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 (TriMix 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.

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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

Translational Relevance

Therapeutic vaccination with activated, tumor-antigen–expressing autologous dendritic cells (DC) might provide a clinical benefit for patients with metastatic malignant melanoma. We have recently shown that DCs pulsed with a MelanA-derived peptide and activated by electroporation with a combination of CD40 ligand, CD70, and constitutively active Toll-like receptor 4 mRNA (TriMix) are far more superior in inducing MelanA-specific T cells than DCs activated 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 TriMix DCs had comparable immunostimulatory capacity as peptide-pulsed TriMix DCs *in vitro*. For melanoma-associated antigens with lower precursor frequencies (such as Mage-A3, Mage-C2, and tyrosinase), we show that TriMix DCs coelectroporated with tumor-antigen–encoding mRNA can also induce specific T cells, both *in vitro* and following therapeutic 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.

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 cytokines 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 determining whether these cells will be potent inducers of an antitumor immune response through vaccination, and there is general belief that the effectiveness of currently used DC vaccination 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 ligand (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 introduction 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 maturation 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 within 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 involved in response to pathogen infection and may therefore lead to enhanced T-cell immunity (12).

Here, we investigate whether TriMix DCs can be coelectroporated with TAA-encoding mRNA instead of being pulsed with antigenic peptides. This approach offers several additional advantages. 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 induce a broader TAA-specific T-cell response, and it is not dependent 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 presentation 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.

Materials and Methods

Genetic constructs. The pGEM-CD40L, pGEM-CD70, and pGEM-caTLR4 plasmids encoding CD40L, CD70, and caTLR4 (containing the intracellular and transmembrane fragments of TLR4, as described in ref. 8); the pGEM-NGFR 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 targeting 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 monocyte–derived DCs*, in vitro *transcription of capped mRNA, and mRNA electroporation of DCs.* Generation, maturation, and cryopreservation of immature and cytokine cocktail–matured DCs, capped mRNA production, and mRNA electroporation of TriMix DCs, either pulsed with MelanA peptide or coelectroporated 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 TAA-encoding mRNA. The following conditions were used for electroporation: 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 melanoma 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 encoding 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 $\pm 12.5 \times 10^6$ TriMix DC per antigen and are administered by four biweekly intradermal injections at four different injection sites (axillar and/or inguinal region).

Synthetic peptides and peptide pulsing. The HLA-A*0201–restricted Mage-A3 (aa 112-120; KVAELVHFL), Mage-C2 (aa 336-344; ALKDVEERV), 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-DPB1*0401–restricted Mage-A3 peptide (aa 243-258; KKLLTQHFVQENYLEY) was purchased from Henogen. For peptide pulsing, DCs or T2 cells were diluted to a final density of 2×10^6/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 (mAb): CD40-allophycocyanin (APC), CD70-phycoerythrin (PE), CD80-PE, CD83-PE, CD86-PE, HLA-ABC-FITC (all from BD Pharmingen), and HLA-DR (purified from clone L243). The anti–HLA-DR antibody was biotin labeled and detected through streptavidin-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 PerCP-conjugated streptavidin (BD Pharmingen). Nonreactive isotype-matched mAbs (BD Pharmingen) were used as controls. Data were collected using a FACSCanto flow cytometer and analyzed using FACSDiva software. Cells were electronically gated according to light scatter properties to exclude dead and contaminating cells.

Cytokine secretion assay. IL-12p70 secretion by DCs during the first 24 h after electroporation was assessed by ELISA using a commercially available kit (eBioscience).

Induction of TAA-specific CD8$^+$ T cells. The induction of MelanA-specific CD8$^+$ T cells from the blood of HLA-A2$^+$ healthy donors has already been described (6). T cells specific for other TAA were stimulated using a slightly adapted protocol. Briefly, CD8$^+$ T cells were isolated from the blood of HLA-A2$^+$ melanoma patients, either before or after vaccination. CD8$^+$ T cells were purified through immunomagnetic selection by using CD8 microbeads (Miltenyi Biotec) and were consistently >90% pure (data not shown). Twenty million CD8$^+$ T cells were cocultured with autologous DCs at a DC/T-cell ratio of 1:10 per 6-wells in 7.5 mL stimulation medium consisting of Iscove's modified Dulbecco's medium containing 1% heat-inactivated AB serum (PAA Laboratories), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 0.24 mmol/L L-asparagine, and 0.55 mmol/L L-arginine (all from Lonza) without any further addition of exogenous cytokines such as interleukin (IL)-2 or IL-7. As stimulator DCs, DCs matured with the cytokine cocktail containing IL-1β, IL-6, tumor necrosis factor (TNF)-α, and prostaglandin E_2 and pulsed with an HLA-A2–restricted Mage-A3–, Mage-C2–, tyrosinase-, or gp100-derived peptide (sequences KVAELVHFL, ALKDVEERV, 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); FLWGPRALV or KVAELVHFL (Mage-A3 derived); ALKDVEERV (Mage-C2 derived); YMDGTMSQV (tyrosinase derived); ITDQVPFSV, YLEPGPVTA, or KTWGQYWQV (gp100 derived); or SLLMWITQC (NY-ESO-1 derived, negative control). Cells were analyzed by flow cytometry.

Intracellular cytokine staining and CD107a/CD137 assay. For intracellular cytokine staining, 2×10^5 primed CD8$^+$ T cells were restimulated with 2×10^4 stimulator cells in the presence of Golgi-plug [brefeldinA, Becton Dickinson (BD)]. After 12 h of incubation at 37°C, CD8$^+$ T cells were then stained with FITC- or APC-Cy7–conjugated anti-CD8 mAb, washed, permeabilized, and stained intracellularly using the BD Cytofix/Cytoperm plus kit with IFN-γ-PE/TNF-α-APC or IFN-γ-PE/TNF-α-FITC, respectively. For the CD107a/CD137 assay, 1×10^5 primed CD8$^+$ T cells were restimulated with 2×10^4 stimulator cells in the presence of Golgi-stop (monensin, BD) and PE-Cy5–labeled anti-CD107a mAb (BD Pharmingen). After 12 h of incubation at 37°C, cells were harvested and stained with FITC-labeled anti-CD8 mAb and PE-labeled CD137 mAb (both from BD Pharmingen). As stimulator cells, TAP-deficient, HLA-A2$^+$ T2 cells pulsed with peptide or cytokine cocktail–matured DCs electroporated with TAA mRNA were used. Cells were analyzed by flow cytometry using a FACSCanto 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, 2×10^4 HLA-DP4$^+$ or control HLA-DP4$^-$ TriMix DCs pulsed with specific peptide or coelectroporated with MageA3-DCLamp mRNA were cocultured with 5,000 T cells from clone R12-C9. The specificity and HLA restriction of these T cells have been described elsewhere (16, 17). Clone R12-C9 was kept in culture as described previously (17), is HLA-DP4 (HLA-DPB1*0401) restricted, and is specific for the Mage-A3 epitope aa 243-258 with sequence KKLLTQHFVQENYLEY. 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 electroporated in comparison with a single mRNA, we investigated

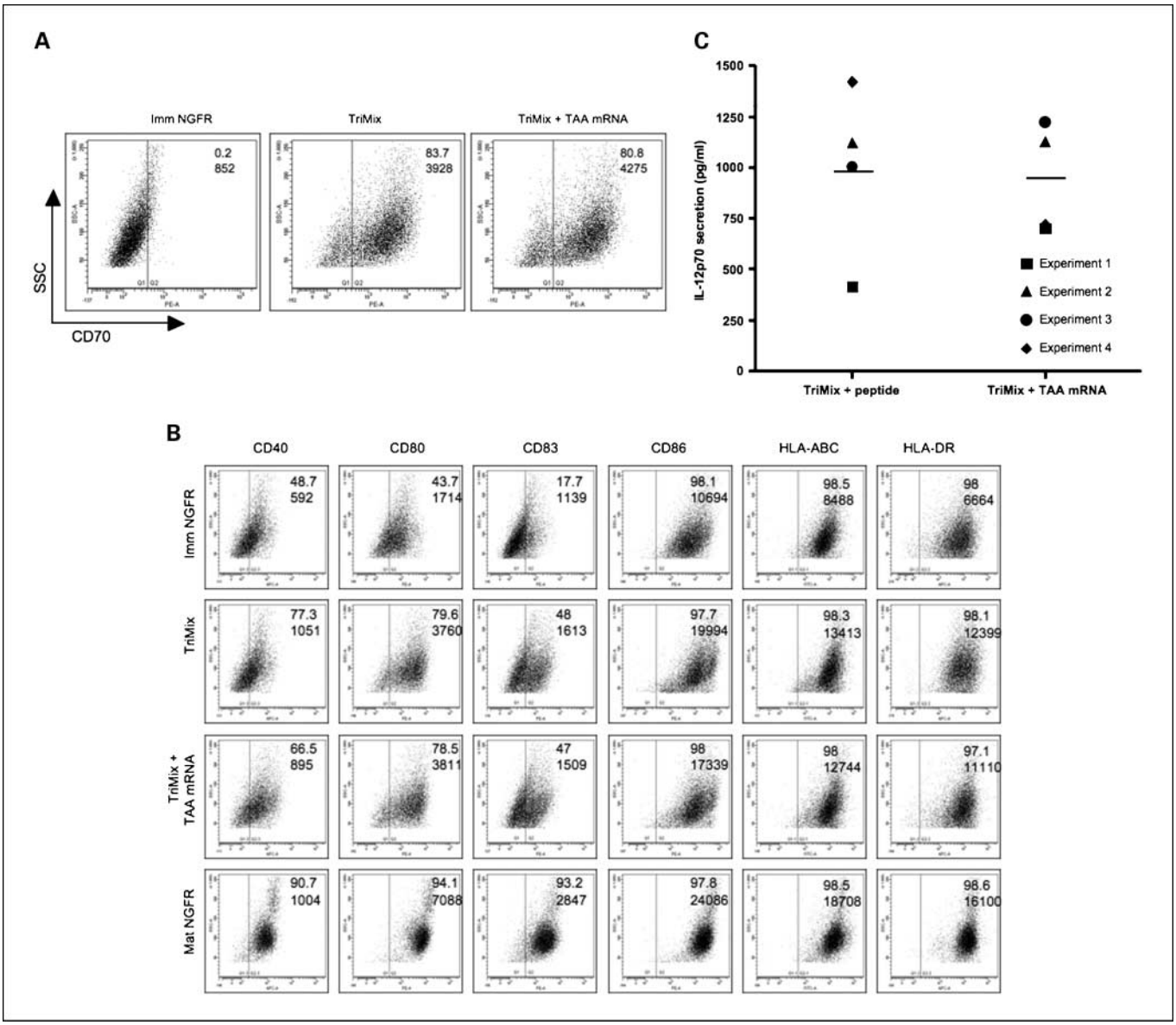

Fig. 1. Electroporation efficiency, phenotype, and IL-12p70 secretion by DCs electroporated with TriMix mRNA alone or in combination with TAA mRNA. *A,* DCs were electroporated with TriMix mRNA alone or in combination with TAA mRNA. Twenty-four hours later, electroporation efficiency was investigated by staining for surface CD70 expression. Immature DCs electroporated with irrelevant NGFR mRNA were used as negative control. The numbers in the top right corners represent the percentage of positive cells and the mean fluorescence intensity of the positive population giving an indication of the amount of molecules expressed per cell. Results are representative for at least five independent experiments. *B,* twenty-four hours after electroporation, DCs were stained for costimulatory molecules CD40, CD80, CD83, and CD86 and for HLA class I and class II molecules. The numbers in the top right corners represent the percentage of positive cells and the mean fluorescence intensity of the positive population giving an indication of the amount of molecules expressed per cell. Phenotype is compared with immature and cytokine cocktail–matured DCs electroporated with irrelevant NGFR mRNA. Results are representative for at least five independent experiments. *C,* IL-12p70 produced within 24 h after electroporation was dosed in the supernatant. Each dot represents one individual experiment and the mean is indicated by a horizontal line.

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 naive MelanA-specific CD8$^+$ T cells. Therefore, DCs from HLA-A2$^+$ healthy donors were

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

	%CD8$^+$ MelanA tetramer$^+$ T cells/no. of CD8$^+$ T cells (10^6)*				Absolute no. of CD8$^+$ MelanA tetramer$^+$ T cells (10^3)†			
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
Imm + MelanA peptide	0.3/1.2	0.1/1.35	1.2/4.2	0.5/3.5	3.5	1.3	50	19
TriMix + MelanA peptide	72.4/6.8	63.2/1.1	52.4/9.3	49.6/13	4922	695	4884	6478
TriMix + MelanA mRNA	72.5/6.3	25.9/1.6	43.3/5.7	44.8/14.7	4572	401	2455	6594
Mat + MelanA peptide	ND	0.1/1.5	ND	2.1/2.4	ND	1.5	ND	52

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 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.
†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.

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 T cells compared with peptide-pulsed TriMix DCs, whereas after three stimulations they were equally potent in two of four experiments.

Next, we assessed the functional and phenotypic properties of CD8$^+$ T cells stimulated thrice with TriMix DCs pulsed with peptide or coelectroporated with TAA mRNA. The main effector mechanisms of stimulated CD8$^+$ T cells (i.e., activation, cytolysis, and cytokine production) were investigated. T cells were restimulated overnight with T2 cells pulsed with MelanA-A2 peptide or gag peptide as a negative control. First we performed a CD107a mobilization assay combined with a CD137 activation assay (Fig. 2B), which measures lytic activity (18) and T-cell activation (19) on antigenic stimulation, respectively. Second, we performed intracellular cytokine staining to enumerate the number of cells secreting IFN-γ and/or TNF-α on antigenic stimulation, both major mediators of the immune response (Fig. 2C). For all donors tested, we observed that the percentage of MelanA-specific T cells correlated with the percentage of lytic/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 effector 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 effector 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 preferably 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

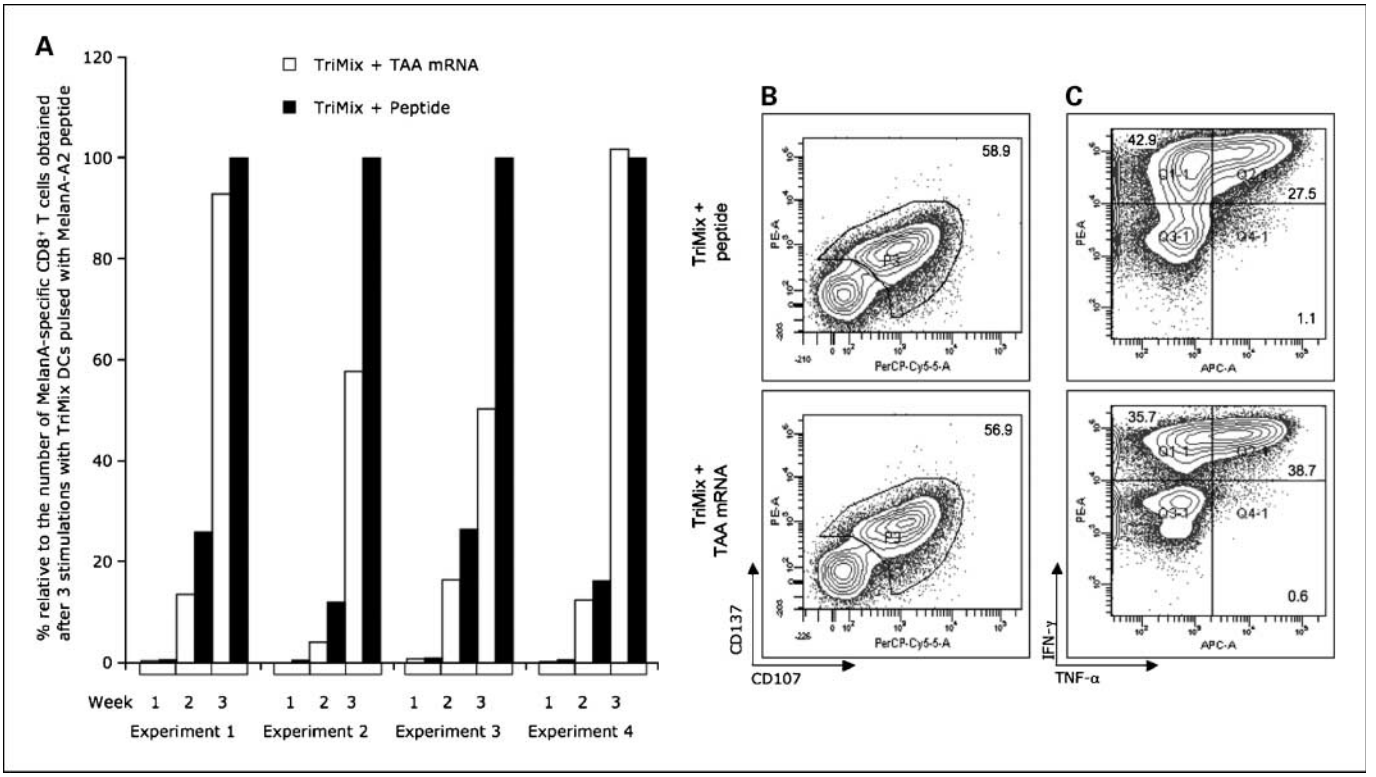

Fig. 2. *In vitro* induction of HLA-A2–restricted MelanA-specific CD8⁺ T cells, activated/cytolytic CD8⁺ T cells, and IFN-γ/TNF-α–secreting CD8⁺ T cells by DCs electroporated with TriMix mRNA pulsed with antigenic peptide or coelectroporated with TAA mRNA. *A,* naive CD8⁺ T cells were stimulated thrice, with a weekly interval with TriMix DCs. Every week, T cells were counted and stained for CD8 and MelanA specificity, and the absolute number of MelanA-specific CD8⁺ cells present in the culture was calculated. Relative percentage in comparison with the number of MelanA-specific CD8⁺ T cells obtained after three stimulations with TriMix DCs pulsed with MelanA-A2 peptide (set at 100%) is shown. *B,* the activation status and cytolytic activity of MelanA-specific T cells were determined by a CD137/CD107a assay. Primed T cells were restimulated with T2 cells pulsed with gag or MelanA peptide in the presence of anti-CD107-PE-Cy5 mAb and Golgi-stop. After overnight culture, 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 after subtraction of background response induced by T2 cells pulsed with gag peptide, which never reached higher than 3%. *C,* intracellular IFN-γ/TNF-α production by MelanA-primed CD8⁺ T cells was measured by flow cytometry. Primed T cells were restimulated overnight with T2 cells pulsed with gag or MelanA peptide 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 after subtraction of background response induced by T2 pulsed with gag peptide, which never reached higher than 3%. Results in *B* and *C* are given for experiment 1. In each experiment, CD137/CD107a positivity and IFN-γ/TNF-α secretion correlated with the percentage of MelanA-specific T cells present in the culture.

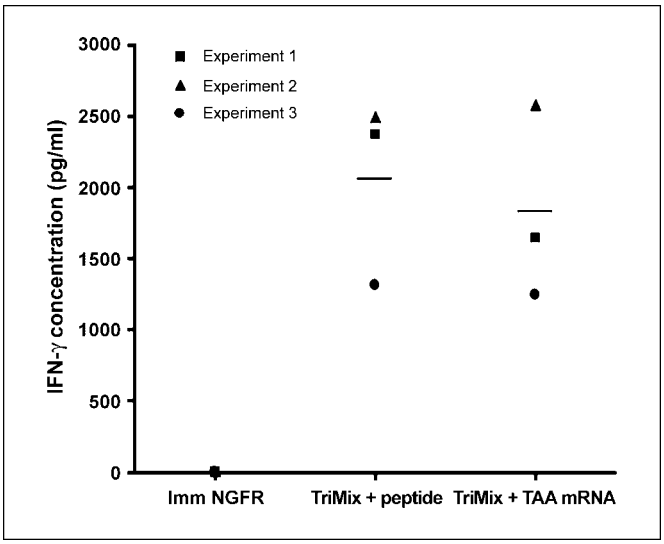

Fig. 3. $CD4^+$ T-cell stimulatory capacity of TriMix DCs pulsed with antigenic peptide or coelectroporated with TAA mRNA. DCs were either pulsed with Mage-A3-DP4 peptide or coelectroporated with MageA3-DCLamp mRNA. Four hours later, the cells were cocultured with Mage-A3–specific, HLA-DP4–restricted T cells for 20 h. Immature DCs electroporated with irrelevant NGFR mRNA were used as a negative control. IFN-γ production is shown. Each dot represents one individual experiment, and the mean is indicated by a horizontal line.

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 $HLA\text{-}A2^+$ 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 caTLR4 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 naive 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 naive 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 Mage-A3–, Mage-C2–, tyrosinase-, or gp100-specific $CD8^+$ T cells by DCs (29–31) 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 $HLA\text{-}A2^+$ 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 had been 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

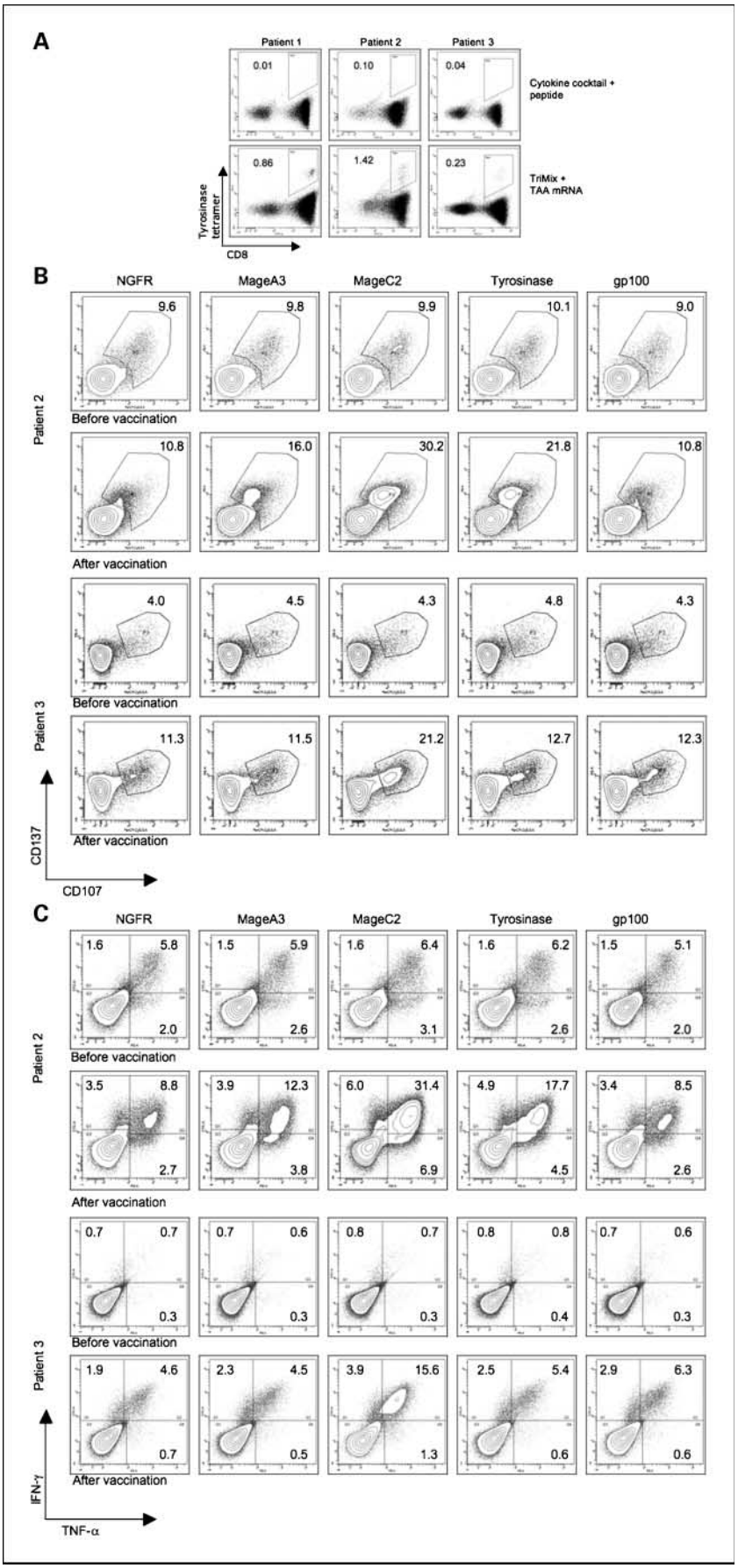

Fig. 4. Induction of CD8$^+$ T cells specific for other antigens than MelanA in melanoma patients both *in vitro* and *in vivo. A,* TriMix DCs as prepared for vaccination were used to stimulate CD8$^+$ T cells isolated from the blood of unvaccinated HLA-A2$^+$ melanoma patients. Cytokine cocktail–matured 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 NGFR 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 TAAs 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 19, 2009 (no. WO2009/034172). Aude Bonehill and Kris Thielemans are named as inventors of this application.

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Clinical Cancer Research

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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