Identification of immunodominant regions among promiscuous HLA-DR restricted CD4+ T cell epitopes on the tumor antigen MAGE-3

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This work was supported by the AIRC (Italian Association for Cancer Research), European Community (grant QLK2-CT2000-00470 to M.P.), the MURST (Ministry of University and Scientific Research), the Ministry of Health, the NIH (grant HL6192 to B.M.C-F) and the NINDS (grant NS23919 to B.M.C-F)

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Abstract

The molecular characterization of the CD4+ T cell epitope repertoire on human tumor antigens would contribute to both clinical investigation and cancer immunotherapy. In particular, the identification of promiscuous epitopes would be beneficial to a large number of neoplastic patients regardless of their HLA-DR type. MAGE-3 is a tumor specific antigen widely expressed in solid and hematological malignancies, therefore is an excellent candidate antigen. We used a MHC class II epitope prediction algorithm, the TEPITOPE software, to predict 11 sequence segments of MAGE-3 that could form promiscuous CD4+ T cell epitopes. In binding assays, the synthetic peptides corresponding to the 11 predicted sequences bound at least 3 different HLA-DR alleles. Nine out of the 11 peptides induced proliferation of CD4+ T cells from both healthy subjects and melanoma patients. Four immunodominant regions (residues 111-125, 146-160, 191-205 and 281-295), containing naturally processed epitopes, were recognized by most of the donors, in association with 3 to 4 different HLA-DR alleles, thus covering up to 94% of the alleles expressed in Caucasians. On the contrary, the other promiscuous regions (residues 161-175 and 171-185) contained epitopes not naturally processed \textit{in vitro}. The immunodominant epitopes identified will be useful in the design of peptide-based cancer vaccines, and in the study of the functional state of tumor specific CD4+ T cells in patients bearing MAGE-3 expressing tumors.
Introduction

Evidences for a critical role of CD4+ T cells in orchestrating the anti-tumor response are increasing (rev. in 1-2). CD4+ T cells provide help for induction and maintenance of anti-tumor CD8+ T cells, and may exert effector functions, both indirectly via macrophages and eosinophils activation, and directly when inflammatory responses produce cytokines such as IFN-γ that induce expression of MHC class II molecules on tumor cells. In addition, IFN-γ produced by anti-tumor CD4+ T cells may function as an anti-angiogenic factor (3-4). CD4+ T cells specific for tumor antigens have been described in humans (rev. 1, 5-6).

MAGE-3 is a tumor specific antigen widely expressed in solid tumors such as melanoma, lung carcinoma, head and neck carcinoma and hematological malignancies, including T cell leukemias and myeloma but not in normal tissues (with the exception of testis) (7). The MAGE gene family comprises several related genes divided into three clusters, named MAGE-A, -B and -C (7). The gene previously known as MAGE-3 belongs to the MAGE-A, and it has received the official name MAGE-A3. We will refer to it, from now on as MAGE-3, in accordance to our previous report (8). Clinical trials in metastatic neoplastic patients using the HLA-A1 restricted MAGE-3 epitope yielded some clinical results (9-13). However, the immune responses were too weak and transient to eradicate tumor cells in the majority of immunized patients. The additional use of CD4 epitopes might help the induction of a memory anti-tumor response, and improve the clinical efficacy of vaccines.

Several allele-specific CD8 and CD4 epitopes of MAGE-3 have been identified (rev. in 14). Promiscuous epitopes, i.e. able to bind to different HLA-DR alleles, are crucial to increase the number of patients eligible for peptide-based cancer vaccination. Up-to now, only MAGE-3146-160 has been described by us (8) and others (15), as a
promiscuous epitope presented in association with HLA-DR11, HLA-DR4 and HLA-DR7.

In this paper, we report the results of the analysis of the MAGE-3 tumor antigen for promiscuous CD4 epitopes by a combined approach of computational identification of candidate T cell-epitopes, followed by *in vitro* biological validation analysis of the predicted sequences as forming natural epitopes recognized by CD4+ T cells.

We identified four immunodominant regions (residues 111-125, 146-160, 191-205, and 281-295), containing naturally processed epitopes, that were recognized by most donors in association with 3 to 4 different HLA-DR alleles. These epitopes, potentially recognized by more than 90% of Caucasians, will be useful for both clinical investigation and cancer immunotherapy.
Methods

T cell epitope prediction and peptides’ synthesis.

We used the TEPITOPE algorithm (16) that allows the identification of i) HLA class II ligands binding in a promiscuous or allele-specific mode and ii) the effects of polymorphic residues on the specificity of HLA class II ligands, to select 11 sequences of the MAGE-3 protein for peptide synthesis. The program permits the prediction and the parallel display of ligands for each of the 25 HLA-DR alleles incorporated in the software (examples are reported in 16). We set the prediction threshold (i.e. the percentage of best scoring natural peptides) at 5%, and we selected the sequences predicted to bind at least 40% of the 25 HLA-DR alleles, plus an additional sequence (residues 21-35) predicted to bind 16%. The MAGE-3 sequences comprising residues 141-155, 146-160, 156-170, 171-185 and 281-295, forming Pool I, were already described (8). The MAGE-3 sequences 21-35, 111-125, 161-175, 191-205, 251-265 and 286-300, forming Pool II were synthesized by manual parallel synthesis as described in (17). The peptide purity was verified by reverse-phase high performance liquid chromatography and electron spray mass spectrometry. The synthetic peptides were lyophilized, reconstituted in DMSO at 10 mg/ml, and diluted in RPMI 1640 (GIBCO, Grand Island, NY) as needed.

Peptide binding assays.

Peptide interactions with detergent-solubilized HLA-DR molecules were measured using an ELISA-based high-flux competition assay (18). HLA-DR molecules were affinity purified from human Epstein-Barr virus transformed lymphoblastoid cell lines (LCL) as described in (19-20). The assays measured the ability of unlabeled peptides to compete with a biotinylated indicator peptide for binding to purified HLA-DR
molecules (21). To determine relative binding affinity, the promiscuous HA\textsubscript{307-319} peptide from influenza hemagglutinin (21) was included in each competition assay.

**Subjects and cells.**

Peripheral blood mononuclear cells (PBMC) were obtained from four healthy subjects (donors #1, #2, #3 and #4) and two melanoma patients (donors #5 and #6). Melanoma cell lines MD TC, OI TC and GF TC were established from two cutaneous and one lymph node metastases respectively from three melanoma patients, and line SK-Mel-24 was purchased from the ATCC (Rockville, MD). MAGE-3 expression by patients’ biopses and melanoma cells lines was verified by reverse transcriptase-polymerase chain reaction (RT-PCR) and in melanoma cells also by intracytoplasmic staining (data not shown). The HLA-DR types of donors and melanoma cell lines were identified by molecular or serologic typing, and are reported in the Figures. Homozygous LCL used were: Bor (DR11) and Mun (DR3), established in our laboratory; Leis (DR4), kindly provided by F. Marincola (NIH, Bethesda, MD); BM21 (DR11) purchased from ATCC; and Pitout (DR7), purchased from the ECCC (Salisbury, UK).

**Propagation of CD4\(^+\) T cells.**

Synthetic peptides corresponding to Pool I and Pool II (see Table 1) were used to stimulate the PBMC from the different donors. Briefly, 20x10\(^6\) PBMC were cultured for 7 days in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with heat inactivated human serum (10%), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (50 µg/ml) (Biowhittaker, Walkersville, MD), containing Pool I or Pool II (1µg/ml of each peptide). The reactive blasts were isolated on a Percoll gradient (22), expanded in IL-2 containing medium (10 U/ml; Lymphocult, Biotest Diagnostic,
Dreieich, Germany) and restimulated at weekly intervals with the same amount of peptides plus irradiated (4000 rad) autologous PBMC as antigen presenting cells (APC).

**Flow cytometry.**

Cytofluorimetric analyses were performed on a FACStarPlus (Beckton Dickinson, Sunnyvale, CA). We used the following monoclonal antibodies (mAbs): anti-CD4-PE and anti-CD8-FITC (Beckton Dickinson), L243 (anti-MHC class II) (ATCC), 57B [originally described as an anti-MAGE-3 mAb (23), and later identified as an anti-MAGE-A4 mAb that cross-reacts with several MAGE-A proteins (24)] kindly provided by G. Spagnoli (Basel, Switzerland), and 20.4 (mAb that recognize the truncated form of the human low affinity nerve growth factor receptor-ΔNGFr) (ATCC). FITC-rabbit anti-mouse Ig antibody (DAKO A/S, Glostrup, Denmark) was used as second step reagent in indirect immunofluorescence stainings. For intracytoplasmic staining, melanoma cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.05% NP40 and then stained as described in (25).

**RT-PCR analysis.** Total RNA was extracted by the use of RNAzolTMB (Biotech, Houston, TX), according to manufacture's instructions. Single-stranded cDNA was synthesized from 2 µg of total RNA, by Moloney murine leukemia virus-derived reverse transcriptase (Life Technologies), in the presence of 20U of RNasin (Promega, Madison, WI). cDNA coding for MAGE-3 was detected by PCR amplification as described in (26). Samples scored positive when a band of the appropriate size was visible on an agarose gel in the presence of ethidium bromide.
**Proliferation assay.**

CD4+ T cells and autologous irradiated PBMC or HLA-DR matched homozygous LCL as APC were diluted at a 1:10 or 1:5 ratio respectively, and used as described in (8). Stimulants are indicated in the Figures. In inhibition experiments, mAb L243 or an isotype matched irrelevant mAb, were added at the concentrations indicated in the Figures. After 48h the cultures were pulsed for 16h with [3H]TdR (1 mCi, well, 6.7 Ci/mol, Amersham Corp., Milan, Italy). The cells were collected with a Skatron Titertek multiple harvester (Skatron Inc., Sterling, VA) and the thymidine incorporated was measured in a liquid scintillation counter.

**Selection of peptide specific CD4+ T cells by magnetic separation.**

To select MAGE-3161-175 specific CD4+ T cells from donor # 5 from the polyclonal cell line, we first incubated the cells with the peptide in the presence of homozygous DR4-PBMC and then we used the MACS Cytokine secretion assay - Cell enrichment and detection kit (Miltenyi Biotec), following the manufacturers’ instructions. MAGE-3161-175 specific CD4+ T cells were then weekly restimulated in the presence of the peptide (1-5µg/ml) and DR4 PBMC as APC.

**Recombinant viruses and infection of LCL.**

The cDNA encoding li.MAGE-3 (27), a fusion protein between the human invariant chain (Ii) and MAGE-3, was kindly provided by P. van der Bruggen (Brussel Belgium). The retroviral vectors M3-CSM and liM3-CSM, which encode the ΔLNGFr, and either the full-length MAGE-3 protein or the fusion protein li.MAGE-3 respectively, were produced as reported previously (28). LCL were transduced by coculture with irradiated packaging cells producing the M3-CSM or the liM3-CSM vectors, produced
by Phoenix cells (GP Nolan, Standford, CA). The percentage of infected cells was evaluated 48 hours after transduction by flow cytometry for ΔLNGFr expression with the mAb 20.4 (28). The ΔLNGFr-positive cells were purified by magnetic cell-sorting, using rat anti-mouse IgG1-coated beads (Dynabeads M-450, Dynal A.S., Oslo, Norway).

**Cytotoxicity assay.**

CD4+ T cells were tested for specific lytic activity in a standard 4-h $^{51}$Cr release assay as described in (29). The following targets were used: melanoma cells MD TC, OI TC, GF TC and SK-Mel-24, LCL and LCL engineered to express MAGE-3 (LCL-M3). In cold target competition assays, unlabeled target cells (cold targets) were added to CD4+ T cells (effectors) and $^{51}$Cr-labeled target cells (hot targets) at serial ratios of cold-to-hot target cells. The percentage inhibition was calculated as follow: $\left[\frac{\left(\% \text{ specific lysis without cold target}\right) - \left(\% \text{ specific lysis with cold target}\right)}{\left(\% \text{ specific lysis without cold target}\right)}\right] \times 100$.

**CD4+ T cell stimulation assay.**

LCL-M3, LCL-IiM3 (LCL expressing Ii.MAGE-3) or melanoma cells were tested for their ability to induce the production of IFN-γ by peptides specific CD4+ T cells, after 24-48h of incubation, using a standard ELISA (Biosource Europe, SA, Nivelles, Belgium), following the manufacturers’ instructions.
Results

We selected 11 MAGE-3 sequences, based on prediction by TEPITOPE, and used synthetic peptides corresponding to the selected sequences to confirm their binding capability in competition assays, using 7 common HLA-DRB1 alleles (Table 1).

Table 1. Determination of HLA-DR binding of MAGE-A3 synthetic peptides corresponding to the sequences predicted by TEPITOPE to form promiscuous epitopes

<table>
<thead>
<tr>
<th>Residues</th>
<th>Sequences</th>
<th>% predicted binding alleles</th>
<th>HLA-DR 1 alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%0101</td>
<td>%1501</td>
</tr>
<tr>
<td>Pool I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>141-155</td>
<td>GNWQYFPVIFS</td>
<td>68</td>
<td>25</td>
</tr>
<tr>
<td>146-160</td>
<td>FFPVIFSKASSSLQL</td>
<td>66</td>
<td>10</td>
</tr>
<tr>
<td>156-170</td>
<td>SSLQLVFGIELMEVD</td>
<td>68</td>
<td>7</td>
</tr>
<tr>
<td>171-185</td>
<td>PIQHLYIFATCLGLS</td>
<td>96</td>
<td>0,3</td>
</tr>
<tr>
<td>281-295</td>
<td>TSVKVLHNMVKISG</td>
<td>80</td>
<td>15</td>
</tr>
<tr>
<td>Pool II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21-35</td>
<td>EALGLYGAQAPATEE</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>111-125</td>
<td>RKVAELVHFLKLYR</td>
<td>76</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>161-175</td>
<td>VFGIELMEVPDGHL</td>
<td>76</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>191-205</td>
<td>GDQNMPKAGLLIV</td>
<td>40</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>251-265</td>
<td>VQNYLEYRVQPGSD</td>
<td>48</td>
<td>&gt;100*</td>
</tr>
<tr>
<td>286-300</td>
<td>VIHNMVKSIGPGHS</td>
<td>88</td>
<td>15</td>
</tr>
<tr>
<td>HA (307-319)</td>
<td>PKYVKQNTKLVAT</td>
<td>84</td>
<td>0,18</td>
</tr>
</tbody>
</table>

The binding data are expressed in terms of relative binding capacity (IC50 µM), calculated as concentration of competitor peptide required to inhibit 50% of the binding of an allele specific biotinylated peptide (indicator peptide). *IC50 values higher than 100 µM are outside the sensitivity limits of the binding assay.

†HA 307-319 = promiscuous sequence from influenza hemagglutinin

‡% of alleles predicted to bind the indicated sequences, with a threshold set at 5% (100% is represented by the 25 alleles incorporated in the software). The threshold is defined as the percentage of best scoring natural peptides, and comprise a range between 10-1%, see text for details.

We grouped the 11 peptides into the Pool I and the Pool II, based on the results of the binding assays. Peptides forming Pool I had the highest promiscuity, peptides forming Pool II bound well to at least 3 different HLA-DR alleles. We used Pool I and Pool II to propagate polyclonal T cell lines from 6 donors. Total PBMC were stimulated with Pool I or Pool II or both in independent cultures for 7 days, activated cells were enriched by a density gradient, expanded in the presence of IL-2 and weekly...
restimulated with irradiated peptide-pulsed autologous PBMC. For five donors, after 2 cycles of stimulation, we obtained lines that comprised only CD4+ T cells. In the case of donors #1, a CD8+ T cells depletion step was necessary to obtain more than 90% CD4+ T cells after four cycles of stimulation (data not shown). CD4+ T cell lines from healthy subjects #1, #2, #4 and from patient #5 could be propagated for several months. The lines from healthy subject #3 and patient #6 could be propagated only for a few weeks, and did not expand well in culture.

**Repertoire of HLA-DR restricted epitopes recognized by polyclonal CD4+ T cells propagated with Pool I and Pool II.**

We determined the epitope repertoire of the CD4+ lines from all donors by testing, after few cycles of stimulation, their proliferative response to each single peptide forming the Pools. Figure 1 summarizes the results obtained. Nine out of 11 peptides tested elicited proliferation of CD4+ T cells in at least two donors. MAGE-3_{111-125}, MAGE-3_{141-155} and the overlapping MAGE-3_{146-160}, MAGE-3_{191-205}, MAGE-3_{281-295} and the overlapping MAGE-3_{286-300}, were strongly recognized by most of the donors, consistent with a high degree of promiscuity. MAGE-3_{156-170} was weakly but significantly recognized by donors #1 and #5, and the overlapping peptide MAGE-3_{161-175} was significantly and strongly recognized by donors #4 and #5. MAGE-3_{171-185} was strongly recognized by donors #1 and #5. Recognition of the MAGE-3 sequences was HLA-DR restricted as demonstrated in vitro by inhibition of the proliferation of CD4+ T cells to the peptides in the presence of an anti-DR antibody in the cultures (data not shown, and Fig. 3B). MAGE-3_{21-35} and MAGE-3_{251-265} were never recognized.
**Figure 1. MAGE-3 sequence segments recognized by CD4+ T cells from six donors and their HLA-DR type.** Polyclonal CD4+ T cell lines propagated in culture with Pool I and Pool II were tested with each single peptides (10 µg/ml) forming the pools in 2-d microproliferation assays. The results are representative of several assays in the case of donor #1, #2, #4 and #5 and, at least 2 experiments, in the case of donor #3 and #6. Peptides that elicited a strong response are indicated in black (p<0.001), peptides that elicited an intermediate response are indicated in hatched segments (0.001<p<0.05), and peptides that elicited a low but still significant response are indicated in white (p<0.05). Responses significantly higher than the blanks (i.e.: the basal level of proliferation of CD4+ T cells in the presence of autologous PBMC as APC) were determined by unpaired, one-tailed Student’s t test. n.t. =not tested.

**HLA-DR restriction molecules for the immunodominant sequences.**

To identify the HLA-DR restricting allele for the immunodominant sequences, CD4+ T cells from donors #1, #2 and #5 were challenged in microproliferation assays with APC, homozygous for each of the two HLA-DRB1 alleles expressed by the donor, pulsed with individual peptides (Figure 2).
We did not perform HLA-DR restriction on donor #4 because HLA-DR11 homozygous, nor on donors #3 and #6, because CD4+ T cells did not expand in culture enough to allow further characterization. MAGE-3\textsubscript{111-125} was recognized in association with HLA-DR1, HLA-DR4 and HLA-DR11 (Fig. 2A). Moreover, MAGE-3\textsubscript{114-127}, which largely overlaps MAGE-3\textsubscript{111-125}, was shown by van der Bruggen (27) to be recognized in association with HLA-DR13. MAGE-3\textsubscript{146-160} was recognized in association with HLA-DR1, HLA-DR4, HLA-DR11 and HLA-DR7 (Fig. 2B). MAGE-3\textsubscript{191-205} was recognized in association with HLA-DR1, HLA-DR4 and HLA-DR11 alleles (Fig. 2C). MAGE-
3281-295 was recognized in association with HLA-DR1, HLA-DR4 and HLA-DR11 (Fig. 2D). The same immunodominant sequence could be recognized in different donors with either one or both the HLA-DRB1 alleles expressed. Moreover, different donors sharing the same alleles might preferentially recognize the same epitope with different alleles, probably due to a difference in precursors’ frequency.

**Immunodominant sequences contain naturally processed epitopes.**

MAGE-3114-127, largely overlapping MAGE-3111-125, MAGE-3146-160 and MAGE-3281-295 were already described to contain naturally processed epitopes recognized in association with HLA-DR13 (27), HLA-DR4 and HLA-DR7 (15), and HLA-DR11 (8), respectively. MAGE-3111-125 contains a natural epitope that is formed after processing through the exogenous pathway (28); we could not test the natural processing pathways for MAGE-3111-125, which most likely contain the same natural epitope, because we failed to obtain CD4+ T cells recognizing exclusively that sequence. MAGE-3146-160 was formed after both endogenous and exogenous pathways (15). In the present work we found that MAGE-3146-160 is recognized by CD4+ cytotoxic T cells (CTL) from donor #2 (HLA-DR3, -DR7) when expressed on HLA-DR7 matched melanoma cells (GTTC) and DR7-LCL engineered to express MAGE-3 (data not shown). MAGE-3281-295 was shown by us (8) to contain an epitope formed after both endogenous and exogenous pathways and recognized by CD4+ CTL.

To verify whether MAGE-3191-205 contains a natural epitope, we used polyclonal CD4+ T cells from donor #4 after 9 weeks of propagation with Pool II, when the cells strongly and exclusively recognized MAGE-3191-205 (Fig. 3A, lower panel). Recognition was HLA-DR restricted as shown in Fig. 3B. DR11-LCL were engineered to express MAGE-3 (DR11-LCL-M3), and used in cytotoxicity assays. As shown in Fig. 3C,
MAGE-3\textsubscript{191-205} specific CD4+ T cells killed DR11-LCL-M3, but not wild type DR11-LCL. Moreover, CD4+ CTL killed the MD TC and OI TC melanoma cells, which express MAGE-3 and HLA-DR11, but not SK-Mel 24, expressing unrelated HLA-DR (Fig. 3D). In addition, we performed cold target inhibition experiments, in which cold targets (DR11-LCL) pre-incubated with peptide MAGE-3\textsubscript{191-205} were added to the hot tumor targets. Killing of MD TC and OI TC was inhibited by addition of peptide pulsed DR11-LCL (up to 75\% and 98\%, respectively), suggesting that this peptide contains an epitope that is presented by the melanoma cells (Fig. 3E and F). The percentage of inhibition of DR11-LCL (Fig. 3E) or DR11-LCL pulsed with an irrelevant peptide (Fig. 3F) was comparable in the two cases, and up to 25-30\%. The high cold/hot ratio (50\%) required observing a specific inhibition of MD TC killing, compared to OI TC, might be related to different binding affinity of the peptide for the two subtypes of DR11-LCL (DR*1104 and DR*1101) used. In the case of MD TC, competition for MHC binding to the cold or hot targets of free peptide released from the LCL in culture might reduced the ability of these LCL to inhibit the killing.
Figure 3. Characterization of the CD4+ T cell response to MAGE-3<sub>191-205</sub>. CD4+ T cells were propagated from donor #4 (HLA-DR11) by weekly restimulation with Pool II. Panel A: Proliferative responses, measured in 2-d microproliferation assays, to each single peptide forming Pool II (10 µg/ml) after 4 and 9 weeks of propagation of the line. The data are means of triplicate determination ± SD. The blank (i.e.: the basal level of proliferation of CD4+ T cells in the presence of autologous PBMC as APC) is expressed as B+DR11-PBMC. Responses significantly higher than the blanks are indicated as **p<0,001, ***p<0,001 (determined by unpaired, one-tailed Student’s t test).

Panel B: Responses to Pool II (5 µg/ml), in the absence or in the presence of L243 mAb or an irrelevant control mAb (0,5 µg/ml). The blank (i.e.; the basal level of proliferation of CD4+ T cells in the presence of autologous PBMC as APC) is expressed as B+APC.

Panels C-F: Recognition of naturally processed MAGE-3 sequences by CD4+ T cells at 9 or more weeks of propagation. (C) Cytolytic activity, measured in a 51Cr release assay, against wild type DR*1104-LCL and DR*1104-LCL engineered to express MAGE-3 (DR*1104-LCL-M3). (D) Cytolytic activity against HLA-DR matched (MD TC and OI TC) and HLA-DR unrelated (SK-Mel 24) melanoma cells. (E-F) Cold target inhibition experiments. (E) Cold targets (MD TC, DR*1104-LCL and DR*1104-LCL pulsed with MAGE-3<sub>191-205</sub>) were used to inhibit the lytic activity of CD4+ cytotoxic T cells against hot MD TC (E/T ratio 40:1). The percentage of specific lysis against MD TC in the absence of cold target was 22 ± 1,3. (F) Cold targets (DR*1101-LCL and DR*1101-LCL pulsed with MAGE-3<sub>191-205</sub> or MAGE-3<sub>21-35</sub>) were used to inhibit the lytic activity of CD4+ cytotoxic T cells against hot OI TC (E/T ratio 40:1). The % lytic activity against OI TC and DR*1101-LCL, as negative control, are shown in black symbols. The data presented in Panels C-F are representative of at least three experiments, and are means of triplicate determination ± SD.
MAGE-3\textsubscript{161-175} and MAGE-3\textsubscript{171-185} contain epitopes not naturally processed \textit{in vitro}.

To verify whether MAGE-3\textsubscript{161-175} contains a natural epitope, we used polyclonal CD4+ T cells from donor #5, after selection of peptide MAGE-3\textsubscript{161-175} specific CD4+ T cells by magnetic sorting (Fig. 4B). CD4+ T cells did not secrete IFN-\gamma after challenge with melanoma cells or DR4-LCL engineered to express MAGE-3 (DR4-LCL-M3), nor DR4-LCL engineered to express Ii.MAGE-3 (DR4-LCL-IiM3), suggesting that MAGE-3\textsubscript{161-175} does not contain a natural epitope produced after processing through both the endogenous and the exogenous pathways (Fig. 4C).

**Figure 4. Characterization of the CD4+ T cell response to MAGE-3\textsubscript{161-175}.** CD4+ T cells from donor #5 (HLA-DR4, -DR11) were propagated by weekly restimulation with Pool II. *Panel A:* Proliferative responses, measured in 2-d microproliferation assays, to each single peptide forming Pool II (10 µg/ml) after 5 weeks of propagation of the line. The blank (i.e.: the basal level of proliferation of CD4+ T cells in the presence of homozygous PBMC or LCL as APC) is expressed as B+DR4-LCL. *Panel B:* Proliferative responses to each single peptide forming Pool II (10 µg/ml) after magnetic selection of MAGE-3\textsubscript{161-175} specific CD4+ T cells. *Panel C:* IFN-\gamma release of CD4+ T cells in the presence of melanoma cells, DR4-LCL, MAGE-3\textsubscript{161-175} pulsed DR4-LCL, DR4-LCL-M3 and DR4-LCL-IiM3. The data in *Panels A-C* are means of triplicate determination ± SD. Responses significantly higher than the blanks are indicated as **p<0.001, ***p<0.001 (determined by unpaired, one-tailed Student’s t test).
To characterize the recognition of peptide MAGE-3_{171-185}, we used polyclonal CD4+ T cells from donor #5, after 12 weeks of propagation in culture, when they strongly and exclusively recognized MAGE-3_{171-185} (Fig. 5B). CD4+ T cells did not recognize melanoma cells or DR4-LCL-M3, nor DR4-LCL-IiM3, suggesting that this sequence does not contain a natural epitope as well (Fig. 5C). To verify that DR4-LCL-M3 and DR4-LCL-IiM3 express a sufficient amount of the MAGE-3 protein to be recognized by CD4+ T cells, we tested its expression by flow cytometry. MAGE-3 intracytoplasmic staining in transduced DR4-LCL and DR11-LCL recognized by CD4+ T cells specific for MAGE-3_{191-205} (Figure 3D), showed that they contain a comparable amount of MAGE-3 (data not shown).

Figure 5. Characterization of the CD4+ T cell response to MAGE-3_{171-185}. CD4+ T cells from donor #5 (HLA-DR4, -DR11) were propagated by weekly restimulation with Pool I. Panels A and B: Proliferative responses, measured in 2-d microproliferation assays, to each single peptide forming Pool I (10 µg/ml) after 3 weeks (A) and after 12 weeks (B) of propagation of the line. The blank (i.e.; the basal level of proliferation of CD4+ T cells in the presence of homozygous PBMC as APC) is expressed as B+DR4-PBMC or B+DR11-PBMC or B-DR4-LCL. Panel C: IFN-γ release of CD4+ T cells in the presence of melanoma cells, DR4-LCL, MAGE-3_{171-185} pulsed DR4-LCL, DR4-LCL-M3 and DR4-LCL-IiM3. The data in Panels A-C are means of triplicate determination ± SD. Responses significantly higher than the blanks are indicated as *p<0.05, **0.001<p>0.05, ***p<0.001 (determined by unpaired, one-tailed Student’s t test).
Discussion

In this study we describe a successful strategy to identify promiscuous CD4 epitopes on candidate tumor antigens. Using a combined approach of computational prediction for promiscuous MHC class II epitopes and in vitro validation by biological assays, we identified four immunodominant regions of MAGE-3, that contain naturally processed epitopes presented by several different HLA-DR alleles.

For most of the predicted epitopes, we found an excellent correlation between prediction and the ability of the peptides to bind promiscuously to HLA-DR molecules and to stimulate CD4+ T cells from different donors. This was especially striking for overlapping sequences MAGE-3\textsubscript{141-155} and MAGE-3\textsubscript{146-160}, and overlapping sequences MAGE-3\textsubscript{281-295} and MAGE-3\textsubscript{286-300}. On the contrary, MAGE-3\textsubscript{111-125} and MAGE-3\textsubscript{191-205} that, as predicted, bound HLA-DR*1101 but not HLA-DR*0101 and HLA-DR*0401, could strongly stimulate proliferation of CD4+ T cells in association with all the 3 HLA-DR alleles, pointing to the importance of the in vitro studies for validation of the predicted sequences. MAGE-3\textsubscript{251-265}, which was predicted to bind 48% of the alleles, did not bind well to the HLA-DR molecules that we tested (Table 1), and was never recognized by CD4+ T cells. MAGE-3\textsubscript{251-265} partially overlaps MAGE-3\textsubscript{243-258}, which is recognized in association with HLA-DP4, but not with HLA-DR (30). MAGE-3\textsubscript{21-35}, which was predicted to bind poorly, never elicited CD4+ T cell responses. In agreement, Kobayashi et al. (15) also reported that the overlapping peptide sequence MAGE-3\textsubscript{22-36} could stimulate T lymphocytes from different donors in association with HLA-DR4, but the CD4+ T cells did not expand in culture (15). All the other sequences activated CD4+ T cells from at least 2 donors, and did so in association with
2 to 4 different alleles (Figure 1 and Table 2), confirming the ability of TEPITOPE to predict promiscuous epitopes.

Most importantly, the immunodominant epitopes identified here are recognized in association with HLA-DR1, HLA-DR4, HLA-DR11 and HLA-DR7 that, with the addition of HLA-DR13 (27) cover up to 94% of the alleles expressed in Caucasians (Table 2). We can not exclude that HLA-DRB3 or HLA-DRB4 molecules in addition to HLA-DRB1 can also present the identified sequences.

Table 2. Summary of CD4 immunodominant MAGE-3 epitopes recognized by CD4+ T cells and their HLA-DR presenting alleles

<table>
<thead>
<tr>
<th>HLA-DR</th>
<th>Residues 111-125</th>
<th>Residues 146-160†</th>
<th>Residues 191-205</th>
<th>Residues 281-295‡</th>
<th>HLA-DR frequency (%) in Caucasians</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>DR4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>24-26</td>
</tr>
<tr>
<td>DR11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>24-27</td>
</tr>
<tr>
<td>DR13</td>
<td>114-127*</td>
<td>n.t.¶</td>
<td>n.t.¶</td>
<td>n.t.¶</td>
<td>19</td>
</tr>
<tr>
<td>DR7</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>26</td>
</tr>
</tbody>
</table>

* described in (27)
† described also in (8-15)
‡ described also in (8)
¶ not tested

The immunodominant regions contain epitopes that are naturally processed from different sources of tumor antigen and by different APC. In our present and previous studies (8), sequences MAGE-3141-160, MAGE-3191-205, and MAGE-3281-295 were recognized by cytolytic CD4+ T cells after endogenous processing of MAGE-3 by LCL and melanoma cells. Moreover, sequence MAGE-3281-295 was recognized by CD4+ T cells after processing and cross-presentation of the recombinant protein by autologous...
APC (8). MAGE-3\textsubscript{141-160} reactive T helper cells were also shown to recognize various form of the MAGE-3 protein (tumor cell lysate, dead/apoptotic tumor cells or recombinant protein) (15). MAGE-3\textsubscript{111-125} largely overlaps MAGE-3\textsubscript{114-127}, that is produced after processing and presentation of recombinant MAGE-3 protein by autologous dendritic cells and by melanoma cells that express an invariant chain-MAGE-3 fusion protein in the endosomal/lysosomal compartment (27). On the contrary, MAGE-3\textsubscript{161-175} and MAGE-3\textsubscript{171-185} specific CD4\textsuperscript{+} T cells did not recognize \textit{in vitro} the MAGE-3 protein after processing through either the endogenous or the exogenous pathways. A possible explanation for this finding is that we failed to detect recognition of the native epitope because peptides bind to HLA molecules in a different conformation when they are loaded exogenously or when they are generated endogenously, as shown by Unanue and colleagues (31, 32). At difference with “naturally processed” epitopes that derive from processing of the whole protein antigen, “cryptic” epitopes are presented after the exposure to the antigen in peptide form, but not after processing of the whole protein antigen (33). Protease activity as well as the nature of the antigen presenting cells during the processing of the protein may have a major influence on the repertoire of naturally processed or cryptic epitopes displayed for T cell recognition (33). It is interesting that, in addition to the immunodominant epitopes, these sequences were strongly and predominantly recognized after long-term culture by CD4\textsuperscript{+} T cells from donor #5, which is a metastatic melanoma patient. We can not completely exclude that priming of CD4\textsuperscript{+} T cells recognizing these sequences occurred \textit{in vivo} as a consequence of epitope spreading during inflammatory conditions and operated by APC not tested in our \textit{in vitro} studies, leading to the formation of “cryptic” epitopes.
The use of pools of peptides for CD4+ T cells stimulation has the advantage that competition among peptides with different binding affinity for the presenting alleles, mimics in culture what occurs in vivo, where different processed peptides compete for MHC class II binding and T cell receptor recognition. Therefore, the finding that some peptides are predominant in our cultures may be related to their higher affinity for the MHC class II molecules, or to the presence of peptide specific CD4+ T cells expressing T cell receptor with high affinity for the peptide-MHC class II complexes. The finding that CD4+ T cells specific for MAGE-3\textsubscript{191-205} and MAGE-3\textsubscript{281-295} proliferate at peptide concentration as low as 1-10 ng/ml (data not shown), supports the latter possibility. The predominant recognition of different immunodominant regions in different donors may also be related to their precursor repertoires.

Animal models have shown that tumor specific CD4+ T cells, in addition to playing a role in the induction of the anti-tumor immune response, have direct effector functions (1-4). The results of the present and previous studies by us and others (8, 33-36), strongly support a potential role of human tumor specific CD4+ T cells as effectors of the anti-tumor immune response. Although the significance of the CD4+ T cells as compared to CD8+ T cells in vivo remains to clarify, lytic function of CD4+ T cells may be relevant when neoplastic cells, constitutively or as a consequence of inflammatory milieu, express MHC class II molecules. It will be interesting to verify the expansion of CD4+ CTL ex vivo from cancer patients, as it has been shown in (37), where CD4+ perforin + T cells were expanded in donors with chronic viral infections.

Clinical trials of vaccination with synthetic peptides corresponding to the HLA-A1 restricted MAGE-3 epitope recognized by CD8+ T cells have started in patients bearing MAGE-3 positive tumors (9-13). The additional use of peptides corresponding to the immunodominant CD4 epitopes identified here would increase the magnitude and life
span of the anti-tumor immune response in these patients. Moreover, the identification of these epitopes will help the study of the number and function of MAGE-3 specific CD4 + T cells, and the reliable monitoring the immune responses in cancer patients before and after vaccination.
Acknowledgments

We thank Cinzia Arcelloni for HPLC analyses, Paolo Dellabona, Matteo Bellone and Angelo Manfredi for enjoyable discussions and critical reading of the manuscript.
References


36. Touloukian CE, Leitner WW, Topalian SL, et al. Identification of a MHC class II-

Footnotes

Abbreviation used: PBMC; peripheral blood mononuclear cells; APC; antigen presenting cells; LCL, Epstein-Barr virus transformed lymphoblastoid cell lines; mAb, monoclonal antibodies; LCL-M3, LCL engineered to express MAGE-3, LCL-IiM3, LCL engineered to express Ii.MAGE-3; ΔLNGFr, truncated form of human low affinity nerve growth factor receptor; CTL, cytotoxic T cells; RT-PCR, reverse transcriptase-polymerase chain reaction
Identification of immunodominant regions among promiscuous HLA-DR restricted CD4+ T cell epitopes on the tumor antigen MAGE-3

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