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Identification of an HLA-DPB1*0501 Restricted Melan-A/MART-1 Epitope Recognized by CD4+ T Lymphocytes: Prevalence for Immunotherapy in Asian Populations

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Summary

CD4+ T lymphocytes play a central role in orchestrating an efficient antitumor immune response. Much effort has been devoted in the identification of major histocompatibility complex class II eptiopes from different tumor-associated antigens. Melan-A/ MART-1 is expressed specifically in normal melanocytes and tumor cells of 75% to 100% of melanoma patients. Melan-A/MART-1 is considered as an attractive target for cancer immunotherapy. In the past, several human leukocyte antigen (HLA) class II restricted epitopes have been identified and characterized, including Melan-A/ MART-$1_{1\text{-}20}$ (HLA-DR11 restricted),Melan-A/MART-$1_{25\text{-}36}$ (HLA-DQ6 and HLA-DR3 restricted), Melan-A/MART-$1_{27\text{-}40}$ (HLA-DR1 restricted), Melan-A/MART-$1_{51\text{-}73}$ (HLA-DR4 restricted), Melan-A/ MART-$1_{91\text{-}110}$ (HLA-DR52 restricted), and Melan-A/MART-$1_{100\text{-}111}$ (HLA-DR1 restricted). Owing to the infrequent expression of the above HLA class II alleles in Asian populations, immunotherapy using these defined Melan-A/MART-1 peptides could potentially only benefit a very small percentage of Asian melanoma patients. In this study, we established several CD4+ T-cell clones by in vitro stimulation of peripheral blood mononuclear cells from a healthy donor by a peptide pool of 28 to 30 amino acid long peptides spanning the entire Melan-A/MART-1 protein. These CD4+ T-cell clones recognized a peptide that is embedded within Melan-A/ MART-$1_{21\text{-}50}$, in a HLA-DPB1*0501 restricted manner. Finally, we demonstrated that this epitope is naturally processed and presented by dendritic cells. HLA-DPB1*0501 is frequently expressed in Asian population (44.9% to 73.1%). Therefore, this epitope

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could provide a new tool and could significantly increase the percentage of melanoma patients that can benefit from cancer immunotherapy.

Keywords

tumor immunotherapy; $CD4^+$ T cells; melanoma; Melan-A/MART-1; HLA-DPB1*0501

In the area of tumor antigen-specific T-cell immunotherapy, most of the studies have focused on the induction of $CD8^+$ cytotoxic T lymphocytes (CTLs),[1] which can efficiently and directly kill tumor cells expressing tumor antigens. However, recent studies have provided direct evidence that $CD4^+$ T lymphocytes can not only act as effector cells but also as regulators for the persistence of long-lasting antitumor responses.[2–4] On the one hand, $CD4^+$ T cells can themselves act as effector cells either by secretion of antitumor cytokines or by direct cytotoxic activity.[5,6] In contrast, $CD4^+$ T cells can prime and maintain CTLs responses by directly secreting cytokines,[7] promoting dendritic cells (DCs) recruitment into the tumor tissues and in secondary lymphoid organs,[8,9] and by maintaining memory cells in the periphery.[10–13] Much effort has been devoted in the identification of novel and high affinity major histocompatibility complex (MHC) class II epitopes. These MHC class II epitopes can be used in active antitumor vaccination, or generation of $CD4^+$ T-cell clones for adoptive therapy.

In 2008, Hunder et al[14] demonstrated the development of an in vitro method for isolating and expanding autologous $CD4^+$ T-cell clones with specificity for the melanoma-associated antigen NY-ESO-1. They infused these cells into a patient with refractory metastatic melanoma who had not undergone any previous conditioning or cytokine treatment. The infusion of the autologous $CD4^+$ T-cell clones caused complete tumor regression of a patient with refractory metastatic melanoma. The adoptively transferred NY-ESO-1-specific $CD4^+$ T-cell clones were detectable for several months in vivo, and mediated a long-term complete clinical remission. Importantly, the infused clonal population of $CD4^+$ T cells could induce T-cell responses to other tumor-associated antigens of his tumor. These observations supported that $CD4^+$ T lymphocytes are also essential for the persistence of a long-lasting antitumor responses and generation of effective, nontoxic immune therapies for cancer.

Recently, a study transferred small numbers of naive tyrosinase-related protein-1-specific $CD4^+$ T cells into lymphopenic recipient mice bearing large established melanoma. Therapy was independent of vaccination, exogenous cytokine support, $CD8^+$, B, natural killer, and natural killer T cells. Transferred $CD4^+$ T cells expanded and differentiated into cytotoxic $CD4^+$ T cells that could eliminate melanoma cells directly. This study confirmed that tumor-specific $CD4^+$ T cells could cause tumor regression in adoptive immunotherapy against melanoma.[15]

Melan-A/MART-1 is a transmembrane protein with 118 amino acids, and is expressed specifically on normal melanocytes and tumor cells of 75% to 100% of melanoma patients.[16] The Melan-A/MART-1 gene product was identified as a tumor-associated antigen by 2 different groups in 1994, simultaneously. Coulie et al[17] identified Melan-A as a melanoma antigen recognized by autologous cytotoxic T-cell clones from peripheral blood

lymphocytes, showing a human leukocyte antigen (HLA)-A2 restriction. Kawakami et al[18] analyzed the target antigen of tumor-infiltrating lymphocytes of HLA-A2$^+$ melanoma patients by cDNA expression cloning, and cloned the same gene, designated MART-1. All of the tumor-reactive CTLs from both groups were observed to recognize the region encompassing residues 26 to 35 of Melan-A/MART-1 in association with HLA-A2.1. In 1995, Castelli et al[19] identified a naturally processed Melan-A/MART-1 peptide containing residues 32 to 40 and binding to HLA-A2.1 with tandem mass spectrometry. Subsequently, epitopes restricted by other HLA class I molecules including HLA-B45, [20] HLA-B35,[21] and HLA-Cw7[22] were described.

According to the cancer immunity peptide database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm), there are 7 known MHC class II restricted epitopes of Melan-A/MART-1, in association with HLA-DR1,[23,24] HLA-DR3,[23] HLA-DR4,[25] HLA-DR11,[26] HLA-DR52,[26] and HLA-DQ6.[23] To identify novel MHC class II-restricted epitopes of Melan-A/MART-1 and exploring epitopes more suitable for cancer immunotherapy for Asian population, in this study, we used a pool of synthetic peptides spanning the entire Melan-A/MART-1 protein sequence to stimulate enriched CD4$^+$ T-cell populations from peripheral blood mononuclear cells (PBMCs) of healthy donors (HDs). Here, we reported the successful isolation, expansion, and characterization of CD4$^+$ T-cell clones, which specifically recognized Melan- A/MART-121-50 peptide. We further demonstrated that these CD4$^+$ T-cell clones recognized this epitope in 12 of 20 PBMCs of HDs in a HLA-DPB1*0501 restricted manner. Finally, we demonstrated that this epitope is naturally processed and presented by DCs pulsed with Melan-A/ MART-1 protein or lysates of melanoma cell lines. HLA-DPB1* 0501 is frequently expressed in Asian population (44.9% to 73.1%).[27] Therefore, this epitope could provide a new tool and could significantly increase the percentage of melanoma patients that can benefit from cancer immunotherapy.

MATERIALS AND METHODS

Cells and Tissue Culture

Peripheral blood was obtained from HDs (PLA 307 hospital, Beijing, China) after obtaining informed consent. Melanoma cell lines, A375, A875, and SK-MEL-1, were purchased from basic medical cell center of China Union Medical University, and were maintained in RPMI 1640 (GIBCO Invitrogen Corp., Rockville, MD) supplemented with 10% heat-inactivated FBS (GIBCO Invitrogen Corp.). Culture medium for lymphocytes was MXFT Medium (IMMUNOTECH UK LTD.) supplemented with recombinant human (rh) IL-2 (Satellite Biological Products Research Institute, Liaoning, China) and, where indicated, rhIL-7 (Peprotech Inc., Rocky Hill, NJ). Every 18 to 20 days, cells were restimulated with allogeneic irradiated PBMCs, 1 μg/mL phytohemagglutinin (Sigma-Aldrich, St. Louis, MO) and 100 IU/mL rh interleukin (IL)-2.

HLA Typing

High resolution typing (4-digit) for HLA-DPB1 loci was done at the HLA typing laboratory of Shenzhen Huada gene technology Co., LTD.

Synthetic Peptides

Peptides were synthesized by Jill biochemical Co., LTD. (Shanghai, China) by standard solid-phase chemistry on a multiple peptide synthesizer (Applied Biosystems) using F-moc for transient NH_2-terminal protection and were analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytic high performance liquid chromatography. Lyophilized peptides were diluted in phosphate buffer saline—10% dimethyl sulfoxide or 100% dimethyl sulfoxide at 2mM and stored at −20°C.

Assessment of Melan-A/MART-1-specific $CD4^+$ T cells in PBMCs and Generation of Specific T-cell Clones

In vitro stimulation of Melan-A/MART-1-specific T cells was conducted as described previously.[28] In brief, 1×10^7 to 2×10^7 $CD4^+$ T cells, highly enriched (>95%) from PBMCs using anti-CD4 microbeads and a Mini- MACS device (Miltenyi Biotec Inc.), were stimulated with irradiated (25 Gy) or mitomycin C (25 μg/mL)-treated autologous $CD4^-$ cells in the presence of a mixture containing 28 to 30 amino acid long and partially overlapping peptides (2 μM each, Jill biochemical Co., LTD, Shanghai, China) spanning the entire Melan-A/ MART-1 protein sequence in MXFT Medium containing rhIL-2 (20 IU/mL) and rhIL-7 (10 ng/mL). After 1 or 2 weeks of in vitro stimulation, cultures were tested for the presence of specific $CD4^+$ T cells using the same pool of peptides (2 μM each) in an intracellular interferon (IFN)-γ secretion assay as follows. T cells were stimulated for 4 hours at 37°C in the absence or in the presence of the peptide pool and GolgiStop protein transport inhibitor (BD Biosciences, San Jose, CA) according to the manufacturers' protocol. After incubation and washing, cells were then stained with anti-CD4 mAb (BD Biosciences) for 30 minutes at 4°C, fixed and permeabilized with fixation/permeabilization solution (BD Biosciences), stained with anti-IFN-γ mAb (BD Biosciences). Samples were analyzed by flow cytometry on FACSCalibur (BD Biosciences, Franklin Lakes, NJ). Data analysis was performed using FACSDako software. $CD4^+$ T cells producing IFN-γ in response to peptide pool stimulation were isolated by cytokine secretion- guided magnetic cell sorting using a cytokine secretion detection kit (Miltenyi Biotec Inc.) and cloned by limiting-dilution culture in the presence of phytohemagglutinin (Sigma-Aldrich), allogeneic irradiated PBMCs, and rhIL-2 (100 IU/mL) as described.[28] Clones were subsequently expanded by periodic stimulation (every 3 to 4 wk) under the same conditions.

Antigen Recognition Assays

To assess the fine specificity of antigen recognition, growing $CD4^+$ T-cell clones (10,000/well) were incubated in the absence or presence of each Melan-A/MART-1 peptides (2 μM) in 200 μL/well of MXFT Medium in 96-well round-bottom plates. Where indicated, antibodies to HLA-DR (clone L243, BD Biosciences), HLA-DP (clone B7/21, abcam, Cambridge, MA), or HLA-DQ (clone SPVL-3, abcam) were used to specifically block MHC class II recognition by specific $CD4^+$ T-cell clones. For recognition of none-related HDs as antigen presenting cells (APCs), equal numbers of cells were preincubated or not with indicated peptide (2 μM) for 1 hour, extensively washed, and then added to T cells. To assess recognition of the native antigen, DCs were incubated with protein (10 μg/ mL) or tumor lysates for 16 hours, intensively washed and used as APCs. After 24 hours of

incubation at 37°C, culture supernatants were collected, and the concentration of IFN-γ released in response to antigen stimulation was assessed by ELISA (dakewe biotech Co., LTD, Shenzhen, Guangzhou, China) following the manufacturer's instructions.

Generation of DCs, Recombinant Proteins, Tumor Lysates, and Ag Loading

Monocyte-derived DCs were generated by isolating $CD14^+$ monocytes from PBMCs by magnetic cell sorting using a miniMACS device (Miltenyi Biotec). Highly enriched $CD14^+$ cells (>95%) were cultured in Iscove Modified Dulbecco Medium (IMDM) (GIBCO Invitrogen Corp.) supplemented with 10% heat-inactivated fetal bovine serum, 1000 IU/mL rh granulocyte macrophage colony-stimulating factor (Beijing medical university united pharmaceutical Co., LTD, Beijing, China) and 1000 IU/mL rhIL-4 (Peprotech Inc.) for 6 days.

Melan-A/MART-1 proteins were expressed in *Escherichia coli* as full-length proteins with a 6-histidine tag at the amino terminus as described previously.[29] The proteins were purified from washed and solubilized inclusion bodies by nickel chelating affinity chromatography using a pH gradient. Proteins were eluted in 8M urea, 100mM phosphate, and 10mM Tris at pH 4.5, followed by step gradient dialysis to 2mM urea in phosphate buffer saline. The purified proteins were reactive with anti-Melan-A/ MART-1 mAbs A103 by western blot analysis; purity was >80% by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Tumor cells (2×10^5) were lysed in 200 μl of Roswell Park Memorial Institute 1640 medium by 10 cycles of rapid freezing and thawing. For antigen loading using peptides, DCs were activated at day 6 with tumor necrosis factor-α (1000 IU/mL). After 24 hours (day 7), mature DCs were pulsed with indicated peptide (2 μM) for 2 to 4 hours. For antigen loading using proteins (10 μg/mL) or tumor lysates (at the equivalent of 3 tumor cells/DC), which required antigen processing, DCs were loaded with antigen at day 5. After overnight incubation, DCs were activated at day 6 with tumor necrosis factor-α (1000 IU/mL) for 24 hours. Mature DCs were washed twice in complete media before their use in stimulation or antigen recognition assay.

Analysis of Melan-A/MART-1 and HLA-DP Expression by Melanoma Cell Lines

To assess the Melan-A/MART-1 expression of tumor cell lines, indirect immunofluorescence labeling was performed. Tumor cell lines were cultured to logarithmic growth phase, then dissociated to single cells by 0.25% trypsin digestion, fixed and permeabilized with fixation/ permeabilization solution (BD Biosciences) for 30 minutes at 4°C, and rinsed with perm/wash buffer (BD Biosciences). Mouse anti-Melan-A/MART-1 mAb (40 to 2300, Zymed Laboratories, Inc., South San Francisco, CA) was used as the primary antibody, and diluted at a concentration of 1 μg/mL. Normal mouse IgG_1 was used for the isotype control. The duration of the primary mAb staining was 1 hour, followed by the secondary antibody, 1:1000-diluted goat antimouse IgG_1-FITC (Santa Cruz Biotechnology, CA) staining for 45 minutes both at room temperature. Samples were washed 3 times with perm/wash buffer for 5 minutes each, and were analyzed by flow cytometry on FACSCalibur. Surface expression of HLA-DP was determined by staining using specific mAb (clone B7/21, Abcam) and flow cytometry analysis.

RESULTS

Isolation and Characterization of Melan-A/ MART-1-specific CD4+ T Cells From Circulating Lymphocytes of HDs

To assess the response of Melan-A/MART-1-specific $CD4^+$ T cells in healthy individuals, we isolated $CD4^+$ T cells from PBMCs of 8 HDs using magnetic cell sorting and stimulated in vitro with a pool of 10 peptides spanning the entire Melan-A/MART-1 protein sequence. These peptides were 28 to 30 amino acid long and partially overlapped by 20 amino acids (Fig. 1A). Autologous irradiated $CD4^-$ cells were added as APCs. After 1 or 2 rounds of stimulation, the presence of Melan-A/MART-1- specific $CD4^+$ T cells was assessed by intracellular staining with IFN-γ and CD4-specific mAbs after stimulation in the presence or in the absence of the Melan-A/MART-1 peptide pool. Data obtained after this analysis for the responding donors are shown in Figure 1B and data obtained for all donors are summarized in Figure 1C. $IFN\gamma^+$ and $CD4^+$ T cells were detected in 4 of the 8 HDs. After the first cycle of in vitro stimulation, specific responses to the Melan-A/MART-1 peptide pool were detected for 2 (HD 005 and HD 106) of 8 health donors. After the second cycle of in vitro stimulation, specific responses could be detected for 2 more HDs (HD 006 and HD 108). For these 4 responding HDs, the frequency of IFN-γ producing $CD4^+$ T cells was 2.5 approximately 7-fold (average 4.6±1.9) higher than the control group (without the Melan-A/ MART-1 peptide pool). The IFN-γ-secreting $CD4^+$ T cells in response to stimulation with Melan-A/ MART-1 peptide pool were isolated by cytokine secretion-guided magnetic cell sorting. The purified fraction was either stimulated as a polyclonal population or was further cloned under limiting dilution conditions by stimulating with phytohemagglutinin in the presence of rhIL-2 (100 IU/ mL) and irradiated feeder cells. Five clones, derived from HD 106, were obtained. They specifically recognized the Melan-A/MART-1 peptide pool. Clone 5 and 7 had enough cell number and were selected for further study.

To determine whether the $CD4^+$ T-cell clones recognize a single peptide, individual peptide was directly pulsed to the 5 CD4 T-cell clones at 2 μM concentration. Twenty-four hours after pulsing, the recognition was measured by IFN-γ production by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 2A, 5 clones from HD 106 specifically recognized Melan-A/MART-$1_{21\text{-}50}$, whereas no significant activity was detected in response to the partially overlapping peptides in the neighboring regions. Peptide titration curves for the active peptide of clone 5 are shown in Figure 2B. Data were shown as mean of triplicate values. Each graph is representative of at least 3 independent experiments giving comparable results. This study demonstrated that Melan-A/MART-$1_{21\text{-}50}$ contains the active epitope that could be recognized by $CD4^+$ T-cell clone 5.

The $CD4^+$ T-cell clone 7 yielded similar results (data not shown).

The Known CD4 Epitopes Melan-A/MART-$1_{25\text{-}36}$ and Melan-A/MART-$1_{27\text{-}40}$ Are Not Recognized by Melan-A/MART-1-specific $CD4^+$ T-cell Clones

Melan-A/MART-$1_{21\text{-}50}$ contains 2 already known HLA class II epitopes, Melan-A/ MART-$1_{25\text{-}36}$ and Melan-A/MART-$1_{27\text{-}40}$. To determine whether any of the 2 epitopes could be recognized by the $CD4^+$ T-cell clones, these 2 epitopes were synthesized and

pulsed to the $CD4^+$ T-cell clone 5 at 2 μM concentration. Twenty-four hours after pulsing, the recognition was measured by IFN-γ production by ELISA. As shown in Figure 3, clone 5 did not recognize Melan-A/MART-1_{25-36} and Melan-A/ MART-1_{27-40} as no significant activity was detected. This study demonstrated that Melan-A/MART-1_{21-50} contains an active epitope that had not been reported previously. The $CD4^+$ T-cell clone 7 yielded similar results (data not shown).

Assessment of MHC Class II Restriction and Identification of the Restricting Allele of the Melan-A/MART-1-specific $CD4^+$ T-cell Clone by Antibody Blocking Experiments

To identify the MHC class II restricting element(s) of Melan-A/MART-1_{21-50}-specific $CD4^+$ T-cell clone from HD 106, the recognition of peptide Melan-A/MART-1_{21-50} was carried out in the presence of either anti-HLA-DR-specific, anti-HLA-DP-specific, or anti-HLA-DQ-specific antibodies. A significant reduction of IFN-γ production was seen with anti-HLA-DP antibody. In contrast, no significant inhibition was observed in the presence of antibodies against anti-HLA-DR antibody. Anti-HLA-DQ antibody and anti-HLA-A, B, C antibody demonstrated little inhibition that was within experimental variations (Fig. 4A). Thus, the Melan-A/MART-1_{21-50}- specific $CD4^+$ T-cell clone from HD 106 recognized the peptide in an HLA-DP-restricted manner.

Each healthy individual contains 2 HLA-DP alleles, and there are more than a dozen HLA-DP alleles reported. We first determined the HLA-DP alleles of HD 106. As assessed by molecular typing, HD 106 was an HLA-DPB1* 0501 homozygote. This suggests that DPB1*0501 is the presenting molecule of HD 106.

To further investigate the association of DPB1*0501 with clone 5 peptide recognition, PBMCs from 20 HDs were randomly selected and tested. Twelve of 20 PBMCs analyzed were able to present Melan-A/MART-1_{21-50} to clone 5 as measured by ELISA (Fig. 4B, C). The 20 HDs were molecularly typed for their HLA-DP alleles. As illustrated in Figures 4B and C, the 12 HDs, which had the ability to present Melan-A/MART-1_{21-50} to specific $CD4^+$ T-cell clone 5, all expressed the DPB1*0501 allele, whereas the remaining 8 health donors did not. It suggested a frequency of the presenting allele in the test population (Chinese) was approximately 60%, which was consistent with the prevalence of DPB1*0501 in Chinese populations.

In conclusion, these results showed that Melan-A/ MART-1_{21-50}-specific $CD4^+$ T-cell response detected in HD 106 was restricted to HLA-DPB1*0501, which is expressed by 44.9% to 73.1% of the Asian population. [27]

The Peptide Epitope Recognized by Clone 7 is a Naturally Processed HLA-DPB1*0501 Restricted Epitope

We then assessed the ability of Melan-A/MART-1_{21-50}- specific HLA-DPB1*0501-restricted $CD4^+$ T-cell clone to recognize the naturally presented antigen on the surface of professional APCs. As illustrated in Figure 5A, >99% of A375, A875, and SK-MEL-1 cell lines expressed Melan-A/ MART-1 protein as detected by flowcytometry. The processing and presentation of Melan-A/MART-1 antigen by DCs after incubation with lysates from tumor cell lines A375, A875, SK-MEL-1 (Melan-A/MART-1-expressing), or A549 (Melan-

A/MART-1-negative) was assessed by ELISA measurement of IFN-γ secretion in the culture supernatant (DCs:T cells=1:10). As shown in Figure 5B, Melan-A/ MART-1-specific $CD4^+$ T cells efficiently recognized the Melan-A/MART-1 native antigen that is present in the tumor lysates from 3 tumor cell lines upon processing and presentation by professional APCs. This result suggests that clone 5 recognized an epitope that was present in Melan-A/ MART-1 positive tumor cells and was naturally processed. To further investigate the specificity of clone 5, soluble recombinant Melan-A/MART-1 protein at the indicated dose was pulsed to monocyte-derived DCs (DCs:T cells= 1:10). Recognition was assessed after 24 hours of incubation by ELISA measurement of IFN-γ secretion in the culture supernatant. Autologous DCs were able to more efficiently process the recombinant Melan-A/MART-1 protein produced in *Escherichia coli* and present the relevant epitope to theMelan-A/MART-1-specific $CD4^+$ T-cell clone, in a dose-dependent manner (Fig. 5C), whereas no significant recognition was obtained using NY-ESO-1 protein used as an internal control, or using paraformaldehyde-fixed DCs pulsed with the Melan-A/MART-1 protein. Specific antigen presentation was also obtained at the different DCs:T cells ratio (Fig. 5D). These results show that the T-cell epitope recognized by Melan-A/MART-1-specific $CD4^+$ T cells from HD 106 is efficiently processed and presented by professional APCs. Recognition of tumor cell lines endogenously express Melan-A/MART-1 antigen by HLA-DPB1*0501-restricted $CD4^+$ T cells could not be assessed because of unavailability of appropriate tumor cell lines that could both express Melan-A/MART-1 and matched HLA-DP molecule.

DISCUSSION

In this study, we used a peptide pool of 28 to 30 amino acid long peptides spanning the entire Melan-A/MART-1 protein sequence and overlapping by 20 amino acids to stimulate enriched $CD4^+$ T cells from a group of 8 HDs with autologous irradiated $CD4^-$ cells. After 1 or 2 rounds of stimulation, the same pool of peptides was used for screening the cultures for the presence of Melan-A/MART- 1-specific $CD4^+$ T cells by intracellular staining with IFN-γ and CD4-specific mAbs. We could detect IFN-γ^+ and $CD4^+$ T cells in 4 of the 8 HDs, thus indicating that about half of HDs mount such T-cell responses.

$CD4^+$ T cells producing IFN-γ in response to peptide pool stimulation were isolated using a cytokine secretion-based magnetic cell sorting and were cloned under limiting-dilution conditions by mitogen. From the growing $CD4^+$ T-cell clones, we identified a new Melan-A/MART-1- derived immunodominant epitope recognized in the context of HLA-DPB1*0501 molecules by a $CD4^+$ T-cell clone. Until recently, HLA-DP-restricted epitope has been poorly characterized. The HLA-DPB1*0501 allele is the most frequent (44.9% to 73.1%) allele at the DPB1 locus found in the Asian population.[27] As consistent with the prevalence of DPB1*0501 in Chinese populations, PBMCs from 12 of 20 HLA-unselected HDs were able to present the Melan-A/ MART-1 epitope to specific clonal $CD4^+$ T cells. The later molecular typing results showed that the 12 HDs, which had the ability to present Melan-A/MART-1_{21-50} to specific $CD4^+$ T-cell clone, all expressed the DPB1*0501 allele, whereas the remaining 8 HDs did not. In contrast with the high expressing frequency in the Chinese population, HLA-DPB1* 0501 allele is rare in whites (2.6% to 5.3%).[27] This big difference in the allele frequency among different ethnic groups may contribute to explain why HLA-

DPB1*0501 restricted epitopes have been reported less frequently in whites than in Asians. As approximately 44.9% to 73.1% of individuals express HLA-DPB1*0501,[27] the identification of this epitope significantly increase the percentage of melanoma patients that can benefit from anticancer immunotherapy.

The peptide recognized by the HLA-DPB1*0501 restricted $CD4^+$ clone is Melan-A/MART-1_{21-50}, encompassing the sequence of the previously defined HLA-A2- restricted (Melan-A/MART-1_{26-35} [18] Melan-A/MART-1_{32-40} [19]), HLA-B35-restricted (Melan-A/MART-1_{26-35} [21]), HLA-B45- restricted (Melan-A/MART-1_{24-33} [20]) $CD8^+$ T-cell epitopes, and HLA-DQ6-restricted (Melan-A/MART-1_{25-36} [23]), HLADR1- restricted (Melan-A/MART-1_{27-40} [23]), HLA-DR3- restricted (Melan-A/MART-1_{25-36} [23]) $CD4^+$ T-cell epitopes. Therefore, Melan-A/MART-1_{21-50} seemed to be an immunodominant epitope in a relatively short amino acid stretch for both $CD8^+$ and $CD4^+$ T cells, which suggests the presence of a potential “hotspot” that can bind to multiple HLA alleles for T-cell recognition in this region of theMelan- A/MART-1 protein. This situation is reminiscent of previous findings with other tumor-associated antigens, such as SSX- 2[30-33] and NY-ESO-1,[34-37] in which immunodominant hotspots also be observed. Immunological hotspots of T-cell epitopes have also been previously described in viral antigens, such as human immunodeficiency virus-1 proteins[38] and severe acute respiratory syndrome coronavirus nucleocapsid. [39] These clusters of T-cell epitopes that bind to multiple alleles of MHC molecules are suitable targets for epitope-based vaccines and cancer immunotherapy because they are relevant to most of the human population and can elicit activation of both $CD4^+$ and $CD8^+$ T cells.[40]

Specific $CD4^+$ T cells can recognize the epitope when recombinant Melan-A/MART-1 protein or lysates of Melan-A/MART-1-expressing tumor cell is processed and presented by autologous DCs. These results show that the T-cell epitope recognized by Melan-A/MART-1-specific $CD4^+$ T cells from HD 106 is efficiently processed and presented by professional APCs through the exogenous pathway. Recognition of tumor cell lines endogenously express Melan-A/MART-1 antigen by HLA-DPB1*0501- restricted $CD4^+$ T cells could not be assessed because of unavailability of appropriate tumor cell lines that could both express Melan-A/MART-1 and matched HLA-DP molecule.

In conclusion, the identification of new epitopes in this study may significantly expand the spectrum of frequently expressed Melan-A/MART-1-derived sequences recognized by specific $CD4^+$ T cells and will be highly instrumental for the evaluation of Melan-A/MART-1-specific $CD4^+$ T-cell responses in cancer patients.

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References

1. Homsi J, Grimm JC, Hwu P. Immunotherapy of melanoma: an update. Surg Oncol Clin N Am. 2011; 20:145–163. [PubMed: 21111964]
2. Muranski P, Restifo NP. Adoptive immunotherapy of cancer using CD4(+) T cells. Curr Opin Immunol. 2009; 21:200–208. [PubMed: 19285848]

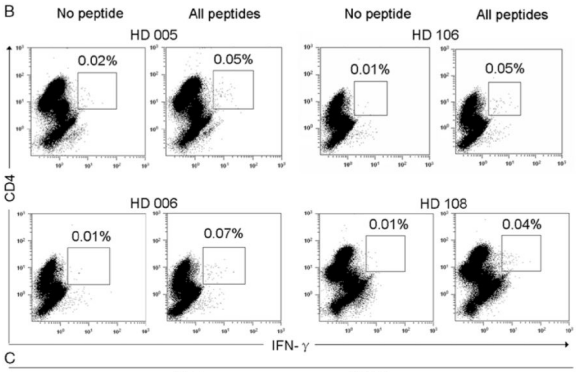
3. Quezada SA, Simpson TR, Peggs KS, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med. 2010; 207:637–650. [PubMed: 20156971]
4. Wang RF. The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. Trends Immunol. 2001; 22:269–276. [PubMed: 11323286]
5. Haigh TA, Lin X, Jia H, et al. EBV latent membrane proteins (LMPs) 1 and 2 as immunotherapeutic targets: LMP-specific CD4+ cytotoxic T cell recognition of EBV-transformed B cell lines. J Immunol. 2008; 180:1643–1654. [PubMed: 18209060]
6. Guo Y, Niiya H, Azuma T, et al. Direct recognition and lysis of leukemia cells by WT1-specific CD4+ T lymphocytes in an HLA class II-restricted manner. Blood. 2005; 106:1415–1418. [PubMed: 15845894]
7. Livingstone AM, Wilson EB, Ontiveros F, et al. Unravelling the mechanisms of help for CD8+ T cell responses. Immunol Res. 2009
8. Zou W, Restifo NP. T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol. 2010; 10:248–256. [PubMed: 20336152]
9. Canderan G, Dellabona P. T helper 17 T cells do good for cancer immunotherapy. Immunotherapy. 2010; 2:21–24. [PubMed: 20635888]
10. Kmieciak M, Worschech A, Nikizad H, et al. CD4+ T cells inhibit the neu-specific CD8+ T-cell exhaustion during the priming phase of immune responses against breast cancer. Breast Cancer Res Treat. 2011; 126:385–394. [PubMed: 20480224]
11. Kennedy R, Celis E. T helper lymphocytes rescue CTL from activation-induced cell death. J Immunol. 2006; 177:2862–2872. [PubMed: 16920921]
12. Rocha B, Tanchot C. Towards a cellular definition of CD8+ T-cell memory: the role of CD4+ T-cell help in CD8+ T-cell responses. Curr Opin Immunol. 2004; 16:259–263. [PubMed: 15134772]
13. Kennedy R, Undale AH, Kieper WC, et al. Direct cross-priming by th lymphocytes generates memory cytotoxic T cell responses. J Immunol. 2005; 174:3967–3977. [PubMed: 15778353]
14. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med. 2008; 358:2698–2703. [PubMed: 18565862]
15. Xie Y, Akpinarli A, Maris C, et al. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. J Exp Med. 2010; 207:651–667. [PubMed: 20156973]
16. Hofbauer GF, Kamarashev J, Geertsen R, et al. Melan A/MART-1 immunoreactivity in formalin-fixed paraffin-embedded primary and metastatic melanoma: frequency and distribution. Melanoma Res. 1998; 8:337–343. [PubMed: 9764809]
17. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J ExpMed. 1994; 180:35–42.
18. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2- restricted tumor infiltrating lymphocytes. J Exp Med. 1994; 180:347–352. [PubMed: 7516411]
19. Castelli C, Storkus WJ, Maeurer MJ, et al. Mass spectrometric identification of a naturally processed melanoma peptide recognized by CD8+ cytotoxic T lymphocytes. J Exp Med. 1995; 181:363–368. [PubMed: 7807017]
20. Schneider J, Brichard V, Boon T, et al. Overlapping peptides of melanocyte differentiation antigen Melan-A/MART-1 recognized by autologous cytolytic T lymphocytes in association with HLA-B45.1 and HLA-A2.1. Int J Cancer. 1998; 75:451–458. [PubMed: 9455808]
21. Benlalam H, Linard B, Guilloux Y, et al. Identification of five new HLA-B*3501-restricted epitopes derived from common melanoma-associated antigens, spontaneously recognized by tumor-infiltrating lymphocytes. J Immunol. 2003; 171:6283– 6289. [PubMed: 14634146]
22. Larrieu P, Renaud V, Godet Y, et al. A HLA-Cw*0701 restricted Melan-A/MART1 epitope presented by melanoma tumor cells to CD8+ tumor infiltrating lymphocytes. Cancer Immunol Immunother. 2008; 57:745–752. [PubMed: 18097665]
23. Bioley G, Jandus C, Tuyaerts S, et al. Melan-A/MART-1- specific CD4 T cells in melanoma patients: identification of new epitopes and ex vivo visualization of specific T cells by MHC class II tetramers. J Immunol. 2006; 177:6769–6779. [PubMed: 17082590]

24. Depontieu FR, Qian J, Zarling AL, et al. Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. Proc Natl Acad Sci USA. 2009; 106:12073–12078. [PubMed: 19581576]
25. Zarour HM, Kirkwood JM, Kierstead LS, et al. Melan-A/ MART-1(51-73) represents an immunogenic HLA-DR4-restricted epitope recognized by melanoma-reactive CD4(+) T cells. Proc Natl Acad Sci USA. 2000; 97:400–405. [PubMed: 10618430]
26. Godefroy E, Scotto L, Souleimanian NE, et al. Identification of two Melan-A CD4+ T cell epitopes presented by frequently expressed MHC class II alleles. Clin Immunol. 2006; 121:54–62. [PubMed: 16814609]
27. Yamasaki K, Horiuchi I, Minohara M, et al. HLA-DPB1* 0501-associated opticospinal multiple sclerosis: clinical, neuroimaging and immunogenetic studies. Brain. 1999; 122(Pt 9):1689–1696. [PubMed: 10468508]
28. Godefroy E, Wang Y, Souleimanian NE, et al. Assessment of CD4+ T cells specific for the tumor antigen SSX-1 in cancer-free individuals. Cancer Immunol Immunother. 2007; 56:1183–1192. [PubMed: 17186289]
29. Chen YT, Stockert E, Jungbluth A, et al. Serological analysis of Melan-A(MART-1), a melanocyte-specific protein homogeneously expressed in human melanomas. Proc Natl Acad Sci USA. 1996; 93:5915–5919. [PubMed: 8650193]
30. Ayyoub M, Stevanovic S, Sahin U, et al. Proteasome-assisted identification of a SSX-2-derived epitope recognized by tumor-reactive CTL infiltrating metastatic melanoma. J Immunol. 2002; 168:1717–1722. [PubMed: 11823502]
31. Ayyoub M, Hesdorffer CS, Montes M, et al. An immunodominant SSX-2-derived epitope recognized by CD4+ T cells in association with HLA-DR. J Clin Invest. 2004; 113:1225–1233. [PubMed: 15085202]
32. Neumann F, Wagner C, Stevanovic S, et al. Identification of an HLA-DR-restricted peptide epitope with a promiscuous binding pattern derived from the cancer testis antigen HOMMEL- 40/ SSX2. Int J Cancer. 2004; 112:661–668. [PubMed: 15382048]
33. Ayyoub M, Merlo A, Hesdorffer CS, et al. Distinct but overlapping T helper epitopes in the 37–58 region of SSX-2. Clin Immunol. 2005; 114:70–78. [PubMed: 15596411]
34. Jager E, Chen YT, Drijfhout JW, et al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med. 1998; 187:265–270. [PubMed: 9432985]
35. Chen JL, Dunbar PR, Gileadi U, et al. Identification of NY-ESO- 1 peptide analogues capable of improved stimulation of tumor-reactive CTL. J Immunol. 2000; 165:948–955. [PubMed: 10878370]
36. Valmori D, Dutoit V, Lienard D, et al. Naturally occurring human lymphocyte antigen-A2 restricted CD8+ T-cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. Cancer Res. 2000; 60:4499–4506. [PubMed: 10969798]
37. Zeng G, Wang X, Robbins PF, et al. CD4(+) T cell recognition of MHC class II-restricted epitopes from NYESO- 1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. Proc Natl Acad Sci USA. 2001; 98:3964–3969. [PubMed: 11259659]
38. Brown SA, Stambas J, Zhan X, et al. Clustering of Th cell epitopes on exposed regions of HIV envelope despite defects in antibody activity. J Immunol. 2003; 171:4140–4148. [PubMed: 14530336]
39. Gupta V, Tabiin TM, Sun K, et al. SARS coronavirus nucleocapsid immunodominant T-cell epitope cluster is common to both exogenous recombinant and endogenous DNA-encoded immunogens. Virology. 2006; 347:127–139. [PubMed: 16387339]
40. Zhang GL, Khan AM, Srinivasan KN, et al. Hotspot Hunter: a computational system for large-scale screening and selection of candidate immunological hotspots in pathogen proteomes. BMC Bioinformatics. 2008; 9(Suppl 1):S19. [PubMed: 18315850]

A

Melan-A/MART-1	1-30	MPREDAHFIY	GYPKKGHGHS	YTTAEEAAGI
	11-40	GYPKKGHGHS	YTTAEEAAGI	GILTVILGVL
	21-50	YTTAEEAAGI	GILTVILGVL	LLIGCWYCRR
	31-60	GILTVILGVL	LLIGCWYCRR	RNGYRALMDK
	41-70	LLIGCWYCRR	RNGYRALMDK	SLHVGTQCAL
	51-80	RNGYRALMDK	SLHVGTQCAL	TRRCPQEGFD
	61-90	SLHVGTQCAL	TRRCPQEGFD	HRDSKVSLQE
	71-100	TRRCPQEGFD	HRDSKVSLQE	KNCEPVVPNA
	81-110	HRDSKVSLQE	KNCEPVVPNA	PPAYEKLSAE
	91-118	KNCEPVVPNA	PPAYEKLSAE	QSPPPYSP

B



C

	IVS 1		IVS 2		
Donor	-Peptides	+Peptides	-Peptides	+Peptides	Fold increase
HD 005	0.02	0.05	ND	ND	**2.5**
HD 006	0.04	0.04	0.01	0.07	**7**
HD 101	<0.01	<0.01	0.02	0.03	1.5
HD 102	<0.01	<0.01	<0.01	<0.01	0
HD 106	0.01	0.05	ND	ND	**5**
HD 108	<0.01	<0.01	0.01	0.04	**4**
HD 109	<0.01	0.01	0.02	0.01	0
HD 110	0.02	0.01	0.03	0.02	0

FIGURE 1.

Assessment of Melan-A/MART-1-specific $CD4^+$ T-cell responses in circulating lymphocytes from healthy donors. A, Amino acid sequences of the 28 to 30 amino acid long and 20 amino acids overlapping peptides encompassing the complete sequence of Melan-A/MART-1 protein were used to stimulate $CD4^+$ T cells. B, $CD4^+$ T cells from 8 healthy donors were stimulated with a peptide pool containing the 10 peptides described in A. The presence of Melan-A/MART-1-specific $CD4^+$ T cells was assessed by staining with anti-CD4 mAb and intracellular staining with anti-IFN-γ mAb after incubation in the absence or in the presence of the same pool of peptides. Numbers in the upper right quadrants are the percentage of cytokine-producing cells among $CD4^+$ T cells. C, Results obtained of 8 healthy donors assessed as in B. $CD4^+$ T cells from healthy donors were stimulated once (in vitro stimulation cycle 1) or twice (in vitro stimulation cycle 2) with a peptide pool containing the 10 peptides described in A. Cultures were then assessed functionally in the absence (–peptides) or in the presence (+peptides) of the Melan-A/MART-1 peptide pool. Values at least 2-fold higher than baseline (–peptides) were considered significant and are shown in bold. IVS indicates in vitro stimulation; ND, not done.

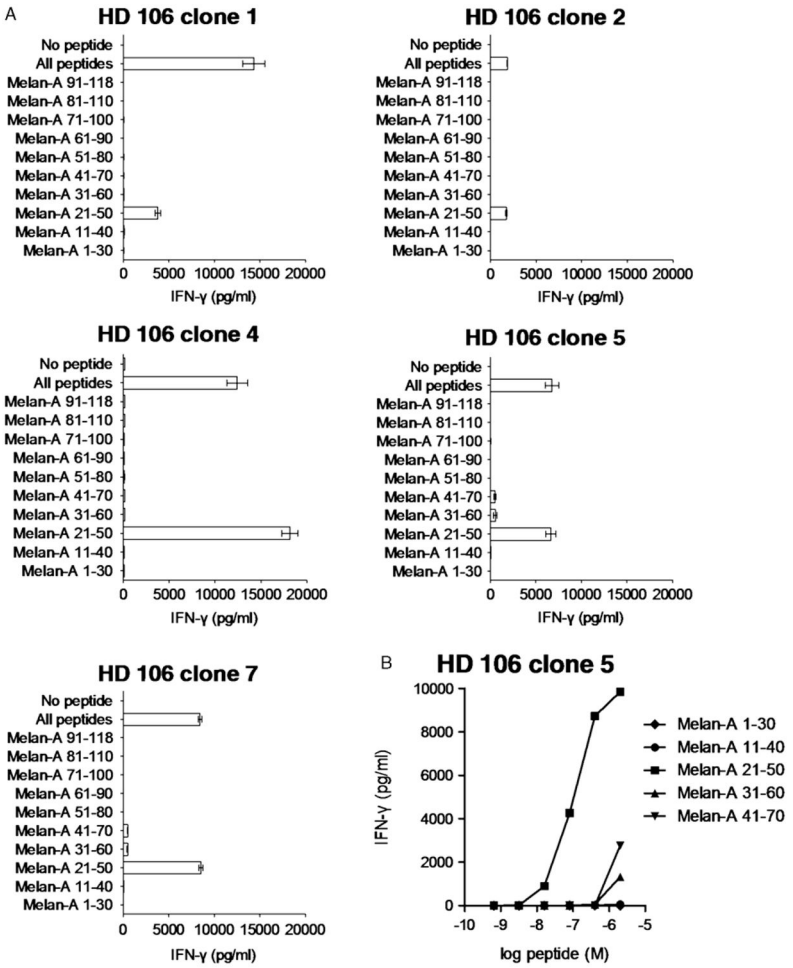


FIGURE 2.

Fine specificity of antigen recognition and MHC restriction of Melan-A/MART-1 specific $CD4^+$ T-cell clones. A, Peptide recognition by 5 Melan-A/MART-1 specific $CD4^+$ T-cell clones from one healthy donor was assessed by stimulating culture aliquots with single Melan-A/MART-1 peptide (2 μM). B, Determination of peptide recognition of $CD4^+$ T-cell clone 5 by peptide titration. Antigen recognition of healthy donor 106 clone 5 was assessed for the active peptide and for neighboring overlapping peptides. IFN-γ secretion in the culture supernatant was measured by ELISA 24 hours after stimulation. Data were shown as mean of triplicate values. Each graph is representative of at least 3 independent experiments giving comparable results. ELISA indicates enzyme-linked immunosorbent assay; IFN, interferon; MHC, major histocompatibility complex.

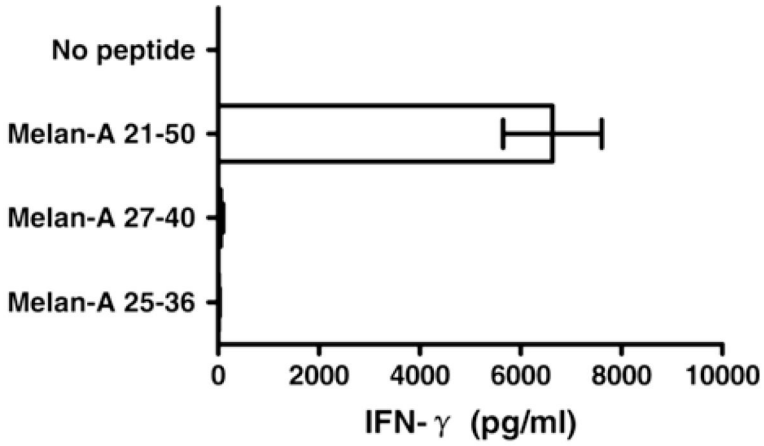
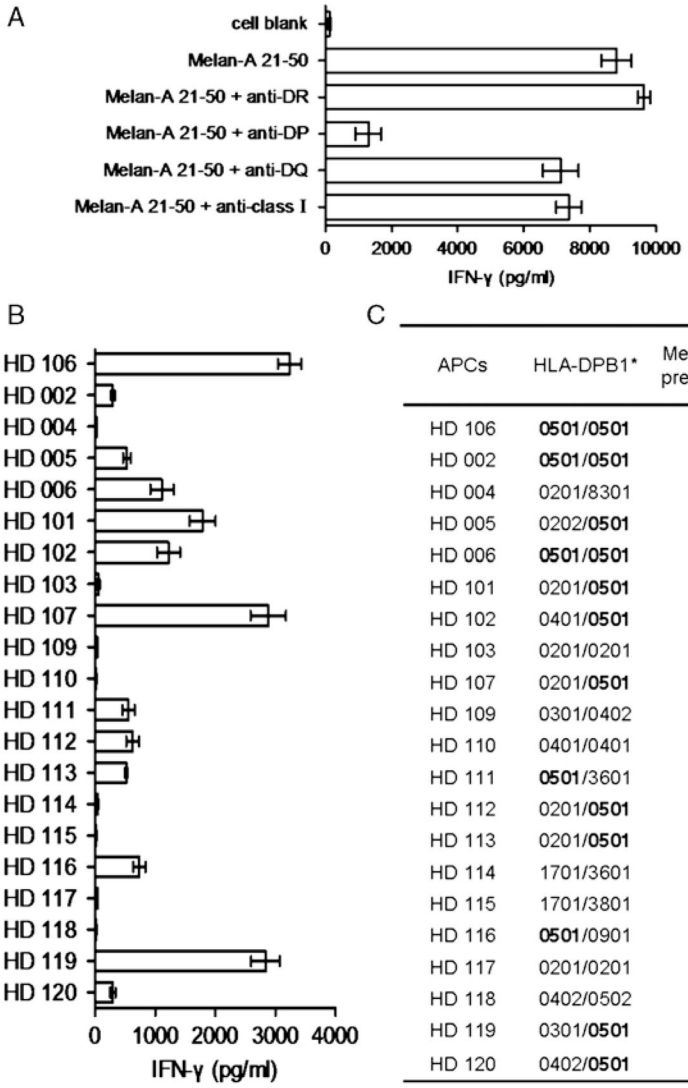


FIGURE 3.

Recognition of 2 already known HLA class II epitopes by Melan-A/MART-1 specific $CD4^+$ T-cell clone. Peptide recognition by Melan-A/MART-1 specific $CD4^+$ T-cell clone 5 from healthy donor 106 was assessed by stimulating culture aliquots with synthetic peptides (2 μM). IFN-γ secretion in the culture supernatant was measured by ELISA 24 hours after stimulation. Data were shown as mean of triplicate values. Each graph is representative of at least 3 independent experiments giving comparable results. ELISA indicates enzyme-linked immunosorbent assay; IFN, interferon; HLA, human leukocyte antigen.



C

APCs	HLA-DPB1*	Melan-A$_{21-50}$ presentation
HD 106	**0501**/**0501**	+
HD 002	**0501**/**0501**	+
HD 004	0201/8301	-
HD 005	0202/**0501**	+
HD 006	**0501**/**0501**	+
HD 101	0201/**0501**	+
HD 102	0401/**0501**	+
HD 103	0201/0201	-
HD 107	0201/**0501**	+
HD 109	0301/0402	-
HD 110	0401/0401	-
HD 111	**0501**/3601	+
HD 112	0201/**0501**	+
HD 113	0201/**0501**	+
HD 114	1701/3601	-
HD 115	1701/3801	-
HD 116	**0501**/0901	+
HD 117	0201/0201	-
HD 118	0402/0502	-
HD 119	0301/**0501**	+
HD 120	0402/**0501**	+

FIGURE 4.

Assessment of MHC class II restriction and identification of the restricting allele of the Melan-A/MART-1-specific CD4$^+$ T-cell clone. A, To determine the MHC restricting element, peptide recognition was assessed either in the presence or in the absence of anti-HLA-I, DR, DP, or DQ antibodies. IFN-γ secretion in the culture supernatant was measured by ELISA 24 hours after stimulation. Data are shown as mean of triplicate values. Each graph is a representative of at least 3 independent experiments yielding comparable results. B and C, The ability of unrelated healthy donor bearing different HLA-DP alleles to present peptide Melan-A/MART-1$_{21-50}$ to specific CD4$^+$ T cell clone was assessed by intracellular IFN-γ secretion. ELISA indicates enzyme-linked immunosorbent assay; IFN, interferon; HLA, human leukocyte antigen; MHC, major histocompatibility complex.

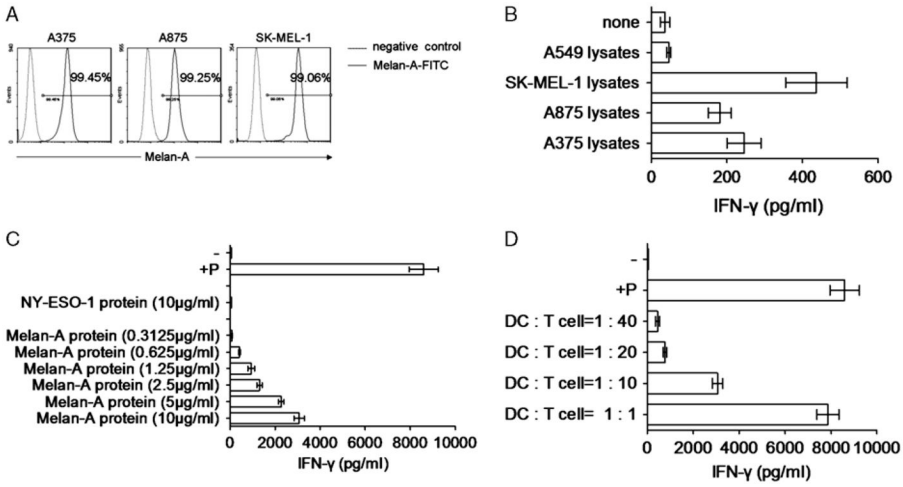


FIGURE 5.

The peptide epitope recognized by clone 5 is a naturally processed HLA-DPB1*0501 restricted epitope. A, Expression of Melan-A/MART-1 protein in melanoma cell lines was assessed using Melan-A/MART-1-specific mAb by flow cytometry. B, Melan-A/ MART-1-specific $CD4^+$ T cells efficiently recognized the Melan-A/MART-1 native antigen that is present in the tumor lysates from 3 tumor cell lines upon processing and presentation by professional APCs. The processing and presentation of Melan-A/MART-1 antigen by DCs after incubation with lysates from tumor cell lines A375, A875, SK-MEL-1 (Melan-A/ MART-1-expressing), or A549 (Melan-A/ MART-1-negative) was assessed by ELISA measurement of IFN-γ secretion in the culture supernatant. DCs:T cells = 1:10. C, Processing and presentation of Melan-A/MART-1 antigen by monocyte derived DCs after incubation with soluble recombinant Melan-A/MART-1 protein at the indicated dose (or NY-ESO-1 protein as a negative control) was assessed after 24 hours of incubation by ELISA measurement of IFN-γ secretion in the culture supernatant. DCs:T cells = 1:10. D, Processing and presentation of Melan-A/MART-1 antigen by monocyte derived DCs after incubation with soluble recombinant Melan-A/MART-1 protein at the 10 μg/mL at the indicated DCs:T cells ratio was assessed after 24 hours of incubation by ELISA measurement of IFN-γ secretion in the culture supernatant. APCs indicates antigen presenting cell; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; HLA, human leukocyte antigen.