[CANCER RESEARCH 63, 5587–5594, September 1, 2003]

Efficient Presentation of Known HLA Class II-restricted MAGE-A3 Epitopes by Dendritic Cells Electroporated with Messenger RNA Encoding an Invariant Chain with Genetic Exchange of Class II-associated Invariant Chain Peptide[1]

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ABSTRACT

For the induction of an optimal immune response against cancer or infections not only $CD8^+$ CTLs but also $CD4^+$ T helper cells must be induced, in particular IFN-γ-secreting type 1 T helper cells. Several strategies have been explored to target tumor-associated antigens to the HLA class II processing compartments. We engineered a genetic construct encoding an invariant chain (Ii) protein where the CLIP region has been replaced by sequences encoding HLA class II-restricted MAGE-A3 epitopes. Monocyte-derived dendritic cells (DCs) were electroporated with *in vitro* transcribed mRNA encoding a modified Ii protein containing the HLA-DP4-restricted MAGE-A3 epitope. The capacity of these electroporated DCs to stimulate a MAGE-A3-specific T-cell clone was compared at different stages of DC maturation with the T-cell stimulatory capacity of DCs pulsed with the synthetic peptide. After electroporation, the T-cell stimulatory capacity was shown to be high and long lasting, whereas the stimulatory capacity of peptide-pulsed DCs decreased rapidly. Upon coculture with epitope-specific T cells, electroporated immature DCs expressed enhanced levels of costimulatory molecules, HLA class II molecules, and CD83, suggesting the induction of maturation. The electroporated DCs can be frozen and thawed without losing their capability to stimulate the specific T-cell clone *in vitro,* and they are able to stimulate unprimed $CD4^+$ T cells specific to the HLA-DP4-restricted MAGE-A3 epitope *in vitro*. Similar results were obtained with a recombinant Ii containing the MAGE-A3 epitope presented in the context of HLA-DR13.

INTRODUCTION

The main goal of cancer immunotherapy is to generate a strong and persistent tumor-specific immune response. Although most attention has been given to $CD8^+$ CTL responses, increasing evidence indicates that $CD4^+$ Th[3] cells, particularly IFN-γ-producing Th1 cells, play a central role in orchestrating an effective antitumor response (1–3). Th cells help to initiate antigen-specific CTLs by activating DCs, they are needed for the maintenance of the $CD8^+$ T-cell number and function, and they may have effector functions through the production of IFN-γ and the recruitment of other effector cells such as macrophages and eosinophils.

Th cells recognize peptides presented in the context of HLA class II molecules on the surface of APCs. The Ii protein plays an essential role in this presentation process (4, 5). The Ii protein associates with HLA class II molecules in the endoplasmic reticulum, mainly through the CLIP region, thus preventing premature loading of the HLA class II molecules with endogenous peptides. Furthermore, Ii works as a molecular chaperone for transport of the Ii/HLA class II complex to the HLA class II processing compartments, where the Ii molecule is degraded and the CLIP region is replaced by antigenic peptides.

Recently, a new strategy has been described for optimal presentation of an epitope of choice to $CD4^+$ T cells. The CLIP region of the Ii can be genetically exchanged with a T-helper epitope. Immunization of mice with such a recombinant Ii induces protective immunity against tumor challenge (6) and *Listeria monocytogenes* infection (7) and can be applied for specific treatment of autoimmunity (8) or allergic disease (9). Several groups have reported that HLA class II^+ human cells transfected with recombinant Ii cDNA efficiently stimulate specific Th cell lines *in vitro* (10, 11). However, none of these studies have made use of the most professional APCs of the immune system, *i.e.,* DCs.

DCs have the ability to stimulate naive and resting memory T lymphocytes. Therefore, these cells are ideal candidates to act as vectors for immunotherapy of various diseases. Depending on their maturation stage and localization, DCs perform different functions within the immune system (12–15). Immature DCs residing in the peripheral tissues are specialized in antigen capture and processing. They carry information about invading pathogens to the T-cell zones of the lymphoid organs. During this migration, the DCs differentiate from an immature to a mature state, with an increased T-cell stimulatory capacity. DCs undergo phenotypical changes during maturation, reflected by increased expression of HLA molecules, costimulatory molecules (CD80, CD86, and CD40), CD83, DC-LAMP, homing receptors, and chemokines. Mature DCs present antigens to $CD4^+$ T cells, and the resulting stimulated $CD4^+$ T cells can then superactivate the DCs through the interaction of CD40L with CD40. These superactivated DCs produce IL-12, which is known to polarize T lymphocytes toward Th1 development and is considered essential for an effective antitumoral immune response (16, 17).

Different methods have been used to load DCs with TAAs (18–20). DCs can be pulsed with TAA-derived protein or peptides, or they can be genetically modified using viral or nonviral gene delivery systems. Nonviral gene delivery systems are preferable because safety issues and the immunogenicity of viral vector-encoded antigens are reduced to a minimum. Recently, Van Tendeloo *et al.* (21) have reported a powerful approach to load DCs with TAAs by electroporation of *in vitro* transcribed mRNA into the DCs. We and others have confirmed these findings (22–24).

In this study, we electroporated DCs with a recombinant Ii where the CLIP region had been replaced by a sequence encoding HLA class II-restricted MAGE-A3 epitopes. We show that these DCs can effi-

Received 1/28/03; revised 6/5/03; accepted 6/20/03.

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[1] This work was supported by a PhD fellowship from the Flemish Institute for Science and Technology (IWT; to A. B.). K. T. was supported by grants from the Fund for Scientific Research-Flanders (FWO-Vlaanderen), the Ministry of Science (IUAP/PAI V), the FORTIS Bank, the Belgische Federatie voor Kankerbestrijding, and the Brussels Region.

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[3] The abbreviations used are: Th, T helper; DC, dendritic cell; APC, antigen-presenting cell; Ii, invariant chain; CLIP, class II-associated invariant chain peptide; IL, interleukin; PE, phycoerythrin; TAA, tumor-associated antigen; UTR, untranslated region; huAB, human AB serum; AP, autologous plasma; IMDM, Iscove's modified Dulbecco's medium; AAG, asparagine-arginine-glutamine; PS, penicillin-streptomycin.

ciently stimulate epitope-specific T cells. Compared with peptide-pulsed DCs, the T-cell stimulatory capacity of the electroporated DCs was more efficient and longer lasting. Unprimed $CD4^+$ T cells from a healthy donor were stimulated with DCs electroporated with recombinant Ii mRNA containing the HLA-DP4- or HLA-DR13-restricted MAGE-A3 epitope. MAGE-A3-specific $CD4^+$ T cell clones were isolated, and these cells recognized the MAGE-A3 peptides.

MATERIALS AND METHODS

Genetic Constructs. A plasmid containing the human Ii cDNA in which the CLIP-coding sequence can be easily replaced by other sequences was kindly provided by W. Vader (Leiden University, Leiden, the Netherlands; Ref. 10). Double-stranded oligonucleotides with sequences encoding MAGE-A3 epitopes presented in the context of HLA-DP4 (amino acids 243–258) or HLA-DR13 (amino acids 121–134) were cloned into the unique *Sfu*I and *Eco*47III sites of the Ii vector. The sequences of these oligonucleotides were as follows: 5′-CGAAGAAGCTTCTCACCCAACATTTCGTGCAGGAAAACTACCTGGAGTACCAAGC-3′ and 5′-GCTTGGTACTCCAGGTAGTTTTCCTGCACGAAATGTTGGGTGAGAAGCTTCTT-3′ for the HLA-DP4-restricted epitope and 5′-CGAAGCTTCTCAAGTATCGAGCCAGGGAACCGGTCACAAAGGCAGAACAAGC-3′ and 5′-GCTTGTTCTGCCTTTGTGACCGGTTCCCTGGCTCGATACTTGAGAAGCTT-3′ for the HLA-DR13-restricted epitope. Both inserts (Ii*M3/DP4 and Ii*M3/DR13, respectively) were checked by DNA sequencing.

Subsequently, the Ii*M3/DP4 and Ii*M3/DR13 inserts were cloned as *Bam*HI-*Xba*I fragments into the pGEM4Z-5′UTR-3′UTR-A64-vector (25), kindly provided by Dr. E. Gilboa (Duke University Medical Center, Durham, NC). This plasmid contains 5′ and 3′ UTRs of the *Xenopus laevis β-globin* gene and a polyA tail (termed A64). At the 3′ end of the A64 stretch, a unique *Spe*I site is present to allow linearization of the plasmid before *in vitro* transcription. The transcription is controlled by a bacteriophage T7 promoter. A schematic representation of the recombinant Ii constructs is shown in Fig. 1.

Generation of Monocyte-derived DCs. Peripheral blood mononuclear cells were used as a source of DC precursors. Peripheral blood mononuclear cells were isolated from buffy coat preparations or leukapheresis products. DCs were generated in tissue culture flasks (Falcon; Becton Dickinson, San Jose, CA) or in a closed culture system using double tray Cell Factories (NUNC, Naperville, IL) as described previously by Tuyaerts *et al.* (26). The DCs were cultured in DC medium consisting of X-VIVO 15 medium (BioWhittaker, Walkerville, MD) supplemented with 1% huAB (PAA Laboratories, Linz, Austria) or AP, 800 units/ml granulocyte/macrophage-colony stimulating factor (Leucomax, Novartis, Basel, Switzerland) and 100 units/ml IL-4 [made in-house, as described by Tuyaerts *et al.* (26)].

***In Vitro* Transcription of Capped mRNA.** For *in vitro* mRNA synthesis, the pGEM4Z Ii*M3/DP4 and pGEM4Z Ii*M3/DR13 plasmids were linearized with *Spe*I. The *in vitro* transcription was performed with T7 polymerase according to the manufacturer's instructions (Ambion mMESSAGE mMACHINE kit, Austin, TX). mRNA concentration was measured spectrophotometrically, and mRNA quality was evaluated by agarose gel electrophoresis.

Electroporation of DCs. Depending on the required number of electroporated DCs, 4, 8, or 50 million DCs were electroporated with 20, 30, or 120 μg of Ii*M3/DP4 or Ii*M3/DR13 mRNA, respectively. Before electroporation, the DCs were washed twice, first with X-VIVO 15 medium without supplements and then with Optimix Solution A (EQUIBIO, Ashford, United Kingdom). After the second wash step, the DCs were resuspended in a final volume of 200 μl (600 μl for the electroporation of 50×10^6 DCs) Optimix Solution B (EQUIBIO) containing the appropriate amount of mRNA. Electroporation was performed in a 4-mm gap electroporation cuvette using the EQUIBIO Easyject Plus® apparatus. The following conditions were used for electroporation: voltage, 300 V; capacitance, 150 microfarads; and resistance, 99 ohms, resulting in a pulse time of about 5 ms. For electroporation of 50×10^6 DCs, these values were 300 V, 450 microfarads, and 99 ohms, respectively. Immediately after electroporation, the DCs were diluted to a final density of 5×10^5 cells/ml in DC medium.

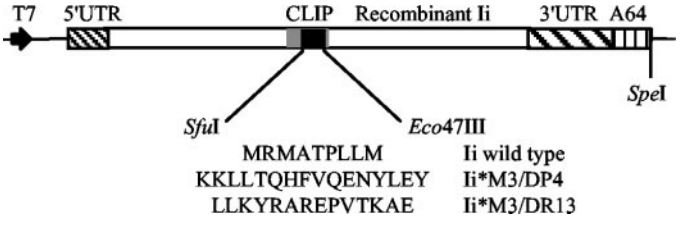

Fig. 1. Schematic representation of the recombinant Ii constructs. The CLIP region is shown as a *gray box* containing the CLIP core region, shown in *black*. The amino acid sequences of the CLIP region and the MAGE-A3 epitopes are shown. The T7 promoter, 5′ UTR, 3′ UTR, A64 stretch, and the unique *Spe*I site are also depicted.

Peptides and Peptide Pulsing of DCs. The lyophilized synthetic peptides corresponding to the MAGE-A3 epitope presented in the context of HLA-DP4 (M3.DP4 peptide, amino acids 243–258, sequence KKLLTQHFVQENYLEY) and HLA-DR13 (M3.DR13 peptide, amino acids 121–134, sequence LLKYRAREPVTKAE) were dissolved in 10 mM acetic acid and 10% DMSO to a final concentration of 5 mg/ml and stored at −20°C. For peptide pulsing, DCs were diluted to a final density of 1×10^6 cells/ml in X-VIVO 15 medium without supplements containing the indicated concentration of the M3.DP4 peptide and were incubated for 4 h at 37°C. Subsequently, the cells were washed and resuspended to a final density of 5×10^5 cells/ml of DC medium.

Maturation of DCs. Immediately after peptide pulsing or 4 h after electroporation, DCs were matured using a maturation mixture containing 100 units/ml of IL-1β (PeproTech, London, United Kingdom), 1000 units/ml IL-6 (PeproTech), 100 units/ml tumor necrosis factor-α (PeproTech), and 1 μg/ml prostaglandin E_2 (Sigma-Aldrich, St. Louis, MO). The cell density of the matured DCs and also of DCs that were left immature was adjusted to 2.5×10^5 cells/ml of DC medium.

Immunophenotyping of DCs. To analyze the expression of surface molecules on the cell surface of the DCs, the following monoclonal antibodies were used: anti-CD80, anti-CD83, anti-CD86, anti-HLA-ABC (all from PharMingen, San Jose, CA), anti-HLA-DP (purified from clone B7/21, a kind gift from Dr. J. Arroyo, Universidad Complutense, Madrid, Spain), and anti-HLA-DR (purified from clone L243). The anti-HLA-DP antibody was biotin labeled and detected through streptavidin-PE or streptavidin-CyChrome C binding (PharMingen). The anti-HLA-DR antibody was biotin labeled and detected through streptavidin-PE binding. The anti-HLA-ABC antibody was FITC conjugated. All other antibodies were PE conjugated. Isotype-matched antibodies (PharMingen) were used as controls. Fluorescence analysis was performed with the FACSCalibur flow cytometer (Becton Dickinson) using CELLQuest software (Becton Dickinson).

Cryopreservation of DCs. DCs were frozen in cryotubes at 1 to 5×10^6 DCs/tube in 1 ml of AP with 10% DMSO and 2% glucose. The DCs were slowly frozen to −80°C using a cryofreezing container (Cryo 1°C freezing container, rate of cooling −1°C/min; Nalgene, Hereford, United Kingdom) and subsequently stored in liquid nitrogen until use. DCs were quickly thawed in a 37°C water bath. Cold HBSS (Invitrogen, Paisley, United Kingdom) was added dropwise, and the cells were pelleted in a precooled centrifuge (4°C). The thawed DCs were resuspended in 5 ml of prewarmed X-VIVO 15 medium supplemented with 1% AP. Cell viability was determined with trypan blue.

$CD4^+$ T-Cell Clones. Two MAGE-A3-specific T-cell clones were used. Clone R12-C9 is HLA-DP4 (HLA-DPB1*0401) restricted and specific for the MAGE-A3 epitope amino acids 243–258 (27). Clone Cl7002 is HLA-DR13 (HLA-DRB1*1302) restricted and specific for the MAGE-A3 epitope amino acids 121–134 (28). The cells, which will be referred to as M3.DP4- or M3.DR13-specific T cells, were cultured in IMDM (Invitrogen) supplemented with 10% huAB, AAG (Invitrogen), PS (Invitrogen), 50 units/ml IL-2 (PeproTech), and 5 ng/ml IL-7 (Peprotech) or 200 units/ml IL-2, respectively. The cells were restimulated every 2 weeks with irradiated allogeneic LG2 EBV-B cells (1.5×10^6 per 24-well) as feeder cells and MZ2 melanoma cells preincubated with synthetic peptide (1×10^5 per 24-well) as stimulator cells.

Antigen Presentation Assays. To investigate the T-cell stimulatory capacity of mRNA electroporated or peptide pulsed DCs, 2×10^4 DCs (unless mentioned otherwise) were cocultured with 5000 M3.DP4- or M3.DR13-specific T cells in 200 μl of IMDM supplemented with 10% huAB, AAG, PS, and 25 units/ml of IL-2. Each coculture was performed in triplicates in round-bottomed microwells. After 20 h of coculture, the supernatant was assessed for the presence of IFN-γ by ELISA using a commercially available kit (Human IFN-γ Cytoset; BioSource International, Camarillo, CA). To

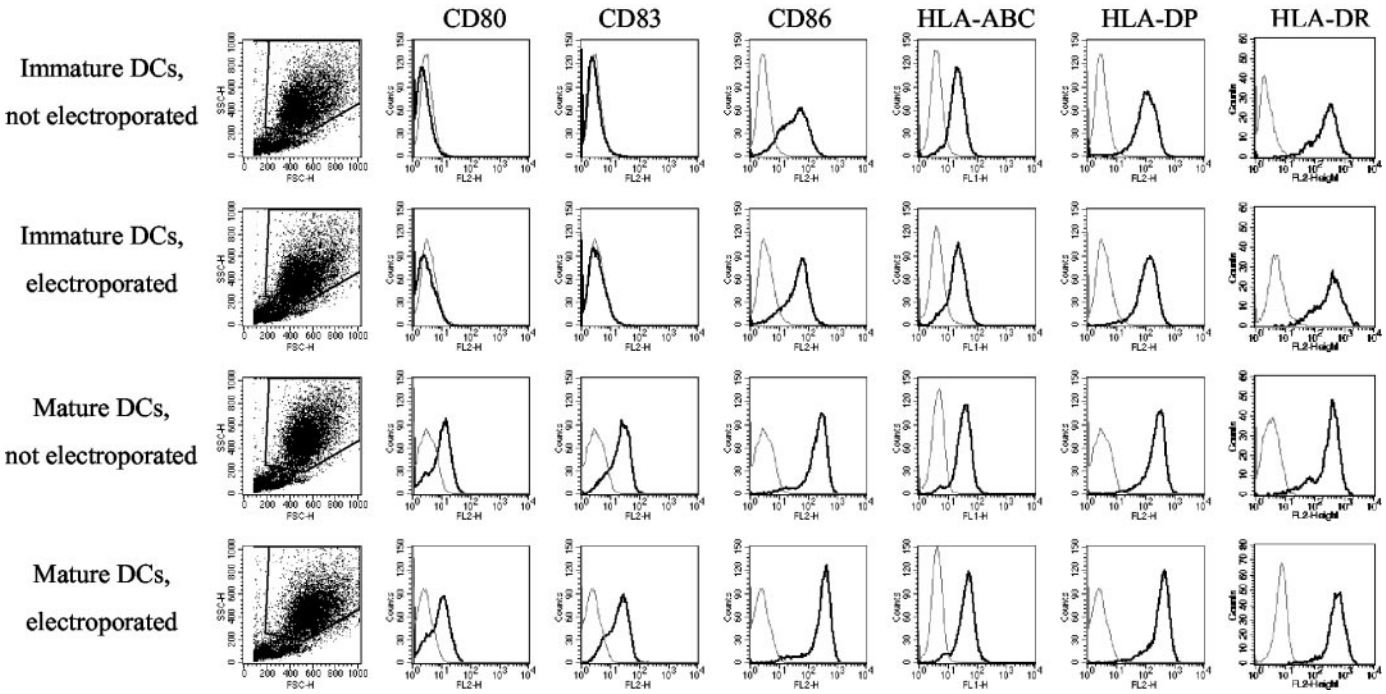

Fig. 2. Phenotype of mature *versus* immature non-electroporated or electroporated DCs. DCs were electroporated on day 6 of DC culture and were matured with the cytokine mixture 4 h after the electroporation. The DCs were cultured for an additional 20 h, after which they were phenotyped by flow cytometry (results represented as *black lines*). *Thin gray lines,* DCs stained with isotype-matched antibodies. This figure is representative of three individual experiments.

investigate the DC immunophenotype after coculture with M3.DP4-specific T cells, 2×10^6 DCs were cocultured for 20 h with 5×10^5 M3.DP4-specific T cells in 2 ml of IMDM supplemented with 10% huAB, AAG, PS, and 25 units/ml of IL-2.

Induction of MAGE-A3-specific T Lymphocytes from the Blood of Individuals without Cancer. Nonadherent cells obtained after adherence of monocytes to plastic were used as a source of unprimed $CD4^+$ T cells. $CD4^+$ T cells of a $HLA\text{-}DP4^+$ and a $HLA\text{-}DR13^+$ normal donor were isolated from the nonadherent fraction by positive selection using anti-CD4 mAb coupled to magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. One hundred thousand $CD4^+$ T cells were cocultured in round-bottomed microwells with 1×10^4 autologous DCs electroporated with Ii*M3/DP4 or Ii*M3/DR13 mRNA in 200 μl of X-VIVO 15 medium supplemented with 1% AP, 1000 units/ml of IL-6, and 10 ng/ml of IL-12 (Peprotech). Four round-bottomed microwell plates were prepared. On days 7, 14, and 21, the $CD4^+$ T cells were restimulated with 1×10^4 thawed Ii*M3/DP4 or Ii*M3/DR13 mRNA electroporated autologous DCs per microwell in X-VIVO 15 medium supplemented with 1% AP, 10 units/ml of IL-2, and 5 ng/ml of IL-7 (PeproTech).

Screening and Subcloning of the MAGE-A3-specific $CD4^+$ T-cell Clones. For the screening and subcloning of the T-cell clones, the protocol previously described by Chaux *et al.* (28) was used with some modifications. Briefly, on day 35 the microcultures were assessed for their capacity to produce IFN-γ when stimulated with autologous EBV-B cells transduced with a retrovirus encoding Ii80-MAGE-A3 (for the $HLA\text{-}DP4^+$ cells) or with allogeneic $HLA\text{-}DR13^+$ EBV-B cells pulsed with 5 μg/ml of M3.DR13 peptide (for the $HLA\text{-}DR13^+$ cells). Approximately 5000 cells of each microculture were cocultured with 2×10^4 EBV-B cells. Nontransduced and non-peptide pulsed EBV-B cells were used as controls. After 20 h of coculture in round-bottomed microwells, IFN-γ released in the supernatant was measured by ELISA. A microculture that tested positive in the screening for MAGE-A3-HLA-DP4 specificity was subcloned. The T cells were restimulated with 2×10^4 Ii*M3/DP4 mRNA electroporated autologous DCs, and 16 h later, the IFN-γ-secreting cells were stained with an IFN-γ Secretion Assay Detection kit according to the manufacturer's instructions (Miltenyi Biotech). Subsequently, the IFN-γ-secreting cells were cloned at one cell per round-bottomed microwell using a FACSVantage flow cytometer (Becton Dickinson). Irradiated allogeneic LG2 EBV-B cells (2×10^4 cells/well) were added as feeder cells, and irradiated autologous Ii80-MAGE-A3 EBV-B cells (1×10^4 cells/well) were added as stimulator cells. The cells were cultured in IMDM supplemented with 10% huAB, 0.1 μg/ml of phytohemagglutinin (Sigma-Aldrich), 50 units/ml of IL-2, 5 units/ml of IL-4, and 5 ng/ml of IL-7. The T cell clones were restimulated every week. Proliferating subclones were tested for their capacity to produce IFN-γ when stimulated with autologous Ii80-MAGE-A3 EBV-B cells or with autologous EBV-B cells pulsed with 5 μg/ml M3.DP4 peptide as described above.

RESULTS

We determined the effect of mRNA electroporation on the immunophenotype of DCs. Immature DCs were electroporated with Ii*M3/DP4 mRNA on day 6 of DC culture. Four h after the electroporation, one-half of the electroporated DCs were isolated and matured with the cytokine mixture for 20 h. Only DCs cultured in the presence of the cytokine mixture showed a fully matured phenotype (up-regulation of CD80, CD83, CD86, HLA class I antigens, HLA-DP antigens, and HLA-DR antigens). No difference was observed between the electroporated and non-electroporated DCs (Fig. 2), indicating that the electroporation step did not alter the expression of surface molecules on the DCs.

To investigate the T-cell stimulatory capacity of DCs electroporated with Ii*M3/DP4 or Ii*M3/DR13 mRNA, we cocultured the DCs with M3.DP4- and M3.DR13-specific T cells. DCs were electroporated on day 6 of DC culture and matured 4 h later. The coculture of DCs and M3.DP4- or M3.DR13-specific T cells started 24 h after the electroporation. IFN-γ released in the supernatant during this 20-h coculture was measured by ELISA (Fig. 3*A*). The results clearly indicate that the HLA-DP4- and HLA-DR13-restricted MAGE-A3 epitopes are efficiently presented by the electroporated DCs and recognized by the M3.DP4- and M3.DR13-specific T cells, respectively. Non-electroporated DCs or DCs electroporated with recombinant Ii mRNA containing a HLA-mismatched MAGE-A3 epitope

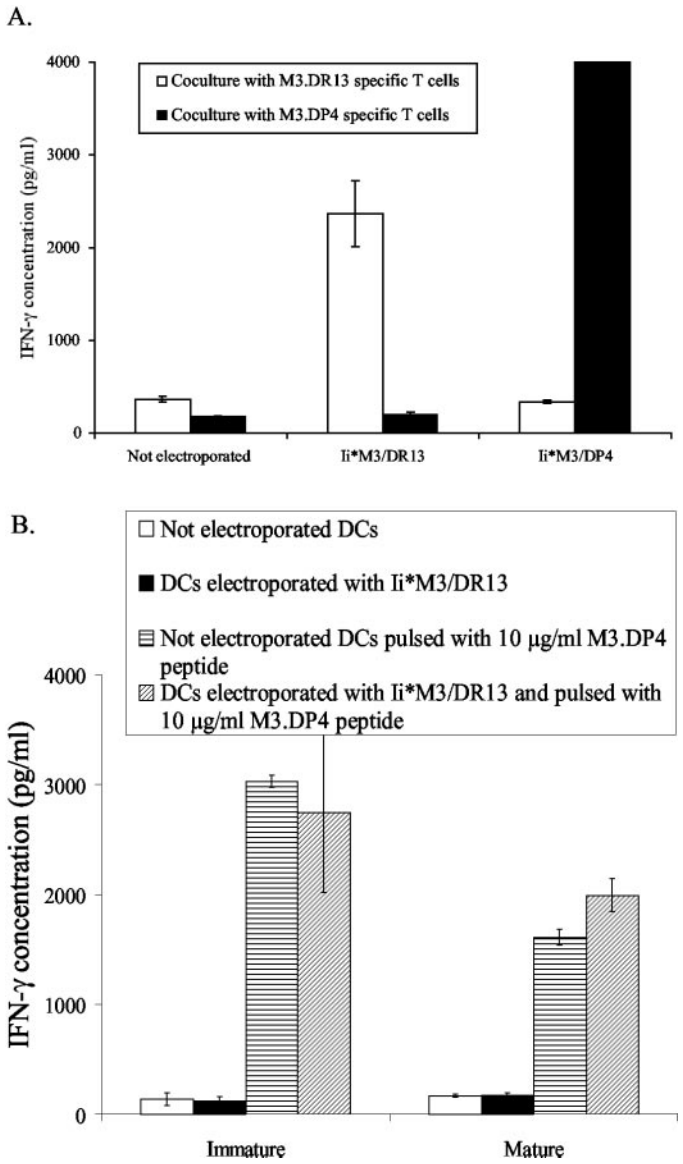

Fig. 3. *A,* T-cell stimulatory capacity of DCs electroporated with Ii*M3/DP4 or Ii*M3/DR13 mRNA. The results are shown as means and are representative of three independent experiments; *bars,* SD. *B,* T-cell stimulatory capacity of electroporated and non-electroporated DCs pulsed with 10 μg/ml of M3.DP4 peptide. The results are shown as means and are representative of three independent experiments; *bars,* SD.

were unable to induce IFN-γ secretion by the MAGE-A3-specific T cells.

We also investigated whether the electroporation of the DCs influences their T-cell stimulatory capacity. Therefore, electroporated and non-electroporated DCs were pulsed with the M3.DP4 peptide and assayed for their capacity to stimulate the M3.DP4-specific T cells. DCs were electroporated on day 6 of DC culture with Ii*M3/DR13 mRNA and were immediately pulsed with 10 μg/ml of M3.DP4 peptide at a density of 1×10^6 cells/ml. Four h after the electroporation, one-half of the DCs were cultured for 20 h in the presence of the cytokine mixture, whereas the other cells were left in the presence of granulocyte/macrophage-colony stimulating factor and IL-4 only. The DCs were then cocultured with the M3.DP4-specific T cells, and 20 h later, IFN-γ released in the supernatant was measured by ELISA. Electroporated and non-electroporated DCs pulsed with the M3.DP4 peptide had a comparable T-cell stimulatory capacity (Fig. 3*B*). The immature DCs were more potent activators of the M3.DP4-specific T cells than the mature DCs. This is probably because of the replacement of the M3.DP4-loaded HLA class II molecules by newly synthesized or stored HLA-DP4 molecules during the maturation process (29–32).

We analyzed the DC immunophenotype of immature DCs electroporated or not with Ii*M3/DP4 or Ii*M3/DR13 mRNA after coculture with the M3.DP4-specific T cells. After 20 h of coculture, the matu-

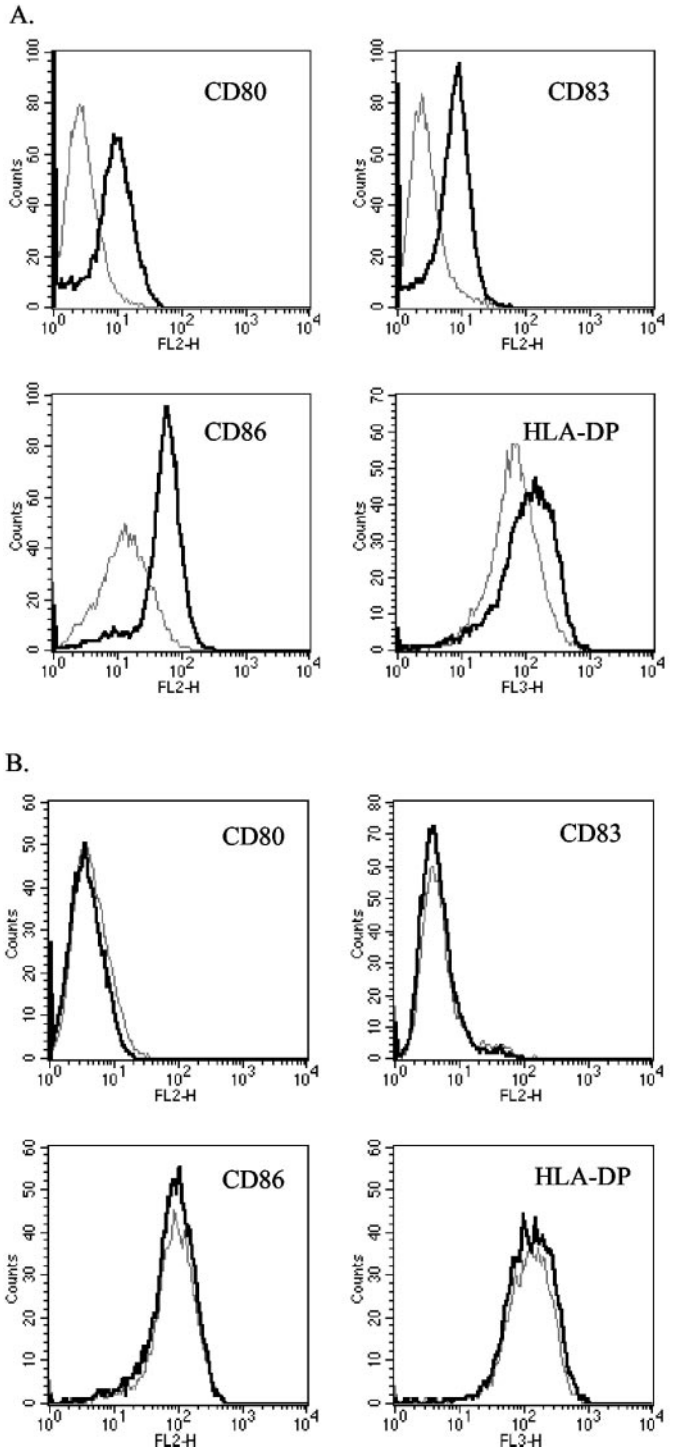

Fig. 4. Phenotype of immature DCs after coculture with specific T cells. *A,* phenotype of immature DCs electroporated or not with Ii*M3/DP4 mRNA after coculture with M3.DP4-specific T cells. *Thin gray lines,* non-electroporated DCs; *thick black lines,* Ii*M3/DP04 mRNA electroporated DCs. *B,* phenotype of immature DCs electroporated or not with Ii*M3/DR13 mRNA after coculture with M3.DP4-specific T cells. *Thin gray lines,* non-electroporated DCs; *thick black lines,* Ii*M3/DR13 mRNA electroporated DCs.

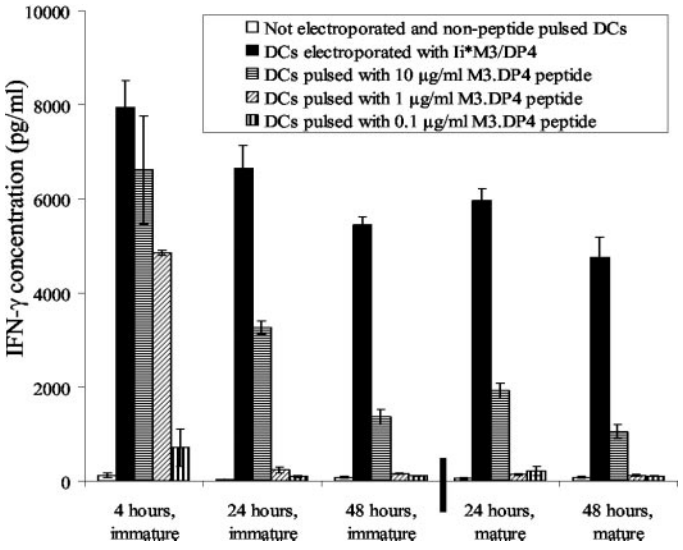

Fig. 5. T-cell stimulatory capacity of DCs electroporated with Ii*M3/DP4 mRNA in comparison with DCs loaded with different amounts of M3.DP4 peptide. The results are shown as means and are representative of three independent experiments; *bars,* SD.

ration status of the DCs was assessed. The histograms shown in Fig. 4*A* clearly demonstrate that coculture of immature Ii*M3/DP4-expressing DCs with M3.DP4-specific T cells induces an up-regulation of the CD80, CD83, CD86, and HLA-DP expression levels, similar to the expression levels of the DCs matured with the cytokine mixture *in vitro* (results not shown). The immunophenotype of DCs electroporated with Ii*M3/DR13 mRNA did not change (Fig. 4*B*), indicating that the maturation of the DCs during coculture depends on specific recognition by the T-cell clone. Moreover, the interaction of M3.DP4-specific T cells with immature Ii*M3/DP4-expressing DCs resulted in the accumulation of IL-12 in the supernatant of this coculture. This IL-12 induction could be totally blocked by the addition of anti-CD40L monoclonal antibodies (results not shown).

A potential advantage of loading cells with an antigenic epitope via genetic modification is a prolonged presentation of the epitope. We compared the T-cell stimulatory capacity of DCs either electroporated with Ii*M3/DP4 mRNA or pulsed with different amounts of the M3.DP4 peptide (ranging from 10 to 0.1 μg/ml). The expression level and therefore the stability of the HLA-DP4/peptide complex was evaluated on both mature and immature DCs. Forty eight, 24, or 4 h after the electroporation or peptide pulsing of the DCs, M3.DP4-specific T cells were added, and the IFN-γ released during the subsequent 20-h coculture was measured (Fig. 5). Our data show that the stimulation of M3.DP4-specific T cells by the Ii*M3/DP4 mRNA electroporated DCs remained high for 48 h, whereas DCs exogenously pulsed with M3.DP4 peptide rapidly lost their T-cell stimulatory capacity.

Because most cancer immunotherapy protocols include several rounds of immunizations, it is essential that electroporated and matured DCs can be frozen and thawed without losing their T-cell stimulatory capacity. DCs were electroporated or not with Ii*M3/DP4 mRNA and were induced to mature in the presence of the inflammatory cytokine mixture. The next day the non-electroporated and matured DCs were pulsed with 30 μg/ml of M3.DP4 peptide, mimicking the situation as used for clinical studies (33). Subsequently, all DCs (including non-electroporated and non-peptide pulsed DCs as negative controls) were frozen and stored in liquid nitrogen. After thawing, the DCs were cocultured with M3.DP4-specific T cells at different stimulator:responder ratios. After 20 h of coculture, IFN-γ released in the supernatant was measured by ELISA (Fig. 6). The results indicate that DCs electroporated with Ii*M3/DP4 mRNA can be efficiently frozen and thawed without losing their T-cell stimulatory capacity. DCs electroporated with 30 μg of mRNA per 8×10^6 cells were more potent activators of the M3.DP4-specific T cells than matured DCs loaded with 30 μg/ml of M3.DP4 peptide.

One major functional characteristic of DCs is their ability to stimulate naive T cells. We investigated whether DCs from healthy donors, electroporated with Ii*M3/DP4 or Ii*M3/DR13 mRNA, were able to induce a primary immune response *in vitro*. Mature DCs electroporated with Ii*M3/DP4 or Ii*M3/DR13 mRNA were frozen in aliquots of 5×10^6 DCs/cryotube. These cells were then used to stimulate autologous $CD4^+$ T cells. After four weekly stimulations, the $CD4^+$ T-cell microcultures were screened for their ability to respond to stimulation by autologous Ii80-MAGE-A3 EBV-B cells (for the HLA-$DP4^+$ cells) or allogeneic EBV-B cells pulsed with M3.DR13 peptide (for the HLA-$DR13^+$ cells). The results for a HLA-$DR13^+$ and a HLA-$DP4^+$ clone that tested positive in the screening are shown in Fig. 7, *A* and *B*, respectively. The HLA-$DP4^+$-positive clone was subcloned, and the proliferating T cells were again tested for stimulation by autologous Ii80-MAGE-A3 EBV-B cells and autologous EBV-B cells pulsed with M3.DP4 peptide. The results for two positive subclones are shown in Fig. 7*C*.

DISCUSSION

Convincing evidence has accumulated that adequate Th cell assistance is crucial for the development of an effective immune response. Although studies on T-cell responses against human tumors initially focused on the identification of epitopes presented to $CD8^+$ CTLs by HLA class I molecules, human tumor antigens recognized by $CD4^+$ T cells are now being identified with increasing frequency (27, 28, 34–38). $CD4^+$ T cells are necessary for the development of tumor immunity after immunization with tumor cells or peptides derived from tumor antigens (1, 39, 40). One of the effector mechanisms of $CD4^+$ T cells during the immunization (priming) phase is the conditioning of the APCs via CD40 ligation by CD40L (16, 41, 42). $CD4^+$ T cells are also needed for the persistence of CTL responses, *e.g.,* by providing cytokines. Several tumor models demonstrated that $CD4^+$ T cells are needed in the effector phase via the recruitment of HLA nonrestricted effector cells such as macrophages, granulocytes, or natural killer cells and the modulation of the tumor environment (2, 43).

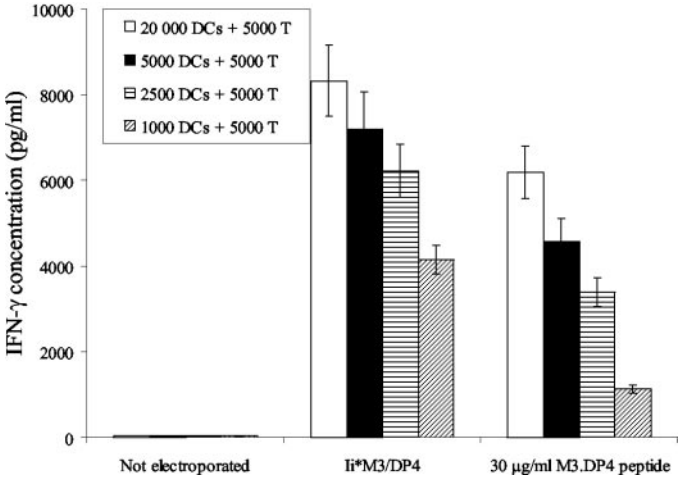

Fig. 6. T-cell stimulatory capacity of DCs electroporated with Ii*M3/DP4 mRNA in comparison with DCs loaded with 30 μg/ml of M3.DP4 peptide after cryopreservation of the DCs. The results are shown as means and are representative of three independent experiments; *bars,* SD.

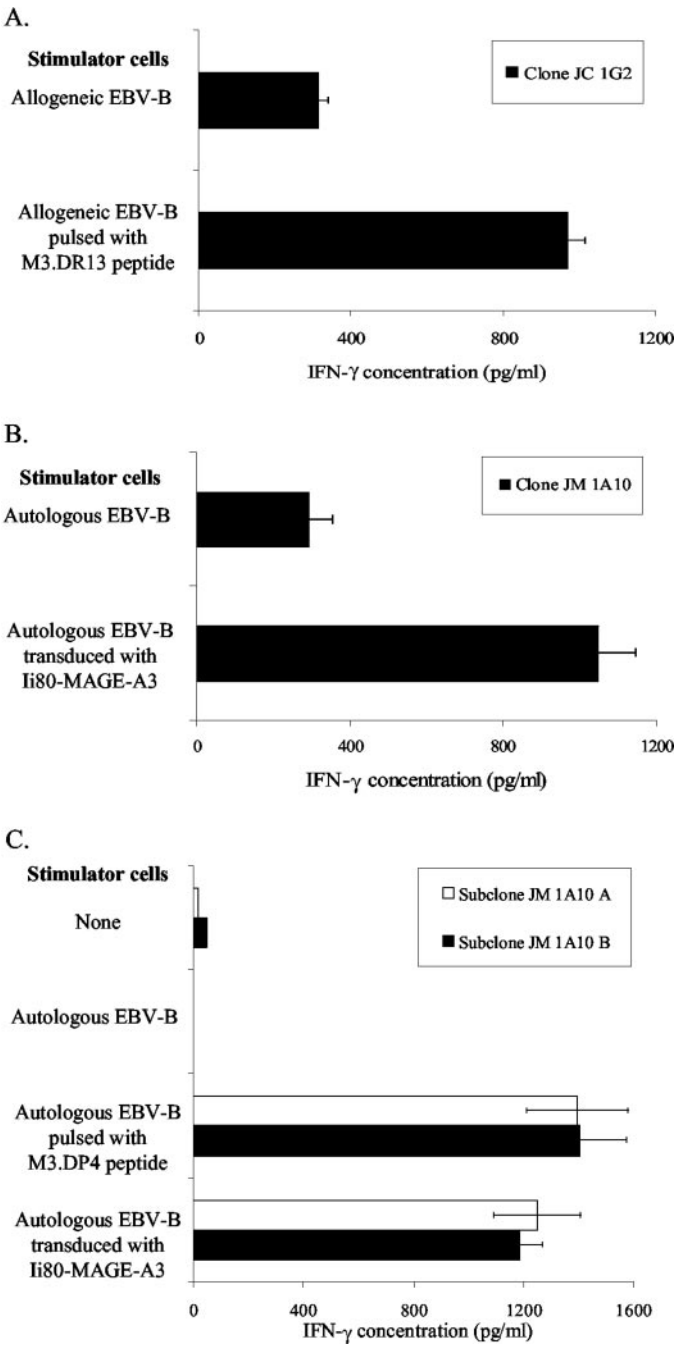

Fig. 7. Induction of unprimed $CD4^+$ T cells. *A,* induction of unprimed $CD4^+$ T cells against the HLA-DR13-restricted MAGE-A3 peptide using DCs electroporated with Ii*M3/DR13 mRNA. Clone JC 1G2 was stimulated with allogeneic EBV-B cells loaded with M3.DR13 peptide. As a negative control, allogeneic EBV-B cells were used. The results are shown as means; *bars,* SD. *B,* induction of unprimed $CD4^+$ T cells against the HLA-DP4-restricted MAGE-A3 peptide using DCs electroporated with Ii*M3/DP4 mRNA. Clone JM 1A10 was stimulated with autologous EBV-B cells transduced with Ii80-MAGE-A3. As a negative control, autologous EBV-B cells were used. The results are shown as means; *bars,* SD. *C,* subcloning of clone JM 1A10, specific for the HLA-DP4-restricted MAGE-A3 epitope. Subclones JM 1A10 A and B were stimulated with autologous EBV-B cells loaded with M3.DP4 peptide or transduced with Ii80-MAGE-A3. As negative controls, culture medium or autologous EBV-B cells were used. The results are shown as means and are representative of three independent experiments for subclone JM 1A10 A and two independent experiments for subclone JM 1A10 B; *bars,* SD.

Numerous methods have been proposed to load APCs used for immunization with tumor antigenic peptides. HLA class I- and class II-restricted, TAA-derived peptides are most commonly introduced into the HLA molecules via the exogenous pathway by peptide pulsing of APCs *in vitro*. However, this approach may have limitations because of the short half-life of the HLA/peptide complexes or because of the destruction of the peptide epitopes by APC-bound enzymes (44, 45). This problem could be resolved by epitope enhancement, whereby modification of the amino acid sequence of the epitopes to enhance binding to the restriction molecules could increase immunogenicity (46). *Ex vivo* pulsing with synthetic peptides may also result in HLA/peptide complexes that are antigenically distinct from those generated by intracellular processing (47).

A continuous encounter of endogenously synthesized peptide with the antigen-binding grooves of HLA class II molecules within the APCs would be expected to enhance presentation of the antigenic epitope. Various strategies have been explored to target endogenously synthesized antigens to the HLA class II loading compartment. Fusion constructs consisting of the Ii-derived (48–50) or LAMP-1-derived (49, 51–53) sorting signals target the antigen to the HLA class II processing pathway. This might result in the presentation of several epitopes in the context of HLA-DR, -DQ, and -DP antigens. Strategies to achieve occupation of a particular HLA class II subtype by a single epitope include constructs placing the peptide at the COOH terminus of the Ii protein (54) or the covalent linking of the peptide to the HLA class II β-chain via a flexible linker (55). We chose the "CLIP replacement" approach, which exploits the natural assembly pathway of Ii/HLA class II complexes to obtain loading of defined T helper epitopes within the endoplasmic reticulum, in an environment where other HLA class II epitopes cannot compete for binding (6, 7, 9–11). Among the wide variety of methods used to introduce relevant antigens into DCs for induction of antitumoral immunity, we chose the recently described mRNA electroporation method. This method offers several advantages: (*a*) most importantly, mRNA electroporated DCs have been proven to be efficient stimulators of the immune system *in vitro* and *in vivo* (25, 56–59); and (*b*) mRNA is not immunogenic, has a relatively short half-life, and lacks the potential to integrate into the host genome, which makes it a very safe tool for clinical trials (60, 61).

Our results show that DCs electroporated with mRNA encoding a recombinant Ii, where CLIP had been replaced by a HLA-DP4- or HLA-DR13-restricted MAGE-A3 epitope, efficiently stimulated MAGE-A3-specific T cells. This T-cell response is epitope specific, because DCs electroporated with a recombinant Ii encoding a HLA-mismatched epitope were unable to stimulate the T cells. The electroporation of DCs had no influence on their T-cell stimulatory capacity because peptide-pulsed DCs, which had been electroporated or not, had the same ability to activate the T cells. Additionally, electroporation had no effect on the phenotype of the DCs or on their capacity to respond to the mixture of inflammatory cytokines.

Although immature DCs are considered to be specialized in antigen capture and processing, whereas mature DCs present antigen and have an increased T-cell stimulatory capacity, our data show a very high T-cell stimulatory capacity for the immature DCs as well. We hypothesize that this is attributable to the maturation of immature DCs during their interaction with antigen-specific T cells. Indeed, although electroporation itself had no influence on the maturation status of the DCs, coculture of specific T cells with antigen-presenting immature DCs induced immunophenotypic changes (comparable with the changes induced by inflammatory cytokines) and secretion of IL-12.

Several groups have reported that the presentation of the HLA class II epitope using CLIP replacement is superior to peptide pulsing (6, 10, 11). Our results are in line with these findings. The T-cell stimulatory capacity of DCs electroporated with the recombinant Ii remained high, whereas it rapidly decreased when using peptide-pulsed DCs. Even when the DCs were pulsed with a very high concentration of synthetic peptide (30 μg/ml), the T-cell stimulatory capacity was less than when electroporated DCs were used.

Efficient induction of an antitumor response will most likely require repeated injections of the vaccine. We show that mature electroporated DCs can be frozen in AP without losing their T-cell stimulatory capacity.

Although several groups have reported the induction of tumor-specific CTLs by mRNA-transfected DCs *in vitro* and *in vivo* (25, 56, 58, 59), only a few studies demonstrated the induction of $CD4^+$ Th cells (57, 62). We show that DCs electroporated with the recombinant Ii constructs are able to induce an epitope-specific immune response in unprimed $CD4^+$ T cells. We successfully isolated IFN-γ-secreting T-cell clones in two independent experiments, using two different known MAGE-A3-derived HLA class II epitopes. Furthermore, for the epitope presented in the context of HLA-DP4, we were able to isolate monoclonal T-cell clones specifically recognizing the epitope.

In conclusion, we show that mRNA electroporation of DCs with CLIP-replacement constructs encoding HLA class II-restricted MAGE-A3 epitopes is a powerful tool for stimulating antigen-specific $CD4^+$ Th cells. This approach of stabilization of the HLA class II/peptide complex might be an alternative for epitope enhancement for HLA class II molecules.

ACKNOWLEDGMENTS

Material from patient R12 (from which clone R12-C9 was derived) was kindly provided by Drs. B. Schuler-Thurner and E. S. Schultz (University Hospital of Erlangen, Erlangen, Germany). We thank Dr. C. De Greef for helpful discussions and D. Carels, C. Huysmans, L. Hollanders, K. Michielsen, E. Vaeremans, P. Verbuyst, J. Volkaert, and C. Wildmann for technical assistance.

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