Endosomal Proteases Influence the Repertoire of MAGE-A3 Epitopes Recognized In vivo by CD4+ T Cells

Jill Marturano, Renato Longhi, Vincenzo Russo, and Maria Pia Protti

Abstract

Little is known about the repertoire of MAGE-A3 CD4+ T-cell epitopes recognized in vivo by neoplastic patients and how antigen processing influences epitope formation. Here, we first show that MAGE-A3–specific CD4+ T cells are present in the blood of advanced melanoma patients. MAGE-A3111–125, MAGE-A3191–205, and MAGE-A3281–300 were recognized by 7, 6, and 3 of the 11 patients tested, respectively. MAGE-A3146–160 and MAGE-A3171–185 were also recognized in two and one cases, whereas no recognition of MAGE-A3161–175 and MAGE-A3243–258 was observed. Cytokines produced were mainly interleukin 5 and/or granulocyte macrophage colony-stimulating factor, suggesting impairment of productive polarized Th1 responses. Secondly, proteases inhibitors were used to modulate in vitro the recognition by CD4+ T-cells clones of dendritic cells loaded with MAGE-A3–expressing cell lysates. We found that formation of MAGE-A3111–125 depended on both leupeptin-sensitive and pepstatin-sensitive proteases. In contrast, we found that MAGE-A3161–175, which was never recognized ex vivo, was formed by leupeptin but destroyed by pepstatin-sensitive proteases. Collectively, our results show that (a) anti–MAGE-A3 CD4+ T-cell immunity develops in vivo in neoplastic patients and is focused toward immunodominant epitopes, (b) the response in advanced disease is skewed toward a Th2 type, and (c) endosomal/lysosomal proteases in dendritic cells influence the repertoire of the epitopes recognized.

Introduction

MAGE-3 is a tumor-specific antigen widely expressed in solid tumors, such as melanoma, lung carcinoma, head and neck carcinoma, and hematologic malignancies, including T-cell leukemia and myeloma but not in normal tissues (with the exception of testis; ref. 1). Evidence for a critical role of CD4+ T cells in the antitumor response is increasing (2, 3). CD4+ T cells provide help for induction and maintenance of antitumor CD8+ T cells and exert effector functions both indirectly via cytokine release and directly on MHC class II molecule (MHC-II)–expressing tumor cells. However, along with productive proinflammatory responses, CD4+ T cells may also exert regulatory functions (4, 5). Several MAGE-A3 CD4+ T-cell epitopes have been identified (6–13). Little is known about the repertoire of CD4+ T-cell epitopes recognized in vivo by neoplastic patients and even less on how antigen processing during priming in dendritic cells influences their formation.

Proteases in the endosomal/lysosomal compartment play an important role in the formation of MHC-II–peptide complexes: they free the MHC-II from the invariant (ii) chain and produce the protein fragments than can be loaded onto it (14–17). A well-studied protease family is the cathepsin family that can be divided into subgroups according to the amino acid in the active site (i.e., cysteine cathepsins, such as cathepsins B, L, and S, and aspartate cathepsins, such as cathepsins D and E; refs. 14–17). Another important lysosomal enzyme is asparagine endopeptidase (AEP), a protease more similar to caspases in structure than to cathepsins (18). Whereas the role of some of these enzymes has been described in the maturation of MHC-II molecules, their exact function in antigen processing is less clear. With the exception of AEP, they have fairly broad cleavage specificity, working mainly as endopeptidases or, in some cases, as carboxypeptidases and aminopeptidases (16). Some epitopes appear produced by one or another in a nonredundant fashion. For example, in mice, cathepsin S has been shown to be essential for the production of the hen egg lysozyme HEL30-44 epitope (19), and cathepsins L and D are important for the production of a distinct subset of ovalbumin peptides (20, 21). AEP has been shown to be necessary for the correct processing of the microbial tetanus toxin C fragment (22). However, other evidence shows that elimination of specific protease activities does not affect presentation of a variety of CD4+ T-cell epitopes. For example, cathepsins B and L were dispensable for presentation of ovalbumin and hen egg lysozyme (23) and a number of endogenous and exogenous antigens (24), respectively. These data suggest that for some antigens, specific protease activity is required, but for others, individual enzymes are dispensable. Up to now, no reports have addressed the requirements for these enzymes in the formation of CD4+ T-cell epitopes from tumor-associated antigens.

In this paper, we first investigated the repertoire of MAGE-A3 CD4+ T-cell epitopes spontaneously recognized by advanced melanoma patients, and secondly, we evaluated by in vitro studies how this repertoire is influenced by endosomal/lysosomal proteases in dendritic cells.

Our results indicate that, depending on the epitope endosomal/lysosomal proteases, formation or destruction of MAGE-A3 CD4+ T-cell epitopes is differently affected and that the repertoire of epitopes recognized by neoplastic patients is strongly influenced by these proteases toward immunodominant epitopes.

Materials and Methods

Subjects and cells. Peripheral blood mononuclear cells (PBMC) were obtained from 11 advanced melanoma patients, and cord blood mononuclear cells (CBMC) were from 10 umbilical cords. The Institutional Ethics

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Committee had approved the study protocol, and informed consent was obtained from all donors before blood sampling. The stage of disease and the HLA class II typing of the patients are reported in Table 1. Expression of MAGE-A3 by the tumor was verified in all patients by reverse transcription-PCR, as described in ref. 25. MAGE-A3146-160, 161-175, 171-185, 191-205, 243-258, and 281-300 were synthesized from a healthy donor and have been described in detail in ref. 13. MAGE-A3111-125-specific CD4+ T cells were generated from melanoma patient 011 (Table 1). Melanoma cell lines were HT144, purchased from the American Type Culture Collection, and MDTC, obtained in our laboratory from a cutaneous metastasis. The LCLs used were wild-type (WT) BM21 and SIMO (kindly provided by K. Fleischhauer at HSR), Mun (established in our laboratory), and DAS (kindly provided by J. Anholts at LUMC). HT144 (HT144IIM3) and DAS (LCL-IIM3) cells expressing MAGE-A3 in the endosomal/lysosomal compartment were described in ref. 13. The HLA-class II type of cells used was identified by molecular typing and is reported in Table 1 for melanoma patients. HLA-DR types for the other cells used were healthy donor (DRB1*1501, *07; DRB4*0401), HT144 (DRB1*04, *07; DRB4*01), MDTC (DRB1*04, *11; DRB4*01, DRB3*02), SIMO (DRB1*10), BM21 (DRB1*11, DRB3*02), Mun (DRB1*13, DRB3*02), and DAS (DRB1*04, DRB4*01).

**Synthesis of MAGE-A3 peptides.** MAGE-A3 sequences 111-125, 114-127, 146-160, 161-175, 171-185, 191-205, 243-258, and 281-300 were synthesized by the stepwise solid-phase method as previously described (26). Synthetic peptides were purified by semipreparative reverse-phase high-performance liquid chromatography (HPLC), the purity of the peptides was confirmed by analytic reverse-phase HPLC, and the mass was determined by matrix-assisted laser desorption/ionization time-of-flight analysis with a Voyager-RII Biospectrometry Workstation (PE BioSystems, Inc.). Observed experimental values were in agreement with the theoretical calculated ones. The peptides were lyophilized, reconstituted in DMSO at 10 mg/mL, and diluted in RPMI 1640 (Life Technologies) as needed.

**In vitro restimulation assay.** CD4+ T cells were purified from total PBMCs and CBMCs by magnetic separation (Miltenyi Biotech) and cultured with total PBMCs and CBMCs by magnetic separation (Miltenyi Biotech) and cultured in X-VIVO 15 medium (BioWhittaker) supplemented with 3% heat-inactivated normal human serum (NHS), penicillin (100 units/mL), and streptomycin (50 μg/mL; Biowhittaker; tissue culture medium) in the presence of irradiated CD4−depleted PBMCs as antigen-presenting cells (APC) at 1:3 ratio in 96-well plates in six replicates for each condition. Stimuli were PHA-L (10 μg/mL; Sigma) as positive control, CD4+ T cells in the presence of the APCs only as baseline (blank), and each single peptide (10 μg/mL). On day 7, half medium from each well was removed and replenished with fresh tissue culture medium containing interleukin 2 (IL-2; 25 units/mL; Proleukin, Novartis) without any further antigen stimulation. On day 14, medium was removed and tested for cytokine production. If cytokine release was above the negative control (i.e., CD4+ T cells in the absence of peptides), the experiment was repeated and the cells were grown in the same conditions for 3 weeks and then tested for specificity in a 2-day proliferation and cytokine release assay (see below).

**Propagation of anti–MAGE-A3 CD4+ T-cell clones.** MAGE-A3141-155-specific CD4+ T cells have been described in ref. 13. The APCs used were autologous CD4−depleted PBMCs (1:10), HLA-DR−matched LCLs (1.5), melanoma cells (1.3), and dendritic cells (1.5). Peptides were added at a final concentration of 10 μg/mL. In peptide titration experiments, the following concentrations of peptides were added: 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 μg/mL. Dendritic cells were obtained from PBMCs after monocyte enrichment via adherence and grown for a week in RPMI 1640 (1% NHS), IL-4 (500 units/mL; Schering Plough), and granulocyte macrophage colony-stimulating factor (GM-CSF; 800 units/mL; Immunex Corp.). On day 6, dendritic cells (2.5 × 105) were fed with 5 × 104 cells (either LCLs or tumors) that had been lysed by three cycles of freeze-thawing and incubated overnight. After removing old medium, 5 × 104 CD4+ T cells were added in fresh tissue culture medium. Duplicate wells with CD4+ T cells alone and APCs alone were used as controls. Two wells with CD4+ T cells plus APCs did not receive any stimulus to determine the basal growth rate. CD4+ T cells and APCs were grown for 48 h, then half of the media was removed for cytokine release assays and cultures pulsed for 16 h with [3H] Thymidine (1 μCi per well, 6.7 Ci/mmol; Amersham Corp.). The cells were collected with a FilterMate Universal Harvester (Packard Instruments) in specific plates (Unifilter GF/C; Packard), and the thymidine incorporated was measured in a liquid scintillation counter (TopCount NXT; Packard). IFN-γ, GM-CSF, and IL-5 release was measured using standard ELISAs (Biosource Europe and MabTech, Miltenyi Biotec) following the manufacturer's instructions.

To study the role of endosomal/lysosomal enzymes in the presentation of MAGE-A3 epitopes APCs (dendritic cells, LCL-IIM3, and HT144IIM3) were treated with specific inhibitors. The inhibitors used were: leupeptin (Sigma) and pepstatin A (Calbiochem). Inhibitors were resuspended in DMSO or water according to the manufacturer's instructions. To study the role of endosomal/lysosomal enzymes in the presentation of MAGE-A3 epitopes APCs (dendritic cells, LCL-IIM3, and HT144IIM3) were treated with specific inhibitors. The inhibitors used were: leupeptin (Sigma) and pepstatin A (Calbiochem). Inhibitors were resuspended in DMSO or water according to the manufacturer's instructions. The APCs were incubated at 37°C for 18 to 20 h before use in proliferation and cytokine release assays. Cell vitality in treated APCs was checked by Trypan blue (Sigma) staining compared with untreated controls, and the expression of HLA-DR molecules was tested by fluorescence-activated cell sorting.

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**Table 1. Stage and HLA class II typing of the patients**

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<th>Patient no.</th>
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<td>–</td>
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<tr>
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<td>*07, *11</td>
<td>*02</td>
<td>+</td>
<td>–</td>
<td>*0402, *1701</td>
</tr>
</tbody>
</table>

Abbreviations: +, expressed; –, not expressed.
(FACS) analysis. Dendritic cells maturation after cell lysate pulsing in the absence or in the presence of the inhibitors was also assessed by FACS analysis.

**Flow cytometry.** Cytofluorimetric analyses were performed on a FACStarPlus (Becton Dickinson). We used the following monoclonal antibodies: anti–DR FITC, anti–CD4-FITC, anti–CD8-PE, anti–CD45RA-FITC, anti–CD45RO-FITC, anti–CD3-FITC, anti–CD40-FITC, anti–CD80-FITC, anti–CD83-PE, anti–CD86-FITC, and anti-DR-PE-Cy5 (Becton Dickinson).

### Results

**Repertoire of MAGE-A3 CD4+ T-cell epitopes recognized by melanoma patients.** To study the presence of MAGE-A3–specific CD4+ T cells in the blood of neoplastic patients and the repertoire of epitopes recognized, we purified CD4+ T cells from PBMCs of 11 advanced melanoma patients and CBMCs from 10 umbilical cords as controls. Patients’ stage and HLA class II types are

![Figure 1](image-url)
were cultured in the presence of CD4+-depleted PBMCs, and each used a protocol established in our laboratory (27). CD4+ T cells A3146-160 and MAGE-A3171-185 were recognized by two and one, respectively. MAGE-A3111-125, MAGE-A3161-175, and MAGE-A3281-300 were recognized by seven, six, and five patients each, respectively. MAGE-A3111-125, MAGE-A3161-175, and MAGE-A3281-300 sequences previously identified by others (8, 9) and us (7, 10, 13) as containing naturally processed epitopes, plus peptide MAGE-A3171-185 which apparently does not contain a natural epitope (10).

To screen for the presence of MAGE-A3–specific CD4+ T cells, we used a protocol established in our laboratory (27). CD4+ T cells were cultured in the presence of CD4+-depleted PBMCs, and each single peptide in several replicates. On day 7, low-dose IL-2 was added in culture, and the cells were cultured for other 7 days without further antigen stimulation. On day 14, cytokine release and T-cell proliferation were assessed. Naive CD4+ T cells from cord bloods were also tested to verify if the culture system induced cytokine production toward one or more peptides. On the contrary, CD4+ T cells from 1 of 10 cord bloods proliferated to MAGE-A3 peptides but did not release any cytokine (data not shown), indicating that the culture conditions used were not favoring in vitro priming.

Effects of leupeptin-sensitive and pepstatin A–sensitive endosomal/lysosomal proteases in the formation or destruction of MIC-II restricted MAGE-A3 epitopes. To investigate the influence of cysteine and aspartic protease in the formation of the repertoire of MAGE-A3 epitopes recognized by melanoma patients, we concentrated on MAGE-A3111-125 and MAGE-A3161-175 that were recognized by 7 of 11 and 0 of 11 patients, respectively. To this aim, we took advantage of CD4+ T-cell clones specific for the two epitopes: MAGE-A3111-125–specific CD4+ T cells were obtained by limiting dilution of a polyclonal cell line from patient 011; MAGE-A3161-175–specific CD4+ T cells were obtained in vitro by repetitive stimulation from a healthy donor and have been described previously (13).

Figure 2 summarizes the characteristics of the clones used. MAGE-A3111-125–specific CD4+ T cells were HLA-DRB1*11–restricted because they specifically proliferated and produced IL-5 in the presence of HLA-DRB1*11 but not HLA-DRB1*10–matched or HLA-DRB1*02–matched LCLs (Fig. 2A, top). We cannot exclude presentation by HLA-DQB1*0301 shared between LCL-DRB1*11 and patient 011. As shown in Fig. 2A (bottom), CD4+ T cells recognized an epitope shared with MAGE-A3144-127, which was previously described by van der Bruggen and collaborators (6), to contain an epitope presented in association with HLA-DRB1*13. This result suggests that the two clones recognize a common promiscuous epitope within the two sequences. MAGE-A3161-175–specific CD4+ T cells were HLA-DRB1*01–restricted as previously shown in ref. 13. Peptide titration curves for the two clones showed that the concentration of peptide requested to reach the half maximal stimulation (EC50) was 1 to 4 μg/mL for MAGE-A3111-125–specific CD4+ T cells and 0.06 to 0.1 μg/mL for MAGE-A3161-175–specific CD4+ T cells (Fig. 2B), demonstrating very low and intermediate affinity, respectively. MAGE-A3111-125–specific and MAGE-A3161-175–specific CD4+ T cells were then tested for recognition of autologous dendritic cells loaded with lysates from MAGE-A3–expressing tumor cells (HT144 and MDTC). MAGE-A3111-125–specific CD4+ T cells significantly lower IL-5 release from MAGE-A3111-125–specific CD4+ T cells specifically produced IL-5 and GM-CSF with the exception of patients 011, 022, and 026 who produced GM-CSF only and patient 008 who produced IFN-γ.

Table 2 summarizes the repertoire of peptides recognized. MAGE-A3111-125 and MAGE-A391-205 were recognized by seven, six, and five patients each, respectively. MAGE-A3146-160 and MAGE-A3281-300 were recognized by two and one, respectively. MAGE-A3243-258 and MAGE-A3161-175 were not recognized. These results confirm the immunodominance of the MAGE-A3 epitopes previously described in ref. 10, except for MAGE-A3146-160.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>111-125</th>
<th>146-160</th>
<th>161-175</th>
<th>171-185</th>
<th>191-205</th>
<th>243-258</th>
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</table>

NOTE: Shaded cells indicate recognition by the patients.
A–sensitive proteases were needed for the epitope's formation. Dendritic cells pretreated with leupeptin (Fig. 3B) similarly elicited a decreased IFN-γ production from MAGE-A3161-175–specific CD4+ T cells. In contrast, pretreatment with pepstatin A dramatically increased recognition (Fig. 3D). This indicates that one or more leupeptin-sensitive proteases are responsible for epitope formation, whereas one or more pepstatin A–sensitive proteases are responsible for epitope destruction. Dendritic cell pretreatment with the inhibitors did not significantly affect the level of expression of maturation molecules (i.e., CD80, CD86, CD40, CD83, and HLA-DR) compared with cell lysate–pulsed dendritic cells as assessed by flow cytometry (data not shown). These results were confirmed when MAGE-A3161-175–specific CD4+ T cells were challenged with LCLs (Fig. 4A) and HT144 cells (Fig. 4B) engineered to express MAGE-A3 in the endosomal/lysosomal compartment. The level of HLA class II molecule on the cell surface, verified by FACS analysis, was not affected by the inhibitors (data not shown).

Discussion

Priming of tumor antigen–specific CD4+ T cells depends on a CD4+ T-cell repertoire after thymic selection and on processing and presentation in the secondary lymphoid organs by dendritic cells after uptake of the antigen at the periphery.

CD4+ T cells specific for MAGE-A3 peptides were found in a high number of patients (8 of 11 tested), demonstrating that a repertoire of naturally occurring MAGE-A3–specific CD4+ T cells exists and that its activation develops in patients.

We previously identified MAGE-A3–specific promiscuous immunodominant CD4+ T-cell epitopes by in vitro priming of CD4+ T cell from healthy donors (10). We report here that three of these immunodominant epitopes are immunogenic in vivo in melanoma patients. Despite the expression of the allele in 7 of the 11 tested, none of the patients recognized the HLA-DP*04–restricted epitope, suggesting that the repertoire of MAGE-A3 CD4+ T-cell epitopes is dictated more by immunodominance than by the expression of a certain allele.

Spontaneous CD4+ T-cell responses have been described for other tumor antigens (29–36). Th1 and mixed Th1 and Th2 responses toward NY-ESO-1 epitopes were found in ref. 32 and refs. 29, 34, respectively. Th1 responses were also found against other cancer testis antigens (33, 35, 36). CAMEL–specific (31) and MAGE-6–specific (30) Th2 CD4+ T-cell responses were associated with disease progression. Most of the patients of our study showed...
formation of MAGE-A3111-125, which was recognized by the MAGE-A3111-125–specific CD4+ T cells in the presence of leupeptin-treated dendritic cells. MAGE-A3161-175–specific and MAGE-A3111-125–specific cells were challenged with dendritic cell pulsed with lysates from control (LCL; open symbols) or MAGE-A3–expressing (HT144; filled symbols) cells in the presence of increasing nontoxic concentrations of the protease inhibitors leupeptin and pepstatin A. Filled squares and triangles refer to the positive controls (response to 10 g/mL of the relevant peptide). A–C, IL-5 release by MAGE-A3111-125–specific CD4+ T cells in the presence of leupeptin-treated (A) and pepstatin A–treated (C) dendritic cells. B–D, IFN-γ release by MAGE-A3161-175–specific CD4+ T cells in the presence of leupeptin-treated (B) and pepstatin A–treated (D) dendritic cell.

The results of the ex vivo studies in the patients agree very well with the results obtained in vitro on antigen processing. Indeed, formation of MAGE-A3111-125, which was recognized by the majority of the patients, depended on both leupeptin-sensitive and pepstatin A–sensitive proteases, whereas proteases of the endosomal/lysosomal compartment were responsible for both formation and destruction of MAGE-A3161-175. The patients did not recognize this epitope, suggesting that destruction more than formation of MAGE-A3161-175 is favored in vivo.

Inhibition of cysteine cathepsins abolished presentation by dendritic cells of MAGE-A3111-125, revealing that at least one of these enzymes is essential for production of this epitope. Similarly, inhibition of cysteine cathepsins considerably reduced presentation of MAGE-A3161-175. Decrease in presentation was seen in the three types of APCs (dendritic cells loaded with lysates from MAGE-A3–expressing tumor cells LCL-liM3 and HT144-liM3) used (Figs. 3 and 4). Specific inhibition of cysteine cathepsins B and L led only to a mild decrease in recognition (data not shown). It is therefore likely that these enzymes are redundant or not involved in processing of this epitope.

Cathepsin S, given its abundance and ubiquitous presence in different cell types, is a good candidate as "antigen processor." Other cysteine cathepsins (such as cathepsins C and H), sensitive to leupeptin, could also be important. It is, however, likely given the broad specificity of cleaved substrates that any of these proteases leads to correct processing of the epitopes; indeed, there are very few examples where epitopes from other antigens (such as lysozyme and tetanus toxin C fragment) have been shown to require specific proteases for proper processing (19, 22, 37).

Within the cell production of epitopes from a given antigen is the result of protein cleavage, and destruction of an epitope may be necessary for the production of others. Negative effects of enzymes on epitope presentation have been described before (38–40). The studied region of MAGE-A3 is rich in hydrophobic residues and aspartic proteases, such as cathepsins D and E, that exhibit a preference for cleaving such regions. (38). Cathepsin D is a good candidate as the protease responsible for destruction of MAGE-A3161-175, as it is abundantly expressed in lysosomes, whereas data about cathepsin E cellular distribution are contradictory (41, 42). Importantly, the destructive effect of aspartic proteases on MAGE-A3161-175 formation was not affected in dendritic cells. This suggests that the low intensity of recognition of these cells by MAGE-A3161-175–specific CD4+ T cells could be due to a higher aspartic protease activity (compared with the other APCs used) other than to a lower amount of protein available for processing. Indeed, cathepsin D activity was higher in dendritic cells than in HT144-liM3 and LCL-liM3 being 797.45, 547.3, and 115.5 ng/mL active protein (per mg/mL total protein.

a Th2 or an unpolarized response, also supporting a skew of the CD4+ T-cell response in advanced disease. Future studies are needed to confirm this hypothesis by evaluation of the anti–MAGE-A3 CD4+ T cells in early stages.
lysate), respectively, for the three APCs used. The difference in basal cathepsin D activity could also explain the reproducible higher increase in IFN-γ production by MAGE–A3161–175–specific CD4+ T cells after challenge with HT144IiM3 compared with LCI-IiM3 (Fig. 4).

The ability of the immune system to focus on a selected number of epitopes of a complex antigen is a distinctive feature of most T-cell immune responses. Immunodominance may be dictated by antigen processing mechanisms, which may vary in different cell types, by intermolecular competition for MHC binding, by HLA-DM molecules and by the existence of a biased T-cell repertoire (43, 44). Our results show that, in the case of MAGE-A3, the content of endosomal/lysosomal proteases also influences the repertoire of immunodominant epitopes. We previously (10) referred to MAGE–A3161–175 as a “cryptic” epitope because CD4+ T cells specific for this peptide could be activated from a melanoma patient after in vitro priming, but the effectors did not recognize the native epitope. Later (13), we showed that MAGE–A3161–175–specific CD4+ T cells from a healthy donor do recognize the native epitope, although poorly, when presented by tumor cell lysate–loaded dendritic cells. It is interesting to note that MAGE–A3111–125–specific CD4+ T cells from patient 011 had an overall low avidity (Fig. 2B), whereas MAGE–A3161–175–specific CD4+ T cells from the healthy donor had intermediate avidity (Fig. 2B; ref. 13). We speculate that, as a result of peptide activity in the thymus, high amounts of MAGE–A3111–125 are produced and presented, leading to deletion of high-affinity cells and establishment of a repertoire of low-affinity T cells. In contrast, due to destructive activity, only a small amount of MAGE–A3161–175 is produced, leaving high-affinity to intermediate-affinity T cells to exit the thymus. At the periphery priming of low-affinity MAGE–A3111–125–specific CD4+ T cells would still be preferred because of the availability of processed antigen, whereas MAGE–A3161–175–specific CD4+ T cells would be activated only in conditions of high-antigen release, such as in conditions of massive tumor necrosis.

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**References**


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