Differential pattern of CD4⁺ and CD8⁺ T-cell immunity to MAGE-A1/A2/A3 in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma

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The factors that determine progression from monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma are unclear but may include the breakdown of immune surveillance. Cancer testis antigens (CTAgs) are expressed by the majority of myelomas and MGUS tumors and are a potential immune target. We have characterized CD4⁺ and CD8⁺ T-cell immune responses to MAGE-A1/A2/A3 in these patients. CD4⁺ T-cell immunity to MAGE proteins is stronger and more frequent in MGUS compared with myeloma with a predominately CD45RA⁺CCR7⁺ effector memory profile and cytotoxicity against MAGE-positive cell lines. In contrast CD8⁺ T-cell immune responses were present almost exclusively in patients with multiple myeloma, correlating with disease, with a CD45RA⁺CCR7⁺ memory phenotype, localizing poorly to the bone marrow but were able to lyse myeloma cell lines in vitro. This suggests that the CD4⁺ CTAg-specific immune response may play a role in controlling tumor growth, whereas the efficacy of the CD8⁺ T-cell response appears to be limited in vivo. Despite this, patients with evidence of a CTAg-specific immune response had a 53% reduction in mortality over a median follow-up of 4 years. These findings have important implications for clinical approaches to CTAg-specific immunotherapy in patients with cancer. (Blood. 2008;112:3362-3372)

Introduction

Multiple myeloma (MM) is a malignant disease of bone marrow plasma cells and remains incurable with current chemotherapy regimens.¹³ Although allogeneic stem cell transplantation is associated with significant morbidity and mortality, a graft-versus-myeloma effect has clearly been demonstrated,¹⁴ and tumor-specific immunotherapy offers potential in the management of this disease.⁶

CTAgs are a family of proteins whose expression is restricted to spermatogenic germ cells,⁷ and more than 50 CTAg genes exhibit mRNA expression in testis with minimal expression in adult somatic cells.⁸⁹ CTAg expression is observed in a wide variety of malignancies including melanoma and epithelial tumors. T-cell tolerance to CTAg peptides appears incomplete and functional T-cell responses may be generated as a consequence of CTAg expression in tumor cells.¹⁰ Expression of several CTAg proteins, including MAGE and NY-ESO, has been described in malignant plasma cells from patients with MM as well as transformed cell lines.¹¹-¹⁸ CTAg expression is detected most commonly in patients with advanced disease but is also found in a significant proportion of patients with MGUS.¹⁵ Recently, aberrant expression of CTAgs has been shown to be a negative prognostic marker for MM¹⁷,¹⁸ and to be associated with a proliferative subset of tumor cells.¹⁵ There is also a correlation in MM¹⁸ and in certain solid tumors²⁰ between the age of the patient and the number of expressed CTAgs. The mechanisms that underlie this expression are unclear but are at least partially related to demethylation of gene promoter sequences.⁷

CTAgs have been the target for several vaccination studies in melanoma with tumor regression reported in approximately 5% to 20% of cases and no reports of vaccine toxicity.²¹-²³ MM is characterized by immune dysfunction and there have been few reports of T-cell immunity to CTAg proteins in patients with MM.¹¹-¹⁸ MGUS is a premalignant precursor of MM with a 1% annual risk of progression to myeloma. The factors that determine progression are uncertain but could include immune control of plasma cell proliferation. Given the potential role of CTAgs as targets for immunosurveillance and immunotherapy strategies, it is essential to understand the cellular and humoral immune responses to these proteins in MM and MGUS to maximize tumor-specific immune responses.⁶ Indeed, both NY-ESO-1–based immunotherapy using protein transduction of dendritic cells²⁴ and MA9E-A3 protein immunizations²⁵ are being developed in multiple myeloma patients.

We have previously reported CTAg-specific CD8⁺ T-cell responses in more than 40% of patients with MM.²⁶ Here we have characterized the CD4⁺ and CD8⁺ T-cell immune responses to MAGE-A1, MAGE-A2, and MAGE-A3 in patients with both MGUS and MM. We show that CD4⁺ T-cell immunity to these proteins is seen more commonly in patients with MGUS, whereas the CD8⁺ T-cell immune response was present almost exclusively in patients with MM. CD4⁺ and CD8⁺ CTAg-specific T-cell clones demonstrated high avidity for CTAg peptides and were cytotoxic for target cells expressing CTAg protein.

Methods

Patient samples

Patients with MM and MGUS were recruited from specialist clinics at the University Hospital NHS Trust and Heart of England NHS Trust (Birmingham,


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increase the sensitivity of detection and further characterize the reactive cells. CSA was performed according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, freshly isolated peripheral blood mononuclear cells (PBMCs) were seeded at a cell density of 10^6/mL in culture media containing RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 10% human serum (HD Supplies, Aylesbury, United Kingdom) and l-glutamine (Invitrogen). The cells were then left unstimulated overnight at 37°C 5% CO2. The next day peptides were added to the cultures for 3 to 6 hours at a final concentration of 10 μg/mL. DMSO and Staphylococcal enterotoxin B (1 μg/mL; Sigma-Aldrich, Poole, United Kingdom) were used as negative and positive controls, respectively. After stimulation, the cells were labeled with IFN-γ catch reagent for 5 minutes and then incubated for 45 minutes at 37°C under continuous rotation. Cells were then labeled with IFN-γ detection antibody (PE-labeled) and then were magnetically labeled, using anti-PE magnetic beads. Enrichment of IFN-γ secretion cells was performed by magnetic separation using the auto–magnetic-activated cell sorting (MACS) separation program Posseld (Miltenyi Biotec). The positively selected cells were labeled with CD4-FITC and CD8 PC5 (Beckman Coulter, High Wycombe, United Kingdom), and propidium iodide (PI; 1 μg/mL; Sigma-Aldrich) was added before analysis to allow exclusion of dead cells. Analysis was performed with a Coulter EPICS XL flow cytometer (Beckman Coulter).

The frequency of cytokine-secreted antigen-specific T cells was calculated as a percentage of the total CD8+ or CD4+ T-cell pool on the basis of the number of cells detected in the positively selected fraction compared with the total number of CD8+ or CD4+ T cells within PBMCs. The frequency was also calculated from the absolute lymphocyte count and expressed as the number of cytokine-secreting antigen-specific T cells/mL.

### Memory phenotype of the reactive cells

The phenotype of CTAg-specific T cells was characterized using antibodies to CCR7-FITC (R&D Systems, Abingdon, United Kingdom) and CD45RA-PC5 (BD Pharmingen, San Diego, CA). The IFN-γ cytokine secretion assay was used initially and the positively selected cells were counterstained with CCR7-FITC and CD45RA-PC5. Analysis was performed with a Coulter EPICS XL flow cytometer (Beckman Coulter).

### Generation of CTAg-specific T-cell clones

After IFN-γ CSA and enrichment, cells were isolated under sterile conditions using 2 MS columns (Miltenyi Biotec) and cloned by limiting dilution assay (LDA) over irradiated (40 Gy) allogeneic PBMCs and LCLs in RPMI 1640 supplemented with 10% human serum (HD Supplies). IL-2 (100 U/mL), and PHA (5 μg/mL), IL-7 (5 μg/mL), IL-15 (2 ng/mL), and IL-21 (2 ng/mL; Peprotech, Rocky Hill, NJ) were added to established cultures.

### ELISA for quantification of IFN-γ cytokine production

Cloned T cells were washed twice in RPMI and incubated in 96-well V-bottom microculture plates with HLA-matched or -mismatched LCLs or MM cell lines U266, JN63, and H929 presupplemented for 1 hour with 5 μM peptide (or DMSO control) or transfected with MAGE-3. Supernatant was harvested onto Maxisorb (NUNC, Roskilde, Denmark) 96-well flat-bottom plates after 18 hours and assayed for IFN-γ by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, MA) in accordance with the manufacturer’s recommended protocol. For peptide titration experiments, the peptide was diluted from 10^−3 M to 10^−11 M and expressed as a percentage of maximum IFN-γ release.

### 51Cr cytotoxicity assay

After 51Cr labeling (1 hour), target cells (2500/well) were added to effector cells (25 000/well) at an E:T ratio of 10:1 in a final volume of 200 μL complete culture medium in 96-well V-bottomed plates. CD4+ T-cell clones were incubated for 12 hours and CD8+ T-cell clones for 6 hours at 37°C. Supernatants were harvested and the mean percentage of specific lysis of triplicate wells was calculated according to the formula: (experimental

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**Table 1. Antigenic peptides encoded by cancer testis antigens used in the cytokine secretion assay**

<table>
<thead>
<tr>
<th>Protein</th>
<th>HLA (I) restriction</th>
<th>HLA frequency (%) within the population</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-A1</td>
<td>A3</td>
<td>23</td>
<td>SLFRAVITK</td>
</tr>
<tr>
<td></td>
<td>A24</td>
<td>19</td>
<td>NYKHCFCPEI</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>26</td>
<td>EADPTGHYS</td>
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<tr>
<td>289-298</td>
<td>B7</td>
<td>17</td>
<td>RVRRFFPSL</td>
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<tr>
<td>MAGE-A2</td>
<td>A2</td>
<td>44</td>
<td>YLQLQVGFIEV</td>
</tr>
<tr>
<td>MAGE-A3</td>
<td>A24</td>
<td>19</td>
<td>TFPDLESEF</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>44</td>
<td>KVAELHVHL</td>
</tr>
<tr>
<td></td>
<td>A1/B35</td>
<td>26</td>
<td>EVDPFLGHLY</td>
</tr>
<tr>
<td>195-203</td>
<td>A24</td>
<td>19</td>
<td>IMPKAGLL</td>
</tr>
<tr>
<td>MAGE-A1/A2/A3/A6</td>
<td>DR13</td>
<td>19</td>
<td>LKYRAREPVTKAE</td>
</tr>
<tr>
<td>MAGE-A3</td>
<td>DR4/7</td>
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<td>VIFSKASSSLQL</td>
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<td>DR11</td>
<td>25</td>
<td>GDNQIMPKAGLLUV</td>
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<td>243-258</td>
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<td>75.63</td>
<td>KKLLOHQPQVENYLEY</td>
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<td>281-295</td>
<td>DR11</td>
<td>25</td>
<td>TSYKVYLVHMVKGIS</td>
</tr>
</tbody>
</table>

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United Kingdom). The study received approval from the local ethics committees at South Birmingham, Birmingham East, North, and Solihull, and informed written consent was obtained in accordance with the Declaration of Helsinki in all cases. Peripheral blood (20 mL) was obtained from patients at various stages of disease and mononuclear cells were purified by density centrifugation using Lymphoprep (Nycomed, Oslo, Norway). MM patients were sampled at a variety of disease stages including presentation and plateau phase (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Freshly isolated lymphocytes were used in all assays, and blood samples were processed within 3 to 6 hours of collection.

### Cell lines and culture conditions

Epstein-Barr virus (EBV)–transformed cell lines were prepared with prototype 1 strain B95.8 or prototype 2 strain Ag876. LCLs were cultured in medium containing 10% fetal calf serum and human leukocyte antigen (HLA) type was obtained from the Anthony Nolan Clinic (London, United Kingdom). To assess the ability of CD4+ T-cell clones to recognize processed antigen, 2 LCL cell lines of different HLA types (KS114 trans M3 DR52b and DESA-M3 DR52a) were transfected with a retrovirus containing the sequence coding for the fusion truncated protein invariant chain (1-80)-MAGE-A3 using established methods and this work was kindly carried out by S. Ottaviani (Ludwig Institute for Cancer Research, Brussels, Belgium).

MM cell lines U266, JJN3, and H929, expressing MAGE, were maintained in medium containing 10% fetal calf serum. The HLA type of these cell lines is listed as follows: U266: HLA A2, 3, B7, 14; DR15, 1, 51, DQ-5, 6. H929: A3, 24, B7, 18, Cw*0701, 60(40); DR15, DR17, DR52a, DR51, DQ-6, 2 DPw-2, DPw-4. JJN2: A3, 33, (19) B7, 14; DR15, 1, 51, DQ-5, 6. H929: A3, 24, B7, 18, Cw*0701, 0702, class II–negative.

### Detection of CTAg-reactive T cells using the cytokine secretion assay

Fourteen peptides derived from CTAg MAGE-A1/A2/A3 (Alta Bioscience, Birmingham, United Kingdom) were chosen from previously described epitopes for CD8+ and CD4+ T cells restricted by a wide variety of HLA alleles (HLA class I: A*0101, A*201, A*301, A*2401, B*702, and B*3501; and HLA class II DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, DPB1*0401, and DQB1*0601)27,28,33 (Table 1). The expression of these 5 CD4+ MAGE-specific peptides is representative of approximately 94% of the alleles expressed by the white population. The IFN-γ cytokine secretion assay (CSA) was used to detect functional responses, and magnetic selection with anti-PE microbeads was used to
release — spontaneous release) / (maximal release — spontaneous release) × 100%. Targets were HLA-matched or -mismatched LCLs or MM cell lines pre-exposed for 1 hour to 5 μM epitope peptide or to an equivalent concentration of DMSO solvent.

Statistical analysis
Mann-Whitney U test was used to test the level of significance between MAGE-specific CD8 versus CD4 T-cell responses in myeloma patients and between MAGE-specific CD8+ T-cell responses in myeloma versus MGUS patients. Linear regression statistical analysis was used to compare the correlation of the frequency of the MAGE-A1–specific CD8+ T-cell response with the paraprotein level over time in patient 7. Both of these were performed using Prism 4 for Windows (GraphPad, San Diego, CA).

The survival of MM patients was studied in relation to the presence or absence of a CTAg-specific CD8+ T-cell response using the R survival package (Vienna University, Vienna, Austria; http://www.r-project.org). The hazard ratio between the 2 groups was calculated using the Cox proportional hazards model and a log-rank test was used to calculate a P value. Statistical analysis of the survival data was performed by Wenbin Wei (Cancer Research UK Institute for Cancer Studies, University of Birmingham).

Results
MAGE-A1–, MAGE-A2–, and MAGE-A3–specific CD8+ T cells are detected more frequently in patients with MM than in patients with MGUS

We have previously reported the use of the IFN-γ CSA to detect functional CD8 T-cell responses to CTAg peptides25 and now extend this cohort to long-term follow-up of 53 patients. A CTAg-specific CD8+ T-cell response was observed in 21 of these patients, of which 19 were specific for MAGE-A1, MAGE-A2, or a MAGE-A3 peptide with a magnitude that represented 0.001% to 0.3% of the CD8+ T-cell pool (Figure 1).

MAGE-A1/A2/A3 expression has also been reported in plasma cells from patients with MGUS15 but there is no information as to whether this triggers a T-cell immune response. We therefore used the IFN-γ CSA to look for evidence of a CTAg-specific CD8+ T-cell response to pools of MAGE-A1/A2/A3 peptides in a cohort of 25 MGUS patients (Table 1). There was a significant difference between the frequency of MAGE-specific CD8+ T-cell responses in MGUS versus MM patients (P = .029). Only 2 responses were seen in this cohort and both were of very small magnitude, representing only 0.001% of the CD8+ T-cell pool (Figure 1). Interestingly, both of these patients also had significant MAGE-A1/A2/A3–specific CD4+ T-cell responses (Figure 1).

MAGE-specific CD4+ T-cell responses are more commonly observed in MGUS than in myeloma patients

Antigen-specific CD4+ T cells can play an important role in regulating CD8+ T-cell immune responses and may also have a direct cytotoxic activity on target cells. We therefore undertook a comprehensive analysis of the CD4+ T-cell response to cancer testis antigens using a panel of 5 T-cell epitopes from MAGE-A1/A2/A3 (Table 1).

The IFN-γ CSA was used to detect functional CD4+ T-cell responses in 32 patients with MM and 30 patients with MGUS. A CD4+ T-cell response was seen in only 1 patient with myeloma (patient 53) and represented a median value of 0.03% of the CD4+ T-cell pool. The absolute magnitude of this CD4+ CTAg-specific T-cell response peaked at 262 cells/mL with a median of 143 cells/mL. In contrast 4 of the 30 MGUS patients demonstrated a CD4+ CTAg-specific T-cell response with
peak and median values of 0.07% and 0.03% of the CD4+ repertoire, respectively.

These results demonstrate that MAGE-specific CD4+ T-cell responses are significantly less common than CD8+ T-cell responses in patients with myeloma ($P = .011$; Figure 1). However, the reverse pattern is observed in MGUS patients in whom MAGE-specific CD8+ T-cell responses are rare, although MAGE-specific CD4+ T-cell responses are more commonly seen (Figure 1).
not been on any treatment for the last 2 years. Remained at plateau stage of disease throughout this period and has included 1 patient (MGUS patient 3) who was monitored over a 2-year period and demonstrated a sustained response. The patient did not demonstrate any evidence of a clinical T-cell response before disease progression, with the IgG paraprotein rising from 17 g/L to 34 g/L. At this time, the magnitude of the CD8+ T-cell response was comparable in both compartments. The clones were isolated from 3 patients who were able to respond to target cells loaded with peptide concentrations down to 10^{-7} M to 10^{-9} M, which is comparable to values obtained for virus-specific CD8+ populations and indicates high-avidity antigen recognition. Importantly, CD4+ T-cell clones were also able to recognize target cells transfected with recombinant MAGE-A3, which demonstrates their ability to respond to naturally processed MAGE-A3 epitopes (Figure 6Aii, Bii). Several CD4+ T-cell clones were isolated from 3 patients who had demonstrated a consistent MAGE-A1289-298–specific CD8+ T-cell response from the point of analysis. Treatment regimens are shown. Thal indicates thalidomide; and Dex, dexamethasone.

**MAGE-specific CD4+ T-cell responses are maintained in patients with stable paraproteinemia**

Serial analysis was performed in all the patients who demonstrated a MAGE-specific CD4+ T-cell response. The patient with myeloma was monitored over a 2-year period and demonstrated a sustained CD4+ T-cell response that fluctuated between 0.01% and 0.06% of the CD4+ T-cell pool (Figure 2A). Importantly, this patient has remained at plateau stage of disease throughout this period and has not been on any treatment for the last 2 years. The 4 patients with MGUS were also studied at several time points and MAGE-specific CD4+ T-cell responses were maintained over an 18-month period. All these patients exhibited stable disease and included 1 patient (MGUS patient 3) who exhibited a stable paraprotein of more than 30 g/L\(^*\) for 9 years (initial level 28 g/L).

**The magnitude of the MAGE-specific CD8+ immune response increases in patients with relapsing disease**

To investigate the magnitude of the CD8+ T-cell response in relation to disease activity, we performed serial analysis of CTAg-specific CD8+ immune responses during several years of patient follow-up (Figure 3). Analysis of patient 7 started 30 months into disease and CD8+ T-cell responses remained relatively stable for the next 40 months ranging from 0.001% to 0.016% of the CD8+ T-cell pool. However, at 74 months after diagnosis the patient demonstrated disease progression, with the IgG paraprotein rising from 17 g/L to 34 g/L. At this time, the magnitude of the CD8+ T-cell response also increased by more than 10-fold, rising from 0.007% to 0.06% of the T-cell pool. In addition MAGE-A1–specific CD8+ T-cell clones could be isolated during this period and demonstrated cytolytic function against target cells in vitro. The patient did not demonstrate any evidence of a clinical response and died 2 months later. Linear regression analysis demonstrated that the frequency of the MAGE-A1–specific CD8+ T-cell response correlated positively with the paraprotein level (\(P = .042\)).

A similar pattern was seen in patient 4, who demonstrated an increase in the CTAg-specific CD8+ T-cell response before disease progression (data not shown). Dual immunohistochemistry staining of bone marrow trephines with primary antibodies to CD138 and MAGE has been previously performed and demonstrated the dual expression of MAGE within the CD138+ cells in 7 patients, including patient 4 and patient 7.

**CD8+ and CD4+ CTAg-specific T-cell responses have a predominant effector memory phenotype**

To examine the memory phenotype of the CTAg-specific immune response in vivo, cells were stimulated with CTAg peptides and costained with antibodies to CD45RA and CCR7. The CTAg-specific CD8+ T-cell response was phenotyped in 5 patients with myeloma and in all cases the population was CD45RA+ but showed no expression of the chemokine receptor CCR7. This CD45RA+CCR7+ subset represents a reuptake effector memory population (T\textsubscript{EMRA}) that has undergone re-expression of the CD45RA isotype due to lack of recent antigen-specific stimulation (Figure 4A).\(^*\) This phenotype is consistent with a previous paper reporting Melan-A and tyrosinase-specific CD8+ T cells in patients with melanoma.\(^*\)

In contrast, the CD4+ CTAg-specific T-cell response in the patient with myeloma showed no expression of CD45RA and had a homogeneous CD45RA–CCR7– effector memory phenotype (Figure 4B). Although the majority of the CTAg-specific CD4+ T cells detected in MGUS patients were also effector memory, there was also a small population of CCR7+ central memory cells (Figure 4C).

**MAGE-A1–specific CD8+ T cells do not show selective recruitment to the bone marrow of patients with myeloma**

Malignant plasma cells in patients with MGUS or myeloma are located almost exclusively within bone marrow, with minimal dissemination within the bloodstream. To determine whether the CTAg-specific immune response was able to home to tumor sites, we then compared the frequency of CTAg-specific CD8+ T cells within paired samples of peripheral blood and bone marrow. Patients 4 and 7 had demonstrated a consistent MAGE-A1298–298–specific CD8+ T-cell response within blood over many months, and paired samples became available from both patients at the time of disease relapse. Despite large numbers of plasma cells within the marrow biopsies, there was no evidence of selective recruitment of CTAg-specific T cells to the marrow microenvironment. In patient 4, the frequency of this T-cell response within marrow was reduced by 50% compared with that seen in blood and represented only 0.005% of the T-cell pool (Figure 5). Analysis of patient 7 showed that the T-cell response was comparable in both compartments. The phenotype of the CTAg-specific CD8+ T-cell immune response within bone marrow was identical (CD45RA–CCR7–) to that in peripheral blood (data not shown).

**MAGE-A3–specific CD4+ T-cell clones from patients with paraproteinemia have high avidity for peptide and recognize endogenously processed antigen**

Several CD4+ T-cell clones were isolated from 3 patients who exhibited a CTAg-specific CD4+ T-cell response, and their avidity assessed by cytokine release in response to target cells loaded with variable concentrations of peptide (Figure 6Ai,Bi). The clones were able to respond to target cells loaded with peptide concentrations down to 10^{-7} M to 10^{-9} M, which is comparable to values obtained for virus-specific CD4+ populations and indicates high-avidity antigen recognition.\(^*\) Importantly, CD4+ T-cell clones were also able to recognize target cells transfected with recombinant MAGE-A3, which demonstrates their ability to respond to naturally processed MAGE-A3 epitopes (Figure 6Aii, Bii). The MAGE-A3–specific CD4+ T-cell response that was detected in the patient with myeloma was specific for peptide MAGE-A3\textsubscript{149-160}, which has previously been reported to be presented by the products of the HLA DRB1*0401 and DRB1*0701
alleles. To confirm the HLA restriction of the T-cell clones, we used several LCLs with defined HLA class II genotype as target cells for peptide presentation. Interestingly, only target cells that expressed DR52b (determined by HLA DRB3*0201) were recognized by the clones (Figure 6Biii), and no recognition was observed when peptides were presented on HLA-DR52a (determined by HLA DRB3*0101)–expressing target cells (Figure 6Bii). This specificity corresponded with the patient’s own HLA-DR type (DR1, 17, 52b), which was negative for DR4 and DR7 but positive for DR52b, and illustrates that the same CD4 peptide can be restricted through several HLA DR alleles. Interestingly, the MAGE-A3191–205–specific CD4+ T-cell clones generated from the MGUS patients (Figure 6Aii) were also able to recognize peptides presented by any target cell that expressed HLA DR52b. This peptide has been reported to be restricted through HLA-DR11, but recognition of the clones from this patient proved to be independent of this allele (Figure 6Aii).
MAGE-A3–specific CD4+ T-cell clones have cytolytic function

To gain some insight into the physiologic relevance of the CTAg-specific CD4+ clones, we tested the ability of the cells to kill target cells loaded with peptide. The CD4+ clones from the patients with MGUS showed strong cytolytic activity against peptide-loaded targets and were also able to kill target cells expressing the recombinant MAGE-A3 protein (Figure 6Aiii).

Interestingly, clones from the myeloma patient were able to kill only peptide-loaded target cells (Figure 6Biv). The mechanism of cytotoxicity was investigated through flow cytometric analysis and demonstrated positive staining for granzyme but only minimal expression of perforin (data not shown).

MAGE-A1–specific CD8+ T-cell clones demonstrate high-avidity recognition of MAGE peptide and can kill myeloma tumor cells

CD8+ T-cell clones were generated from 2 patients with MM (patients 4 and 7) to allow a detailed analysis of their functional capacity and antigenic specificity. The avidity of the clones was assessed by cytokine response in the presence of limiting peptide concentrations (Figure 6Cia,Cib). MAGE-A1289-298–specific CD8+ T-cell clones from both patients were able to recognize target cells loaded with peptide concentrations down to $10^{-9}$ M to $10^{-11}$ M. The concentration that sensitized for half-maximal IFN-γ release was $10^{-8}$ M, which is comparable to values obtained for virus-specific CD8+ populations.

We next investigated the capacity of MAGE-A1289-298–specific CD8+ T-cell clones to recognize HLA-matched MM cell lines with endogenous expression of MAGE-A1. CD8+ T-cell clones were restricted through HLA-B7, which is also expressed by 3 MM cell lines U266, JIN3, and H929, all of which have previously been shown to express MAGE-A1.12,28 Both sets of clones were able to recognize endogenously processed antigen in all 3 cell lines as determined by IFN-γ ELISA assay (Figures 6Cib,Cibi).

The chromium release assay was then used to assess the cytotoxic capacity of the CD8+ CTAg T-cell response and confirmed that all 3 MM cell lines were killed by the T-cell clones. (Figure 6Cic,Ciic).

Patients with MM who demonstrate a CTAg-specific T-cell response have an improved clinical outcome.

The clinical significance of a CTAg-specific immune response in patients with paraproteinemia is unknown, and we therefore compared the survival rates in 52 patients in whom a CD8+ CTAg-specific T-cell response could be detected and compared it with patients who exhibited no CTAg-specific immunity (Figure 7; Table S1). The presence of a CTAg-specific immune response was associated with a 53% reduction in mortality (hazard ratio: 0.457; 95% confidence interval [CI] 0.203-1.03), although this failed to reach statistical significance (P value of log-rank test = .052).

Discussion

CTAg protein expression has been observed in a wide range of tumors and frequently induces a cellular and humoral immune response that may help to control tumor growth.10 T-cell immunity to CTAg proteins has been identified in association with several different cancers, although the frequency of CTAg-specific T cells is often of a lower magnitude than the immune response against other tumor-associated antigens such as differentiation antigens.43,44 CTAg is expressed in many cases of myeloma and MGUS, and an immune response to these proteins may play a role in controlling disease progression.14,15,45 Indeed, there is precedent for this principle as patients with MGUS often exhibit an immune response to the embryonal antigen SOX2.45 CD8+ immune responses against CTAg proteins have been documented in patients with myeloma,14,16,18,24,26,45 but there is currently no information regarding CTAg-specific immunity in MGUS. In this study, we performed a comprehensive study of CD8+ and CD4+ T-cell immunity to cancer testis antigens in patients with paraproteinemia by long-term study of cohorts of patients with MM or MGUS.

A striking feature of the current study is that although CD8+ immune responses were seen in many patients with myeloma, they were observed most strongly in late stages of disease and CTAg-specific T-cell clones could be isolated readily from patients with terminal disease. These observations suggest that expanded CD8+ CTAg-specific immune responses have limited value in controlling tumor growth in vivo despite the potent activity of T-cell clones against tumor cells in vitro. The biologic basis for this is unclear, but potential factors have been addressed in this study. The localization of CD8+ CTAg-specific T cells to the site of tumor is clearly impaired as no selective recruitment to bone marrow was observed. In contrast, previous studies have shown that the bone marrow can act as a reservoir for T cells that have the ability to lyse malignant plasma cells.37,48 Our study focused on CTAg-specific T cells rather than whole effector T-cell populations, and the observation that CTAg-specific CD8+ T cells are not sufficient to eradicate the tumor may partly be explained by their low frequency in the tumor microenvironment. In addition, samples were taken at the time of clinical relapse, which may relate to their poor localization to marrow.

The phenotype of CD8+ CTAg-specific T cells was that of CD45RA+CCR7+ resting memory cells that represent a population that has not recently undergone antigenic stimulation.39,49 This phenotype has been reported on Melan-A and tyrosinase tumor-reactive CD8+ T cells in melanoma patients40 and indicates that CTAg-specific CD8+ T cells appear to be prevented from interacting with tumor cells in vivo. This is supported by the finding that such clones revert to CD45RO expression after stimulation with...
Figure 6. Functional analysis of CD4 and CD8 clones generated from MM and MGUS patients. (A,B) IFN-γ production and cytotoxicity properties of generated CD4+ T-cell clones (TCC) from MGUS and MM patients. (i) Peptide titration analysis of CD4+ T-cell clones from MM (Bi) and MGUS (Ai) patients. (ii) MAGE-A3–specific CD4+ T cells were stimulated with peptide-loaded or DMSO-loaded HLA-matched or -mismatched LCL target cells or target cells transfected with a recombinant MAGE-A3 protein (KS114d transfected with MAGE-A3 [DR52b+] or DESA mismatched DR52b– LCL trans MAGE-A3). (Biii) T-cell clones from MM patient were screened against a panel of different LCLs to identify class II restriction of T-cell clones specific to MAGE-A3 145-160. Cytolytic activity of the CD4+ T-cell clones by standard 12-hour chromium release assay at an effector:target ratio of 10:1 (Biv) and (Aiii) against peptide-loaded HLA-matched target cells or target cells transfected with MAGE-A3. (Ci,ii) IFN-γ production and cytotoxicity properties of generated CD8+ TCC from MM patients 4 and 7: (a) Peptide titration analysis of CD8+ T-cell clones from MM patients 4 and 7 represented as a percentage of IFN-γ release. The line represents 50% recognition of peptide concentration, (b) MAGE-A1 289-298–specific CD8+ T cells were stimulated with peptide-loaded or DMSO-loaded HLA-matched or -mismatched LCL target cells or MM cell lines U266, JJN3, and H929. (c) Cytolytic activity of the CD8+ T-cell clones by standard 6-hour chromium release assay at an effector:target ratio of 10:1 against peptide or DMSO-loaded HLA-matched target cells or MM cell lines.
also exhibited a small population of CCR7− frequency of 0.03% of the CD4+ T-cell response could be detected; MM patients who were negative. The hazard ratio between the CTAg-positive and -negative groups is 0.457 (95% CI 0.203-1.03) with P = .052 by log-rank test.

Interestingly, this was present in a patient with stable plateau phase from MAGE-A1/A2/A3. Twenty-two patients with MM were found that 4 of them exhibited a CD4+ T-cell response in vivo, and a recent study showed that MAGE-A3 vaccine-induced CD8+ T cells expressed a CD45RO25 phenotype in many patients with MGUS rather than myeloma, the breadth, magnitude, and phenotype of the CTAg-specific CD4+ T-cell response reflects the magnitude of tumor burden in myeloma but is not sufficient to control disease. Many experimental models have demonstrated that effective CD8+ T-cell immunity is dependent on the presence of a CD4+ T-cell response, and we therefore undertook a comprehensive analysis of the CD4+ T-cell response to CTAg in patients at different stages of paraproteinemia using T-cell epitopes derived from MAGE-A1/A2/A3. Thirty-two patients with MM were screened and only a single CD4+ T-cell response was observed. Interestingly, this was present in a patient with stable plateau phase disease and was durable on 7 occasions during 21 months of observation. We then went on to study 30 patients with MGUS and found that 4 of them exhibited a CD4+ CTAg-specific T-cell response. Although present in only a minority of patients, it is important to remember that the study used only 5 CTAg peptides and, as more than 50 CTAg genes have been described, the frequency of CTAg-specific CD4+ T cells is likely to have been considerably underestimated. As well as being more common in patients with MGUS rather than myeloma, the breadth, magnitude, and phenotype of the CTAg-specific CD4+ T-cell response were also unique in this group. Responses were directed against all the 5 CD4+ peptides used in the study and demonstrated a mean frequency of 0.03% of the CD4+ T-cell pool. Finally, although the majority of CTAg-specific CD4+ cells in all patients exhibited a CD45RA−CCR7+ effector memory phenotype, patients with MGUS also exhibited a small population of CCR7+ central memory cells, which were absent in the patient with myeloma. The functional capability of these cells was demonstrated by the observation that CTAg-specific CD4+ T-cell clones could be isolated from both myeloma and MGUS patients and demonstrated cytolytic activity against endogenously processed MAGE-A3 protein.

The current study has used a peptide approach to investigate the detection of CD4+ T-cell responses in MM and MGUS patients, and it could be argued that our results are underestimating the frequency of responses. Indeed a whole-protein approach could potentially be more comprehensive. However, the peptides chosen were all previously described and discovered using whole-protein screening, and therefore are likely to represent the most immunodominant regions of the protein. Therefore, although the cohort size is small, these data suggest that CD4+ CTAg-specific T-cell responses may play a more important role in the control of tumor progression than CD8+ immunity. Indeed, there is substantial precedent for this model in other diseases, and the presence of a CD4+ T-cell response has been shown to be predictive of control of Papillomavirus infection in malignant cervical disease. A CD4+ immune response has been shown to play an important role in the control of plasma cell proliferation in murine models, and potential mechanisms could include growth suppression through cytokine release, direct cytotoxicity, or maintenance of functional CD8+ immunity.

These observations on the differential induction and function of the CD4+ and CD8+ immune response to cancer testis antigens during tumor progression have important implications for immunotherapy. If CTAg-specific CD4+ T-cell responses do indeed play a role in controlling the progression of paraproteinemia, then effort should be given to maintaining or re-establishing their presence in patients with progressive disease. In addition, although the CD8+ CTAg-specific T-cell response may have limited therapeutic value in vivo, at least in patients with advanced disease, it should be possible to activate these cells in vivo through the use of vaccination or dendritic cell therapy. Indeed, patients with myeloma have been shown to harbor a tumor-specific CD8+ T-cell response that can lyse primary tumor cells after stimulation with tumor cells in vitro, although the antigenic specificity of this population has not been documented. Patients who developed a CTAg-specific CD8+ T-cell immune response demonstrated an improved long-term survival, but it is unclear whether this reflects a specific CTAg-specific CD8+ role in controlling tumor progression. Previous studies indicate that the expression of CTAgS by malignant plasma cells in MM is indicative of poor prognosis but did not address the prognostic role of CTAg-specific immune responses. In our study, many patients were analyzed during or after therapy and received varied treatments including immunomodulatory drugs such as thalidomide, and this is clearly relevant in the detection of immune responses and interpreting our data. Further work is required to look at T-cell responses to other CTAgS, particularly MAGE-C1, which is widely expressed in myeloma, although no T-cell epitopes from MAGE-C1 have been described to date. Our data indicate that cancer testis antigens represent an important target for future immunotherapeutic intervention in MM.

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References


Differential pattern of CD4+ and CD8+ T-cell immunity to MAGE-A1/A2/A3 in patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma

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