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J Immunol 2009; 183:4800-4808; Prepublished online 4 September 2009;
doi: 10.4049/jimmunol.0900903
http://www.jimmunol.org/content/183/7/4800

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/09/04/jimmunol.0900903.DC1

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Characterization of Preexisting MAGE-A3-Specific CD4⁺ T Cells in Cancer Patients and Healthy Individuals and Their Activation by Protein Vaccination

Takemasa Tsuji,* Nasser K. Altorki,† Gerd Ritter,* Lloyd J. Old,* and Sacha Gnjatic2*

Vaccination with cancer/testis Ag MAGE-A3 in the form of recombinant protein often induces specific humoral and cellular immune responses. Although Ag-specific CD4⁺ T cells following vaccination are detectable by cytokine production after a single in vitro stimulation, their detection before vaccination is difficult because of low frequency. In this study, we have applied a sensitive method using CD154 (CD40L) staining to detect MAGE-A3-specific CD4⁺ T cells. MAGE-A3-specific T cell responses were analyzed in four healthy donors, two lung cancer patients with spontaneous serum Abs to MAGE-A3, and two baseline seronegative lung cancer patients throughout vaccination with MAGE-A3 protein. MAGE-A3-specific CD4⁺ T cells were detected in all individuals tested, at low frequency in healthy donors and seronegative cancer patients and higher frequency in patients seropositive for MAGE-A3. Polyclonal expansion of CD154-expressing CD4⁺ T cells after cell sorting generated a large number of MAGE-A3-specific CD4⁺ T cell lines from all individuals tested, enabling full characterization of peptide specificity, HLA-restriction, and avidity. Application of this method to cancer patients vaccinated with MAGE-A3 protein with or without adjuvant revealed that protein vaccination induced oligoclonal activation of MAGE-A3-specific CD4⁺ T cells. It appeared that MAGE-A3 protein vaccination in the presence of adjuvant selectively expanded high avidity CD4⁺ T cells, whereas high avidity T cells disappeared after multiple vaccinations with MAGE-A3 protein alone. The Journal of Immunology, 2009, 183: 4800–4808.

C ertain tumor Ags are known to frequently induce spontaneous immune responses in cancer patients who have Ag-expressing tumors but not in patients with Ag-negative tumors or healthy individuals (1–4). In addition, vaccination with various forms of tumor Ags successfully induced humoral and cellular immune responses, even in patients who had no spontaneous immunity against the Ag (5–7). Such spontaneous and vaccine-induced tumor Ag-specific T cells were detectable after in vitro stimulation (presensitization) with synthetic Ag peptides or other forms of Ags, such as recombinant viral vectors or proteins. In contrast, detection of tumor Ag-specific T cells in patients without spontaneous immunity before vaccination or in healthy individuals is difficult because of low frequency and/or suppression by regulatory T cells (8–11). In general, multiple stimulations of T cells from healthy donors with tumor Ag-loaded dendritic cells (DC)³ are required to induce tumor Ag-specific T cells (12). However, this method is not always applicable to cancer patients because of the requirement of large numbers of PBMC to generate DC. In addition, multiple stimulations by DC could induce a clonal expansion of a rare tumor Ag-specific T cell not representative of the donor’s T cell precursors against the Ag.

MAGE-A3 is a cancer/testis Ag with expression in normal tissues limited to testis but that shows high level of expression in various types of tumor (13). Because of its high expression in a wide array of tumor types, MAGE-A3 is an attractive target for cancer vaccines. Furthermore, MAGE-A3 has been shown to be immunogenic and induce specific T cell responses. Spontaneous CD4⁺ T cell responses against MAGE-A3 in melanoma patients were detectable after two consecutive stimulations of CD4⁺ T cells with selected MAGE-A3 peptides by proliferative response and cytokine release against the peptides (14). Interestingly, five of eight responders indicated MAGE-A3-specific IL-5 production, in contrast to two of eight responders that showed IFN-γ production, indicating that MAGE-A3-specific CD4⁺ T cells were differentiated to Th2 in advanced melanoma patients in vivo. MAGE-A3-specific T cell responses in healthy donors were also reported and significant MAGE-A3-specific proliferative response over background proliferation required four to six weekly stimulations (15). We and others have reported the results of immunomonitoring cancer patients who received MAGE-A3 protein with or without adjuvant AS02B, a formulation of saponin QS21 and monophospho- lipid A (16–18). None of the non-small cell lung cancer patients enrolled in the study showed significant spontaneous humoral or cellular immune responses before vaccination (16, 17). However, significant humoral and CD4⁺ T cell responses were developed after a single presensitization in most patients who were vaccinated with MAGE-A3 and AS02B. In contrast, most of patients vaccinated with MAGE-A3 protein alone showed no immune responses against the Ag. We also found a striking difference in the induction of immune responses after the second course of vaccination with MAGE-A3 with AS02B to both groups of patients (17). Patients first vaccinated with MAGE-A3 protein alone failed
to respond to the second course of vaccination with MAGE-A3 protein with the adjuvant whereas patients who had previously received MAGE-A3 with AS02B continuously responded to the vaccines. It is very important to investigate the detailed characteristics of MAGE-A3-specific immune responses in both groups of patients to explain the different induction of immune responses depending on the adjuvant. However, a more sensitive assay to analyze specific T cell responses has to be developed because low frequency of MAGE-A3-specific CD4+ T cells prevents detailed analyses, especially in patients first vaccinated with MAGE-A3 protein alone.

The main objective of this study was to establish a protocol to fully characterize low frequency tumor Ag-specific CD4+ T cells, such as those in healthy donors and seronegative cancer patients before vaccination. Such information is considered to be very important to understand the effect of vaccination by comparison with that of vaccine-induced CD4+ T cells. Recently, Ag-specific CD4+ T cells were shown to be detected by CD154 expression. In addition, using this method, Ag-specific T cells were able to be isolated by cell sorting. In the present study, we have applied this sensitive method to detect low-frequency tumor Ag-specific CD4+ T cells in cancer patients or healthy donors who showed no spontaneous immune responses against the Ag in conventional immunomonitoring assays. Using this method, MAGE-A3-specific CD4+ T cells were detected and fully characterized in four healthy donors and four cancer patients, two with spontaneous Ab response to MAGE-A3 and two seronegative. In addition, CD4+ T cells before vaccination were characterized and compared with specific CD4+ T cells observed after vaccination in the two seronegative cancer patients. It was found that MAGE-A3 protein, especially when formulated in AS02B, efficiently stimulated some preexisting CD4+ T cells and also induced newly elicited MAGE-A3-specific CD4+ T cells. By using this method, it was also possible to compare peptide specificities, avidity, and their HLA-restrictions for all MAGE-A3-specific CD4+ T cells detected before and after vaccination.

Materials and Methods

Patient and donor samples

Non-small cell lung cancer patients undergoing surgery at New York Presbyterian Hospital (New York, NY) consented to participate in a clinical study. UD1999-010, sponsored by the Ludwig Institute for Cancer Research (16, 17). PBMC samples collected throughout the study from two representative patients, WS07 and GO17, were used in the current study. WS07 and GO17 had stage IB (T2N1M0) squamous carcinoma and stage IB (T2N0M0) adenocarcinoma, respectively, and both patients were male. Additionally, PBMC and serum specimens were collected with informed consent from non-small cell lung cancer patients under a protocol approved by the Institutional Review Board of Weill Medical College of Cornell University, and samples from two patients spontaneously seropositive for MAGE-A3, LU-649, and LU-729 were used in the current study. Samples from healthy donors with no history of autoimmune disease were collected with informed consent at the New York Blood Center, and PBMC from four individuals, NC173, NC193, NC229, and NC236, were used in the current study.

In vitro sensitization (presensitization)

PBMC were collected from peripheral blood using Ficoll gradient and stored in liquid nitrogen. CD4+ and CD8+ T cells were isolated from typically 1 × 10^7 PBMC by using Dynabeads (Invitrogen). In some experiments, CD4+ T cells were further separated into CD45RA+ and CD45RO+ T cells by CD45RO beads (Miltenyi Biotec). Remaining CD4+ T cell-depleted PBMC were pulsed overnight with overlapping peptides for MAGE-A3 and were irradiated. CD4+ T cells (5 × 10^5) were stimulated by peptide-pulsed and irradiated T cell-depleted PBMC (1 × 10^6) in 96-well round-bottom plate in RPMI 1640 medium supplemented with 10% human AB serum (Gemini Bio-Products), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (RPMI 1640 + 10%SAB). The cultures were maintained for 20 days in the presence of 10 U/ml IL-2 (Roche) and 20 ng/ml IL-7 (R&D Systems) to allow the expansion of specific T cells. Separately, a part of CD4+ T cells were stimulated with 10 μg/ml PHA (REMEI) and cultured in RPMI 1640 + 10%SAB with IL-2 and IL-7, to be used as APC (T-APC) (19). Autologous EBV-transformed B (EBV-B) cells were generated from T cell-depleted PBMC by supernatant from B95–8 cells. Allogeneic HLA-compatible EBV-B cells had been generated and stored in our cell bank. EBV-B cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (RPMI 1640 + 10%FCS).

ELISPOT assay

IFN-γ ELISPOT assay was performed as described previously (16). Briefly, nitrocellulose-coated microtiter plates (Millipore) were coated overnight with 2 μg/ml peptide with 10% human serum (Sigma-Aldrich) in RPMI 1640 medium. Indicated number of effector T cells were cocultured with Ag-pulsed target cells, such as 5 × 10^6 EBV-B cells or 1 × 10^5 T-APC, for 24 h in RPMI 1640 medium without serum. Plates were developed using 0.2 μg/ml biotinylated anti-IFN-γ mAb (7-B6-1-biotin, Mabtech), 1 U/ml streptavidin-alkaline phosphatase conjugate (Roche Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich). The number of spots was evaluated using C.T.L. Immunospot analyzer and software (Cellular Technology). Results were shown as the number of spots without subtracting the number of background spots.

Detection and isolation of MAGE-A3-specific CD4+ T cells

MAGE-A3-specific CD4+ T cells were isolated by using CD154 expression sorting as described (20). Briefly, presensitized CD4+ T cells were restimulated for 6 h in 500 μl X-Vivo15 (BioWhittaker) with T-APC that were pulsed overnight with MAGE-A3 overlapping peptide pool (30 peptides with 20 amino acids length; 3 μg/ml for each peptide) and labeled with CFSE (Invitrogen-Molecular Probes) in the presence of 1 μM of PE-conjugated anti-CD154 mAb and 0.3 μl GolgiStop (BD Biosciences). CFSE+ /CD154+ MAGE-A3-specific effector T cells were sorted by FACSaria instrument and FACSdiva software (BD Biosciences). Sorted cells were stimulated with 10 μg/ml PHA in the presence of irradiated allogeneic PBMC. Cells were expanded for 20 days in RPMI 1640 + 10%SAB in the presence of 10 U/ml IL-2 and 20 ng/ml IL-7.

Measurement of cytoplasmic cytokines

MAGE-A3-specific CD4+ T cell lines were cocultured for 6 h in 250 μl X-Vivo in the presence of 0.17 μl GolgiStop with autologous EBV-B cells that were pulsed overnight with MAGE-A3 peptide pool and labeled with CFSE. Cyttoplasmic cytokines were stained using a BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer’s instructions with PE-conjugated anti-IL-2, -IL-4, -IL-13, and -GM-CSF mAbs, and allophycocyanin-conjugated anti-IFN-γ mAb, and allophycocyanin-conjugated anti-IL-5 mAb (anti-IL-4 mAb was purchased from eBioscience; all other mAbs were purchased from BD Biosciences). CFSE− effector CD4+ T cells were measured and analyzed for their fluorescent intensity by FACScalibur instrument with CellQuest software (BD Biosciences).

Measurement of cytokine levels

To evaluate the cytokine production from MAGE-A3-specific CD4+ T cell lines, autologous or HLA-compatible allogeneic EBV-B cells were pulsed overnight with MAGE-A3 peptides. Peptide-pulsed EBV-B cells (5 × 10^5) and effecter CD4+ T cell line (5 × 10^5) were cocultured for 20 h in 96-well round-bottom plate in 250 μl RPMI 1640 + 10%FCS and supernatant was harvested. Cytokine levels in the supernatant were measured by ELISA. Unlabeled and biotin-conjugated mAbs against GM-CSF, IFN-γ, and IL-4 were purchased from BD Biosciences. Unlabeled anti-IL-13 mAb and biotin-conjugated anti-IL-13 polyclonal Ab were purchased from eBioscience. ELISA kits for the measurement of IL-5, IL-10, TGF-β, and IL-17 were obtained from eBioscience.

To calculate apparent avidity of peptide-specific T cells, GM-CSF production was measured against graded amounts (from 10,000 to 1 ng/ml) of the peptide pulsed on autologous EBV-B cells. The dose-dependent curve for GM-CSF production was fitted by nonlinear regression analysis by using Prism 5 software (GraphPad Software) and the concentration to induce half of the amount of GM-CSF production against EBV-B cells pulsed with 10,000 ng/ml peptide was calculated by interpolation.
characterization of preexisting MAGE-A3-specific CD4+ T cells in cancer patients and healthy donors

To increase the chance of detecting MAGE-A3-specific CD4+ T cells in cancer patients and healthy donors, CD4+ T cells from patients and healthy donors were presensitized with MAGE-A3 overlapping peptides (nos. 1–30). After 20 days, MAGE-A3-specific IFN-γ producing T cells were evaluated by ELISPOT assays (A) or CD154 expression (B) for patient LU649 and healthy donor NC236. Error bars represent SD of two wells. Autologous T-APC were used as APC. Percentage of CD154-expressing CD4+ T cells from healthy donors after coculture with nos. 1–30 peptides pulsed and unpulsed target cells.

Results

Detection of preexisting MAGE-A3-specific CD4+ T cells in cancer patients and healthy donors

To increase the chance of detecting MAGE-A3-specific CD4+ T cells from PBMC, we first focused on patients with rare spontaneous Ab response against MAGE-A3. CD4+ T cells from two non-small cell lung cancer patients, LU-649 and LU-729, seropositive for MAGE-A3 (titers 1/13,000 and 1/8,000 respectively, data not shown) were stimulated with a pool of 30 MAGE-A3 overlapping peptides that were 20 amino acids long and had a 10 amino acids overlap (supplemental Table S1 for peptide sequences). After 20 days, IFN-γ producing MAGE-A3-specific T cells were evaluated by IFN-γ ELISPOT assay using autologous T-APC as target cells. Strong CD4+ T cell responses were observed against MAGE-A3 peptides in these seropositive cancer patients against multiple epitopes (Fig. 1A and supplemental Fig. S1). We then evaluated MAGE-A3-specific T cell precursors in four healthy donors whose CD4+ T cells were similarly presensitized and analyzed by IFN-γ ELISPOT assay. In contrast to seropositive patients, no significant IFN-γ-producing CD4+ T cells were observed in healthy donors, consistent with a previous observation that no measurable proliferative response over the background proliferation could be obtained after two consecutive Ag stimulations of CD4+ T cells from healthy donors (15). However, the results of IFN-γ ELISPOT assays could not exclude the existence of non-IFN-γ-producing, MAGE-A3-specific CD4+ T cells in healthy donors. Thus, we took advantage of CD154 (CD40L) staining, a recently described method to detect Ag-specific CD4+ T cells with any cytokine-producing capability (20). When presensitized CD4+ T cells from seropositive patients were stimulated with target cells pulsed with overlapping peptides in the presence of anti-CD154 mAb, significant number of CD4+ T cells up-regulated CD154 compared with those stimulated with unpulsed target cells (Fig. 1B and Figure S1). CD154-expressing cells in LU729 (2.4%) were much less frequent than that in LU649 (29%). Because serum from LU729 also showed reactivity against another MAGE family member, MAGE-A4, it is possible that serum Ab titer against MAGE-A3 was due to a cross-reaction of anti-MAGE-A4 Ab and patient LU729 did not show as strong spontaneous CD4+ T cell responses to MAGE-A3. The same method also detected CD154-expressing CD4+ T cells from healthy donors, albeit with much lower percentages of frequency (Fig. 1B). Fig. 1C summarizes the percentages of CD154-expressing CD4+ T cells of four healthy donors after stimulation with MAGE-A3 peptides-pulsed and unpulsed target cells. Increased CD154-expressing CD4+ T cells were found in all healthy donors tested after stimulation with MAGE-A3 peptide pool compared with unpulsed target cells, although the percentage of specific cells was lower (0.4–1.3%) than seropositive cancer patients (29 and 2.4%). The percentage of CD154-expressing T cells is expected to correlate with frequency of preexisting MAGE-A3-specific T cells in healthy donors and cancer patients. However, because cells were expanded for 20 days after peptide stimulation and the doubling time for specific CD4+ T cells in bulk culture was very difficult to estimate, specific T cell frequency could not be precisely determined without more complex experiments such as limiting dilution.

Characterization of MAGE-A3-specific CD4+ T cells in cancer patients and healthy donors

To fully characterize MAGE-A3-specific CD4+ T cell responses in healthy donors and seropositive cancer patients, CD154-expressing cells were sorted after stimulation with MAGE-A3 peptide pool by flow cytometry and polyclonally expanded with PHA. After 2–3 wk of culture, a large number of stable T cell lines were established from all healthy donors tested. Their specificity against MAGE-A3 peptides was first confirmed by ELISPOT assays (Fig. 2A). A CD4+ T cell line established from donor NC173 produced significant number of spots against MAGE-A3 peptide pool nos. 16–20, while IFN-γ production against other peptide pools was minimal (<6 spots).

This MAGE-A3 peptide-specific IFN-γ production was confirmed by intracellular cytokine staining (ICCS, Fig. 2B). A MAGE-A3-specific CD4+ T cell line from donor NC173 indicated IL-2 and GM-CSF production against MAGE-A3 nos. 11–20 peptides in addition to IFN-γ (Fig. 2B), whereas the production of these cytokines against other peptide pools or unpulsed target cells was negligible. In addition, IL-4, −5, and −13 production was detected by ICCS (Fig. 2B, data not shown) and ELISA (Table I), indicating that this T cell line was a mixture of Th1 and Th2 cells. Table I summarizes cytokine levels in the supernatant of CD4+ T cell lines stimulated by MAGE-A3 peptide pool-pulsed or unpulsed autologous EBV-B cells for four healthy donors. All T cell lines produced IFN-γ, IL-13, and GM-CSF specifically against MAGE-A3 peptides. In addition, IL-10 was found in several T cell lines. From these results, MAGE-A3-specific T cell lines generated from healthy donors were mainly a mixture of Th1-, Th2-, and/or IL-10-producing regulatory T cells. Analyses of MAGE-A3-specific T cells from two healthy donors (NC173 and NC236) were repeated twice to address the reproducibility of the method. MAGE-A3-specific CD4+ T cell lines were similarly generated from all three replicate experiments and reproducibly produced...
Autologous EBV-B cells were pulsed overnight with 20 pmoles of MAGE-A3 peptide and expansion with PHA. CD154-expressing cells after stimulation with MAGE-A3 peptides and expansion with IL-4, IL-5, and/or IL-10 were much less or negligible (data not shown). Large amount of IFN-γ was produced against unpulsed target cells.

We previously demonstrated that NY-ESO-1-specific CD4+ T cell precursors in healthy donors are present in naive T cell population, CD45RA+ naive and CD45RO+ memory CD4+ T cells from NC173 and NC236 were presensitized and MAGE-A3-specific CD4+ T cell lines were generated. In contrast to NY-ESO-1-specific CD4+ T cells, MAGE-A3-specific CD4+ T cells were detectable from both naive and memory populations in both healthy donors (data not shown). NY-ESO-1-specific CD4+ CD45RA+ T cell precursors were shown to have high avidity and recognize naturally processed NY-ESO-1 protein (8). Interestingly, only MAGE-A3-specific CD4+ T cells obtained from naive population of both healthy donors were able to recognize MAGE-A3 protein, compared with memory populations (Fig. 2C and data not shown).

MAGE-A3-specific CD4+ T cell lines established from seropositive lung cancer patients were evaluated by ELISPOT assay (data not shown), ICCS (Fig. S2), and measurement of cytokine levels in the supernatant by ELISA (Table I). As apparent from Table I, CD4+ T cell lines from these cancer patients were characterized by very strong IFN-γ production compared with healthy donors-derived T cells. In addition, Th2 cytokines (IL-4, IL-5, and IL-13) were also produced. ICCS also showed strong IFN-γ staining in addition to IL-4 staining after stimulation with MAGE-A3 peptide pools, indicating that MAGE-A3-specific CD4+ T cells in seropositive cancer patients were a mixture of Th1 and Th2 cells (Fig. S2).

**Frequency of MAGE-A3-specific CD4+ T cells in cancer patients before and after vaccination with MAGE-A3 protein**

Detection and characterization of MAGE-A3-specific T cell precursors in healthy donors with CD154 staining as mentioned above enabled us to detect preexisting MAGE-A3-specific T cells in cancer patients before vaccination, which were difficult to measure by conventional immunomonitoring methods such as ELISPOT assay or ICCS. We applied this method to two cancer patients vaccinated with a fusion protein of MAGE-A3 and protein D with or without adjuvant AS02B. Patients WS07 and GO17, who were previously shown to have no spontaneous immune response before vaccination, received MAGE-A3 protein alone intradermally and MAGE-A3 plus AS02B i.m., respectively (16, 17). As reported previously, patient GO17 developed strong humoral and cellular immune responses after vaccination, whereas WS07 showed no immune response during vaccination with MAGE-A3 protein alone (17). After completing this first cycle of vaccination, both patients received an additional course of vaccination with MAGE-A3-protein D fusion protein formulated in

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**Table I. Cytokine levels (ng/ml) in supernatant of MAGE-A3-specific CD4+ T cell lines of healthy donors and seropositive cancer patients**

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-13</th>
<th>GM-CSF</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy donors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC173a</td>
<td>17.1</td>
<td>1.1</td>
<td>1.8</td>
<td>6.4</td>
<td>16.3</td>
<td>0.2</td>
</tr>
<tr>
<td>NC236a</td>
<td>2.4</td>
<td>0.0</td>
<td>0.3</td>
<td>2.9</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>NC193b</td>
<td>4.7</td>
<td>0.4</td>
<td>3.2</td>
<td>10.0</td>
<td>13.6</td>
<td>3.3</td>
</tr>
<tr>
<td>NC229c</td>
<td>5.2</td>
<td>0.0</td>
<td>0.1</td>
<td>3.0</td>
<td>6.1</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Seropositive cancer patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU649a</td>
<td>68.0</td>
<td>2.2</td>
<td>0.5</td>
<td>9.1</td>
<td>28.8</td>
<td>0.4</td>
</tr>
<tr>
<td>LU729d</td>
<td>72.8</td>
<td>2.3</td>
<td>1.6</td>
<td>11.0</td>
<td>27.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a MAGE-A3-specific T cell lines (50,000 cells) and peptides-pulsed autologous EBV-B cells (50,000 cells) were cocultured for 20 h and cytokines levels in the supernatant were measured by ELISA. Numbers in parentheses indicate background cytokine production against unpulsed autologous EBV-B cells. MAGE-A3-specific cytokine production exceeding the limit of detection and five times higher than background production was considered to be significant and is shown in boldface.

b Sum of cytokine levels produced against nos. 1–14 and 15–30 pools.

c Sum of cytokine levels produced against nos. 1–10, 11–20, and 21–30 pools.
AS02B. Interestingly, although GO17 continuously responded to the second course of vaccination, WS07 did not show any immune response against additional course of vaccination with MAGE-A3 protein in the presence of AS02B (17). In contrast, both patients developed strong humoral immune response against protein D, indicating that a selective suppressed or nonresponsive state was induced during protein vaccination without adjuvant (17).

To evaluate MAGE-A3-specific CD4+ T cells before and after vaccination, CD4+ T cells from selected time points were presensitized with MAGE-A3 peptide pool and CD154 expression was evaluated by flow cytometry after restimulation with MAGE-A3 peptides, as shown in Fig. 3. Although conventional IFN-γ ELISPOT assays with CD4+ T cells from WS07 and GO17 showed no specific response before vaccination, there were significantly more CD154-expressing cells (2.4 and 5.8%, respectively) after restimulation with MAGE-A3 peptide pool compared with unstimulated cells (0.5 and 0.6%, respectively), indicating the presence of MAGE-A3-specific T cells in these two seronegative cancer patients. Although both patients were seronegative before vaccination, these percentages of CD154-expressing cells before vaccination were slightly higher than those in healthy donors, which possibly indicated that MAGE-A3-specific CD4+ T cell precursors in these cancer patients were primed in vivo by tumor cell-derived MAGE-A3. There was a striking change in the frequency of CD154-expressing cells in WS07 during vaccination with MAGE-A3 protein alone. After a single vaccine injection, CD154-expressing cells dramatically increased to ~20% in CD4+ T cells and then rapidly decreased to baseline levels after the next injections. In our previous study, similar transient induction of MAGE-A3-specific CD4+ T cells after vaccination with MAGE-A3 protein alone was observed in another patient, ST-04, by IFN-γ ELISPOT assay and ICCS (16). In sharp contrast, when MAGE-A3 protein was formulated with adjuvant AS02B and administered to patient GO17, CD154-expressing MAGE-A3-specific CD4+ T cells were increased from 5.2% before vaccination to 9.6% after the first vaccination (Fig. 3B), and were further expanded after the second vaccination at day 43 (16.5%) and the third vaccination at day 64 (36.1%), confirming previous observations for most patients who received MAGE-A3 protein in AS02B by IFN-γ ELISPOT assay and/or ICCS (16, 17).

During the second course of vaccination with MAGE-A3 protein with AS02B, MAGE-A3-specific CD4+ T cells in GO17 were boosted. However, as consistent with our previous study, although patient WS07 received the same treatment with adjuvant for the second course, MAGE-A3-specific CD4+ T cells remained at baseline level (Fig. 3).

Characterization of MAGE-A3-specific CD4+ T cells before and after vaccination

To fully characterize MAGE-A3-specific CD4+ T cells observed in each vaccination point, we generated MAGE-A3-specific CD4+ T cell lines by isolating specific cells based on their CD154 expression and then evaluated by flow cytometry after restimulation with MAGE-A3 overlapping peptides, even those derived from samples taken before vaccination, which are considered to be precursors of MAGE-A3-specific T cells (Tables II and III).

Specificity

As shown in Table II, MAGE-A3-specific T cell lines obtained from WS07, who received MAGE-A3 protein alone, recognized multiple epitopes as measured by GM-CSF, which is produced by both Th1 and Th2 cells (21) and was thus selected to show the specificity of MAGE-A3-specific Th1 and Th2 cells. GM-CSF production against a pool of MAGE-A3 overlapping peptides pulsed on autologous EBV-B cells. All CD4+ T cell lines showed strong reactivity against MAGE-A3 overlapping peptides and polyclonal expansion with PHA. Obtained MAGE-A3-specific CD4+ T cell lines were tested by cytokine release for their reactivity against MAGE-A3 overlapping peptides pulsed on autologous EBV-B cells. All CD4+ T cell lines showed strong reactivity against a pool of MAGE-A3 overlapping peptides, even those derived from samples taken before vaccination, which are considered to be precursors of MAGE-A3-specific T cells (Tables II and III).

Table II. GM-CSF production by MAGE-A3-specific CD4+ T cell lines generated from patient WS07a

<table>
<thead>
<tr>
<th>Peptides (no.)</th>
<th>MAGE-A3 alone (ng/ml)</th>
<th>MAGE-A3 + AS02B (ng/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Pre d22 d43 d64</td>
<td>Pre d22 d43 d85</td>
</tr>
<tr>
<td>7</td>
<td>0.0 0.0 0.1 0.0</td>
<td>0.2 0.0 2.7 0.0</td>
</tr>
<tr>
<td>11</td>
<td>0.6 3.1 2.1 1.2</td>
<td>0.1 0.8 0.2 0.1</td>
</tr>
<tr>
<td>12</td>
<td>0.7 3.1 2.2 1.9</td>
<td>0.1 0.7 0.2 7.8</td>
</tr>
<tr>
<td>13</td>
<td>0.0 5.5 0.1 0.0</td>
<td>0.0 0.1 0.0 0.0</td>
</tr>
<tr>
<td>15</td>
<td>1.9 0.4 1.5 0.0</td>
<td>0.0 1.2 0.0 0.6</td>
</tr>
<tr>
<td>17</td>
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<td>0.0 1.2 0.0 0.6</td>
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</tr>
<tr>
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<td>2.8 2.0 1.3 1.5</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>1–30</td>
<td>4.5 9.9 4.7 2.2</td>
<td>3.0 8.3 3.8 8.1</td>
</tr>
</tbody>
</table>

a Sequences are given in Table S1. Peptides that induced less than 1.0 ng/ml GM-CSF are not shown. GM-CSF production exceeding the limit of detection (0.6 ng/ml) and five times higher than background production was considered to be significant and is shown in bold.

Pre, Before vaccination; d, day.
response against no.17 peptide before vaccination disappeared after the first injection.

**Th1/Th2 differentiation during vaccination**

Vaccine adjuvant is known to influence T cell differentiation. Especially, monophospholipid A in AS02B is reported to activate APC through TLR4 to induce IL-12, which causes Th1 differentiation of naive T cells (22). Thus, we wanted to characterize Th1/Th2 polarization of MAGE-A3-specific CD4+ T cells after vaccination by measuring IFN-γ and IL-4 levels produced by MAGE-A3-specific T cell lines. As shown in Fig. 4A, IFN-γ production by MAGE-A3-specific CD4+ T cells from WS07 against the pool of overlapping peptides was more than six times greater after the first injection of MAGE-A3 protein alone, but gradually decreased after the second injection. In contrast, IFN-γ levels in patient GO17 were nearly eight times greater after the first injection of MAGE-A3 protein and AS02B adjuvant and remained high during the following vaccinations. We also compared IL-4 levels and found that changes in IL-4 production during the first course of vaccination were more modest (three times greater), transient, and similar in both patients. In addition, IL-5 and IL-13 levels did not show significant changes during the first course of vaccination in both patients (data not show). These results indicate that MAGE-A3 protein vaccination with the adjuvant system AS02B had a greater effect on polarizing specific CD4+ T cells to Th1.

We also found that IL-10, which is a cytokine sometimes associated with regulatory T cells in addition to Th2 cells, was produced by some vaccine-induced MAGE-A3-specific CD4+ T cells from both patients (data not shown). IL-10 production from vaccinated patients’ specific CD4+ T cells was always associated with IL-4 production, indicating that IL-10 was probably produced by Th2 cells. In addition to IL-10, TGF-β is considered to be a main soluble factor produced by regulatory T cells to exert their suppressive effect. TGF-β levels in the supernatant of all T cell lines stimulated with MAGE-A3 peptide pool were under the limit of detection. Thus, the present vaccination protocol appears not to induce suppressive regulatory T cells or it is possible that they did not expand in the experimental condition. Recently, a newly discovered IL-17-producing helper T cell subset, Th17, was paid much attention to with regard to autoimmune disorders. However, IL-17 was not detected in any T cell lines (not shown).

**Avidity**

Because these patients were vaccinated with MAGE-A3 protein, vaccine-induced T cells were expected to have high affinity TCR that can recognize naturally processed Ag. To confirm this, functional avidity of all T cell lines against each reactive peptide was determined. MAGE-A3-specific CD4+ T cells before vaccination in WS07 had low or modest avidity that could recognize 1000 to 100 nM peptides (Fig. 5A). In comparison, the avidities of T cell lines from healthy donors were within a similar range (data not shown).

To compare the avidities for each peptide response of T cell lines obtained from different vaccination points, the peptide concentration required to induce half the amount of GM-CSF production against 10,000 nM peptides (apparent avidity) was calculated in immunized patients. The avidity of no. 12 peptide-specific T cells in WS07 slightly increased after the first injection but significantly decreased after the second injection of MAGE-A3 protein alone (Fig. 5B). Another consistently observed response against no. 25 was low avidity before and during vaccination. Generally, most CD4+ T cells observed after vaccination had low avidity (Fig. 5C).

Preexisting CD4+ T cells in GO17 also had low to modest avidity (Fig. 5A). In contrast to WS07, the avidity of sustained T cell responses against peptides nos. 12 and 16 increased during vaccination compared with those before vaccination (Fig. 5B). In addition, most newly induced responses after vaccination were of medium to high avidity to recognize 100 or 10 nM peptides, respectively (Fig. 5C).

These changes in avidity could influence the recognition of naturally processed protein. MAGE-A3-specific CD4+ T cell lines from both patients before vaccination did not efficiently recognize MAGE-A3 protein pulsed on autologous EBV-B cells (Fig. 5D). Consistent with the changes in avidity against peptides, CD4+ T cell line from patient WS07 after the first injection of MAGE-A3 protein alone (day 22) clearly recognized MAGE-A3 protein but that obtained after the second injection (day 43) lost the ability to recognize protein. In contrast, CD4+ T cell lines from GO17 after vaccination with MAGE-A3 protein in the presence of AS02B

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**Table III. GM-CSF production by MAGE-A3-specific CD4+ T cell lines generated from patient GO17**

<table>
<thead>
<tr>
<th>Peptides (no.)</th>
<th>MAGE-A3 alone (ng/ml)</th>
<th>MAGE-A3 + AS02B (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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</tr>
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<tr>
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</tr>
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</tr>
<tr>
<td>17</td>
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<tr>
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<tr>
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<tr>
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<td>9.2</td>
<td>14.8</td>
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<tr>
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</table>

*Sequences are given in Table S1. Peptides that induced less than 1.0 ng/ml GM-CSF are not shown. GM-CSF production exceeding the limit of detection (0.6 ng/ml) and five times higher than background production was considered to be significant and is shown in bold.

Pre. Before vaccination; d, day.
autologous EBV-B cells. GM-CSF production against unpulsed EBV-B
during the first course of vaccination against MAGE-A3 protein-pulsed
responses was determined and HLA restrictions were found to be
distributed to most of patients’ HLA types.

Discussion
Monitoring T cell response against vaccinated Ag is important to
developing better vaccine formulations or protocols. Recently,
full-length tumor Ags, such as recombinant protein or viral vec-
tors, instead of defined short peptides were used for vaccination
because they were considered to activate multiple T cell precursors
without limitation by HLA types (5, 6, 16, 23–25). Many studies
reported successful induction of multiple T cell responses against
the vaccine Ag, however, functional comparison to preexisting T
responses before vaccination was hampered because of the low
frequency of specific T cells in nonvaccinated patients without
spontaneous immunity against the Ag. Thus, it is not clear which
repertoire of T cells preexist in patients and which are activated or
recruited by tumor Ag vaccination. Furthermore, comparison of
the functional difference of preexisting tumor Ag-specific T cells
and vaccine-induced T cells is important for better understanding
the effect of vaccination. In the present study, we have applied a
sensitive method using CD154 staining to detect and isolate pre-
existing tumor Ag-specific T cells in healthy individuals and can-
cer patients who showed no significant T cell response in conven-
tional immunomonitoring methods.

Staining with anti-CD154 mAb detected MAGE-A3-specific
CD4+ T cells from all eight individuals tested, with low frequency
in four healthy donors and two seronegative cancer patients before
vaccination, but higher percentages in two cancer patients with
spontaneous seropositivity for MAGE-A3. The precursor fre-
cency is expected to be higher than 2 × 10^-6 from the initial
number of CD4+ T cells (5 × 10^5), although the precise estimation
of precursor frequency is difficult because cells were expanded for
20 days after peptide stimulation. In addition, when peptide spec-
ificity of MAGE-A3-specific CD4+ T cell lines from three inde-
dependent cultures of healthy donors, NC173 and NC236, were compared,
some peptides-specific T cells were detected only in one T
line (data not shown), indicating that the precursor frequency for
some epitopes is less than 2 × 10^-6 in healthy individuals.
This indicated the requirement of multiple cultures to estimate the
whole spectrum of peptides-specificity and their frequencies in
healthy donors in the present protocol, similarly to other tech-
niques. However, this precursor frequency for healthy donors was
within the range of 1.4 × 10^-6–3.1 × 10^-7 in healthy donors,
determined for several HLA-DP4-restricted peptides from other
MAGE-A gene family proteins (26). Their estimation is based on
the detection rate of IFN-γ-producing CD4+ T cells from multiple
wells containing a limited number of CD4+ T cells after stimula-
tion four times with peptide-loaded DC (26). NY-ESO-1- and
SSX-2-specific CD4+ T cell precursor frequency in healthy donors
was estimated to be 1.1–9.1 × 10^-6 and 5–25 × 10^-6, respec-
tively, by IFN-γ ICCS (27, 28), which is similar to that of anti-
MAGE-A4 CD4+ T cell frequency. With some modification, such
as limiting dilution, the present protocol is expected to become an
alternative method to estimate precursor frequency of tumor Ag-
specific CD4+ T cell precursors that produce any cytokines. No
significant MAGE-A3-specific CD8+ T cells were detected in
healthy donors or cancer patients tested in the present study by
IFN-γ ELISPOT assays. In addition, isolation and expansion of
CD154-expressing CD8+ T cells after presensitization and re-
stimulation with MAGE-A3 overlapping peptides from two

HLA-restriction
Finally, the generation of MAGE-A3-specific T cell lines allowed
us to precisely determine HLA-restriction of CD4+ T cell re-

efficiently recognized MAGE-A3 protein-pulsed EBV-B cells even
after multiple injections (Fig. 5D).

FIGURE 5. Avidity of MAGE-A3-specific CD4+ T cells before and
during the courses of vaccination. A, CD4+ T cell lines established from
WS07 and GO17 before vaccination were stimulated with autologous
EBV-B cells pulsed with graded concentration of MAGE-A3 peptides and
GM-CSF levels in the supernatant were determined ELISA. Results are
shown against the GM-CSF level induced by 10,000 ng/ml peptide. B, Change
in the avidity of CD4+ T cell lines during the first course of vaccination by
MAGE-A3 protein in the absence (WS07) or presence (GO17) of AS02B. Peptide concentration to induce 50% of GM-CSF pro-
duction against 10,000 ng/ml peptides (apparent avidity) was calculated by
interpolation of dose-dependent GM-CSF production shown in A. Re-
results are presented as relative change of apparent avidity of CD4+ T cell
lines compared with apparent avidity of CD4+ T cell lines before vac-
cination (Pre). Positive and negative values indicate fold decrease and
increase in apparent avidity, respectively. C, Apparent avidities were
determined for all significant responses against peptides observed be-
fore and during vaccination. D, GM-CSF production by MAGE-A3-
specific CD4+ T cell lines obtained from cancer patients before and
during the first course of vaccination against MAGE-A3 protein-pulsed
autologous EBV-B cells. GM-CSF production against unpulsed EBV-B
cells was below 0.1 ng/ml. d, day.
healthy donors did not generate MAGE-A3 peptide-reactive CD8\(^+\) T cell lines, indicating either low efficacy of CD154 staining in CD8\(^+\) T cells or lower precursor frequency of MAGE-A3-specific CD8\(^+\) T cells in healthy donors.

Isolation of CD154-expressing cells after presensitization and restimulation with MAGE-A3 overlapping peptides enabled high enrichment of MAGE-A3-specific CD4\(^+\) T cells. Polyclonal expansion of MAGE-A3-specific T cells by PHA allowed the generation of a large number of MAGE-A3-specific T cell lines that could be used to characterize peptide specificity, cytokine producing profile, avidity, and HLA-restriction. Such information on pre-existing tumor Ag-specific CD4\(^+\) T cells against multiple Ags before vaccination may be useful for the determination of target Ags or the selection of vaccine peptides or truncated protein when the full-length protein is not available for vaccination. The major advantage of the present method is the full characterization of pre-existing low frequency T cell responses, which is difficult in standard immunomonitoring methods, such as ELISPOT assays and ICCS, after a short term in vitro presensitization. Although similar information could be drawn by repeated Ag stimulations with autologous APC such as DC, it is difficult to obtain sufficient numbers of PBMC for the preparation of APC for repeated stimulations, especially from cancer patients. Furthermore, the use of CD154 staining for the isolation of specific CD4\(^+\) T cells enabled us to analyze multiple subsets of CD4\(^+\) T cells with different functions, in contrast to the methods that detected or selected cells with a specific function, such as IFN-\(\gamma\)-secreting cells. In addition, because of the requirement of a relatively small number of PBMC and short culture period to establish T cell lines (20 days for presensitization and 20 days for expansion), this method is applicable to adoptive CD4\(^+\) T cell therapy (29).

Application of this method to monitor CD4\(^+\) T cell responses in cancer patients who were vaccinated with MAGE-A3 protein allowed us to compare preexisting CD4\(^+\) T cells with vaccine-activated T cells. We found that protein vaccination activated selected preexisting MAGE-A3-specific CD4\(^+\) T cells and maintained the response during the course of vaccination, whereas some preexisting CD4\(^+\) T cells were not expanded by vaccination. In addition, MAGE-A3 protein vaccination induced strong CD4\(^+\) T cell responses that were not detectable before vaccination. We have previously shown that MAGE-A3 protein vaccination with adjuvant system AS02B induced long-lasting Ab and T cell responses against MAGE-A3, whereas MAGE-A3 protein in the absence of adjuvant induced no immune response in most patients. Furthermore, patients vaccinated with MAGE-A3 protein alone did not develop humoral or cellular immune responses after additional vaccination with MAGE-A3 protein plus AS02B (16, 17). Because only one patient from each vaccination protocol was analyzed, it is difficult to draw general conclusions about the effect of adjuvant. However, MAGE-A3-specific CD4\(^+\) T cells in two vaccinated patients showed significant quantitative and qualitative differences that could give hints to understand the effect of adjuvant. By comprehensive analysis of MAGE-A3-specific CD4\(^+\) T cell lines established from vaccinated patients, it was found that there is a trend of differentiation to Th1 type after vaccination with the adjuvant. In addition, although the avidity of MAGE-A3-specific CD4\(^+\) T cells before vaccination in both patients were similar, MAGE-A3-specific CD4\(^+\) T cell lines obtained after vaccination with MAGE-A3 protein alone clearly showed lower avidity than those before vaccination. The low avidity for MAGE-A3-specific CD4\(^+\) T cells after vaccination with MAGE-A3 protein alone could explain the failure to respond to booster vaccination of MAGE-A3 protein with adjuvant AS02B. In contrast, MAGE-A3 protein with AS02B increased the avidity of specific CD4\(^+\) T cells, suggesting that inclusion of the adjuvant helped expanding high avidity CD4\(^+\) T cells that are expected to continuously respond to MAGE-A3 protein. Indeed, MAGE-A3-specific CD4\(^+\) T cells obtained from WS07 after the second injection of MAGE-A3 protein alone failed to recognize MAGE-A3 protein but those from GO17 after vaccination with MAGE-A3 protein with AS02B efficiently recognized MAGE-A3 protein in vitro (Fig. 5D). The change in avidity of specific CD4\(^+\) T cells by protein Ag with adjuvant is in contrast to our observation for peptide vaccination: although high avidity NY-ESO-1-specific CD4\(^+\) T cells were detectable in cancer patients before vaccination by removing CD25\(^+\) regulatory T cells, low avidity NY-ESO-1-specific CD4\(^+\) T cells were preferentially expanded after HLA-DP4 binding NY-ESO-1 peptide vaccination (8). Several explanations are possible for the difference in the avidity of MAGE-A3-specific T cells activated in the presence or absence of adjuvant, i.e., MAGE-A3-specific CD4\(^+\) T cells with high avidity TCR were not detectable after repeated vaccination with MAGE-A3 protein alone, whereas the inclusion of adjuvant selected high avidity T cells for expansion. Relevant observations were made in mice, where animals were immunized with peptides in the presence or absence of LPS after transferring peptide-specific CD4\(^+\) T cells. Interestingly, transferred T cells expanded in mice immunized with peptide and LPS, whereas peptide immunization alone decreased the number of transferred T cells compared with unimmunized mice (30). Maxwell et al. also found that tight T cell-DC interactions were formed by coimmunization with LPS (30), which was recently shown to be required for memory T cells (31). Thus, it is possible that vaccination in the presence of immunostimulatory adjuvant induced Ag-specific memory T cells and the lack of APC activation by absence of adjuvant induced T cell unresponsiveness because of lack of costimulation (32). We recently observed that induction of IL-6 and glucocorticoid-induced TNF-receptor ligand in APC restored the function of Ag-specific T cells from suppression by regulatory T cells (33). In addition, we have demonstrated that high avidity CD4\(^+\) T cells were more susceptible to suppression by regulatory T cells than low avidity CD4\(^+\) T cells (8). Thus, it is possible that TLR signaling provided by the adjuvant blocked the suppression of MAGE-A3-specific CD4\(^+\) T cells with high avidity TCR by regulatory T cells. Analyses of more patients who were vaccinated with MAGE-A3 protein in the presence or absence of adjuvant are required for a general understanding of the effects of adjuvant on the avidity of T cells and the mechanism of unresponsiveness in patients immunized with MAGE-A3 protein alone.

Many Ags have been discovered and shown to be highly expressed in various types of tumors with limited expression in normal tissues (34). Many of these Ags were found to be immunogenic to induce spontaneous Ab and T cell responses in cancer patients with Ag-expressing tumors. These observations indicate that T cell precursors against tumor Ags are present in humans and tumor Ags produced by tumors may activate and expand them. However, little is known about the frequency and function of preexisting tumor Ag-specific T cells in healthy individuals and cancer patients without spontaneous immunity. Using presensitization by synthetic tumor Ag peptides and isolation of specific cells with CD154 expression, it becomes possible to evaluate frequency of CD4\(^+\) T cell precursors for any tumor Ag protein. It will be important to compare the frequency of CD4\(^+\) T cell precursors against various tumor Ags in healthy individuals to determine the most interesting target Ags for human cancer vaccines.
Acknowledgments
We thank E. Ritter for the measurement of MAGE-A3-specific serum Ab titer in cancer patients.

Disclosures
The authors have no financial conflict of interest.

References