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*J Immunol* 2009; 183:4800-4808; Prepublished online 4 September 2009; doi: 10.4049/jimmunol.0900903

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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.

Certain 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 Ag-loaded dendritic cells (DC)2 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 monophosphoryl 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

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Received for publication March 23, 2009. Accepted for publication July 22, 2009.

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1This work was supported in part by the Cancer Vaccine Collaborative, funded by Cancer Research Institute and Ludwig Institute for Cancer Research.

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Abbreviation used in this paper: DC, dendritic cells; EBV-B cells, EBV-transformed B cells; ICSCS, intracellular cytokine staining; T-APC, PHA-activated CD4+ T antigen presenting cells.

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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, as 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 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. 1LD1990–010, 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 IIB (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 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 × 10^5) in 96-well round-bottom plate in RPMI 1640 medium supplemented with 10% human AB serum (Gemini Bio-Products), 2 mM L-glutamine, 100 μM 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 (REME) 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 μM 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 peptides 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 × 10^5 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–biontin, Mabtech), 1 μg/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 Dynabeads (Invitrogen) and CD154 expression. In some experiments, Ag-specific T cells were further separated into CD45RA+ and CD45RO+ 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.

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 and labeled with CFSE. Cytoplasmic cytokines were stained using a BD Cytofix/Cytperm kit (BD Biosciences) according to manufacturer’s instructions with PE-conjugated anti-CD154 mAb and 0.3 μg/ml 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 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 effector 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, INF-γ, and IL-4 were purchased from BD Biosciences. Unlabeled anti-IL-13 mAb and anti-IL-17 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).
characterization of preexisting MAGE-A3-specific CD4+ T cells

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 (ICS, 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 ICS (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 cells from patients and healthy donors after coculture with nos. 1–30 peptides pulsed and unpulsed target cells.

Characterization of 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 patients and healthy donors 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).

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*The online version of this article contains supplemental material.*
Autologous EBV-B cells were pulsed overnight with 20 ng/ml of MAGE-A3 peptides and expansion with PHA. A, MAGE-A3-specific IFN-γ production was evaluated by ELISOPOT assays. Error bars represent SD of two wells. B, Cytokine production was evaluated by cytoplasmic cytokine staining after stimulation with nos. 10–20 peptides. Autologous EBV-B cells were used as APC. Numbers in each quadrant indicate the percentage of cells and parenthesized numbers indicate the background production of cytokines against unpulsed target cells. C, Recognition of naturally processed MAGE-A3 protein by MAGE-A3-specific T cell lines derived from CD45RA+ and CD45RO+ CD4+ T cell subsets was evaluated by GM-CSF production. Autologous EBV-B cells were pulsed overnight with 20 μg/ml MAGE-A3 protein, washed, and cocultured with CD4+ T cell lines. After 24 h, supernatant was harvested and GM-CSF level was measured by ELISA.

A large amount of IFN-γ, IL-13, and GM-CSF, whereas the levels of IL-4, IL-5, and/or IL-10 were much less or negligible (data not shown).

We previously demonstrated that NY-ESO-1-specific CD4+ T cell precursors were detectable from CD45RA+ naïve T cell subset after a single presensitization by removal of CD25+ regulatory T cells (9). To find out whether MAGE-A3-specific CD4+ T cell precursors in healthy donors are present in naïve T cell population, CD45RA+ naïve 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 naïve 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 naïve 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 ELISOPOT 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, −5, and −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).

**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>Healthy donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC173&lt;sup&gt;a&lt;/sup&gt;</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>NC236&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4</td>
<td>0.0</td>
<td>0.3</td>
<td>2.9</td>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NC193&lt;sup&gt;c&lt;/sup&gt;</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>NC229&lt;sup&gt;d&lt;/sup&gt;</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>Seropositive cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU649&lt;sup&gt;e&lt;/sup&gt;</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>LU729&lt;sup&gt;f&lt;/sup&gt;</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>

<sup>a</sup> 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.

<sup>b</sup> Sum of cytokine levels produced against nos. 1–10, 11–20, and 21–30 pools.

<sup>c</sup> Sum of cytokine levels produced against nos. 1–14 and 15–30 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-expressing cells after restimulation with T-APC loaded with the same peptide pool was evaluated by flow cytometry. Pre, Before vaccination; d, day.

As shown in Table II, GM-CSF production by MAGE-A3-specific CD4\(^+\) T cells from patient WS07 (Pre), before and after vaccination, was evaluated by flow cytometry after restimulation with MAGE-A3 overlapping peptides and CD154-expressing cells after restimulation with T-APC loaded with the same peptide pool was evaluated by flow cytometry after restimulation with MAGE-A3 overlapping peptides and CD154-expressing cells after restimulation with T-APC loaded with the same peptide pool was evaluated by flow cytometry. Pre, Before vaccination; d, day.

Table II. GM-CSF production by MAGE-A3-specific CD4\(^+\) T cell lines generated from patient WS07.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>MAGE-A3 alone (ng/ml)</th>
<th>MAGE-A3 + AS02B (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(no.)</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 0.1 0.2 0.0</td>
</tr>
<tr>
<td>17</td>
<td>0.0 0.0 0.1 0.0</td>
<td>0.0 1.2 0.0 0.6</td>
</tr>
<tr>
<td>22</td>
<td>0.0 9.4 1.0 0.1</td>
<td>0.0 0.1 0.0 0.0</td>
</tr>
<tr>
<td>25</td>
<td>3.2 3.0 1.6 1.2</td>
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<td>3.0 8.3 3.8 8.1</td>
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*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.

## 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 after restimulation with 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).

### 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 some peptides, such as nos.11, 12, and 25 in patient WS07, were consistently observed before and during the course of vaccination. In addition, some responses were observed transiently after vaccination. Similar observations were made for GO17, who was vaccinated in the presence of AS02B (Table III). GM-CSF production against no.12 was already seen before vaccination as well as during the vaccination period. Similarly, no. 29 peptide-specific T cells were marginally detected before vaccination but became one of the dominant responses after the first injection of MAGE-A3 with adjuvant. In contrast, the strongest
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 shown). 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>
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<tbody>
<tr>
<td></td>
<td>Pre</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.

---

**FIGURE 4.** Th1/Th2 polarization of MAGE-A3-specific T cell response after vaccination. CD4⁺ T cell lines obtained from WS07 and GO17 before and during the first course of vaccination were restimulated with MAGE-A3 overlapping peptide pool using EBV-B cells as APC. Cytokine levels in the supernatant were measured by ELISA. Results are presented as fold increase against the cytokine level of the prestudy sample. The IFN-γ (A) and IL-4 (B) levels of pre-CD4⁺ T cell lines (Pre) were 9.8 and 0.2 ng/ml for WS07 and 7.0 and 1.5 ng/ml for GO17, respectively. The levels of cytokines against unpulsed target cells were below the limit of detection (0.5 ng/ml for IFN-γ and 0.1 ng/ml for IL-4), d, day.
cells was below 0.1 ng/ml. d, day.

d, day.

autologous EBV-B cells. GM-CSF production against unpulsed EBV-B during the first course of vaccination against MAGE-A3 protein-pulsed EBV-B cells pulsed with graded concentration of MAGE-A3 peptides and GM-CSF levels in the supernatant were determined ELISA. Results are presented as relative change of apparent avidity of CD4\(^+\) T cells before and during vaccination.

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 production against 10,000 ng/ml peptides (apparent avidity) was calculated by interpolation of dose-dependent GM-CSF production shown in A. Results are presented as relative change of apparent avidity of CD4\(^+\) T cell lines compared with apparent avidity of CD4\(^+\) T cell lines before vaccination (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 before 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.

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. d, day.

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 vectors, 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 cell 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 preexisting tumor Ag-specific T cells in healthy individuals and cancer patients who showed no significant T cell response in conventional 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 frequency is expected to be higher than 2 \(\times 10^{-6}\) from the initial number of CD4\(^+\) T cells (5 \(\times 10^{5}\)), although the precise estimation of precursor frequency is difficult because cells were expanded for 20 days after peptide stimulation. In addition, when peptide specificity of MAGE-A3-specific CD4\(^+\) T cell lines from three independent cultures of healthy donors, NC173 and NC236, were compared, some peptides-specific T cells were detected only in one T cell line (data not shown), indicating that the precursor frequency for some epitopes is less than 2 \(\times 10^{-6}\) 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 techniques. However, this precursor frequency for healthy donors was within the range of 1.4 \(\times 10^{-6}\)–3.1 \(\times 10^{-7}\) 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-\(\gamma\)-producing CD4\(^+\) T cells from multiple wells containing a limited number of CD4\(^+\) T cells after stimulation 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 \(\times 10^{-6}\) and 5–25 \(\times 10^{-6}\), respectively, by IFN-\(\gamma\) 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-\(\gamma\) 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

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

HLA-restriction

Finally, the generation of MAGE-A3-specific T cell lines allowed us to precisely determine HLA-restriction of CD4\(^+\) T cell responses, which was difficult to do by using bulk T cells obtained by presensitization because of limited number of cells and high background reactivity against allogeneic EBV-B cells. As shown in supplemental Fig. S3, HLA-restriction of most peptide responses was determined and HLA restrictions were found to be distributed to most of patients’ HLA types.
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 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-γ-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 low 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-1 157–170 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.