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Transcutaneous immunization with hydrophilic recombinant gp100 protein induces antigen-specific cellular immune response

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Abstract

The objective of this study was to evaluate the potential of transcutaneous immunization with tumor antigen to induce cell-mediated immunity. For this purpose, hydrophilic recombinant gp100 protein (HR-gp100) was topically applied on human intact skin *in vitro*, and used as a vaccine in a mouse model. We demonstrate that HR-gp100 permeates into human skin, and is processed and presented by human dendritic cells. In a mouse model, an HR-gp100-based vaccine triggered antigen-specific T cell responses, as shown by proliferation assays, ELISA and intracellular staining for IFN- γ . Transcutaneous antigen delivery may provide a safe, simple and effective method to elicit cell mediated immunity.

Keywords

transcutaneous immunization; gp100; cancer vaccine

1. Introduction

Active immunization strategies for the immunotherapy of patients with cancer are based on the ability to increase the number of T lymphocytes capable of reacting against tumor antigens (Ags). The feasibility of anti-melanoma vaccination using tumor cells, proteins, peptides or DNA as immunizing agents, was demonstrated in numerous pre-clinical and clinical settings (reviewed in [1]). Antigen presenting cells (APC) are a mandatory link between the site of Ag introduction and secondary lymphoid organs where effector cell activation will occur [2]. It was shown that T cells primed by APCs which had acquired their Ag in the skin express skin homing molecules[3]. This signature is valuable for the treatment of tumor deposits in the skin

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and subcutaneous tissues, and may explain why objective regressions attained by melanoma vaccines are mainly in skin metastases [4]. Because dendritic cells (DCs) are the most potent APC, these cells have been used for therapeutic vaccines against different tumor types by loading them with various sources of tumor antigens (peptides, proteins, tumor lysates, DNA and mRNA). Although some encouraging results have been observed [5,6], the *ex vivo* generation of DCs for therapeutic purposes is time-consuming, expensive, and requires trained personnel. Thus, a more practical approach to generate antitumor CTL responses would be to efficiently deliver an antigen directly to skin-resident DCs.

Transcutaneous immunization (TCI) is a simple, compelling and relatively new method of vaccination that targets the skin as the gate for antigen delivery to the immune system. The use of the skin as a target for vaccination has been prompted by the fact that it is populated by densely distributed and potent APCs, mainly Langerhans cells (LC) and dermal dendritic cells [7]. Epidermal LCs, upon encounter with external Ags and followed by their uptake and digestion, mature and migrate to the draining lymph nodes, where they present their antigenic load and consequently activate T- and B-cells [8]. Immunization strategies of antigen application to the skin have proven to be feasible and to elicit both systemic and mucosal immunity [9,10]. Vogt et al showed that transcutaneous anti-influenza vaccination in humans promotes both CD4 and CD8 T cell immune responses [11]. In murine studies, CD4⁺ and CD8⁺ T cell responses were observed after peptide immunization through the skin [12,13]. Data on TCI for the treatment of cancer are rather limited. Unlike viral proteins, most cancer antigens are self proteins of low immunogenicity. The generation of strong, reactive T cells to these antigens is often hindered by the lack of self-reactive progenitors following thymic deletion [14].

In a recent clinical trial, melanoma patients immunized with peptides on barrier-disrupted skin developed cytotoxic CD8⁺ T cell responses and showed regression of some lesions [15], highlighting the attractiveness of this approach for tumor immunotherapy.

Native gp100 is a member of a family of melanoma/melanocyte differentiation Ags strongly expressed in most melanomas. It is a hydrophobic glycoprotein of 661 amino acids with a molecular mass of 70 kD (GenBank Acc No. NM_006928). Gp100 protein includes a variety of immunogenic epitopes that are recognized by cytotoxic T lymphocytes (CTLs) recovered from peripheral blood of melanoma patients and from tumor infiltrating lymphocytes (TILs) [16]. While gp-100-derived peptides have been used in many melanoma vaccination studies [17–19] the full-length gp100 protein was rarely used. Compared with the peptide-based approach, there are several notable advantages to the use of protein vaccines: they are non HLA-restricted, can contain multiple antigenic epitopes, and may stimulate both CD4⁺ and CD8⁺ T cells. One of the reasons that full-length gp100 protein was rarely used has been the difficulty of purification due to its hydrophobic nature. In an attempt to produce a recombinant protein that is less hydrophobic, gp100 was cloned with the exclusion of two hydrophobic regions: the signal peptide at the N-terminus and the transmembrane motifs at the C-terminus, while retaining all of the peptides that have been reported to bear immunologic properties [20]. The resultant protein was termed HR-gp100. Our group has shown in the past that HR-gp100 is effectively taken up by dendritic cells (DC) and presented to CD8⁺ lymphocytes [21], and that transcutaneous immunization with HR-gp100 induces specific antibody production [22]. The present study focused on evaluating the potential of transcutaneous immunization with HR-gp100 to induce Ag-specific cellular immune responses. We show that HR-gp100 is absorbed through healthy human skin and elicits a measurable cellular immune response *in vivo*, as demonstrated by antigen-specific CD8 lymphocyte activation.

2. Materials and methods

2.1 Mice and cell lines

Female BALB/c and C57/B6 mice (8–12 wk old) were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were maintained under specific pathogen free conditions. All experiments were conducted in accordance with Hadassah-Hebrew University Hospital Animal Facility and NIH guidelines. T2 is a TAP-2-deficient lymphoblastoid line of HLA-A2 genotype. The 624mel (HLA-A2⁺) melanoma cell line was provided by M. Parkhurst (Surgery Branch, NIH, Bethesda, MD). Melanoma cell lines M171, and M579 (all HLA-A2[−]) were established in the Sharett Institute of Oncology/Hadassah-Hebrew University Hospital laboratory as described [23]. The M579-A2 clone is a stable HLA-A2 transfectant of M579 cells [23]. All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Fetal calf serum), 2 mmol/L L-glutamine, and combined antibiotics. M579-A2 cells were maintained in the same medium, supplemented with 1 mg/mL geneticin (Life Technologies, Carlsbad, CA). Human lymphocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 25 mmol/L HEPES (pH 7.4), 50 μmol/L β-mercaptoethanol, and combined antibiotics.

2.2 Histology

Fresh skin fragments (5–7mm diameter) obtained from eyelids of anonymous healthy donors undergoing blepharoplasty was used within two hours of removal to examine the ability of HR-gp100 to penetrate human skin. HR-gp100 (30μg/30μl PBS/0.5cm² skin) or PBS, as a control, was applied on the skin for 2 hours at 37°C, followed by freezing to optimal cutting temperature. Immunostaining was performed on acetone-fixed frozen sections according to SuperPicTure polymer detection system (Zymed, Invitrogen). Anti-gp100 monoclonal antibody HMB45 was from DakoCytomation.

2.3 Generation of peptide-specific cytotoxic T-cell lines

Generation of peptide-reactive T cell populations from patients with metastatic melanoma was performed as previously described, with minor modifications [24]. gp100_{209–217(210M)}, gp100_{280–288(288V)} or MART-1_{27–35(27L)} peptides (all commercially synthesized and purified (>95%) by reverse-phase HPLC by BiomerTechnology) were used to stimulate HLA-A2⁺ bulk melanoma patient-derived lymphocytes. Commercially available ELISA reagents (R&D Systems, Minneapolis, MN) were used to detect IFN-γ secretion as an estimate for specific recognition of peptides by T-cells following their co-incubation with peptide-pulsed T2 cells or HLA-A2⁺ melanoma cells (624mel, M579-A2) versus irrelevant peptide or HLA-A2[−] melanoma cells (M579, M171). To obtain T cell lines, peptide-reactive cultures were expanded with 30 ng/ml ortho-anti-CD3 (eBioscience, San-diego, CA), recombinant IL-2 (rIL-2, 300 IU/ml, Chiron B.V., The Netherlands) and 5 × 10⁶ allogeneic PBMCs as feeder cells.

2.4 Generation of monocyte-derived dendritic cells (DC)

HLA-A2.1-positive healthy volunteers were recruited for in vitro generation of monocyte-derived DC, as described [25]. All volunteers gave written informed consent to participate in the study. Briefly, PBMCs obtained by leukapheresis were purified by centrifugation on Ficoll-Paque Plus gradients (Amersham, Uppsala, Sweden). Immature dendritic cells were generated from adherent PBMC by incubation in RPMI 1640 with 10% heat-inactivated human AB serum, supplemented with IL-4 (1,000 units/mL, R&D, Minneapolis, MN) and GM-CSF (1,000 units/mL; R&D) on days one and four. Immature DC were harvested on day five and used in the following experiments.

2.5 Cytokine secretion assay

Immature DC were loaded with HR-gp100 (50 μ g/ml) for 10h at 37°C in OPTIMEM (Invitrogen, Paisley, UK) followed by maturation in low-serum (1%) complete medium supplemented with a cocktail of IL-1 β , IL-6, tumor necrosis factor α (each at 10 ng/mL, R&D), and prostaglandin E2 (1 μ g/mL, Alexis, Carlsbad, CA) for additional eight hours. After extensive washing, 10⁵ DC were co-cultured in complete medium with gp100-derived or irrelevant peptide-specific T cell lines at an effector-to-target ratio of 1:1, for 24h at 37°C. T cell reactivity was evaluated by IFN- γ secretion, as measured by ELISA, according to the manufacturer's instructions (R&D).

2.6 Transcutaneous immunization (TCI)

Anesthetized BALB/c or C57BL/6 mice (by i.p. injection of ketamine-HCL, 100 mg/kg) underwent transcutaneous immunization (TCI) by topical application of HR-gp100 on both sides of the ears (200 μ g/mouse). Mice were treated twice at a 3-day interval and five days after the last immunization they were sacrificed and the lateral retro-pharyngeal lymph nodes, from 8 mice per group, were harvested and pooled. A negative control group received BSA in the same mode as HR-gp100. As a positive control, mice were injected once into the foot pads with 50 μ g/mouse HR-gp100 in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, Saint Louis, MO), sacrificed after 8 days, and inguinal lymph nodes were harvested. Single cell suspensions were prepared, washed and suspended to 5 $\times$ 10⁶/ml in RPMI 1640 supplemented with 10% fetal calf serum, 50 μ mol/L β -mercaptoethanol and combined antibiotics.

2.7 Proliferation assay

To test Ag-specific T cell proliferation, lymphocytes from immunized or naïve mice, were cultured in 96-well plates (10⁵/well) and re-stimulated at the onset of the culture period, by adding 50 μ g/ml HR-gp100 or BSA. Cultures were incubated for 72 hrs, pulsed with one microcurie of methyl-[3H]thymidine (5 Ci/mM; Amersham, Buckinghamshire, UK) for the last 18 hr of incubation, and harvested. Cultures were harvested to a 96 GF/C Unifilter (Perkin Elmer, Waltham, MA), and Microscint-20 scintillation fluid (Perkin Elmer) was added. The plates were read using a Topcount microplate scintillation and luminescence counter (Packard, Ramsey, MN). The results are expressed as mean counts/min (c.p.m.) with standard error bars.

2.8 Cytokine secretion assay

To test Ag-specific IFN- γ production, lymphocytes from immunized or naïve mice were cultured in 96-well plates (10⁵/well) and re-stimulated at the onset of the culture period by adding 50 μ g/ml of HR-gp100 or BSA. Cultures were incubated for 24h at 37°C. T cell reactivity was evaluated by IFN- γ secretion, as measured by ELISA (BioLegend).

2.9 Intracellular cytokine staining and flow cytometry

Primed or naïve lymphocytes were stimulated in vitro with 50 μ g/ml HR-gp100 or BSA for 16h. Brefeldin A (10 μ g/mL, GolgiPlug) was added for the last 4 hours to enable intracellular proteins to accumulate. IFN- γ -producing cells were detected using the Cytotfix/Cytoperm Plus Kit (BD) according to the manufacturer's protocol. Briefly, 10⁶ the cells were harvested, suspended in staining buffer (PBS with 1% FCS and 0.1% sodium azide) and blocked with purified anti-Fc γ R2/3 monoclonal antibody (2.4G2, eBioscience) for 15 minutes at 4°C, and stained with FITC-conjugated anti-CD4 or CD8 (e-Bioscience) for 30' at 4°C. The cells were washed with staining buffer, fixed with 4% paraformaldehyde for 20 minutes at 4°C and washed twice. Cells were then permeabilized in PBS containing 5% FCS, 0.1% saponin and 0.1% sodium azide for 15' at 4°C and incubated in the presence of allophycocyanine (APC)-conjugated anti-mouse IFN- γ (XMG1.2) or isotype control (all from eBioscience). The data were analyzed by LSRII (BD) and "FCS express" software (De Novo).

3. Results

3.1 HR-gp100 permeates human epidermis and is taken-up by resident cells

With the aim to establish a transcutaneous method for anti-melanoma vaccination, the ability of HR-gp100 protein to penetrate human skin was evaluated. To this end, skin from eyelids obtained from human donors undergoing cosmetic surgery was used. Following cleansing with 70% ethanol, HR-gp100 was directly applied on the skin (without prior tape stripping), that was then sectioned and labeled for anti-human gp100 immunohistochemistry. Figure 1 shows that epidermal cells were positively stained with a mouse anti-human gp100 antibody (HMB-45). The labeling was clearly detectable in the cytoplasm. M624 melanoma cell line expressing endogenous gp100 served as positive control (not shown). This experiment shows that HR-gp100 penetrated intact human epidermis, without any further need for stripping or disruption.

3.2 HR-gp100-loaded DCs stimulate peptide-specific T cell lines

In this experiment the ability of human dendritic cells loaded with HR-gp100 to process the protein was evaluated using peptide-specific T cell lines. It was expected that T cells against gp100-derived peptides will detect processed peptides, be activated and secrete IFN- γ . The gp100 peptide-specific T cell lines were generated by repeated peptide stimulation and their activity evaluated as described in the Material and Methods section. Gp100-specific T cells were co-incubated with HR-gp100-loaded DCs from HLA-A2.1-positive healthy volunteers, and IFN- γ secretion was measured by ELISA. Table 1 shows increased IFN- γ secretion in response to DC and the other loaded with HR-gp100 by two gp100-specific T cell lines, one against gp100_{209–217} against gp100_{280–288}. MART-1-specific T cells and irrelevant antigen-loaded DCs served as negative controls. In conclusion, DCs took up and processed HR-gp100 followed by presentation of the protein-derived peptides in conjunction with MHC class I molecules and these complexes could be detected by their cognate T cells.

3.3 Cellular immune responses in vivo

3.3.1 Topically applied HR-gp100 induces antigen-specific T cell proliferation—

In the past we showed that transcutaneous delivery of HR-gp100 applied on mice ears induces a humoral immune response [22]. This time we set to examine whether transcutaneous delivery can also activate gp100-specific T cells using the same transcutaneous immunization murine model [22]. HR-gp100 was topically applied on the ears of mice at two time points. Five days after the last immunization, lateral retro-pharyngeal lymph nodes were harvested and lymphocytes were re-stimulated *in vitro* for 72 hrs in the presence of HR-gp100. T cell proliferation was examined using ³H-thymidine incorporation assays. As shown in Fig. 2, transcutaneous immunization with HR-gp100 caused vigorous proliferation of draining lymph node-derived, *in vitro* re-stimulated T cells. Topical application of BSA induced background proliferation levels.

3.3.2 Topically applied HR-gp100 induces antigen-specific IFN- γ secretion—

To further characterize the immune response elicited by TCI, HR-gp100 was topically applied on the ears of mice at two time points. Five days after the last immunization, lateral retro-pharyngeal lymph nodes were harvested and lymphocytes were re-stimulated *in vitro* for 24 hrs in the presence of HR-gp100. IFN- γ secretion by lymphocytes was evaluated using an ELISA assay. As shown in Fig. 3, high levels of IFN- γ were secreted (up to 650pg/ml) by lymphocytes from HR-gp100 immunized mice following re-stimulation with HR-gp100. In contrast, low IFN- γ secretion by naïve or BSA-primed lymphocytes was detected after *in vitro* re-stimulation with HR-gp100. These results further support the development of an antigen-specific response following skin application of HR-gp100.

3.3.3 Topical application of HR-gp100 induces IFN- γ production by antigen-specific CD8 $^{+}$ T lymphocytes—To determine whether CD8 $^{+}$ and/or CD4 $^{+}$ lymphocytes are involved in the immune response documented above, IFN- γ intracellular staining (ICS) of Ag-specific T cells was performed. For this purpose, HR-gp100 was applied on the ears of mice as described above, and lymphocytes derived from regional lymph nodes were harvested and re-stimulated in vitro for 16 hrs with HR-gp100. As a positive control, in this experiment we injected HR-gp100 with IFA – a strong adjuvant - into the animals' foot pad. As shown in Fig. 4, following re-stimulation of lymphocytes with HR-gp100, an increased percentage of IFN- γ -producing CD8 $^{+}$ cells was found among lymphocytes from HR-gp100 immunized mice (0.5% out of total CD8 $^{+}$ cells), in comparison with un-stimulated cells or with lymphocytes from naïve animals (0.1%). The positive control mice, injected with HR-gp100-IFA gave a maximal response of IFN- γ producing CD8 $^{+}$ cells reaching 2% (Fig. 4). IFN- γ producing CD4 $^{+}$ cells could only be identified at low levels in the control IFA and HR-gp100-intrafootpad immunized mice following in vitro re-stimulation with HR-gp100, and not in lymphocytes from transcutaneously immunized mice. These results support the involvement of CD8 $^{+}$ lymphocytes as the primary IFN- γ producing cell population in response to topical HR-gp100 application.

4. Discussion

Transcutaneous immunization is an attractive method of antigen delivery, because application of antigens onto bare skin is a simple procedure that exploits the abundance of skin-resident dendritic cells. These potent APCs are necessary for the initiation of a CTL response and the use of the natural cutaneous source overcomes the logistic difficulties of ex vivo manipulation and transfer of DC. The novelty of the present study lies in the selection of a full-length cancer self-antigen (hydrophilic recombinant gp100) modified for transcutaneous immunization, and in the advantage achieved by targeting the unrestricted reservoir of dermal antigen presenting cells. To the best of our knowledge, this is the first evidence of successful induction of cell-mediated immune responses against a cancer-associated antigen following transcutaneous immunization with a full-length protein.

Previously, our group has shown that HR-gp100, an antigenic melanoma-derived protein, activates the immune system following transcutaneous delivery, as shown by Langerhans cell activation and antibody production in immunized mice [22]. In this study we further characterized the immune response that developed as a result of immunization using HR-gp100 protein. First, it was demonstrated that the HR-gp100 protein, applied on human skin, entered the epidermis and was traced in epidermal cells. Thus, we were reassured that this large protein essentially penetrates intact human skin without the need for a further carrier.

As uptake of HR-gp100 by antigen presenting cells was already shown [21], it was now mandatory to verify that this antigen was also effectively presented to lymphocytes in a manner that leads to their activation. Due to the difficulty of isolating sufficient numbers of human skin Langerhans cells for in vitro studies, we used human monocyte-derived DCs as alternative APC. Using these cells, we were able to demonstrate activation of two gp100-reactive T cell lines by mDCs loaded with the full length protein. Although the response levels, as measured by IFN- γ secretion, were relatively low, it is clear that mDCs processed the protein and presented HR-gp100-derived peptides to the cognate CD8 $^{+}$ T cell lines. The low response level could be explained by the decreased efficiency of cross presentation of soluble proteins in conjunction with MHC class I molecules to CD8 $^{+}$ T cells, which was encountered in DC [26].

For in vivo studies, a mouse model was used to evaluate whether TCI with full length hydrophilic gp100 can elicit cellular immune responses. In spite of the fact that no adjuvant

was used, skin application of soluble HR-gp100 induced vigorous cell proliferation in an antigenic-specific manner (Fig. 2). In addition, the IFN- γ intracellular staining assay verified that CD8+ T cells are a subset that produced substantial amount of IFN- γ which was secreted in response to the antigenic stimulation. The secretory activation of murine CD8+ T cells was not accompanied by increased cytotoxic activity (data not shown). The mild potency of the vaccine could account for this result.

Transcutaneous immunization with HR-gp100 has several advantages: the antigen can be produced and delivered effectively, it is readily absorbed by the skin, and thus reaches unlimited numbers of skin resident APCs with no need for ex vivo generation and loading of these cells. Following application to the skin, epidermal APCs participate in induction of the immune response. Topically applied HR-gp100 is capable of evoking lymphocyte proliferation and cytokine secretion by CD8+ T cells.

In order to increase its therapeutic potency, HR-gp100 could be administered in conjunction with an adjuvant that would add a co-stimulatory component and subsequently drive stronger cytotoxic responses. Since the prime objective of this study was to evaluate the immunogenic potency of HR-gp100 in the particular setting of TCI, we refrained from adding a potent adjuvant that might divert or shade the net effect of the recombinant molecule. Thus, the results reflect the basic immunogenicity of HR-gp100.

In summary, we have demonstrated that TCI is a safe, easy and effective way to generate antigen specific T cells which are the most important effector cells that mediate tumor regression. Since TCI is an attractive strategy of immunization, this approach could be used for other tumor associated antigens, for the induction of anti-tumor immunity.

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Figure 1.

HR-gp100 permeates human epidermis. HR-gp100 (left panel) or PBS (right panel) were applied on eyelids skin followed by freezing of the samples. Acetone-fixed frozen sections were immunostained with anti-gp100 monoclonal antibody HMB45. *Red*, epidermis-resident cells stained by HMB45 MAb (depicted with arrowheads); *blue*, hematoxylin staining. Representative images from two independent experiments are shown.

Figure 2.

Specific lymphocyte proliferation following topical HR-gp100 application *in vivo*. Mice (8 mice per group) were immunized by TCI with HR-gp100. BSA-treated mice were used as a control. Five days after the last immunization, lymphocyte cultures prepared from the pooled draining lymph nodes were re-stimulated *in vitro* with HR-gp100 (black columns) or BSA (open columns) for 72 h. Cell proliferation was measured by ^3H incorporation. Columns represent mean $\pm$ SE (n=8). Statistical significance was determined by Student *t* test. $P=0.019$ for HR-gp100-re-stimulated lymphocytes obtained from mice immunized transcutaneously with HR-gp100 versus BSA. Results are representative of two independent experiments.

Figure 3.

IFN- γ secretion following topical HR-gp100 application in vivo. Mice (8 mice per group) were transcutaneously immunized with HR-gp100, as described. Five days following the last immunization, lymphocyte cultures were prepared from pooled lateral retro-pharyngeal lymph nodes and re-stimulated in vitro by HR-gp100 (black columns) or BSA (open columns) for 24 h. Naïve mice and BSA- treated mice served as negative control. For the positive control, mice were immunized intra footpad with HR-gp100 emulsified in incomplete Freund's adjuvant. IFN- γ secretion was measured by ELISA. Columns represent mean $\pm$ SE (n=8). Statistical significance was determined by Student *t* test. $P=0.001$ for HR-gp100-re-stimulated lymphocytes obtained from HR-gp100 transcutaneously immunized versus naive mice. Comparable results were obtained in three separate experiments.

Figure 4.

Transcutaneous immunization with HR-gp100 induces IFN- γ production by CD8⁺ T cells in an antigen-specific manner. Pooled lymph node-derived lymphocytes from naïve or HR-gp100-immunized (topically or intra footpad) - mice were re-stimulated in vitro with HR-gp100 or BSA, as indicated. Numbers within quadrants indicate the percentages of IFN- γ -expressing cells among CD8⁺ or CD4⁺ lymphocytes.

Table 1

IFN- γ production by gp100-specific T cell lines following stimulation with HR-gp100-loaded dendritic cells.

T cell line	DC loaded with		
	None	HR-gp100	Cognate peptide ^a
Anti-gp100 _{209–217}	0	161	1480
Anti-gp100 _{280–288}	0	352	1867
Anti-MART 1 _{27–35}	0	0	2011

Immature mDCs were loaded with HR-gp100 followed by incubation with a cytokine cocktail (IL-1 β , IL-6, TNF α and PGE₂) to induce maturation. Loaded mature DCs were co-cultured with peptide-specific T cell lines for 24h. IFN- γ (pg/ml) secretion was measured by ELISA.

^a cognate peptide: gp100_{209–217} peptide- loaded DCs used for stimulation of an anti- gp100_{209–217} T cell line; gp100_{280–288} peptide-loaded DCs were used for stimulation of anti- gp100_{280–288}, and MART 1_{27–35} peptide-loaded DCs were used for stimulation of anti-MART 1_{27–35}.