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Vaccine-Induced CD4^{+} T Cell Responses to MAGE-3 Protein in Lung Cancer Patients[1]

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MAGE-3 is the most commonly expressed cancer testis Ag and thus represents a prime target for cancer vaccines, despite infrequent natural occurrence of MAGE-3-specific immune responses in vivo. We report in this study the successful induction of Ab, CD8^{+}, and CD4^{+} T cells in nonsmall cell lung cancer patients vaccinated with MAGE-3 recombinant protein. Two cohorts were analyzed: one receiving MAGE-3 protein alone, and one receiving MAGE-3 protein with adjuvant AS02B. Of nine patients in the first cohort, three developed marginal Ab titers and another one had a CD8^{+} T cell response to HLA-A2-restricted peptide MAGE-3 271–279. In contrast, of eight patients from the second cohort vaccinated with MAGE-3 protein and adjuvant, seven developed high-titered Abs to MAGE-3, and four had a strong concomitant CD4^{+} T cell response to HLA-DP4-restricted peptide 243–258. One patient simultaneously developed CD8^{+} T cells to HLA-A1-restricted peptide 168–176. The novel monitoring methodology used in this MAGE-3 study establishes that protein vaccination induces clear CD4^{+} T cell responses that correlate with Ab production. This development provides the framework for further evaluating integrated immune responses in vaccine settings and for optimizing these responses for clinical benefit. *The Journal of Immunology,* 2004, 172: 3289–3296.

The MAGE-3 Ag was identified during analysis of CD8^{+} T cell reactivity against an autologous melanoma cell line and was found to be encoded by a member of a multigene family located on the X chromosome (1). MAGE-3 belongs to the growing class of cancer testis (CT)[3] Ags that are expressed only in testicular germ cells and no other normal tissue, yet aberrantly found in a broad variety of tumors (2). A major characteristic of many CT Ags is their capacity to elicit spontaneous immune reactions in cancer patients (3, 4). However, even though ~40% of nonsmall cell lung cancers express MAGE-3 (1, 2, 5, 6), patients with naturally occurring immune responses to MAGE-3 actually appear to be very rare (7–9).

Still, several CD8^{+} T cell epitopes of MAGE-3 have been identified in vitro (10–18), including HLA-A1-restricted epitope 168–176 (1) and HLA-A2-restricted epitope 271–279 (19). Based on these findings, synthetic peptides corresponding to these epitopes have been introduced into clinical vaccination studies in which they were associated with regression of melanoma in individual cases (20). However, circulating anti-MAGE-3 CD8^{+} T cells have been very difficult to detect, even in patients with tumor regressions (21–24).

Studies have indicated that CD4^{+} Th cells in vivo have the capacity to enhance CD8^{+} T cell activity (25–27) and, most importantly, help to maintain the immune response for sustained periods of time (27–29). Therefore, it seems likely that optimal antitumor activity can only be achieved if both CD4^{+} and CD8^{+} tumor-specific T cells are induced (30, 31). The inclusion of CD4^{+} epitopes into MAGE-3 vaccination studies has recently been facilitated by the identification of several HLA-DR-restricted (32–35) and one HLA-DP4-restricted epitope (36, 37).

Clinical vaccination studies using full-length recombinant proteins have the advantage that this form of Ag potentially includes the full range of epitopes for CD4^{+} and CD8^{+} T cells. In addition, it is likely that protein vaccination leads to presentation of epitopes in the context of various HLA alleles, and therefore this type of vaccine should be applicable to any patient regardless of HLA restriction. To date, only one clinical study using MAGE-3 protein as a vaccine has been reported (38). Using a cloning approach, one patient was shown to have a CD4^{+} T cell response to HLA-DR1-restricted peptide 267–282 (39).

We have recently introduced new methodologies for monitoring CD8^{+} (3) and CD4^{+} (4, 40) T cell responses in uncloned populations at the single cell level, to explore the repertoire of naturally occurring T cells against another CT Ag, NY-ESO-1. This is particularly important in the analysis of CD4^{+} T cells, in which high background precluded the interpretation of the specificity of responses. We now applied our experience to MAGE-3 monitoring and show that vaccination with rMAGE-3 protein results in the production of anti-MAGE-3 Ab and the generation of peptide-specific CD4^{+} and CD8^{+} T cells in patients with nonsmall cell lung cancer.

Materials and Methods

Patients

Seventeen patients with MAGE-3-expressing stage I or II nonsmall cell lung cancer were analyzed in this study. All patients had undergone surgical resection of their primary lung tumor at the Department of Cardio-Thoracic Surgery, Weill Medical College of Cornell University, and had no

[3] Abbreviations used in this paper: CT, cancer testis; ASC, Ag-stimulating cell; NP, nucleoprotein.

evidence of disease at the onset of the study. Tumor expression of the gene MAGE-3 was assessed by RT-PCR. Patients provided informed consent to participate in the experimental vaccination study and to donate blood for immunological monitoring. The study was approved by the Institutional Review Board of Weill Medical College of Cornell University and sponsored by the Ludwig Institute for Cancer Research under a Ludwig Institute IND (Investigational New Drug).

Vaccine

The MAGE-3 protein preparation used in this trial was a DNA recombinant fusion protein (ProteinD MAGE-3/His) expressed in *Escherichia coli* AR58 (GlaxoSmithKline, Rixensart, Belgium), as described (38). This experimental vaccine was injected alone or in combination with adjuvant AS02B (Adjuvant System 2B; GlaxoSmithKline). Adjuvant AS02B contains monophosphoryl lipid A and QS21, a saponin extracted from the South American tree Quillaja Saponaria Molina.

Study design

The first nine consecutive patients received 300 μg of MAGE-3 protein alone; the remaining eight patients received MAGE-3 protein combined with AS02B adjuvant containing 100 μg of monophosphoryl lipid A and 100 μg of QS21 in oil/water emulsion in a final formulation volume of 500 μl. The vaccination schedule of this phase II study was as follows: four intradermal injections (protein alone cohort) or four i.m. injections (protein with adjuvant cohort) at 3-wk intervals (days 1, 22, 43, and 64). Blood for immunomonitoring purposes was drawn at five different time points (pre-study, and days 22, 43, 64, and 85).

MAGE-3 Ab

Serum IgG Ab against MAGE-3 protein was measured by ELISA using MAGE-3 full-length protein (GlaxoSmithKline) and rMAGE-3 truncated protein (aa 57–219) purified from *E. coli*, as described before (7). We used a protein from a different source than the vaccine agent, to avoid potential reactivity with protein D, and found consistent results when cotyping reactivity against the MAGE-3 protein used for vaccination (7). MAGE-1 and NY-ESO-1 recombinant proteins were used as negative controls, to rule out reactivity against bacterial contaminants (7). Positive results were defined from titration curves, as described before (7). In summary figures, to facilitate comparison of Ab responses between patients, all sample OD values at 1/400 serum dilution were normalized according to positive and negative control sera using the following formula: $(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})$. Positive control serum from patient A25 ranged from 1000 to 1900 absorbance units, and negative control serum from patient NW29 ranged from 100 to 300 absorbance units at 1/400 dilution.

Peptides and viral vectors

MAGE-3.DP4 peptide 243–258 (KKLLTQHFVQENYLEY) was provided by Clinalfa AG (Läufelfingen, Switzerland). MAGE-3.A1 peptide 168–176 (EVDPIGHLY) and MAGE-3.A2 peptide 271–279 (FLWGPRALV) were synthesized by Multiple Peptide Systems (San Diego, CA). Influenza A nucleoprotein (NP) peptide 206–229 (FWRGENGRKTRIAYERMC NILKGK), NY-ESO-1 peptides 159–167 (LMWITQCFL), and 80–109 (ARGPESRLLEFYLAMPFATPMEAELARRSL) were obtained from Bio-Synthesis (Lewisville, TX). All peptides had a purity >90%. Vaccinia virus recombinant for full-length NY-ESO-1 was obtained from THERION Biologics (Cambridge, MA) and was constructed, as described (41). Vaccinia virus recombinant for full-length MAGE-3 was kindly provided by V. Cerundolo (Weatherall Institute of Molecular Medicine, Oxford, U.K.).

In vitro presensitization

PBMC were collected using a Ficoll gradient and were frozen in RPMI 1640 containing 10% FCS and 10% DMSO in liquid nitrogen until further processing. HLA typing of donor PBMCs or derived cell lines was done by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures. CD4$^+$ and CD8$^+$ T lymphocytes were separated from PBMC of healthy donors and cancer patients using Ab-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) and seeded into round-bottom 96-well plates (Corning, NY) at a concentration of 5×10^5 cells/well in RPMI 1640 medium with 10% human AB serum (Gemini Bio-Products, Woodland, CA), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% nonessential amino acids. As Ag-stimulating cells (ASC) for presensitization, PBMC depleted of CD4$^+$ and CD8$^+$ T cells were pulsed with 10 μM of peptide overnight at 37°C in 500 μl of serum-free medium (X-VIVO-15; BioWhittaker, Walkersville, MD). Pulsed CD4$^-$/CD8$^-$ ASC were then washed, irradiated, and

added to plates containing CD4$^+$ or CD8$^+$ T cells at a concentration of 1×10^6 ASC/well. After 20 h, IL-2 (10 U/ml; Roche Molecular Biochemicals, Indianapolis, IN) and IL-7 (20 ng/ml; R&D Systems, Minneapolis, MN) were added. Subsequently, one-half of medium was replaced by fresh complete medium containing IL-2 (20 U/ml) and IL-7 (40 ng/ml) twice per week.

Generation and culture of target cells

A fraction of CD4$^+$ T cells remaining from the initial separation (see above) was seeded into 24-well plates (Corning Glass, Corning, NY) at a concentration of $2–4 \times 10^6$ cells/well in complete medium supplemented with 10 μg/ml PHA (PHA HA15; Murex Diagnostics, Dartford, U.K.). Cells were fed and expanded twice per week with complete medium containing IL-2 (10 U/ml) and IL-7 (20 ng/ml). The activated T cell APCs (T-APC) were typically harvested and used as target cells after 20–30 days of culture. EBV-transformed B lymphocytes (EBV-B cells), the mutant TAP-deficient cell line T2 (CEM $\times$ 721.174.T2), and the HLA-A1$^+$/ MAGE-3$^+$ melanoma cell line SK-MEL-128 were cultured in RPMI 1640 medium supplemented with 10% FCS (Summit Biotechnology, Ft. Collins, CO), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ ml), and 1% nonessential amino acids. In all assays, target cell APC were washed twice in X-VIVO-15 medium to remove serum and were resuspended in appropriate medium for testing.

Tetramer staining

HLA-A1 tetramer assembled with MAGE-3.A1 peptide 168–176 (EVD-PIGHLY) was a kind gift from D. Colau from the Ludwig Institute for Cancer Research (Brussels, Belgium). HLA-A2 tetramers assembled with MAGE-3.A2 peptide 271–279 (FLWGPRALV) were obtained from I. Luescher at the Ludwig Institute core facility (Lausanne, Switzerland). Presensitized CD8$^+$ T cells in 50 μl of PBS containing 3% FCS (Summit Biotechnology) were stained with PE-labeled tetramer for 15 min at 37°C before addition of Tricolor-CD8 mAb (Caltag Laboratories, South San Francisco, CA) and FITC-conjugated anti-CD62L mAb (Caltag Laboratories) or fluorescein-conjugated anti-CCR7 mAb (R&D Systems) for 15 min on ice. After washing, results were analyzed by flow cytometry (FACS-Calibur; BD Biosciences, San Diego, CA).

ELISPOT assays

ELISPOT assays for the determination of Ag-specific effector cells were usually performed on day 10 of presensitizing culture for CD8$^+$ T cells and on day 20 for CD4$^+$ T cells. Flat-bottom, 96-well nitrocellulose plates (MultiScreen-HA; Millipore, Bedford, MA) were coated with IFN-γ mAb (2 μg/ml, 1-D1K; Mabtech, Stockholm, Sweden) and incubated overnight at 4°C. After washing with RPMI 1640, plates were blocked with 10% human AB-type serum for 2 h at 37°C. Target cells were pulsed at 37°C in 500 μl of serum-free medium with 10 μM peptide for 1 h (target cells for CD8$^+$ effectors) or overnight (target cells for CD4$^+$ effectors). In some assays, target cells were infected overnight with 20 PFU/cell vaccinia virus recombinant either for NY-ESO-1 or for MAGE-3. Target cells were washed twice and were resuspended in RPMI 1640 medium without serum. A total of 5×10^4 or 1×10^4 presensitized CD4$^+$ or CD8$^+$ T effector cells and 1×10^5 targets cells (T2 cells, T-APC, or EBV-B cells) was added to each well and incubated for 20 h. Plates were then washed thoroughly with water containing 0.05% Tween 20, and anti-IFN-γ mAb (0.2 μg/ml, 7-B6-1-biotin; Mabtech) was added to each well. After incubation for 2 h at 37°C, plates were washed and developed with streptavidin-alkaline phosphatase (1 μg/ml; Mabtech) for 1 h at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma-Aldrich, St. Louis, MO) was added and incubated for 5 min. Plate membranes displayed dark-violet spots that were scanned and counted using C.T.L. ImmunoSpot analyzer and software (Cellular Technologies, Cleveland, OH).

Measurement of intracellular cytokines (CYTOSPOT)

Pulsed T-APC were stained for 10 min at 37°C in 500 μl of X-VIVO-15 with 0.2 μM CFSE (Molecular Probes, Eugene, OR). Target cells were then washed with cold complete medium and were resuspended in X-VIVO-15. Presensitized CD4$^+$ effector T cells were incubated with pep-tide-pulsed CD4$^+$ T-APC at a 1:2 ratio in 200 μl of X-VIVO-15 at 37°C for 2 h. Brefeldin A (Sigma-Aldrich) at 10 μg/ml was added to each sample and cells were incubated for an additional 5-h period. Cells were then fixed using FACS Lysing Solution (BD Biosciences) diluted 1/10, permeabilized using Permeabilizing Solution 2 (BD Biosciences), and stained with Tri-color-labeled anti-CD4 mAb (Caltag Laboratories), APC-labeled anti-IFN-γ mAb, and PE-labeled anti-IL-2, anti-TNF-α, anti-IL-4, anti-IL-5,

and anti-IL-10 mAb (BD PharMingen, San Diego, CA) at room temperature for 15 min. Cells were subsequently analyzed by flow cytometry with gating on morphologically defined lymphocytes and CD4-positive and CFSE-negative cells.

Results

Vaccination with MAGE-3 protein in combination with adjuvant AS02B results in the generation of anti-MAGE-3 Abs

Of nine patients who had been vaccinated with MAGE-3 protein in the absence of adjuvant, three (WS-07, AS-08, and SG-09) developed a modest, but significant increase in Abs against MAGE-3 protein, as measured by ELISA (Fig. 1, *left*). In contrast, of eight patients who received MAGE-3 protein in combination with adjuvant AS02B, seven showed a marked increase in serum concentrations of anti-MAGE-3 (Fig. 2, *left*). Increases in Ab titers usually became significant on sample day 43, 3 wk after the patients had received the vaccine for the second time (see Fig. 3 for representative patients in titration experiments). Maximum serum levels were reached at day 85, the end of the observation period after four vaccine injections.
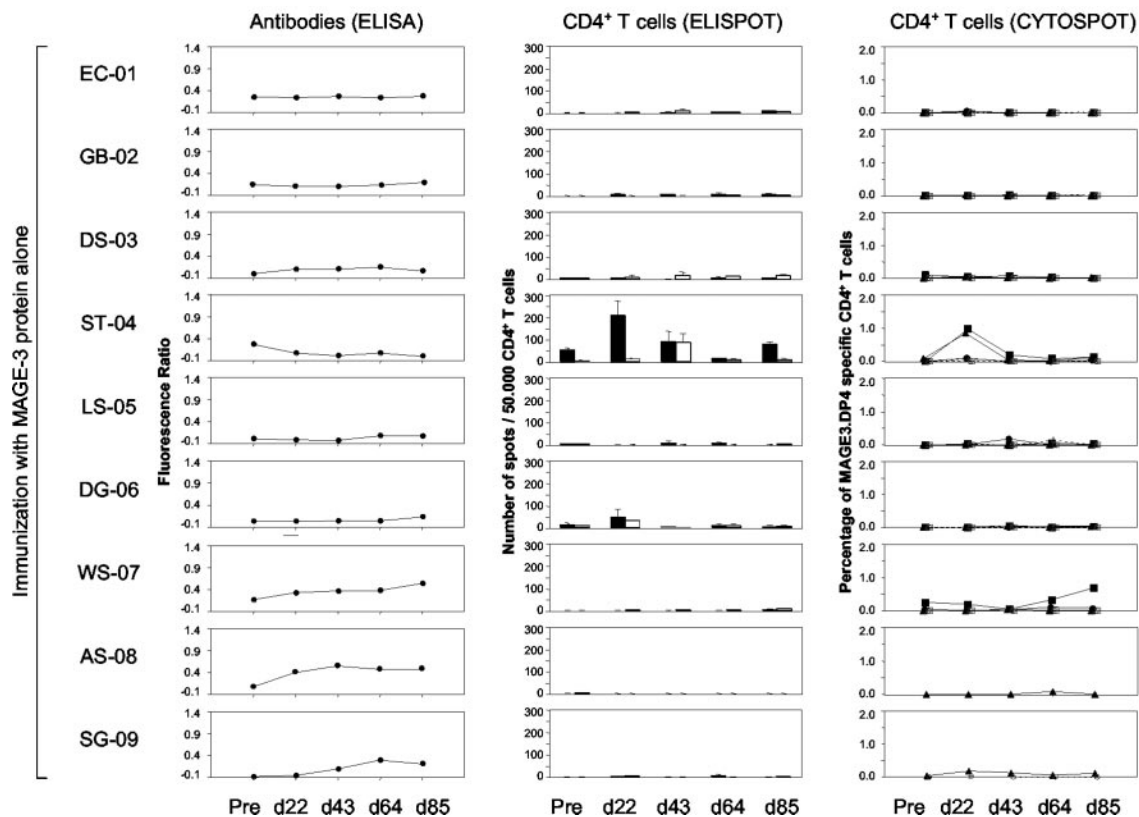
Vaccination with MAGE-3 protein in combination with adjuvant AS02B evokes a strong CD4+ T cell response

We have previously introduced activated T cells (T-APC) as targets in a modified IFN-γ ELISPOT assay (40). In this highly sensitive assay, Ag-specific CD4+ T cells can be detected on the basis of their cytokine secretion over a very low background. Using the same technique, we analyzed CD4+ T cell responses against MAGE-3 in all patients.

To examine whether these patients had in principle the capacity to develop a CD4+ T cell response, or whether T cell immunity might have been compromised by malignant disease, we first examined CD4+ T cell responses directed against a promiscuous epitope of influenza NP. All patients, with the exception of DS-03, showed good responses (mean: 399 spots/50,000 CD4+ T cells) against T-APC pulsed with NP peptide 206–229 (data not shown).

We then examined CD4+ T cell responses against peptide MAGE-3.DP4 in all 17 patients. This epitope was chosen for the frequent distribution of its restriction allele HLA-DP4 and its proven immunogenicity (37). We observed that only one of the patients who had received MAGE-3 protein without adjuvant AS02B showed a CD4+ T cell response against MAGE-3.DP4 in ELISPOT assays (Fig. 1, *middle*). It seemed that in this patient (ST-04) expressing the HLA-DP4 haplotype (Table I), a pre-existing immunity against MAGE-3 was present, because his CD4+ T cells secreted IFN-γ in response to MAGE-3.DP4-pulsed T-APC even before he had received the first vaccination. Also, the same patient had low-titered pre-existing Ab titers against MAGE-3 protein. However, repeated immunizations with MAGE-3 protein in the absence of AS02B adjuvant did not have a boosting effect on the level of MAGE-3.DP4-specific T cell immunity or the level of anti-MAGE-3 Abs in this specific patient.

All eight patients who had received MAGE-3 protein in combination with adjuvant AS02B were found to have the HLA-DP4 allele (Table I). Of these patients, four (WG-13, ER-14, GT-15, and GO-17) showed a marked increase in CD4+ T cell responses against MAGE-3.DP4 (Fig. 2, *middle*). All responding patients had

FIGURE 1. Monitoring of Ab and CD4+ T cell responses in patients vaccinated with MAGE-3 protein without adjuvant. *Left*, Ab responses to MAGE-3 by ELISA at different vaccination time points. To facilitate comparison between patients, all sample OD values at 1/400 serum dilution were normalized according to positive and negative controls. *Middle*, CD4+ T cell responses as measured by IFN-γ ELISPOT. Presensitized CD4+ T cells (50,000) were tested against T-APC pulsed with MAGE-3.DP4 (■) or irrelevant peptide (□). *Right*, CD4+ T cell responses as measured by staining of intracellular cytokines (CYTOSPOT). Percentage of presensitized CD4+ T cells specifically producing TNF-α (■), IFN-γ (▲), IL-2 (●), IL-10 (□), IL-4 (△), or IL-5 (○) against MAGE-3.DP4.
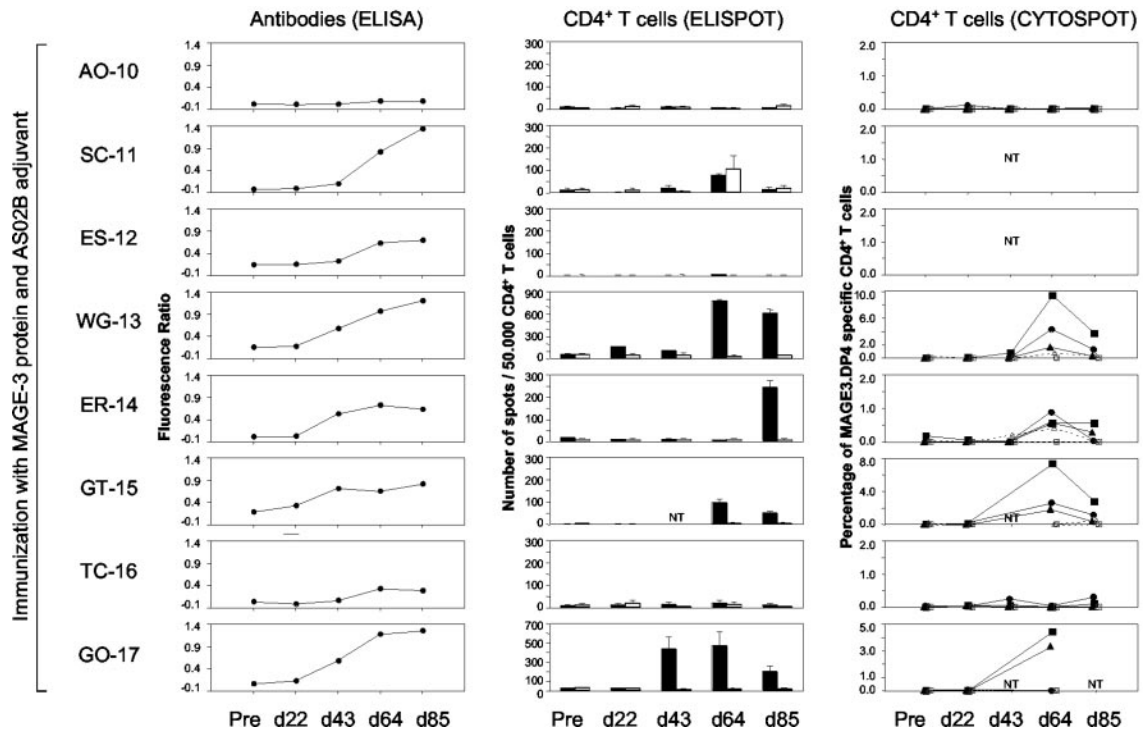
FIGURE 2. Induction of strong Ab and CD4^{+} T cell responses in patients vaccinated with MAGE-3 protein plus adjuvant. See Fig. 1 for legends.

a concomitant humoral response against MAGE-3. CD4^{+} T cells specific for the tumor Ag appeared between days 43 and 64 and were always clearly detectable until day 85, the last sampling date. A presensitization with the irrelevant peptide NY-ESO-1 80–109 as a specificity control did not lead to the generation of effector cells specific for this epitope in any of the patients (data not shown). Numbers of IFN-γ-producing spots after presensitization with positive control peptide NP 206–229 were comparable to number of spots for MAGE-3.DP4 in responding patients (mean = 866 spots for NP/50,000 CD4^{+} T cells for patient WG-13; 921 spots for ER-14; 71 spots for GT-15; 733 spots for GO-17).

CD4^{+} T cells induced by vaccination with MAGE-3 protein in combination with adjuvant AS02B produce Th1-, but no Th2-type cytokines

We next performed a more detailed analysis of the pattern of cytokines produced by patients' CD4^{+} T cells in response to MAGE-3.DP4. We had previously determined that our in vitro culture conditions were likely to reflect the pre-existing cytokine profile of effectors (40). Using T-APC as targets, we performed a flow cytometry analysis of a variety of intracellular Th1-type (IFN-γ, IL-2, TNF-α) or Th2-type (IL-4, IL-5, IL-10) cytokines in all but two patients (Figs. 1 and 2, *right*).
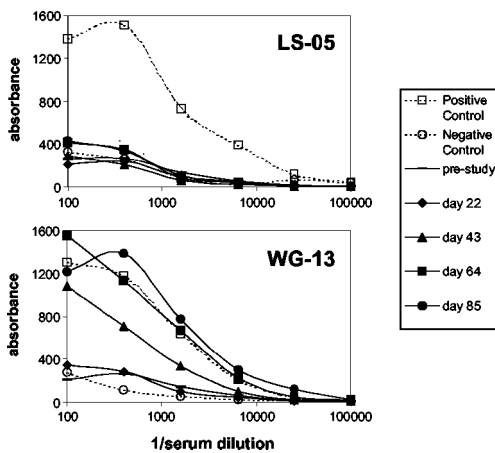
FIGURE 3. Characteristic titration curves by ELISA of patient sera responding (WG-13) or not (LS-05) to MAGE-3 protein at various study dates. Positive control serum (□) was from melanoma patient A25 with spontaneous humoral response to MAGE-3, and negative control serum (○) was from patient NW29.

Table I. *HLA type of all patients included into the MAGE-3 protein vaccination study[a]*

	MHC Class I HLA-A		MHC Class II HLA-DP4 status
EC-01	0301	**0205**	−
GB-02	**0101**	0301	+
DS-03	2402	2402	+
ST-04	**0205**	2301	+
LS-05	0301	2402	+
DG-06	**0201**	3001	+
WS-07	**0201**	3001	−
AS-08	1101	3101	+
SG-09	**0201**	**0201**	−
AO-10	**0101**	0304	+
SC-11	**0201**	2301	+
ES-12	**0201**	0301	+
WG-13	**0101**	2501	+
ER-14	2301	3101	+
GT-15	**0101**	**0201**	+
TC-16	**0101**	0301	+
GO-17	**0201**	6801	+

[a] Shown are the HLA-A alleles and the status of HLA-DP4 expression by the given patient. Bold characters highlight patients with HLA-A1 or HLA-A2 expression.

We found that results in the ELISPOT assay were closely paralleled by those observed after staining of intracellular cytokines. Of the eight patients who received MAGE-3 protein in combination with adjuvant, the same four patients that had clear CD4$^+$ responses in the ELISPOT assays also showed MAGE-3.DP4 peptide-induced increase in intracellular cytokines (Fig. 2).

The cytokines that were produced in response to MAGE-3.DP4 Ag were almost exclusively of Th1 type. None of the patients showed significant increases in the intracellular concentrations of IL-4, IL-5, or IL-10 in response to MAGE-3. In contrast, we observed marked increases in the intracellular concentration of IFN-γ and IL-2 following exposure to T-APC pulsed with the MAGE-3.DP4 peptide. TNF-α, however, seemed to be by far the most sensitive parameter for the detection of MAGE-3-specific CD4$^+$ T cells, as shown in a representative patient (Fig. 4).

Vaccination with MAGE-3 protein induces MAGE-3.A1- and MAGE-3.A2-specific CD8$^+$ T cells

We looked for the presence of MAGE-3.A1- or MAGE-3.A2-specific T cells in patients that had been vaccinated with MAGE-3 protein by performing tetramer analyses (Table II) and ELISPOT assays (Table III).

Of nine HLA-A2$^+$ patients analyzed, one (DG-06) showed a significant increase in tetramer-positive MAGE-3.A2-specific CD8$^+$ T cells on day 85. These T cells were negative for CD62L and CCR7, and therefore expressed an effector phenotype (Fig. 5A). When an ELISPOT assay was performed using these effector cells, they recognized T2 cells or T-APC pulsed with the HLA-A2-restricted epitope of MAGE-3 (Fig. 5B).

Of five HLA-A1$^+$ patients analyzed, one (GT-15) showed a marked increase in tetramer-positive MAGE-3.A1-specific CD8$^+$ T cells from day 43 onward. These T cells expressed intermediate levels of CD62L and were negative for CCR7 (Fig. 6A). However, an IFN-γ ELISPOT assay performed using this effector cell line revealed that these CD8$^+$ T cells did not recognize EBV-B cells, whether they were pulsed with MAGE-3.A1 peptide or infected

FIGURE 4. Vaccination with MAGE-3 protein induces a strict Th1 type of response against peptide MAGE-3.DP4. The figure shows the typical pattern of intracellular cytokine staining in a patient (WG-13) responding to the vaccine. PBL were obtained on day 64 after study onset, and MAGE-3.DP4-specific CD4$^+$ T cells were expanded in a single cycle of peptide-driven stimulation. FACS analysis of intracellular concentration of Th1-type (IFN-γ, IL-2, TNF-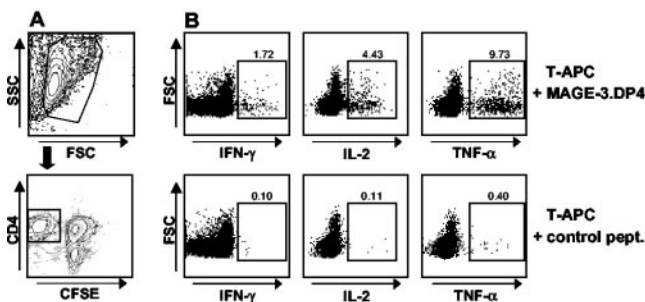α) and Th2-type (IL-4, IL-5, IL-10) cytokines was performed on day 25 postculture initiation. To distinguish them from effector cells, CD4$^+$ T-APC were stained using the intracellular dye CFSE before the assay was started. Targets were later excluded from the analysis using double gating on morphologically defined lymphocytes and gating on CFSE-negative and CD4$^+$ cells. Responding patients usually showed strong Th1-type responses against peptide MAGE-3.DP4-pulsed T-APC. Th2-type cytokine concentration did not exceed background levels (data not shown). Background levels were determined using T-APC pulsed with the irrelevant control NY-ESO-1 peptide 80–109 and were typically below 0.1% of effector CD4$^+$ T cells.

Table II. *CD8$^+$ responses against MAGE-3 as indicated by tetramer analysis*[a]

		Pre	Day 22	Day 43	Day 64	Day 85
MAGE-3.A1	GB-02	0.09	0.07	0.04	0.06	0.03
	LS-05	0.08	0.08	0.08	0.05	0.14
	AO-10	0.10	0.11	0.05	0.07	0.10
	WG-13	0.23	0.14	0.12	0.13	0.14
	GT-15	0.31	0.11	**3.23**	**6.59**	**0.79**
	TC-16	0.14	0.14	0.09	0.1	0.08
MAGE-3.A2	EC-01	0.00	0.02	0.00	0.00	0.02
	DS-03	0.01	0.01	0.01	0.01	0.00
	DG-06	0.10	0.05	0.14	0.16	**1.60**
	WS-07	0.10	0.00	0.06	0.00	0.03
	SG-09	0.06	0.00	0.01	0.19	0.10
	SC-11	0.00	0.05	0.03	0.02	0.00
	ES-12	0.08	0.14	0.07	0.12	0.07
	GT-15	0.00	0.00	0.00	0.04	0.01

[a] All five HLA-A1* patients and eight HLA-A2* patients were analyzed CD8$^+$ T cells were expanded by a single peptide-driven stimulation with peptide MAGE-3.A1 (168–176) or MAGE-3.A2 (271–279), respectively. Effector cells were tested on day 10 against using the given tetramer. Numbers given indicate the percentage of tetramer-positive CD8$^+$ T cells. Bold numbers represent significant positive responses.

with vaccinia virus recombinant for MAGE-3. Furthermore, the same CD8$^+$ T cells did not recognize a MAGE-3-expressing HLA-A1$^+$ melanoma cell line even after this tumor cell line had been pulsed with MAGE-3.A1 peptide (Fig. 6B).

Discussion

Successful vaccination of animals with tumor Ags has been shown to be largely dependent on CD8$^+$ T cells, and adoptive transfer of purified CD8$^+$ T cell fractions can mediate tumor regression in mice (42). However, it has recently become clear that CD4$^+$ T cells play an important role in the antitumor response following vaccination (43, 44). Although CD4$^+$ T cells seem to also possess the potential for an immediate effector function against tumor targets, in vitro (45–47) and in vivo (48), their main role is still widely believed to be that of a helper cell. Thus, activated CD4$^+$ T cells help to initiate, amplify, and maintain CD8$^+$ T cell responses. They do so by providing important costimulation via a variety of surface molecules (49), by secretion of a network of cytokines (50), and by activating professional APC (51–56). In addition, studies have suggested that CD4$^+$ T cells have the capability to recruit eosinophils as well as macrophages into the tumor tissue. These cells, activated by neighboring tumor-specific CD4$^+$ T cells, might then contribute to an effective antitumor function by producing factors such as superoxide and NO (57).

We have used a rMAGE-3 protein as a vaccine in patients with nonsmall cell lung cancer. This approach, in addition to the generation of MAGE-3-specific CD8$^+$ T cells, theoretically allows the introduction of specific CD4$^+$ T cell help into the T cell-mediated antitumor reaction. We show in this study that vaccination with a protein of a CT Ag indeed provides a strong peptide-specific CD4$^+$ T cell response. The occurrence of CD4$^+$ T cell responses correlated with Ab responses. Importantly, we monitored this protein-based vaccination study using peptides as Ag in our assays. This excludes the possibility of T cell responses against contaminants in the protein batch that have been observed in assays using MAGE-3 protein as stimulating Ag and in the readout assay (32).

The MAGE-3 protein-induced CD4$^+$ T cell responses were of the Th1 type, suggesting a supporting role of these Th cells for anti-MAGE-3 CD8$^+$ T cell responses in vivo. In addition, it has previously been shown that MAGE-3.DP4-specific CD4$^+$ T are in principle capable of lysing HLA-DP4$^+$ tumor cells expressing

Table III. *CD8$^+$ responses against MAGE-3, as indicated by ELISPOT analysis*[a]

		Pre	Day 22	Day 43	Day 64	Day 85
MAGE-3.A1	GB-02	113 (113)	28 (26)	36 (50)	32 (26)	3 (3)
	AO-10	45 (38)	38 (36)	38 (39)	28 (25)	44 (40)
	WG-13	106 (123)	65 (89)	106 (74)	128 (111)	99 (89)
	GT-15	18 (15)	11 (3)	15 (22)	54 (53)	31 (45)
	TC-16	165 (174)	139 (144)	115 (109)	158 (181)	129 (162)
MAGE-3.A2	EC-01	12 (0)	7 (5)	3 (7)	6 (4)	13 (4)
	DS-03	0 (1)	1 (0)	0 (3)	0 (0)	0 (1)
	DG-06	67 (18)	11 (13)	2 (0)	139 (109)	**214** (53)
	WS-07	3 (2)	2 (2)	4 (0)	7 (0)	4 (9)
	SG-09	9 (2)	9 (8)	10 (8)	23 (5)	5 (2)
	SC-11	3 (6)	1 (6)	3 (5)	1 (1)	4 (14)
	ES-12	28 (62)	11 (18)	2 (11)	18 (12)	17 (13)
	GT-15	6 (3)	3 (0)	0 (4)	2 (0)	1 (8)
	GO-17	4 (16)	4 (5)	8 (54)	8 (3)	16 (14)

[a] All five HLA-A1$^+$ patients and nine HLA-A201$^+$ patients were analyzed. CD8$^+$ T cells were expanded by a single peptide-driven stimulation with peptide MAGE-3.A1 (168–176) or MAGE-3.A2 (271–279). respectively. Effector cells were tested on day 10 against the HLA-A1$^+$ EBV-transformed B cell line from healthy donor NC 32 or T2 cells, respectively. Targets were pulsed with the relevant MAGE-3 peptide. Numbers given indicate specific IFN-γ spots per 50,000 CD8$^+$ effector T cells. Numbers in brackets indicate background reactivity against unpulsed targets (for HLA-A1$^+$ patients) or targets pulsed with the irrelevant NY-ESO-1 peptide 159–167 (for HLA-A*0201$^+$ patients). Bold numbers represent significant positive responses.

MAGE-3 (36), indicating that this epitope, in contrast to other class II MAGE-3 epitopes (32), may be presented on the surface of tumor cells.

In addition to the strong CD4$^+$ T cell responses in 50% of patients receiving MAGE-3 protein plus adjuvant, we also observed peptide-specific CD8$^+$ T cell responses in two patients. Previous in vitro studies have indicated that the HLA-A1-restricted MAGE-3 peptide 168–176 is naturally processed by professional (58) and nonprofessional APC (1, 59), and the same peptide has been eluted from cancer cell lines (60). In this study, we show that the MAGE-3.A1 peptide is naturally processed in vivo and that this leads to a strong increase in peripheral numbers of CD8$^+$ T cells specific for this epitope, in coordination with MAGE-3 CD4$^+$ T cell and Ab responses. The vaccine-induced MAGE-3.A1-specific CD8$^+$ T cells could easily be expanded using a single cycle of peptide-driven stimulation. De novo CD8$^+$ T cells were not generated during this in vitro expansion phase because reactivity to MAGE-3.A1 was not detected before the second vaccination.

Rather, CD8$^+$ T cells appeared as a result of vaccination, concomitantly to Ab and CD4$^+$ T cell responses in an individual patient. Following this 10-day culture period, the MAGE-3.A1 tetramer-positive cells expressed an effector cell phenotype. However, these CD8$^+$ T cells did not produce IFN-γ in response to target cells pulsed with MAGE-3.A1 peptide or MAGE-3-expressing HLA-A1$^+$ tumor cells. We did not examine whether the MAGE-3.A1-specific T cells produced other cytokines (i.e., T cytotoxic type 2 cytokines) in response to their respective epitope, and it remains to be further examined whether these cells have to be considered partially nonresponsive. MAGE-3.A1-specific CD8$^+$ T cells have previously been shown to kill MAGE-3-expressing tumor cells (1, 59), and future vaccination studies will
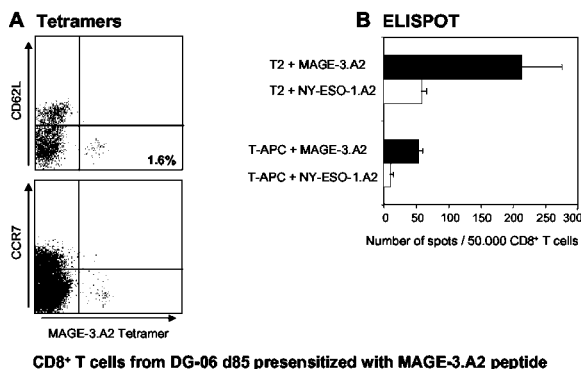
FIGURE 5. MAGE-3 protein vaccination-induced generation of fully functional MAGE-3.A2-specific CD8$^+$ T cells. PBL of patient DG-06 were obtained on day 85 after study onset, and MAGE-3.A2-specific CD8$^+$ T cells were expanded in a single cycle of peptide-driven stimulation. Tetramer analysis (A) was performed on day 10 postculture initiation. The CD8$^+$ T cells were costained for CD62L and CCR7. An ELISPOT assay (B) was performed on the same day using this tetramer-positive CD8$^+$ T cell line. Effector cells were tested against T2 cells or T-APC pulsed with MAGE-3.A2 peptide or the irrelevant NY-ESO-1 peptide 159–167.
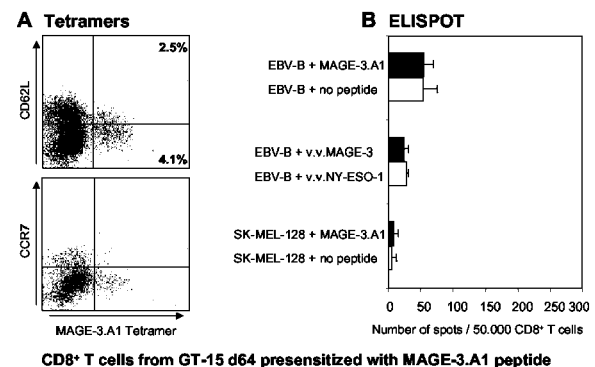
FIGURE 6. MAGE-3 protein vaccination-induced generation of non-IFN-secreting MAGE-3.A1-specific CD8$^+$ T cells. PBL of patient GT-15 were obtained on day 64 after study onset, and MAGE-3.A1-specific CD8$^+$ T cells were expanded in a single cycle of peptide-driven stimulation. Tetramer analysis (A) was performed on day 10 postculture initiation. The CD8$^+$ T cells were costained for CD62L and CCR7. An ELISPOT assay (B) was performed on the same day using this tetramer-positive CD8$^+$ T cell line. Effector cells were tested against autologous EBV-transformed B cells unpulsed or pulsed with MAGE-3.A1 peptide. The same tetramer-positive line was also tested against autologous EBV-B cells infected with vaccinia virus recombinant for full-length MAGE-3 or recombinant for NY-ESO-1 as irrelevant Ag. Finally, reactivity of the MAGE-3.A1-specific CD8$^+$ T cell line was tested against the peptide-pulsed or unpulsed HLA-A1$^+$, MAGE-3-expressing melanoma cell line SK-MEL-128.

show whether stronger adjuvants delivered with MAGE-3 protein or more prolonged immunization might lead to the in vivo generation of fully functional T cytotoxic type 1 MAGE-3.A1-specific CTL.

In vitro studies have indicated that the HLA-A2-restricted MAGE-3 epitope 271–279 is not naturally processed by nonprofessional APC, including most tumor cells (61). This seems to be caused by cleavage of the MAGE-3 protein at position 278 during its processing by the proteasome (62, 63). In contrast, it has been shown that professional APC using the immunoproteasome are capable of generating antigenic MAGE-3 peptides that are not produced by a standard proteasome (18), possibly including MAGE-3.A2. Accordingly, it has been shown that the processing of MAGE-3-expressing tumor cells by dendritic cells may result in the generation of $CD8^+$ T cells specific for peptide MAGE-3 271–279 (64–66).

We observed that the MAGE-3.A2 epitope seems to be naturally processed in vivo because the vaccination with rMAGE-3 protein resulted in the appearance of MAGE-3.A2-specific $CD8^+$ T cells in the peripheral blood of one patient. These $CD8^+$ T cells could easily be expanded using a single peptide-driven stimulation and were only detected after vaccination. Although MAGE-3.A2-specific $CD8^+$ T cells were seen in the absence of $CD4^+$ T cell or Ab response to MAGE-3, they were fully functional in the sense that they secreted IFN-γ in response to target pulsed with their respective peptide. Therefore, we suggest that in vivo MAGE-3 protein might have been taken up and processed in patients by professional APC, resulting in the generation of $CD8^+$ T cells specific for MAGE-3.A2 peptide 271–279. It remains questionable, however, whether these T cells will have clinical efficacy against nonprofessional APC, such as autologous tumor cells, even if these cells express the MAGE-3 gene.

In conclusion, we show in this study that vaccination with the recombinant protein of a CT Ag provides strong Ag-specific $CD4^+$ T cell help along with Ab and $CD8^+$ T cell responses, and leads to integrated immunity comparable to what is observed in patients with spontaneous responses to NY-ESO-1 (4). It is likely that responses against a range of $CD4^+$ and $CD8^+$ epitopes other than the ones we examined in this study were generated, and application of general methodologies (40, 41) will allow the identification of this repertoire.

The current study design included patients with no evidence of disease at the onset of the trial, precluding assessment of clinical efficacy at this early stage. The data presented in this work lay the grounds for the design of vaccine constructs and immunization protocols to define conditions for maximal immunogenicity and answer the most important question in tumor immunology: can immunization affect the course of human cancer?

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