Processing of Tumor-Associated Antigen by the Proteasomes of Dendritic Cells Controls In vivo T-Cell Responses

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Abstract

Dendritic cells are unique in their capacity to process antigens and prime naive CD8+ T cells. Contrary to most cells, which express the standard proteasomes, dendritic cells express immunoproteasomes constitutively. The melanoma-associated protein Melan-AMART1 contains an HLA-A2-restricted peptide that is poorly processed by melanoma cells expressing immunoproteasomes in vitro. Here, we show that the expression of Melan-A in dendritic cells fails to elicit T-cell responses in vitro and in vivo because it is not processed by the proteasomes of dendritic cells. In contrast, dendritic cells lacking immunoproteasomes induce strong anti-Melan-A T-cell responses in vitro and in vivo. These results suggest that the inefficient processing of self-antigens, such as Melan-A, by the immunoproteasomes of professional antigen-presenting cells prevents the induction of antitumor T-cell responses in vivo. (Cancer Res 2006; 66(10): 5461-8)

Introduction

The specificity of a CTL response is determined by the peptide-MHC complexes present at the surface of antigen-presenting cells. MHC class I-restricted peptides are produced by the proteasome, a multicatalytic protease complex that degrades proteins into peptides ranging from 3 to >20 amino acids in length. The eukaryotic 20 S proteasome particles are composed of 14 different α and β subunits, arranged in four heptameric rings α7β7γ7α7 (1, 2). Three subunits of each β ring (β1, β2, and β5) possess proteolytic activities. In mammalian cells, these three subunits are constitutively expressed and incorporated into the so-called standard proteasomes. However, treatment of cells with IFN-γ leads to the expression and incorporation of three different catalytic subunits, denoted βi (LMP2), β2i (MECL-1), and β5i (LMP7), resulting in the formation of the so-called immunoproteasomes (3). Although immunoproteasomes degrade proteins as efficiently as standard proteasomes (4), subtle differences in the cleavage specificities of the two types of proteasome have been described (5). Because proteasomes produce most MHC class I antigenic peptides, the distinct proteolytic specificities of the two types of proteasomes have a direct effect on the presentation of many CTL epitopes (6–10). In mice, CTL responses against several epitopes were shown to be differentially affected by the presence of LMP2 or LMP7 (7, 11, 12).

CD8+ T-cell responses are initiated by professional antigen-presenting cells, in particular dendritic cells, which stimulate the proliferation of naive antigen-specific CD8+ T cells by presenting MHC class I-restricted peptides and delivering costimulatory signals. Infection, inflammation, or tissue lesion leads to maturation and migration of dendritic cells from peripheral tissues to secondary lymphoid organs, where activation of CD8+ T cells occurs (13). Dendritic cells are endowed with the capacity of presenting MHC class I-restricted peptides that are derived from proteins synthesized by the dendritic cells themselves or by other cells or microorganisms. Activation of T cells against peptides of the first category is called “direct priming” and against those of the second category is called “cross-priming.” In both cases, the proteasomes of dendritic cells play an essential role in the production of peptides (10, 14). Interestingly, dendritic cells have been shown to express immunoproteasomes constitutively (10, 15–17), together with standard proteasome. However, the effect of the constitutive expression of both types of proteasome by dendritic cells on the selection of tumor-derived CTL epitopes in vivo has not been studied. Thus, it is unknown if the efficacy by which particular peptides are produced by the proteasomes of dendritic cells will affect the magnitude of the T-cell responses.

We have addressed these questions by analyzing the anti-MelanA26-35/HLA-A2 T-cell response in HLA-A2/H-2Kb (A2Kb) transgenic mice (10, 18). This epitope constitutes a particularly attractive target for melanoma immunotherapy for two reasons: first, it has been shown to be immunodominant in HLA-A2 individuals (19). Second, adoptive transfer of Melan-A26-35-specific T cells has been shown to lead to significant tumor regressions in melanoma patients, and immunizations of patients with Melan-A26-35 peptides elicit vigorous T-cell responses (20, 21). Melan-A26-35 is derived from the melanoma-associated antigen Melan-A/MLT1, which is constitutively expressed in melanocytes and melanomas. Melan-A26-35 is efficiently processed by standard proteasomes of melanoma cells (22). However, melanoma cells expressing immunoproteasomes are not recognized by anti-Melan-A CTLs (10). Here, we show that the absence of anti-Melan-A T-cell responses in vivo can be directly linked to the presence of immunoproteasomes in dendritic cells. Indeed, dendritic cells do not process and present Melan-A26-35 are not recognized by specific CTLs in vitro, and do not elicit detectable T-cell responses in vivo, in A2Kb LMP2−/− mice. In contrast, dendritic cells derived from A2Kb LMP2−/− mice, which lack bona fide immunoproteasomes, process Melan-A26-35 are recognized by specific CTLs in vitro, and induce strong T-cell responses in vivo. These results have broad implications for the design of therapeutic vaccines.
implications for antitumor immunotherapy and might explain why spontaneous anti-Melan-A T-cell responses are rare in humans despite high levels of circulating anti-Melan-A T cells (23).

**Materials and Methods**

Human dendritic cells, IFN-γ release assay, and ELISA. Human monocyte–derived dendritic cells were generated by culturing CD14+ cells isolated from circulating lymphocytes of healthy donor using magnetic cell sorting in medium containing granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN; 1,000 IU/mL) and interleukin-4 (IL-4; R&D Systems; 1,000 IU/mL) during 5 days. At this time, dendritic cells and tumor cell lines were electroporated with plasmids encoding or not tumor antigens or with a green fluorescent protein (GFP)–encoding plasmid as an internal control, using Human Dendritic Cell Nucleofector, according to the manufacturer's instructions. After electroporation, cells were cultured for an additional day before being used as stimulators in IFN-γ secretion assays. At this point, 30% to 40% of the cells in the control expressed GFP, as assessed by flow cytometry. Melan-Ag3-35- or SSX-241-49-specific CTL clones (refs. 24, 25; 15) were obtained by transducing the above-mentioned cells with recombinant lentiviruses. The recombinant viruses were modified Dulbecco's medium containing 25 IU/mL recombinant human IFN-α. Twenty-four hours after addition, cells were electroporated with cychrome 5.5-conjugated anti-CD8 (Becton Dickinson). The pellet, which was enriched in proteasomes, was resuspended in 90 mmol/L glycerol and resuspended in 90 mmol/L glycerol before the addition of CTLs at an effector-to-target (E/T) ratio of 3:1 in DMEM containing 10% FCS, PSN/HEPES, 5% IL-2, and 2% 2-mercaptoethanol. Twenty-four hours later, supernatants were harvested, and the concentration of IFN-γ in the supernatant was measured by ELISA as described previously (26).

**Mice.** Immunizations were done using A2Kb (27) or A2Kb LMP2−/− mice. A2Kb LMP2−/− mice were produced by crossing A2Kb mice with LMP2−/− mice (kindly provided by L. Van Kaae). Expression of A2Kb was assessed by flow cytometry (see below). Deletion of LMP2 was tested by reverse transcription-PCR, using genomic tail DNA as template and two different couples of primers. LMP2−/− mice are characterized by the absence of a band of 580 bp using the following primers: LMP2 forward, 5'-GGGATCC-AGGACCAGGAAAG-3' and LMP2 reverse, 5'-CCCGTGGTCCCTCGGATAC-3' and the presence of a band of 1.3 kb using Neo-LMP2 forward, 5'-GTTCCTCTGCTTACGG-3' and Neo-LMP2 reverse, 5'-CAGAGGGGAGGCTTCG-3'. Animal experiments were conducted in pathogen-free facilities and in compliance with the rules of the local veterinary office.

**Construction of lentivectors.** All melan-A sequences, except for Fig. 1, contained the Aα to Leu substitution at position 27 to increase the affinity of the peptide for HLA-A2 (28). Recombinant lentivectors (rev. iOS) were generated by inserting the DNA sequence coding for EGFP-ubiquitin-Melan-Ag3-35 or Melan-A between the BamHI and XbaI sites of the lentiviral transfer vector pBRLsin18PPT.CMV. The production of recombinant lentiviruses has been described previously (29–31). The recombinant viruses expressing the VP6 technology described earlier (22, 32).

**Flow cytometry.** Flow cytometry was done on a FACSCalibur using CellQuest software (Becton Dickinson, Sunnyside, CA). Expression of A2Kb was monitored by staining peripheral blood mononuclear cells (PBMC) with the anti-HLA antibody B9.12.1 (produced in our laboratory). PBMCs obtained by tail bleedings were incubated with phycoerythrin-conjugated Melan-Ag3-35/A2Kb tetramers (prepared in our laboratory) for 40 minutes at 20°C and washed before incubation with cyclohexane-conjugated anti-CD8 monoclonal antibody (mAb) 53-6-7 (BioScience, Indianapolis, IN), and FITC-conjugated anti-CD62L antibodies (Mel-14, produced in our laboratory) for 20 minutes at 4°C. Other antibodies used were phycoerythrin-cytochrome 5.5-conjugated anti-CD8α (SH101; Caltag Laboratories, Burlingame, CA) and anti-CD4 (GK1.5, produced at our institute). Erythrocytes were lysed using the Fluorescence-Activated Cell Sorting Lysing solution (Becton Dickinson).

**Dendritic cells and transduction.** Mouse bone marrow–derived dendritic cells (BMDC) were generated in medium containing GM-CSF (Prepotech, Rock Hill, NJ) and IL-4 (Prepotech) according to standard procedures (33). Briefly, bone marrow was collected from tibiae and femurs of either A2Kb or A2Kb LMP2−/− mice, passed though a nylon mesh, and resuspended in complete DMEM (Life Technologies, Gaithersburg, MD). After a panning step to remove macrophages, the cells were cultured in the presence of 200 units/mL GM-CSF (Prepotech, Juro). Twenty-four hours later, nonadherent cells were discarded. On day 5, medium-containing GM-CSF was supplemented with IL-4. At this time, transductions were carried out by adding concentrated virus stocks at an expression forming unit (EFU) of 2 or 5. At those EFU, between 3% and 6% of the dendritic cells were transduced. Dendritic cells culture was continued in presence of virus until day 7, when cells were harvested, washed thrice, and used as targets for CTL assays or transfected into mice. Based on the enhanced GFP (EGFP) expression of transduced cells, the mean fluorescence was in average 10+ higher than nontransduced cells. The transduction efficiency was similar between the dendritic cells derived from A2Kb or A2Kb LMP2−/−, and the expression of EGFP was comparable between the dendritic cells transduced with rec. lv. Neogen-encoding plasmid as an internal control, using Human Dendritic Cell Nucleofector, according to the manufacturer's instructions. After electroporation, cells were cultured for an additional day before being used as stimulators in IFN-γ secretion assays. At this point, 30% to 40% of the cells in the control expressed GFP, as assessed by flow cytometry. Melan-Ag3-35 or SSX-241-49-specific CTL clones (refs. 24, 25; 15) were obtained by transducing the above-mentioned cells with recombinant lentiviruses. The recombinant viruses were modified Dulbecco's medium containing 25 IU/mL recombinant human IFN-α. Twenty-four hours after addition, cells were electroporated with cyanochrome 5.5-conjugated anti-CD8 (Becton Dickinson). The pellet, which was enriched in proteasomes, was resuspended in 90 mmol/L glycerol and resuspended in 90 mmol/L glycerol before the addition of CTLs at an effector-to-target (E/T) ratio of 3:1 in DMEM containing 10% FCS, PSN/HEPES, 5% IL-2, and 2% 2-mercaptoethanol. Twenty-four hours later, supernatants were harvested, and the concentration of IFN-γ in the supernatant was measured by ELISA as described previously (26).

**Western blot analysis.** Cells were lysed in lysis buffer containing 20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 5 mmol/L EDTA, and 1% NP40, in the presence of a cocktail of proteases inhibitors (Complete Mini, EDTA-free, Roche, Indianapolis, IN). The lysate was cleared by centrifugation at 11,000 × g for 15 minutes at 4°C. Lysates were separated by SDS-12% PAGE and subjected to immunoblotting. For BMDCs, lysates were obtained by adding 20 mmol/L HEPES buffer followed by three cycles of freezing and thawing. Lysates were cleared by centrifugation at 11,000 × g for 15 minutes at 4°C. The loaded lysates correspond to 2 × 106 cells. The membrane was revealed with rabbit polyclonal antibodies anti-LMP2 (1:1,000; Biomol International, Plymouth Meeting, PA) and anti-MELC-1 (1:1,000; Biomol International), mouse mAb anti-LMP7 (1:1,000; Biomol International) and anti-tet (1:1,000; Biomol International), and horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibody (1:2,500; Amersham Biosciences, Uppsala, Sweden).

All immunoblots were revealed using ECL detection kit (Amersham Biosciences, Uppsala, Sweden) or supersignal west femto (Pierce, Rockford, IL).

**Proteasome purification.** Homogeneous cell suspensions from spleen of A2Kb and A2Kb LMP2−/− mice were prepared using nylon cell strainer (BD Biosciences, Bedford, MA). Cells were counted and resuspended in 20 mmol/L HEPES at a concentration of 6 × 107 per mL and lysed by three cycles of freezing and thawing. Thereafter, sucrose was added to a final concentration of 250 mmol/L. The lysate was cleared by centrifugation at 11,000 × g for 15 minutes 4°C. The cleared supernatant was then centrifuged at 100,000 × g for 5 hours 4°C, as reported previously (11). The pellet, which was enriched in proteasomes, was resuspended in 20 mmol/L Tris-HCl (pH 7.6), 2 mol/L NaCl so as to correspond to the equivalent of ~3 × 1010 cells per mL and dialyzed overnight against 20 mmol/L Tris-HCl (pH 7.6). The solution was then centrifuged at 11,000 × g for 15 minutes 4°C to remove aggregates.

**Fluorogenic assay.** The proteasome-enriched fraction and unfractionated cytosol were diluted in 90 mL of 20 mmol/L Tris-HCl (pH 7.6) in the presence or absence of 50 mmol/L lactacystin (Biomol International) for 15 minutes at room temperature in 96-well plates (Nalge Nunc International, Naperville, IL). The fluorogenic peptides Bz-VGR-AMC (Bachem, Bubendorf, Switzerland), Z-LLL-AMC (Bachem), and Suc-LLVY-AMC (Calbiochem, La Jolla, CA) were added on the proteasomes at a final concentration of 100 μmol/L and incubated at 37°C in the dark. The fluorescence emitted by the released fluorogenic group AMC was measured after 4 hours by spectrofluorimetry (excitation/emission = 380/460 nm).
Peptide digestion by proteasome, high-performance liquid chromatography separation, and mass spectrometry. Purified synthetic peptides were quantitated by amino acid analysis (620 pmol of peptide Melan-A26-35) and digested in 25 μl proteasome purified from spleen of A2Kb mice or A2Kb LMP2−/−, which correspond to 7.5 × 10⁶ cell equivalents. For each condition, an aliquot was removed at time 0 and served as reference for quantitative analysis. Digestions were allowed to proceed for 4 hours at 37°C. At each point in time, the reaction was stopped with 1% trifluoroacetic acid. The digests were diluted with acetic acid/water (50:50, v/v) to a final volume of 110 μl, and 100 μl were injected on the high-performance liquid chromatograph (HPLC). HPLC separations were done as previously described (22). Fractions were collected and subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Results

Human dendritic cells do not present Melan-A26-35 and fail to stimulate specific CTLs. In melanoma cells expressing immunoproteasomes, the presentation of Melan-A26-35 by HLA-A2 was previously shown to be impaired (10). Because of the inefficient processing of Melan-A26-35 by human dendritic cells, we tested if the processing and presentation of Melan-A26-35/HLA-A2 by murine dendritic cells was also affected by the type of proteasome expressed in those cells. BMDCs were isolated from HLA-A2/H-2Kb (A2Kb) transgenic mice. Isolated BMDCs were transduced with recombinant lentivectors (rec. lv) coding for the preprocessed peptide Melan-A26-35 (rec. lv/Egfp; Melan-A26-35). Similar levels of Egfp were detected, indicating that the expression of proteasome LMP2 subunit. Because of the inefficient processing of Melan-A26-35 by human dendritic cells, we tested if the processing and presentation of Melan-A26-35/HLA-A2 by murine dendritic cells was also affected by the type of proteasome expressed in those cells. BMDCs were isolated from HLA-A2/H-2Kb (A2Kb) transgenic mice. Isolated BMDCs were transduced with recombinant lentivectors (rec. lv) coding for the preprocessed peptide Melan-A26-35 (rec. lv/Melan-A26-35), the full-length protein Melan-A (rec. lv/Melan-A), or, as a control, Egfp (rec. lv/Egfp; Fig. 2A). The advantages of rec. lv have been described earlier (30, 37). In those constructs, the Melan-A sequence contained an Ala to Leu substitution at position 27, which was shown to increase affinity of the antigenic peptide to HLA-A2 (28). Moreover, Melan-A was produced as linear fusion to Egfp-ubiquitin (Fig. 2A). The characteristics and advantages of the Egfp-ubiquitin fusion system have been explained elsewhere (22, 32, 38). BMDCs were transduced with the different rec. lv at an EFU of 2 and incubated with a murine CTL line specific for Melan-A26-35/HLA-A2. Recognition was assessed by measuring the amount of IFN-γ released by the CTLs. BMDCs transduced with rec. lv/Melan-A26-35 were efficiently recognized by specific CTLs, as indicated by the elevated amount of secreted IFN-γ (Fig. 2B). In contrast, four to five times less IFN-γ was produced by CTLs incubated with BMDCs transduced with rec. lv/Melan-A. Similar level was obtained after incubation of CTLs with nontransduced dendritic cells. This result confirmed our findings with human dendritic cells (Fig. 1) and lead us to conclude that the processing machinery of murine BMDCs prevented the efficient production of Melan-A26-35.
To unambiguously identify the contribution of immunoproteasome on this process, we crossed A2Kb mice with LMP2\(^{-/-}\) mice, which were shown to lack bona fide immunoproteasomes (11). As expected, expression of LMP2 was abolished in BMDCs of these mice, whereas the expression of LMP7 remained unaffected (11). As expected, expression of LMP2 was abolished in BMDCs of A2Kb mice. Spleens were shown to contain BMDCs expressing Melan-A, A2Kb LMP2\(^{-/-}\) mice were transduced either with rec. lv/Melan-A or rec. lv/Melan-A. Dendritic cells were incubated with melan-A-specific CTLs at an E:T ratio of 3:1, and the production of IFN-\(\gamma\) by the CTLs was measured by ELISA. As controls, nontransduced dendritic cells were loaded (+P) or not (-P) with saturating amounts of synthetic peptide Melan-A\(_{26-35}\). Columns, average of one of three independent experiments; bars, SD.

**Figure 2.** Recognition of dendritic cell (DC) A2Kb and dendritic cell A2Kb LMP2\(^{-/-}\) expressing Melan-A by specific CTLs. A, schematic representation of the rec. lv constructs used. Lentivectors coding for a linear fusion containing HA-tagged EGFP, ubiquitin, and Melan-A\(_{26-35}\) or Melan-A were generated. After rapid cleavage of EGFP-ubiquitin by specific ubiquitin proteases, these constructs produce equimolar amounts of two distinct polypeptides, EGFP-ubiquitin and Melan-A\(_{26-35}\) peptide or protein. The arrow indicates the cleavage site of the ubiquitin-specific protease. B, BMDCs derived from A2Kb or A2Kb LMP2\(^{-/-}\) mice were transduced either with rec. lv/Melan-A\(_{26-35}\) or rec. lv/Melan-A. Dendritic cells were incubated with melan-A-specific CTLs, and the production of IFN-\(\gamma\) by the CTLs was measured by ELISA. As controls, nontransduced dendritic cells were loaded (+P) or not (-P) with saturating amounts of synthetic peptide Melan-A\(_{26-35}\). Columns, average of one of three independent experiments; bars, SD.
or A2Kb LMP2−/− mice with rec. lv/Melan-A or, as positive control, rec. lv/Melan-A26-35. As previously shown (37), mice immunized with rec. lv/Melan-A26-35 mounted robust T-cell responses that were readily detectable ex vivo with A2Kb tetramers (tet) incorporating the Melan-A26-35 peptide (Fig. 4). An average of 12% to 15% activated (CD62Llow) CD8+ T-cell population showed similar results in vivo with A2Kb and A2Kb LMP2−/− mice. Administration of rec. lv/Melan-A to A2Kb mice elicited a much weaker T-cell response, which reached a maximum of 4% tet+ cells among total CD8+ T cells at the peak of the response. In stark contrast, immunization of A2Kb LMP2−/− mice with rec. lv/Melan-A elicited a specific T-cell response that was of similar magnitude as the one elicited by rec. lv/Melan-A26-35 (Fig. 4B). Tet staining among activated (CD62Llow) CD8+ T-cell population showed similar results (data not shown). Taken together, these results show that the absence of LMP2 favors the processing of Melan-A and leads to the induction of a better specific T-cell response.

**Adoptive transfer of LMP2−/− Melan-A+ dendritic cells but not LMP2+/+ Melan-A+ dendritic cells induces CD8+ T-cell response in vivo.** To formally show that the ineffective T-cell response elicited by immunization with rec. lv/Melan-A was due to the composition of the proteasomes of dendritic cells and to exclude the effect of potential differences in the T-cell repertoire of A2Kb and A2Kb LMP2−/− mice, we derived BMDCs from A2Kb and A2Kb LMP2−/− mice and transduced them either with rec. lv/Melan-A or rec. lv/Melan-A26-35 at an EFU of 5. Because these viruses are nonreplicative (42), they could not spread by infecting other cell types. As previously described, transduction of dendritic cells induced their maturation, evidenced by the increased cell surface expression of MHC class II, CD80 and CD86 molecules (30). Three days after transduction, 5 × 10^6 cells were adoptively transferred into A2Kb recipient mice, and the specific T-cell response was followed ex vivo over 20 days (Fig. 5A). At the peak of the response, an average of 2% to 2.5% tet+ cells among CD8+ T cells were detected after transfer of A2Kb and A2Kb LMP2−/− dendritic cells transduced with rec. lv/Melan-A26-35 into A2Kb mice (Fig. 5B). In contrast, significant differences were observed after adoptive transfer of A2Kb or A2Kb LMP2−/− dendritic cells transduced with rec. lv/Melan-A. Whereas specific T-cell responses were elicited by transduced A2Kb LMP2−/− dendritic cells, no response was detected after transfer of transduced A2Kb dendritic cells.

Altogether, we conclude that the proteasomes expressed in dendritic cells impair the correct processing of Melan-A and thus prevent the induction of anti-Melan-A26-35 T-cell response in vivo.

**Discussion**

We have shown for the first time that, in vivo, the induction of T-cell responses against a major melanoma-associated antigen, such as Melan-A26-35/HLA-A2, is impaired due to the poor processing of that antigen by the proteasome of dendritic cells. These proteasomes are derived from A2Kb and A2Kb LMP2−/− mice. A. Western blot analysis of the content of BMDCs and spleen cells derived from A2Kb and A2Kb LMP2−/− mice. After 1 week of culture, BMDCs were lysed, and lysates corresponding to 2 × 10^6 cells were loaded on SDS-12% PAGE (left). Purified proteasomes derived from spleen of A2Kb and A2Kb LMP2−/− mice were loaded on SDS-12% PAGE (right). Immunoblots were stained with anti-LMP2, anti-LMP7, anti-MECL-1, and anti-α5 antibodies. B, proteasomal activities using fluorogenic peptides. Proteasomes purified from spleen of either A2Kb or A2Kb LMP2−/− mice were incubated with 50 µmol/L lactacystine (Lc) or not before incubation with the three different fluorogenic peptides for 4 hours. Peptide hydrolysis was detected by monitoring the increased fluorescence, in arbitrary units (A.U.), emitted by the released fluorogenic group AMC. Bz-VGR-AMC tests the tryptic-like activity; Z-LEL-AMC tests the caspase-like activity; and Suc-LLVY-AMC tests the chymotryptic-like activity of proteasomes. As negative control, peptides without proteasomes were used. C, HPLC profile (A2Kb) of the fragments produced by the degradation of the synthetic peptide Melan-A15-35 by proteasomes derived from spleen cells of either A2Kb or A2Kb LMP2−/− mice. The peaks corresponding to the precursor are indicated (P). The values above individual peaks correspond to the integration of the surface under the peak and are proportional to the relative abundance of the particular peptide species. Gray box, fragment Melan-A15-35. See text for details.
findings also underscore the importance of immunoproteasomes in the priming of T cells reactive against tumor-associated antigens.

Earlier biochemical analyses have measured the ratio of standard proteasomes versus immunoproteasomes present in dendritic cells, at different stages of maturation. It was shown that immature dendritic cells express similar levels of standard and immunoproteasomes (16). During maturation, the synthesis of immunoproteasomes is stimulated, but this increased synthesis does not seem to translate into an increased content of immunoproteasomes (17). Therefore, the contribution of immunoproteasomes on the priming of antigen-specific T-cell responses has remained unclear. Our data indicate that the presence of immunoproteasomes in dendritic cells profoundly affects the processing and presentation of Melan-A<sub>26-35</sub>-specific T cells. This interpretation is supported by our in vitro studies (44–46). These results seem to contradict ours because we could neither detect in vitro CTL recognition of dendritic cells expressing Melan-A nor induce any measurable T-cell response after adoptive transfer of transduced dendritic cells. Common to all the in vitro studies was the use of large doses of vectors for the transduction of dendritic cells (in ref. 44, dendritic cells were infected with rec. adenovirus/Melan-A at an MOI of 1,000). It is likely that the amount of protein produced under these conditions was so high that even if only a small percentage of the antigen was processed by the few standard proteasome particles present in mature dendritic cells, this remained sufficient to elicit immunoproteasomes is significantly shorter than that of standard proteasomes (43). It is, therefore, possible that the increased synthesis of immunoproteasome subunits produces indeed more immunoproteasome particles in mature dendritic cells and affects Melan-A processing, whereas the shorter half-life of the immunoproteasome might explain why the overall proportion of the two types of proteasomes seems to remain unchanged. Hence, biochemical measurement of proteasome composition might not be sufficiently sensitive to predict the functional contribution of proteasome on antigen processing.

Transductions or transfections of dendritic cells with vectors encoding the full-length Melan-A protein were shown previously to elicit specific T-cell responses against the HLA-A2-restricted Melan-A epitope in vitro (44–46). These results seem to contradict ours because we could neither detect in vitro CTL recognition of dendritic cells expressing Melan-A nor induce any measurable T-cell response after adoptive transfer of transduced dendritic cells. Common to all the in vitro studies was the use of large doses of vectors for the transduction of dendritic cells (in ref. 44, dendritic cells were infected with rec. adenovirus/Melan-A at an MOI of 1,000). It is likely that the amount of protein produced under these conditions was so high that even if only a small percentage of the antigen was processed by the few standard proteasome particles present in mature dendritic cells, this remained sufficient to elicit specific T cells. This interpretation is supported by our in vitro peptide digestion assay, which showed that the preparation of immunoproteasome isolated from the spleen of A2Kb mice, which most probably also contains a small amount of standard proteasomes, produced minute amounts of the fragment with the proper COOH terminus (Fig. 3C). However, absence of detectable T-cell responses after adoptive transfer of transduced dendritic cells transduced with rec. lv/Melan-A suggests that the processing of Melan-A by the proteasomes of dendritic cells is a limiting factor during priming of CD8<sup>+</sup> T cells in vivo.

Finally, our results could also explain why an unusually high frequency of naive anti-Melan-A T cells, which are potentially...
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Autoreactive, circulate in HLA-A2 individuals (23). Potentially autoreactive thymocytes undergo negative selection in the thymus. This process is mediated by thymic dendritic cells and medullary thymic epithelial cells (mTEC). The mTEC are specialized cells, in which genes normally expressed in peripheral tissues are transcribed (47, 48). Interestingly, both dendritic cells and mTECs have been shown to express immunoproteasomes constitutively (15). Thus, it is tempting to speculate that anti-Melan-A thymocytes will not be eliminated in the thymus, due to the poor processing of that antigen by immunoproteasomes, and will be released at high frequency in the periphery. Once in the periphery, these T cells remain naive because the cells capable of eliciting them express immunoproteasomes. Nevertheless, spontaneous anti-Melan-A T-cell responses in melanoma patients have been documented (49). It is, therefore, possible that direct priming by tumor cells might occur. Contrary to dendritic cells, melanoma cells normally express standard proteasomes and process Melan-A efficiently. As shown previously, replacement of standard proteasomes by immunoproteasomes in cells not expressing immunoproteasomes constitutively takes several days (10). Therefore, within the environment of a lymph node into which tumor cells might have migrated, it is possible that these tumor cells could activate specific T cells directly. Incidentally, direct priming by cells expressing standard proteasomes might also explain why the immunization of A2Kb mice with rec IV-Melan-A still induces a weak anti-Melan-A T-cell response (Fig. 4). Taken together, our results suggest that differential antigen processing prevents spontaneous T-cell responses against self peptides in healthy individuals and imply that direct priming by tumor cells might occur in cancer patients.

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