Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming

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We previously reported results of a phase II trial in which recombinant MAGE-A3 protein was administered with or without adjuvant AS02B to 18 non-small-cell lung cancer (NSCLC) patients after tumor resection. We found that the presence of adjuvant was essential for the development of humoral and cellular responses against selected MAGE-A3 epitopes. In our current study, 14 patients that still had no evidence of disease up to 3 years after vaccination with MAGE-A3 protein with or without adjuvant received an additional four doses of MAGE-A3 protein with adjuvant AS02B. After just one boost injection, six of seven patients originally vaccinated with MAGE-A3 protein plus adjuvant reached again their peak antibody titers against MAGE-A3 attained during the first vaccination. All seven patients subsequently developed even stronger antibody responses. Furthermore, booster vaccination widened the spectrum of $CD4^+$ and $CD8^+$ T cells against various new and known MAGE-A3 epitopes. In contrast, only two of seven patients originally vaccinated with MAGE-A3 protein alone developed high-titer antibodies to MAGE-A3, and all these patients showed very limited $CD4^+$ and no $CD8^+$ T cell reactivity, despite now receiving antigen in the presence of adjuvant. Our results underscore the importance of appropriate antigen priming using an adjuvant for generating persistent B and T cell memory and allowing typical booster responses with reimmunization. In contrast, absence of adjuvant at priming compromises further immunization attempts. These data provide an immunological rationale for vaccine design in light of recently reported favorable clinical responses in NSCLC patients after vaccination with MAGE-A3 protein plus adjuvant AS02B.

antibody | $CD4^+$ T cell | $CD8^+$ T cell | immunization | non-small-cell lung cancer

Cancer/testis (CT) antigens all share a common expression pattern: They are found frequently in a large variety of human tumors but not in normal tissue, except immunoprivileged germ-line tissues (1). MAGE-A3 may well be the most commonly expressed gene among CT antigens and is detected in >50% of primary NSCLC (2, 3). A small proportion of these patients naturally develop antibody responses to MAGE-A3, indicating that this tumor antigen is capable of evoking spontaneous immune responses. Active immunotherapy holds the potential to boost such preexisting immune responses and to induce *de novo* MAGE-A3-specific immunity targeting MAGE-A3-expressing tumor cells.

MAGE-A3 vaccination using recombinant protein is likely to have several advantages when compared with vaccines consisting of commonly used "short" peptides. Such peptides are potentially presented by unprofessional antigen-presenting cells (APCs) in the absence of appropriate costimulation, notably from helper T cells, thus generating a less efficient immune response (4). In contrast, long peptides and proteins are much more likely to elicit an integrated immune response made of a variety of $CD4^+$ and $CD8^+$ T cell as well as B cell responses, after being taken up, processed, and presented by professional APCs (5, 6).

We previously reported the immunological results of a MAGE-A3 protein vaccination study in non-small-cell lung cancer (NSCLC) patients (7). Stage I/II patients without evidence of disease after resection of their MAGE-A3-expressing primary tumor received four injections at 3-week intervals of a recombinant MAGE-A3 fusion protein (MAGEA3/ProtD/His). Of 18 patients that completed the study, half received the protein alone (cohort 1) and half received the protein in the presence of AS02B, a saponin-based adjuvant containing monophosphoryl lipid A (cohort 2). By analyzing humoral immunity and T cell responses to selected MAGE-A3 peptides, we showed that vaccination with recombinant MAGE-A3 protein was able to induce antibody and $CD4^+$ T cell responses but that the presence of adjuvant AS02B was a prerequisite for the development of MAGE-A3-specific immunity.

In light of these encouraging immunological data, we sought to further define the requirements for immunological and clinical efficacy of this vaccine by exploring the initial impact of immunological adjuvant on long lasting memory responses after additional MAGE-A3 protein vaccination.

We describe here the immunological results of booster vaccination with MAGE-A3 protein. Of the 18 patients enrolled in study LUD99–010, 14 had no evidence of disease for up to 3 years after completing their original vaccine regimen. The 14 patients agreed to receive a new cycle of four tri-weekly injections of MAGE-A3 fusion protein; but this time, patients in both cohort 1 (originally vaccinated without adjuvant, $n = 7$) and cohort 2 (originally vaccinated with adjuvant, $n = 7$) received the MAGE-A3 protein in the presence of adjuvant AS02B. In addition, we evaluated a patient with pancreatic neuroendocrine cancer and a patient with pediatric osteogenic sarcoma, enrolled in compassionate single-patient protocols (SPP), who received eight consecutive injections every 3 weeks of MAGE-A3 fusion protein with AS02B adjuvant.

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Conflict of interest statement: GlaxoSmithKline has licensed from the Ludwig Institute for Cancer Research certain patents relating to the compositions used in the clinical trial reported in this article. E.R., G.R., E.W.H., L.J.O., and S.G. are employees of the Ludwig Institute for Cancer Research; however, none of them are inventors of the relevant patents.

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The immunological objectives of the current study were to: (*i*) investigate the breadth of vaccine-induced B and T cell responses by using comprehensive monitoring techniques with full-length MAGE-A3 antigen; (*ii*) analyze whether MAGE-A3 protein vaccination elicited long lasting immunological memory by measuring the impact of booster vaccinations; and (*iii*) address the impact of adjuvant AS02B in the initial vaccine preparation on long-term immunity and tolerance.

Results

Antibody Responses to MAGE-A3 Vaccination. Antibody responses against the MAGE-A3 and protein D components of the fusion protein vaccine were analyzed by ELISA. To prevent false positive results due to reactivity against potential bacterial contaminants within the vaccine preparation, we analyzed serological responses against unrelated proteins made in the same bacterial vector and against MAGE-A3 protein prepared from insect cells transfected with a baculovirus vector encoding for MAGE-A3.

We now confirmed and extended our previously published results of immune responses elicited during the first cycle of vaccination with MAGE-A3 fusion protein: Of seven patients in cohort 1 who received the MAGE-A3 fusion protein in saline, only one patient (AS-08) developed an antibody response to MAGE-A3 protein after four injections (Fig. 1). Interestingly, patient EC-01, who had a preexisting antibody response to MAGE-A1, a CT antigen that shares 66% amino acid sequence homology with MAGE-A3, did not show an increase in his antibody titer against MAGE-A1 nor seroconverted to recognize MAGE-A3. None of the patients in cohort 1 reacted with bacterially derived truncated MAGE-A3$_{64–226}$, including patient AS-08 [supporting information (SI) Fig. 5*A*]. In contrast, of seven patients in cohort 2 who received one cycle of vaccination with MAGE-A3 protein plus adjuvant AS02B, all except patient AO-10 developed very strong antibody titers to both full-length baculovirus-derived MAGE-A3 (Fig. 1) and *Escherichia coli*-derived truncated MAGE-A3$_{64–226}$ (SI Fig. 5*A*).

Next, we analyzed antibody responses to MAGE-A3 elicited during the second cycle of vaccination with MAGE-A3 protein plus adjuvant AS02B in both cohorts. Only two patients in cohort 2 (SC-11 and LK-19) still had detectable MAGE-A3 antibodies before repeat vaccination (Fig. 1), whereas all other patients had no detectable MAGE-A3 antibodies after an average period without vaccination of 945 days (±34) for cohort 1 and 598 days (±82) for cohort 2. Patient AO-10, the only patient in cohort 2 who had not reacted to MAGE-A3 after the first vaccine cycle, seroconverted after the second vaccine cycle. The six remaining patients in cohort 2 reached the maximum antibody titers to MAGE-A3 attained during the first vaccination after just one new injection and subsequently developed even stronger antibody responses. These responses were typical of a booster vaccination, indicating persistence of memory B cell precursors generating recall antibody responses upon reimmunization.

In striking contrast, only one of seven patients from cohort 1 (DS-03) developed a high-titered antibody response to MAGE-A3 after receiving the MAGE-A3 protein vaccine plus adjuvant (Fig. 1). Another four patients (EC-01, DG-06, WS-07, and SG-09) developed low-titer antibodies against MAGE-A3 after the second vaccine cycle (mean titer 1/500), whereas patient AS-08 gradually recovered the antibody titer originally seen after the first vaccine cycle without adjuvant. In comparison with what is expected from patients receiving MAGE-A3 protein and AS02B for the first time, the immunogenicity of the vaccine and adjuvant was markedly decreased in patients previously vaccinated with protein alone (mean reciprocal titer for cohort 1 at day 85b = 1,475 vs. mean reciprocal titer for cohort 2 at day 85 = 5,890, $P = 0.003$ by t test, SI Fig. 5*B*).

It is important to note that this decreased capacity to mount

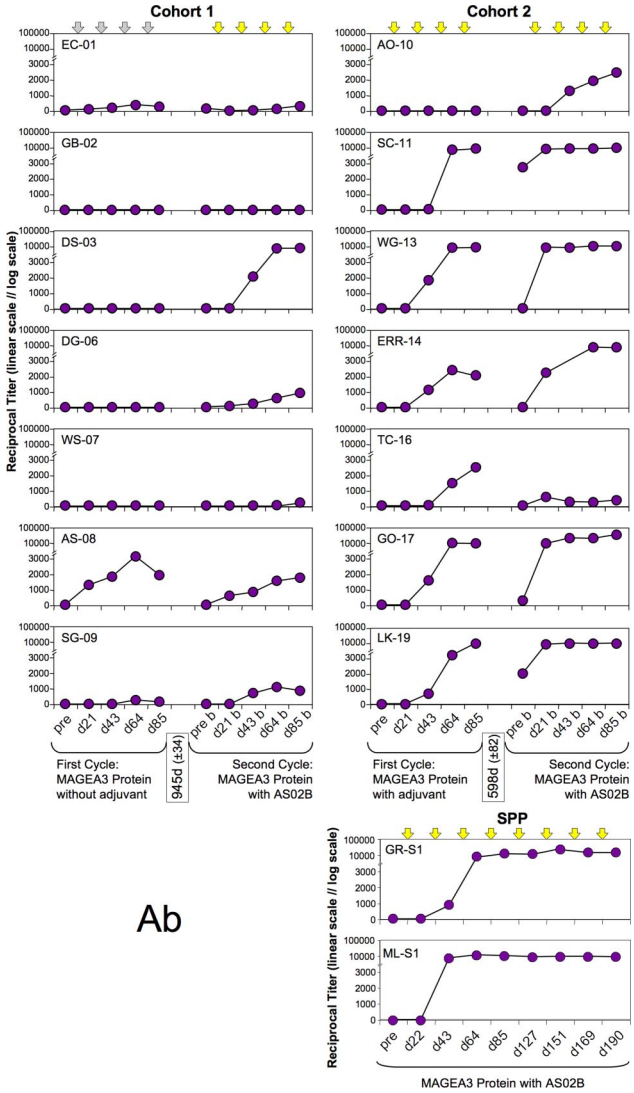


Fig. 1. Antibody responses to MAGE-A3 in vaccinated patients. Reciprocal antibody titers against baculovirus-derived MAGE-A3 protein were measured by ELISA and are shown for each time point from prestudy (pre) to day (d) 85 for both vaccine cycles in the presence (yellow arrows) or absence (gray arrows) of adjuvant. Each box represents one patient, with cohort 1 patients on the left, cohort 2 patients on the right, and SPP patients at the bottom. None of the patients developed any significant reactivity against control proteins NY-ESO-1, LAGE-1, or p53. Results are representative of at least three independent experiments.

antibody response in cohort 1 was observed only for MAGE-A3 but not for the protein D part of the vaccine (SI Fig. 5*C*). Remarkably, all patients developed high-titer antibodies against influenza protein D when receiving the vaccine with AS02B, regardless of their original cohort, indicating a higher intrinsic immunogenicity for this foreign antigen and a selective inhibition of MAGE-A3-specific immunity after vaccination in the absence of adjuvant.

The two additional patients, GR-S1 and ML-S2, who received continuous MAGE-A3 protein plus AS02B vaccination as single-patient protocols (SPP), both developed a strong antibody response to MAGE-A3 and protein D and even showed an extension of antibody reactivity to other MAGE family member sharing homology with MAGE-A3 but not to other tumor antigens such as p53 or NY-ESO-1 (Fig. 1 and SI Fig. 5*D*). Such a widening of seroreactivity specifically against other MAGE

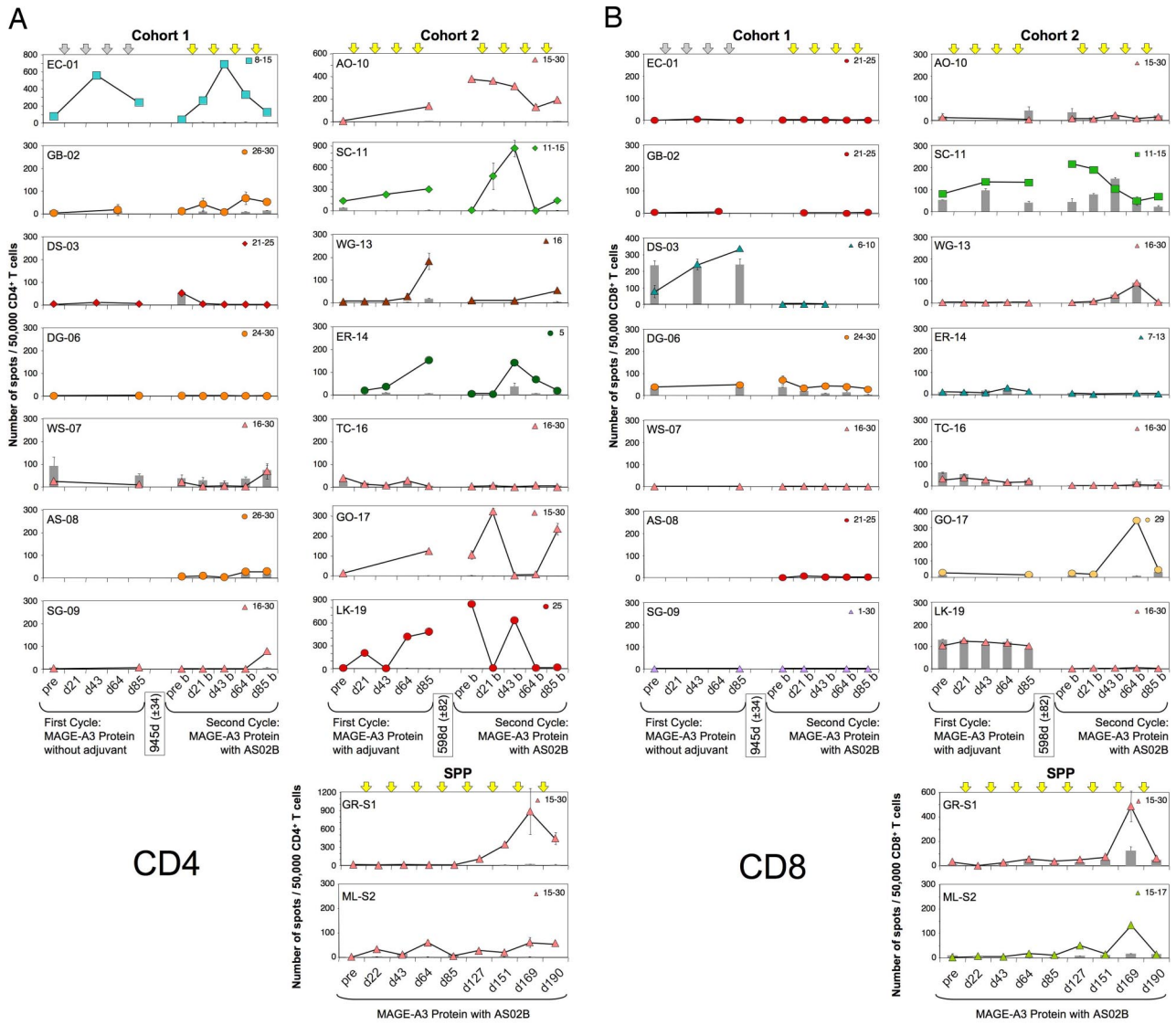


Fig. 2. CD4$^+$ and CD8$^+$ T cell response to MAGE-A3 in vaccinated patients. Mean numbers of IFN-γ producing cells of 50,000 presensitized CD4$^+$ (*A*) or CD8$^+$ (*B*) T cells were measured by ELISPOT against indicated individual or pooled MAGE-A3 long overlapping peptide(s) as indicated by color symbols and are shown for each time point from prestudy (pre) to day (d) 85 for both vaccine cycles in the presence (yellow arrows) or absence (gray arrows) of adjuvant. If detectable, responses to irrelevant peptide-pulsed targets are shown as gray bars. Each box represents one patient, with cohort 1 patients on the left, cohort 2 patients on the right, and SPP patients at the bottom. Results are representative of at least two independent experiments, and error bars represent SD of replicates.

antigens was also observed in patients SC-11 and GO-17 (data not shown).

Analyzing the isotypes of the immunoglobulins induced by vaccination, we observed a mixed profile of responses, with half of the responders generating preferentially IgG1 against MAGE-A3 and protein D, whereas the other half developed more IgG2 against both. Most patients, however, developed a strong IgG4 response against MAGE-A3 and protein D after a second cycle of vaccination, indicating that prolonged antigen exposure may favor this isotype subclass (SI Fig. 5*E*).

T Cell Responses to MAGE-A3 Vaccination. We have adapted a protocol, developed for tumor antigen NY-ESO-1 (8–10), for the monitoring of MAGE-A3-specific T cell responses. Specific T cell precursors are expanded, and T cell activity is visualized by ELISPOT. This *in vitro* sensitization protocol is not strong enough to induce *de novo* responses, and detection of specific T cell responses reflects *in vivo* priming. Compared with our previous analysis, we expanded the cellular monitoring to now include the repertoire of potential T cells to all possible MAGE-A3 epitopes in any HLA restriction context. CD4$^+$ and CD8$^+$ T cell responses were analyzed by using MAGE-A3 antigen in the form of either long overlapping peptides covering the entire sequence of MAGE-A3 or recombinant adenovirus encoding full-length MAGE-A3.

In Fig. 2*A*, a representative CD4$^+$ T cell response of each patient is shown for individual peptides or peptide pools. Similar to what was observed for antibodies, there were major differences between the two cohorts. Of seven patients from cohort 1, only patient EC-01 showed a strong CD4$^+$ T cell response against a peptide pool from MAGE-A3, which was already detectable in the first vaccine cycle and was maintained during the second cycle. It cannot be excluded that the response in this MAGE-A1 seropositive patient was due to cross-reactivity of potential preexisting CD4$^+$ T cell responses against MAGE-A1. Another two patients (SG-09 and GB-02) developed weak CD4$^+$ T cell responses to their respective MAGE-A3 peptide pool at the very end of the second vaccine cycle. There was no correlation of

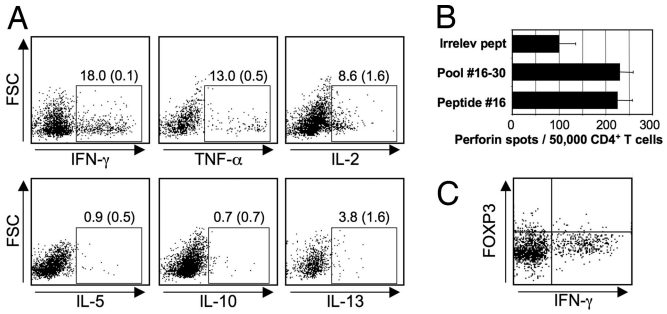


Fig. 3. Functional analyses of vaccine-induced MAGE-A3-specific CD4$^+$ T cells. (*A*) Intracellular cytokine staining of CD4$^+$ T cells of representative patient LK-19 on day 43b responding to vaccination with MAGE-A3 protein. CD4$^+$ T cells were presensitized for 14 days by using pooled MAGE-A3 peptides, and intracellular cytokine staining of Th1 (*Upper*) and Th2 (*Lower*) cytokines was performed after reexposure to single MAGE-A3 peptides. To differentiate effector from target cells, T-APC were stained beforehand by using the intracellular dye CFSE and gated out. Percentages of CD4$^+$ effector T cells expressing the given cytokine in response to MAGE-A3 peptide 141–160 are indicated. Background levels were determined by using T-APC pulsed with irrelevant peptide (irrelev pept) and are shown in parentheses. (*B*) Perforin ELISPOT assays for cytolytic activity of MAGE-A3-specific CD4$^+$ T cells. Representative results are shown for CD4$^+$ T cells obtained on day 85 from patient WG-13 responding against pooled MAGE-A3 peptides (#16–30) and single MAGE-A3 peptide 151–170 (#16). (*C*) After *in vitro* sensitization with pooled peptides, MAGE-A3-specific CD4$^+$ T cells did not coexpress Treg marker FOXP3. Representative results of intracellular costaining of IFN-γ and FOXP3 are shown for MAGE-A3 141–160-specific CD4$^+$ T cells of patient LK-19 obtained on day 43b.

CD4$^+$ T cell responses with antibody elicited against MAGE-A3 in cohort 1. None of the patients from cohort 1 developed any CD8$^+$ T cell response in either vaccine cycle (Fig. 2*B*).

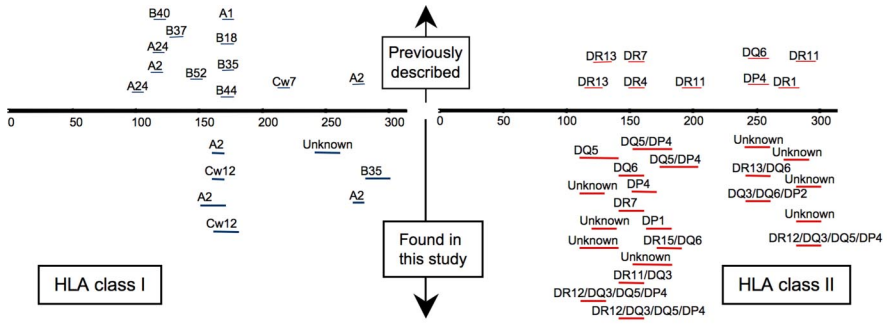
In contrast, six of seven patients in cohort 2 developed a CD4$^+$ T cell response to MAGE-A3 that could be detected in most cases after the first vaccine cycle. In all six patients, the CD4 responses could be recalled during the second vaccine cycle (Fig. 2*A*). In addition, two of seven patients (SC-11 and GO-17) in cohort 2 had CD8$^+$ T cell responses to MAGE-A3 during the second vaccine cycle (Fig. 2*B*). Interestingly, these two patients showed some of the strongest antibody titers to MAGE-A3 (Fig. 1) and serological cross-reactivity to other MAGE antigens (data not shown).

By analyzing intracellular expression of 6 different cytokines in individual responding patients, we observed, as in our previous study (7), that vaccine-induced CD4$^+$ T cells specifically produced Th1-type (IFN-γ, IL-2, TNF-α) but not Th2-type (IL-5, IL-6, IL-10) cytokines (Fig. 3*A*). We also observed cytolytic activity of MAGE-A3-specific CD4$^+$ T cells in Perforin ELISPOT assays (Fig. 3*B*). These MAGE-A3-specific CD4$^+$ T cells did not coexpress FoxP3, a marker for T regulatory cells (Tregs; Fig. 3*C*) even after *in vitro* sensitization.

Finally, the two patients that had received continuous vaccination with MAGE-A3 protein plus AS02B developed CD4$^+$ and CD8$^+$ T cell responses to MAGE-A3 peptide pools after extensive vaccination (Fig. 2 *A* and *B*). In addition, both patients developed antibodies against MAGE-3 with titers even higher than patients in cohort 2 (Fig. 1).

Mapping of Previously Uncharacterized T Cell Epitopes Naturally Processed from MAGE-A3. Fig. 2 showed the strongest representative T cell responses of each patient against single peptides or peptide pools from MAGE-A3, and these appeared to have dynamic characteristics, possibly reflecting shifting immunodominance or mobility in the localization and accessibility of T cell effectors from peripheral blood. Yet many patients developed polyclonal multiepitopic CD4$^+$ T cell responses that were mapped by testing reactivity with individual peptides. HLA restriction was assayed by using partially histocompatible targets in ELISPOT assays. Altogether, almost all patients who developed CD4$^+$ T cell responses to MAGE-A3 did so against more than one epitope. For example, patient EC-01 had a CD4$^+$ T cell response that was mapped to peptide MAGE-A3 141–160 and appeared restricted by HLA-DR07 whereas patient SC-11 CD4$^+$ T cells reacted to the same epitope but in the context of HLA-DR11 (SI Fig. 6*A*). This patient also had detectable CD4$^+$ T cell responses to MAGE-A3 peptides 111–130 and 281–300 in HLA contexts that remain to be determined (data not shown). Collectively, we were able to identify a large number of previously unknown CD4$^+$ epitopes of MAGE-A3 by analyzing vaccine-induced T cell responses in our patients after protein vaccination (Fig. 4).

We also mapped CD8$^+$ T cell responses against MAGE-A3 in patient GO-17 to peptide 281–300 restricted by HLA-B35 and in patient ML-S2 to peptide 160–169 with promiscuous HLA restriction (Fig. 4). Notably, CD8$^+$ T responses were also seen in these MAGE-A3 protein-vaccinated patients after a sensitization using adenovirus encoding full-length MAGE-A3 (SI Fig. 6*B*). This indicates that the MAGE-A3 antigen was naturally processed by antigen presenting cells and allowed the stimulation of specific precursors primed *in vivo* by the vaccine. Although no tumor cell lines with the proper HLA restriction and antigen expression could be tested for direct T cell recognition, the results with recombinant adenovirus sensitization argue in favor of a vaccine-induced repertoire capable of recognizing naturally processed MAGE-A3 antigen.



MAGE-3 peptide sequence	HLA class I potential restriction element
151-170	A*02
160-169	A*02
160-169	Cw*12
161-180	Cw*12
241-260	Unkwown
281-300	B*35

MAGE-3 peptide sequence	HLA class II potential restriction element
110-140	DQB1*05
110-140	Unknown
111-130	DRB1*12/DQB1*03/DQB1*05/DPB1*04
141-160	DQB1*06
141-160	DRB1*12/DQB1*03/DQB1*05/DPB1*04
151-170	DPB1*04

Fig. 4. Summary of T cell epitopes from MAGE-A3 previously described and found in this study. (*Left*) Epitopes in the context of HLA class I (left, blue bars) or class II (right, red bars) are shown aligned to scale along the amino acid sequence of full-length MAGE-A3 protein (black graduated bar). Whenever defined, a selection of potential HLA restriction alleles is indicated. (*Right*) Summary of HLA class I- or class II-restricted epitopes of MAGE-A3 defined by analyzing T cell responses after vaccination with full-length MAGE-A3 protein.

Discussion

The potential clinical impact of the vaccine formulation described in this article has been reported recently (11). Results from a randomized phase II placebo-controlled multicentered trial indicated efficacy in improving disease-free survival of stage II NSCLC patients vaccinated with MAGE-A3 protein plus AS02B in the adjuvant setting. Based on this finding, a phase III study has been proposed.

A detailed analysis of antibody as well as $CD4^+$ and $CD8^+$ T cell responses in patients immunized with MAGE-A3 protein is indispensable for further improving our understanding of the immunological basis of this approach and for optimizing future vaccination therapies. We showed here that vaccination with a recombinant MAGE-A3 fusion protein plus adjuvant AS02B induces consistent high-titered antibody and broad polyclonal $CD4^+$ T cell responses in patients with NSCLC. We also detected MAGE-A3-specific $CD8^+$ T cell responses in patients who developed the highest-titered broadly specific antibody responses after vaccination. So far, few trials have been conducted in which recombinant proteins were used as immunogen in cancer vaccines (12–16), and if T cell responses have been reported, they consisted mainly of $CD4^+$ T cells (17, 18). Two reports have shown that vaccination with CT antigen NY-ESO-1 as a recombinant protein, formulated either with saponin-based adjuvant ISCOMATRIX or with cholesterol-bearing hydrophobized pullulan, induced strong antibody as well as $CD4^+$ and $CD8^+$ responses in the majority of patients (19, 20). In another recent trial with NY-ESO-1 protein, $CD8^+$ T cell responses correlated with strong antibody responses in half of patients vaccinated with NY-ESO-1 protein mixed with Incomplete Freund's Adjuvant and CpG (21). In these studies and in the study reported here with MAGE-A3 protein, induction of strong antibody responses against the cancer antigen were prominent features of the vaccine. A possible role for antibodies may be to form immune complexes with the vaccine antigen and thus facilitate its cross-presentation to $CD8^+$ on MHC class I molecules, as we have shown *in vitro* (22). Additionally, inflammatory signals, i.e., mediated by Toll-like receptors, may further enhance cross-presentation (23, 24).

$CD4^+$ T cells have been shown to also play a decisive role in antitumor responses after vaccination (25, 26). Importantly, although a main function of $CD4^+$ T cells in this setting is to provide help for the initiation, the amplification, and the maintenance of $CD8^+$ T cell responses (27, 28), $CD4^+$ T cells are also capable of activating effector cells other than $CD8^+$ cells. For instance, tumor-infiltrating cells such as eosinophils and macrophages contribute to an effective antitumor response after activation by neighboring tumor-specific $CD4^+$ T cells (29–32). We showed that vaccine-induced $CD4^+$ T cells were capable of producing the cytolytic molecule perforin and, accordingly, may have an immediate effector function against tumors, as has been shown *in vitro* (33, 34) and *in vivo* (35). Finally, IFN-γ secreted by tumor-infiltrating $CD4^+$ T cells or activated bystander cells has the potential to promote tumor recognition and elimination by up-regulating expression of MHC molecules and might also contribute to the inhibition of tumor angiogenesis (36, 37).

One major goal of every tumor vaccine approach must be to generate persisting T cell memory to guarantee continuous surveillance of tumor development and progression. However, the question of whether persistence of antigen is necessary for maintaining T cell memory is still a subject of intense debate (38). Furthermore, information on the durability of vaccine-specific T cell responses during the months and years after discontinuation of vaccination has been extremely limited. A slow decline of specific T cells has been described only in individual patients over the period of several months after repeated peptide vaccination (39–41). In our current study, we demonstrate that vaccination with the recombinant protein of a tumor antigen is capable of inducing memory T and B cell responses that persists for at least 2 years after the last application of the vaccine. Importantly, such immune responses could be boosted by a single readministration of the recombinant MAGE-A3 protein antigen leading to a rapid reactivation of MAGE-A3-specific immune responses in those patients who had been primed in the right immunological context.

We have also reported that absence of adjuvant in the vaccine during priming resulted in the failure of the vaccine to induce detectable antibody and T cell responses, an observation confirmed by others (42, 43). Our study now demonstrates that immune memory was also persistent in patients primed with MAGE-A3 protein without an inflammatory adjuvant. However, this memory effect was manifested by a profound antigen-specific tolerance that compromised further vaccination attempts to convert and rescue an effective MAGE-A3-specific immune response. Although we cannot exclude the possibility that the route of administration (i.d. versus i.m.) contributed to the induction of tolerance in patients receiving MAGE-A3 protein without adjuvant, we are convinced that the absence of adjuvant represented the critical point. Our findings, therefore, support the view that the longevity of T cells memory and its biological characteristics are irreversibly imprinted at the time of immune priming. Our data also highlight the need for the addition of an adjuvant to the initial priming phase for the tumor antigen but not for foreign proteins, such as influenza virus protein D in our fusion protein construct, suggesting that there may be an additional level of regulation or tolerance to overcome when targeting MAGE-A3-specific immunity and that the presence of noncognate immunogenic epitopes from protein D did not help establishing MAGE-A3 T cell responses.

How can future tumor vaccine approaches be further optimized? Although there are only very limited data on the effect of multiple cycles of vaccination in humans, a few studies support the idea of progressive strengthening of tumor-specific immunity over prolonged courses of vaccination (18, 20, 44). As we observed in this study with broadening of immune responses upon repeated vaccination, the application of serial vaccinations might indeed improve the immunological, and hopefully also clinical, efficacy of cancer vaccines. Another way to further improve future tumor vaccine approaches might be to counteract systemic or local immunosuppressive influences. In addition to their role in suppressing autoimmune responses, Tregs represent a main obstacle of an effective antitumor T cell response (45) and might even be induced by tumor vaccination, thus undermining a clinically relevant immune response. An ideal vaccine would generate effectors with intrinsic resistance to regulatory mechanisms, and this probably needs to occur at priming, as suggested by our data on immune imprinting. Although limited patient material kept us from performing a more detailed analysis of the role of Tregs in the immune response developed after vaccination with MAGE-A3 protein, we hypothesize that the majority of $CD4^+$ T cells induced in vaccinated patients represent conventional memory T cells. First, MAGE-A3-specific $CD4^+$ T cells were, even after *in vitro* stimulation, negative for FoxP3, the most specific Treg marker to date (46–48). Second, as in our first analysis (7), MAGE-A3-specific $CD4^+$ T cells strongly produced IL-2 and TNF-α, cytokines not associated with Tregs (49). In addition, none of the $CD4^+$ produced cytokines such as IL-10, which is found in certain Treg subtypes (50, 51).

Together, results from this study highlight critical parameter for a successful cancer vaccine, which include: use of antigen formulations consisting of recombinant proteins or long peptides, addition of a proper adjuvant for priming, generation of long lasting memory, application of serial vaccinations, and the design of effective booster strategies. In conjunction with additional measures using knowledge on important costimulatory

mechanisms to counteract immune regulation, vaccine strategies will hopefully transform into an effective weapon against human cancer.

Materials and Methods

Patients and Vaccine Composition and Administration. The vaccine containing 300 μg of a MAGE-A3 fusion protein (consisting of a His-tagged full-length MAGE-A3 protein and influenza protein D (GlaxoSmithKline), was administered in saline or AS02B (monophosphoryl lipid A and QS21; GSK) once every 3 weeks for four consecutive injections (equal to one cycle). Patients without evidence of disease after surgical resection of stage I or II NSCLC were split into two cohorts as part of protocol LUD99–010 approved by the IRBs of the Ludwig Institute for Cancer Research and of Weill Medical College of Cornell University (SI Fig. 7). Cohort 1 first received one cycle of the fusion protein alone i.d. in the absence of adjuvant, and up to 3 years later (average 945 $\pm$ 68 days) received another cycle of the fusion protein i.m. with adjuvant AS02B. Cohort 2 received two cycles of fusion protein i.m. with adjuvant AS02B spaced by up to 2 years (average 598 $\pm$ 164 days). In addition, two patients with pancreatic neuroendocrine cancer and pediatric osteogenic sarcoma, respectively, who were enrolled in IRB-approved compassionate single-patient protocols, received eight consecutive triweekly i.m. injections of MAGE-A3 protein with adjuvant AS02B. Peripheral blood mononuclear cells (PBMC) and plasma were collected and cryopreserved before each vaccination as well as 3 weeks after the last injection.

Serological Analyses Against Recombinant Proteins. Patient plasma samples were analyzed by ELISA for seroreactivity to various recombinant full-length protein antigens (baculovirus-derived MAGE-A3, *E. coli*-derived protein D, MAGE-A4, NY-ESO-1, LAGE-1, and p53), to recombinant truncated proteins (MAGE-A3 64–226, MAGE-A1 57–219), and to the vaccine fusion protein MAGEA3/ProtD/His itself as described (7) with modifications *SI Materials and Methods* for details and titer calculations).

Monitoring of CD4$^+$ and CD8$^+$ T Cell Responses. Monitoring of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells specific for MAGE-A3 was performed by ELISPOT and CYTOSPOT after a single *in vitro* presensitization as described (7–10, 52) with modifications *SI Materials and Methods* for details).

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