account for this. It is possible that the tyrosinase-specific T cells that were stimulated in vitro from the blood CD8+ T cells of unvaccinated patients issued from a naive T-cell response, which was not induced in vivo, or it concerns here a tyrosinase-specific T-cell response that was already present in vivo before vaccination but that was lost afterward; or the tyrosinase-specific T-cell response is still present after vaccination, but can simply no longer be detected because the T cells were overgrown during the in vitro culture by other vaccine-specific T cells.

Concluding remarks. We show that TriMix DCs coelectroporated with whole tumor-antigen mRNA can stimulate antigen-specific T cells against the MelanA-antigen and against TAA’s with lower precursor frequencies and this both in vitro and through vaccination. They thus form a promising new approach for the therapeutic vaccination of cancer patients with a potentially broad clinical application whereby in one simple and effective step both the TAA and an activation signal are given to the DCs. Moreover, the results documented here provide justifications for the conduct of a larger clinical trial on the therapeutic vaccination of advanced melanoma patients with a TriMix DC vaccine. Further study should establish the clinical antitumor activity of TriMix DC therapeutic vaccination as well as further documentation of the immunologic responses in delayed-type hypersensitivity skin biopsies and in the blood.

Disclosure of Potential Conflicts of Interest

Data and results of this manuscript are part of an international patent application filed by The Vrije Universiteit Brussel, entitled ‘Enhancing the T-cell stimulatory capacity of human antigen-presenting cells and their use in vaccination’ and was published on March 18, 2009 (no. WO2009/034172). Aude Bonehill and Kris Thielemans are named as inventors of this application.

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