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 β1i (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 immunoproteasome 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-Melan-A26-35/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-A1MART1, 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 transgenic 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 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 donors 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 electrophoretically run 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-A26-35 or SSX21-34–specific CTL clones (refs. 24, 25; 10 × 10^4 well) were cocultured with APC (15 × 10^4 per well) for 24 hours at 37°C in Iscove’s modified Dulbecco’s medium containing 25 IU/mL recombinant human IL-2 (rhIL-2). Supernatants were then harvested and the concentration of IFN-γ 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 Kaer). 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 primer pairs. LMP2−/− mice are characteristic of the absence of a band of 580 bp using the following primers: LMP2 forward, 5'-GGGATCCC-AGGACCGAAGAAG-3' and LMP2 reverse, 5'-CCCGTGTCCTCCGGGAT-AC-3' and the presence of a band of 1.3 kb using Neo-LMP2 forward, 5'-GGC-TTCTGCGCTTACGG-3' and Neo-LMP2 reverse, 5'-CAGAGGGAGAG-CTTGTG-3'. Animal experiments were carried out in pathogen-free facilities and in compliance with the rules of the local veterinary office.

Construction of lentivectors. All melan-A sequences, except in Fig. 1, contained the Ala to Leu substitution at position 27 to increase the affinity of the peptide to HLA-A2 (28). Recombinant lentivectors (rec. lv) were generated by inserting the DNA sequence coding for EGFP-ubiquitin-Melan-A26-35 or Melan-A between the BsrAI and XbaI sites of the lentiviral transfer vector pbRRLsin18PPT.CMV. The production of recombinant lentiviruses has been described previously (29–31). The recombinant viruses were screened using the UPR technology described earlier (22, 32).

Flow cytometry. Flow cytometry was done on a FACS Calibur using CellQuest software (Becton Dickinson, San Jose, CA). Expression of A2Kb was monitored by staining peripheral blood mononuclear cells (PBMC) with the anti-HLA antibody B912.1 (produced in our laboratory). PBMCs obtained by tail bleedings were incubated with phycoerythrin-conjugated Melan-A26-35/A2Kb tetramers (prepared in our laboratory) for 1 hour at 20°C and washed before incubation with cytokine-conjugated anti-CD8 monoclonal antibody (mAb) 53-6.7 (eBioscience, San Diego, CA), and FITC-conjugated anti-CD62L antibodies (Mel-14, provided in our laboratory) for 20 minutes at 4°C. Other antibodies used were phycoerythrin-conjugated anti-CD8a (SK1; Caltag Laboratories, Burlingame, CA) and anti-CD4 (GK1.5, produced at our institute). Erythrocytes were lysed using the Fluorescence-Activated Cell Sorting Lysis 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 were collected from tibia and femur 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 coding for Melan-A26-35 or Melan-A.

In vitro T-cell stimulation. Specific anti-Melan-A CD8+ T cells derived from spleens of immunized A2Kb mice were stimulated in vitro. Cytotoxic T cells (10^6) were cocultured with 2 × 10^5 irradiated (100 Gy) EL-4 A2Kb cells and pre pulsed with 1 μmol/L Melan-A26-35 peptide for 1 hour at 37°C, in DMEM supplemented with 10 mmol/L HEPES, 50 μmol/L 2-mercaptoethanol, 10% FCS, and conditional medium containing 30 units/mL IL-2. CTLs were used on day 4 after stimulation.

CTL assay, IFN-γ release assay, and ELISA. Transduced BMDCs were seeded at a density of 2 × 10^5 per well in 96-well plates. Half of the cells were pulsed with exogenous Melan-A26-35 peptide (1 μmol/L) for 30 minutes at 37°C before the addition of CTLs at an effector-to-target (E:T) ratio of 3:1 in DMEM containing 10% FCS, 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 (22).

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 protease inhibitors (Complete Mini, EDTA-free, Roche, Indianapolis, IN). The lysate was centrifuged at 11,000 × g for 15 minutes at 4°C. Lysates were separated by SDS-PAGE and subjected to immunoblotting. For BMDCs, lysates were obtained by adding 20 μmol/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 × 10^5 cells. The membrane was revealed with rabbit polyclonal antibodies anti-LMP2 (1:1,000; Biomol International, Plymouth Meeting, PA) and anti-MEL-1 (1:1,000; Biomol International), mouse mAb anti-LMP7 (1:1,000; Biomol International) and anti-tp60 (1:2,000; 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. Homogenous 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 μmol/L HEPES at a concentration of 6 × 10^7 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 × 10^6 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.

Fluorigenic assay. The proteasome-enriched fraction and unfractonated cytosol were diluted in 90 μL of 20 mmol/L Tris-HCl (pH 7.6) in the presence or absence of 50 μmol/L lactacysteine (Biomol International) for 15 minutes at room temperature in 96-well plates (Nalge Nunc International, Naperville, IL). The fluorescent peptides Br-VG-AMC (Bachem, Bubendorf, Switzerland), Z-LLL-AMC (Bachem), and Suc-LLL-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 fluorigenic group AMC was measured after 4 hours by spectrophotometry (excitation/emission = 380/460 nm).
Peptide digestion by proteasome, high-performance liquid chromatography separation, and mass spectrometry. Purified synthetic peptides were quantified by amino acid analysis; 620 pmol of peptide Melan-A15-40 was digested in 25 µL proteasome purified from spleen of A2Kb or A2Kb LMP2−/− mice, corresponding to 7.5 × 10^6 cell equivalents. 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 time point, the reaction was stopped with 1% trifluoroacetic acid. The digests were diluted with acetic acid/water (50:50, v/v) to a final concentration of one of two independent experiments; bars, SD.

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 human dendritic cells constitutively express standard and immunoproteasomes (10, 16, 17), we assessed the ability of HLA-A2 expressing tumor cells to stimulate the production of IFN-γ by clonal CTL populations specific for Melan-A26-35 or SSX-241-49 as indicated by the elevated amount of secreted IFN-γ (Fig. 2A). The characteristics and advantages of the EGFP-ubiquitin fusion system have been explained elsewhere (22, 32, 38). 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 of one of two independent experiments; bars, SD.

Figure 1. Recognition of human dendritic cells expressing tumor antigens by specific CTLs. HLA-A2 transgenic mice. Isolated BMDCs were transduced with recombinant lentivectors (rec. lv) coding for the full-length Melan-A or SSX-2 protein, and used to stimulate clonal CTL populations as described previously (36). As illustrated in Fig. 1, Melan-A- and SSX-2-specific CTLs produced similar amounts of IFN-γ after incubation with melanoma cells expressing the corresponding antigen. However, whereas SSX-2-specific CTLs were efficiently stimulated by dendritic cells transected with the SSX-2 plasmid, dendritic cells transected with the plasmid coding for Melan-A did not stimulate Melan-A-specific CTLs. Note that the plasmid used here encoded the wild-type sequence of Melan-A. Similar levels of EGFP were detected, indicating that the expression levels of both proteins were comparable (data not shown). We conclude that human dendritic cells are deficient in generating the Melan-A26-35/HLA-A2 epitope in vitro.

Processing of Melan-A by dendritic cells is influenced by the expression of proteasome LMP2 subunit. Because of the inefficent 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-A2/2-Kb (A2Kb) transgenic mice. Isolated BMDCs were transduced with recombinant lentivectors (rec. lv) for the expresion of proteasome LMP2 subunit. 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 led 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−/− (p31i−/−) 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 mice, which were shown to lack bona fide immunoproteasomes. This mechanism causing the inefficient processing of Melan-A26-35 by the immunoproteasomes, we purified proteasomes from spleens of A2Kb mice (Fig. 3A). As before, we derived BMDCs from A2Kb LMP2−/− mice and transduced them with rec.Iv/Melan-A26-35 and rec.Iv/Melan-A. Contrary to what was observed with A2Kb-derived BMDCs expressing Melan-A, A2Kb LMP2−/− BMDCs expressing Melan-A or the preprocessed Melan-A26-35 peptide were recognized with equal efficiency (Fig. 2B). This result shows that the expression of the immunoproteasomes subunit LMP2 in BMDCs drastically reduced the processing of Melan-A26-35.

Inefficient release of the COOH terminus of Melan-A26-35 by immunoproteasomes. To better delineate the molecular mechanism causing the inefficient processing of Melan-A26-35 by the immunoproteasomes, we purified proteasomes from spleens of A2Kb and A2Kb LMP2−/− mice. Spleens were shown to contain high levels of immunoproteasomes (39). Western blot analyses revealed that the proteasome isolated from A2Kb mice contained indeed the three subunits LMP2, LMP7, and MECL-1 (Fig. 3A). In contrast, proteasome particles purified from A2Kb LMP2−/− not only lacked the LMP2 subunit but also MECL-1. The LMP7 subunit was still incorporated. These results confirm previous findings, which showed cooperative incorporation of the two subunits LMP2 and MECL-1 (40), and the incorporation of LMP7 in the absence of LMP2 and MECL-1 (11, 41). Proteolytic activities measured with short fluorogenic substrates indicated that the proteasomes purified from A2Kb LMP2−/− mice cleared more efficiently after acidic amino acids (caspase-like activity) than proteasomes isolated from A2Kb mice (Fig. 3B). No significant differences were observed with peptides testing the tryptic- and chymotryptic-like activities. As control, we repeated the digestions in presence of the proteasome inhibitor lactacystin and found a drastic reduction in all three activities. Some residual caspase-like activities of the proteasomes isolated from A2Kb LMP2−/− mice could be observed. However, we previously reported that lactacystin inefficiently blocks this activity (32).

Next, we analyzed the consequence of these differences on the processing of Melan-A26-35. Previous studies had shown that the generation of the COOH terminus of Melan-A26-35 was mediated by the proteasome (22). We, therefore, digested the synthetic precursor peptide Melan-A15-40, which encompasses the antigenic peptide sequence, with proteasomes purified from spleens of A2Kb and A2Kb LMP2−/− mice. The peptide was digested for 4 hours and fractionated by HPLC, and the fragments were identified by mass spectrometry. We focused on the fragment Melan-A15-35, which is generated by the cleavage at the COOH terminus of Melan-A26-35. The degradation of the precursor and the production of an irrelevant fragment with a retention time of ~21 minutes were comparable between the two types of proteasomes. The analytic conditions used here did not allow us to identify the fully processed Melan-A26-35 peptide (data not shown).

Altogether, these data show that the COOH terminus of the antigenic peptide Melan-A26-35 is only produced by proteasomes lacking LMP2 and MECL-1.

Comparison of the anti-Melan-A T-cell response in A2Kb and A2Kb LMP2−/− mice. To study the effect of these findings on the induction of primary T-cell responses in vivo, we immunized A2Kb
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. CD8+ T cells at the peak of the response. In stark contrast, immunization of A2Kb and 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 × 105 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
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

**Figure 4.** Kinetic of the CD8+ T-cell response induced by rec. lv/Melan-A26-35 and rec. lv/Melan-A in A2Kb and A2Kb LMP2−/− mice. A, the anti-Melan-A CD8+ T-cell response in HLA-A2/Kb was measured ex vivo at different time points after immunization by enumerating the number of tet+ T cells among total CD8+ lymphocytes in blood. Response induced by rec. lv/Melan-A26-35 (●, n = 10 mice) or rec. lv/Melan-A (○, n = 10 mice). Rec. lv/EGFP was used as negative control (△, n = 7 mice). Points, mean; bars, SE.

**Figure 5.** T-cell response in A2Kb mice induced by lentivirus transduced BMDCs derived from A2Kb or A2Kb LMP2−/− mice. A, BMDCs from A2Kb (● and ○) and A2Kb LMP2−/− (△ and ▲) were transduced with rec. lv/Melan-A26-35 (● and △) or rec. lv/Melan-A (○ and ▲). Three days later, dendritic cells (DC) were injected into A2Kb mice s.c., and the kinetic of the specific T-cell response in PBMC was analyzed ex vivo by flow cytometry. Tet+ cells among CD8+ cells. Points, mean of four mice that are representative of at least three independent experiments. B, percentage of tet+ cells among total CD8+ T cells at the peak of the response (day 14 after immunization). Columns, mean of n mice per condition; bars, SE. n.s., not significant; ***, P < 0.001 (calculated by the Student’s t test).
Proteasomes of Dendritic Cells Control T-Cell Responses

autoimmune, circulate in HLA-A2 individuals (23). Potentially auto-
reactive 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 naïve 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
come. Contrary to dendritic cells, melanoma cells normally express
standard proteasomes and process Melan-A efficiently. As shown
previously, replacement of standard proteasomes by immunopro-
"donors in not expressing immunoproteasomes constitu-
tively takes several days (10). Therefore, within the en-
vironment of a lymph node into which tumor cells might have migrated, it is
possible that these tumor cells could activate specific T cells di-
c. 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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