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A randomized pilot trial testing the safety and immunologic effects of a MAGE-A3 protein plus AS15 immunostimulant administered into muscle or into dermal/subcutaneous sites

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Abstract

Introduction—Methods to induce T cell responses to protein vaccines have not been optimized. The immunostimulant AS15 has been administered with the recombinant MAGE-A3 protein (recMAGE-A3) i.m. but not i.d. or s.c. This study tests hypotheses that the i.d./s.c. route is safe and will increase CD4⁺ and CD8⁺ T cell responses to MAGE-A3.

Patients and Methods—Twenty-five patients with resected stage IIB-IV MAGE-A3⁺ melanoma were randomized to immunization with recMAGE-A3 combined with AS15 immunostimulant (MAGE-A3 immunotherapeutic) either i.m. (Group A, n=13), or i.d./s.c. (Group B, n=12). Adverse events were recorded. Ab responses to MAGE-A3 were measured by ELISA. T cell responses to overlapping MAGE-A3 peptides were assessed in PBMC and a sentinel immunized node (SIN) after 1 in vitro stimulation with recMAGE-A3, by IFN γ ELISPOT assay and by flow cytometry for multifunctional (TNF α /IFN γ) responses.

Results—Both routes of immunization were well tolerated without treatment-related grade 3 adverse events. All patients had durable Ab responses. For all 25 patients, the T cell response rate by ELISPOT assay was 30% in SIN (7/23) but only 4% (1/25) in PBMC. By flow cytometry, multifunctional CD8⁺ T cell responses were identified in 1 patient in each group; multifunctional

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Conflict of Interest

Dr. Slingluff has the following relationships directly related to this work: The recombinant MAGE-A3 protein, overlapping peptides, and AS15 were provided to the University of Virginia for this trial by GlaxoSmithKline, and the trial was funded largely by a grant to the University of Virginia from GlaxoSmithKline. The remaining authors have no conflicts.

CD4⁺ T cell response rates for Groups A and B, respectively, were 31% and 64% in SIN, and 31% and 50% in PBMC.

Conclusion—The MAGE-A3 immunotherapeutic was well tolerated after i.d./s.c. administration, with trends to higher CD4⁺ T cell response rates than with i.m. administration. This study supports further study of AS15 by i.d./s.c. administration.

Keywords

Vaccine adjuvants; Cancer vaccines; Melanoma/im; T-Lymphocytes; TLR agonists; Human

Introduction

Traditional vaccines against infectious pathogens often employ all or part of the target pathogen, which can directly activate TLRs and other pattern recognition receptors. However, purified or synthetic Ag preparations alone lack those immune-stimulatory components and require addition of agents to activate DC and to signal through pattern recognition receptors. Such immunostimulants function as vaccine adjuvants, which are required to augment T cell responses to purified cancer Ags in vaccines [1–3]. The cellular and molecular events induced by such adjuvants are incompletely understood, and optimal vaccine adjuvants remain to be defined [4]. A general concern with protein vaccines has been the low rate of CD8⁺ T cell responses, often with low magnitude CD4⁺ T cell responses [5,6], but effective induction of Ab. Even the FDA-approved prostate cancer vaccine sipuleucel-T, which encodes the full-length protein prostatic acid phosphatase (PAP), has not induced convincing T cell responses to that target [7]. Thus, better approaches to improve T cell responses to protein vaccines are needed.

TLR agonists have demonstrated value as vaccine adjuvants [8–13]. Monophosphoryl lipid A (MPL) activates TLR4 in DC in a TRIF-biased manner, induces a Th1 microenvironment, and augments induction of protective cytotoxic memory CD8⁺ T cells [12,14]. Also the TLR9 agonist CpG 7909 added to incomplete Freund's adjuvant (IFA) increased immunogenicity of a peptide vaccine for induction of CD8⁺ T cells about 10-fold [8]. AS15 is an immunostimulant formulation that incorporates these agonists for TLR4 and TLR9 plus the saponin adjuvant QS-21 Stimulon® (*Quillaja saponaria* Molina, fraction 21; licensed by Glaxo Smith Kline (GSK) from Antigenics Inc., a subsidiary of Agenus Inc., Lexington, MA, USA). QS-21 has been a potent adjuvant for promoting CD8⁺ T cell responses to subunit Ag vaccines [15] and to a long (69mer) malarial peptide [16]. QS-21 was superior to IFA as an adjuvant for vaccines using Class I-MHC-restricted melanoma peptides from tyrosinase and gp100 [17]. Adjuvants combining MPL and QS-21 can induce potent CD4⁺ and CD8⁺ T cell responses to a protein vaccine, with multifunctional Th1-dominant CD4⁺ responses, inducing IFN γ and IL-2 production [18]. As an adjuvant for a recMAGE-A3 vaccine, the combination immunostimulant AS15 induced favorable clinical outcome compared to another MPL/QS-21 combination, AS02B [5]. AS15 is a strong immunostimulant and when administered to mice, monkeys and humans with recombinant protein, can support induction of Th1 responses and protection against tumor challenge [5,19–21].

The MAGE gene family was the first discovered Ag recognized by human T lymphocytes in an MHC-restricted manner and is a cancer-testis Ag [22]. Vaccination with recMAGE-A3 showed promise for clinical benefit in phase II trials, in melanoma and non-small cell lung cancer [5]. However, in recent reports, phase III trials with the MAGE-A3 immunotherapeutic have not met primary endpoints for improving OS [23–25]. Thus, the clinical impact of this and other protein vaccines have not yet met expectation. Nonetheless, experience with recMAGE-A3 immunotherapeutic provides a good model for studying adjuvant effects of the AS15 immunostimulant.

In prior preclinical studies and human trials, the MAGE-A3 immunotherapeutic has been administered i.m. However, other immunization routes may also be effective. Ag can be presented effectively to T cells by skin DC, which include at least 3 populations: CD1a⁺CD207⁺ Langerhans cells arising in the epidermis, CD1a⁺CD207^{neg}CD14^{neg} dermal DC, and CD14⁺ DC [26]. Langerhans cells are immature DC that are optimal for uptake of protein and subsequent presentation of Ag to T cells. When activated by innate immune signals in the skin, Langerhans cells mature and migrate to draining nodes [26,27]. Dermal DC can also be very effective at activating CD8⁺ T cells [26]. Thus, there is rationale for i.d. or s.c. administration of Ag and immunostimulants. However, i.d./s.c. administration had not previously been studied, in the absence of safety data by that route. The present study was initiated to evaluate the safety of i.d./s.c. administration, as compared to i.m., and to obtain pilot data on the magnitude of Ab and CD4⁺ and CD8⁺ T cell responses to MAGE-A3 epitopes by the i.d./s.c. and i.m. routes of immunization with MAGE-A3 immunotherapeutic in patients with high-risk melanoma. We hypothesized that the i.d./s.c. route would be safe and would increase the CD4⁺ and CD8⁺ T cell responses to MAGE-A3. The trial incorporated biopsies of a vaccine-draining node to test whether immune monitoring in the vaccine-draining node is a more sensitive approach than monitoring T cell responses in the blood.

Materials and Methods

Study design

This was an open-label randomized single institution pilot study to evaluate the safety and immunologic response to MAGE-A3 immunotherapeutic administered by either of two injection routes (i.m. or i.d./s.c.). Patients were studied following IRB approval (IRB #15398) and documentation of informed consent. The trial was registered in clinicaltrials.gov (NCT01425749), and was performed at the University of Virginia. A schema is shown in Figure 1.

MAGE-A3 immunotherapeutic (0.5 ml) was administered five times (weeks 0, 3, 6, 9, 12) in extremities uninvolved with melanoma. Vaccines 1 and 3 were administered at the same site; other vaccine sites were rotated among available extremities. Subjects were randomized 1:1, within each stratum (AJCC stage II/III or IV), to i.m. (Group A) or i.d./s.c. (Group B) administration. The randomization code was generated by the study statistician using varying block sizes of 2 to 4. For group B patients, half of the dose was injected s.c., then the needle was withdrawn to the dermis, then advanced intradermally from that same

puncture site and the remaining half dose was injected i.d. Immune responses were evaluated in a SIN and PBMC.

Primary endpoints included

1. Safety of cutaneous administration (i.d./s.c.) of MAGE-A3 immunotherapeutic,
2. CD4⁺ and CD8⁺ T cell reactivity to MAGE-A3 epitopes in the SIN (IFN-gamma ELISPOT, tetramer, proliferation).

Secondary endpoints included

CD4⁺ and CD8⁺ T cell reactivity to MAGE-A3 epitopes in peripheral blood, and circulating Ab to MAGE-A3.

Lymph node biopsy

The SIN, which drained the 1st and 3rd injection sites, was identified by lymphoscintigraphy using technetium 99m-sulfur colloid (Tc99m-sc) [28] to be injected i.m. (group A) or i.d. (group B). The node was sectioned immediately after excision and divided as follows: a) 30% for viable cell suspensions, b) 20% formalin-fixed and paraffin-embedded (FFPE), c) 25% in RNeasy Lysis Buffer (Qiagen, Life Technologies, Grand Island, NY) for gene expression profiling, and d) 25% bisected, with each half quick-frozen in liquid nitrogen.

Skin biopsies

For patients in group B, skin at the MAGE-A3 immunotherapeutic injection sites was biopsied with three 4-mm punch biopsies, one week after dose 1 and 3 (days 8 and 50). Data on inflammatory changes in the vaccine site biopsies are reported separately (Pinczewski J, et al, manuscript in preparation).

Sample size considerations

We hypothesized that both immunization routes would be safe. However, there was concern that i.d./s.c. administration may induce local tissue necrosis and skin ulceration in some patients. Thus, monitoring dose limiting toxicities guided decisions about accrual. To assess safety and to enable estimation of immune response rates in the SIN and PBMC, with 90% confidence that the estimates would be accurate $\pm 26\%$, target enrollment was 12 eligible subjects per group. Limited additional enrollment was allowed for patients in screening at the time target enrollment was reached. Twelve patients per group also allowed 80% power to test for an 87% immune response rate in the SIN versus a null immune response rate of 56% with a one-sided 5% level test. The historical rate of 56% was based on prior experience with detection of T cell responses in the SIN after vaccination with multiple peptides in IFA [29]. We expected both immunization routes to be immunogenic, but if the immune response rate for one study group was low, (eg, 2%, 5% or 10%), we would have approximately 87%, 83% and 79% power, respectively, to detect a difference in immune response rates of 50% between study groups.

Eligibility criteria

Patients were eligible if they had histologically or cytologically proven stage IIB-IV melanoma (7th edition AJCC, at original presentation or at recurrence) rendered clinically free of disease within 6 months. Patients with up to 3 small treated brain metastases were eligible. Participants were required to have: age 18 years or above; Eastern Cooperative Oncology Group performance status 0–1; adequate hematologic, liver and renal function; negative serology for HIV and HCV, and ability to give informed consent. Patients with diabetes were required to have adequate glucose control (HgbA1c < 7.5%). Multiple primary melanomas were allowed, as were cutaneous, mucosal, or unknown primary sites. Patient tumors were screened for MAGE-A3 mRNA expression on FFPE tissue based on quantitative RT-PCR (performed by GSK [30]).

Adverse events were recorded using Common Terminology Criteria for Adverse Events v4.

Ab responses to MAGE-A3 were assessed in serum by ELISA, as described [31]. Seroconversion was defined as a detectable Ab response by ELISA (>20 EU/ml).

Evaluation of T cell responses to MAGE-A3

T cell responses to overlapping 15-mer MAGE-A3 peptides were measured after one in vitro sensitization. Lymphocytes ($5-10 \times 10^6$) from PBMC or SIN were stimulated 2h (37°C, 5%CO₂) with MAGE-A3 Ag/Ab complexes (25 mcg/ml recMAGE-A3 plus pooled MAGE-A3-reactive serum (1:200) from several patients) in 1 ml complete medium (RPMI-1640 + pen/strept + 10% human AB serum (Gemini, West Sacramento, CA)). IL-2 (10 U/ml) and IL-7 (20 ng/ml) were added in complete medium, for a final volume of 2ml. Cells were incubated 14d. Cytokines were replenished day 7, and proliferating wells were split as indicated.

ELISPOT assay—The cells were assayed day 12–14 for IFN γ production by ELISPOT assay. Autologous Epstein-Barr virus-transformed B cells (EBV-B) were pulsed 2h with a) overlapping MAGE-A3 15-mer peptides (1 mcg/ml each, GSK, Rixensart, Belgium), b) overlapping PRAME 15-mer peptides (1 mcg/ml each, GSK) as negative control, or c) complete medium only, and plated. The T cells were then washed and plated in quadruplicate wells at 25,000 and 12,500 cells per well and assayed as reported [29,32]. HIV gag 9-mer peptide (4 mcg/ml) served as a negative control, and PMA (Sigma-Aldrich, St. Louis, MO; 50ng/mL) plus ionomycin (Sigma-Aldrich, 1 mM) provided a positive control.

Flow cytometry—CD4⁺ and CD8⁺ T cells also were assayed for production of IFN γ and TNF α by flow cytometry. Cultured lymphocytes (100,000) were combined, in round bottom polypropylene 96-well cluster dishes (CoStar), with an equal number of autologous EBV-B pulsed with test and control Ags in 200 μ L AIM V medium (Life Technologies, Grand Island, NY) plus 5% human AB serum. Brefeldin A (BD Biosciences, San Jose, CA; 20 μ L of a 1:100 dilution) was added 2h later. After 5h more incubation, cells were washed and stained with the live/dead indicator Aqua (Life Technologies) and with Abs (BD

Biosciences unless specified otherwise) to CD3 (v450, clone UCHT1), CD19 (PE, clone HIB19) and CD8 (APC, clone RPA-T8) for 30 minutes followed by washing.

The cells were fixed and permeabilized (Cytotfix/Cytoperm, BD Biosciences), followed by incubation 45 min at 4°C with Abs for the intracellular detection of IFN γ (FITC, Clone B27), TNF α (PE-Cy7, clone MAb11) and CD4 (PerCP-Cy5.5, clone OKT4; BioLegend, San Diego, CA). Cells were evaluated on a Canto II flow cytometer (BD Biosciences), and data were acquired with DIVA software and analyzed with TreeStar FlowJo software using custom analysis templates to warrant reproducible gating strategy and accuracy of gating with curated checkpoints.

The analysis determined the proportion of CD4⁺ (and/or CD8⁺) T cells producing IFN γ or TNF α , or both, in response to MAGE-A3 peptide pools (with irrelevant peptide as negative control). T cell response was defined when T cells producing both IFN γ and TNF α in response to MAGE-A3 peptides exceeded (a) twice the maximum of 2 negative controls (PRAME peptides, media only), corrected for pre-existing response; and (b) exceeded the negative controls by at least 0.2% of the T cell population. These criteria also were used to define immunogenicity by ELISpot (IFN γ only), and are similar to criteria used in prior trials [29,32]. High T cell responses were also defined as a five-fold increase and at least a 1% difference. If the negative control values for a given sample were zero, a meaningful fold-increase could not be calculated; so, in those cases, we used the minimum detectable value among all similar assays (0.06%) as the negative control value for that sample.

Enumeration of immune subsets in SIN—FFPE sections of SIN were assessed by IHC using mouse Abs to CD4 (1:120, Vector Labs, Burlingame, CA), CD8 (1:200, Dako, Carpinteria, CA), CD1a (1:50, Dako), CD83 (1:20, Leica Biosystems, Buffalo Grove, IL), GATA3 (1:200, Biocare Medical, Concord, CA), T-bet (1:40, Santa Cruz, Dallas, TX), ROR γ t (1:300, EMD Millipore, Billerica, MA), and rat Ab to FOXP3 (1:125, eBioscience, San Diego, CA). Stained slides were imaged at 20x using the Leica SCN400 slide scanner. For all but CD1a counts, stained cells were enumerated per mm², using Digital Image Hub Tissue IA software and the stained cell algorithm (Leica Biosystems, Buffalo Grove, IL). Annotations were drawn on large areas of the SIN, excluding tissue folds. Automated cell counts were audited and verified manually. CD1a cell data were obtained by manual counting by two coauthors (ISM, CMR), with mean values reported.

Statistics—Point estimates and 90% confidence intervals (CIs) were estimated to summarize immune response rates. For the analysis of Ab responses to MAGE-A3, a repeated measure model [33] was fit to log₁₀ transformed data to assess change in slope in weeks 0–7 compared to weeks 7–13, and weeks 7–13 compared to >13, and to determine if there was a difference over time by group. F-tests based upon the repeated measure model were used to assess statistical significance.

RESULTS

At final analysis, total enrollment was completed with 25 participants. Enrollment spanned 18 months (July 2011 to January 2013). The two groups were well-matched for disease stage

and other clinical parameters (Table 1). All had been rendered clinically free of disease by surgery. None had received prior cytotoxic chemotherapy or checkpoint blockade therapy. Prior therapies included IFN (3A, 1B), vaccine (1A), and radiation therapy (5A, 4B)

Adverse events

Treatment-related adverse events were limited to grade 1–2 (Table 2 and Supplemental Table 1), including local injection site reactions, without skin ulceration, that resolved almost completely within a week, plus transient systemic constitutional symptoms, usually observed for 1–2 days after each vaccine. Three patients experienced elevations in circulating anti-nuclear Ab, but all were asymptomatic (grade 1), listed as autoimmune disorders. Grade 2 injection site reactions occurred in 10 patients in Group A (77%, 90%CI (51,93%)) and 7 patients in Group B (58%, 90%CI (32,82%)).

Ab responses to MAGE-A3

One of 25 patients (4%) had detectable Ab to MAGE-A3 (46 EU/ml, group A) in prestudy blood. By week 1, six of 25 (24%) had seroconverted (less than 100 EU/ml Ab, Figure 2). Ab increased markedly by week 7, exceeding 250 EU/ml for all evaluable samples (Supplemental Table 2). In both groups, the rate of change from week 0 to 7 was approximately 0.3 log per week, with flattening of the rate of change to week 13 (slope 0.1), a significant change ($p < 0.001$), followed by a slight but significant decrease ($p < 0.01$) after week 13 (Supplemental Figure 1). The average rate and level of response does not differ between the two study groups (Supplemental Table 3) though Ab responses may have been slightly earlier in Group A, with seroconversion by week 1 in 33% (90%CI(12,61%)) vs. 8% in Group B (90%CI(0.5,34%)) Supplemental Table 2).

Identification and biopsy of SIN

SIN were identified and biopsied for 24 of 25 patients (96%). One patient did not undergo SIN biopsy to enable workup of reported adverse events. Lymphatic channels from the injection site to the SIN were evident from the i.m. site (Supplemental Figure 2a), or the i.d. site (Supplemental Figure 2b). Overall, the lymphoscintigraphy signal in the SIN appeared slightly less intense after i.m. injection than after i.d. injection. Logistical and technical challenges arose because lymphatic mapping from i.m. sites is not otherwise routine. For 3 patients in Group A, Tc99m-sc was not injected i.m., but was injected i.d. in skin overlying the i.m. immunization site, and SIN were identified and removed. In 8 patients, Tc99m-sc was injected i.m., and a SIN was identified in 7 (VMM1079, 1080, 1091, 1100, 1103, 1107, 1109); in the one patient injected i.m. for whom a SIN was not identified by lymphoscintigraphic imaging, additional Tc99m-sc was injected i.d. directly overlying the i.m. immunization site, and a node was identified and removed. In 2 patients, Tc99m-sc was injected both i.d. and i.m., and a single node was highlighted (Supplemental Figure 2c), suggesting that nodal drainage patterns may be comparable from skin and muscle in the same site.

T cell responses to MAGE-A3

Initially, we tested for T cell responses to MAGE-A3 peptides by ex vivo ELISPOT assay; however, responses were not detected for the first 3 patients tested (not shown). Thus, we developed a more sensitive assay by which lymphocytes were stimulated with MAGE-A3 protein and then cultured 14 days prior to testing by ELISPOT assay and flow cytometry, for response to autologous EBV-B pulsed with a pool of MAGE-A3 peptides, compared to unpulsed EBV-B or EBV-B pulsed with PRAME peptides.

T cell responses by ELISPOT assay

IFN γ responses were detected by ELISPOT assay in 7 patients (28%, 90%CI(14,46%)), with similar proportions in Groups A (4/13 patients, 31%) and B (3/12, 25%), shown in Table 3. All responses, but one, were limited to the SIN. The hypothesis of improved immune response in the SIN compared to historical controls was rejected within both groups. In all 7 patients with ELISPOT responses, CD4⁺ T cell responses were detected in the SIN by flow cytometry (both by measuring multifunctional T cells and by measuring all cytokine-secreting CD4 T cells).

T cell responses by flow cytometry

The primary analysis was based on the percent of multifunctional T cells producing both IFN γ and TNF α . Additional analyses included measures of T cells producing either of those cytokines alone. The gating strategy is illustrated in Figure 3a, and examples of the data are shown in Figure 3.

CD4⁺ T cell responses by flow cytometry

In the SIN, multifunctional CD4⁺ T cell responses to MAGE-A3 were detected in 11 of 24 patients (46%, 90%CI(28, 64%)) and were more frequent for Group B (7/11 patients, 64%, 90%CI(35, 86%)) than Group A (4/13 patients, 31%, 90%CI(11, 57%)) (Table 3). Multifunctional high responders in the SIN were detected in 3 (31%) and 4 (36%) patients in groups A and B, respectively (Figure 3b,c).

In PBMC, multifunctional CD4⁺ T cell responses were detected for 31% in Group A and 50% in Group B. None of the PBMC responses met criteria for high responses.

Considering all patients with either a PBMC or SIN response, multifunctional CD4⁺ T cell responses were detected in 64% of patients (16/25), including 54% (90%CI(29, 78%)) of Group A (7/13) and 75% (90%CI(47, 93%)) of Group B (9/12, Table 3). Responses in both PBMC and SIN were detected in just 1 patient (8%) in Group A and in 4 (36%) in Group B (examples in Figure 3b,c).

CD8⁺ T cell responses by flow cytometry

CD8⁺ T cell responses met criteria for positivity in 4 patients (1 in group A (8%); 3 in group B (25%)) but were of low magnitude and isolated. For example, one patient in Group A met criteria for response in PBMC at week 1, and approximated a response at week 26 (1.9x, >1% difference), but other samples were negative; the pattern for that patient includes

substantial sample-to-sample variability compared to background (Figure 3d) and is not as convincing as the measured CD4 T cell responses (Figure 3b,c).

Detection of immune responses in SIN as a function of route of Tc99m-sc injection

Among patients in Group A, T cell responses were detected in the SIN in similar proportions of patients with Tc99m-sc injected i.d. (2/6), or only i.m. (2/7) both by ELISPOT and by flow cytometry.

Cellular composition of sentinel immunized nodes

We also evaluated for differences in the cellular composition of the SIN by IHC, depending on the route of vaccination (Supplemental Figure 3). There were no significant differences in the density of any of these cell populations between study groups. There is variability among patients in all of these measures. On average, numbers of CD4⁺ and CD8⁺ T cells were similar, and numbers of T cells greatly exceeded numbers of DC. T-bet⁺ cells were more common than those expressing GATA3 or ROR γ t. There were similar numbers of Foxp3⁺ cells between groups, and FOXP3⁺ cells were more numerous overall than T-bet⁺ cells. The numbers of FOXP3⁺ cells per mm² did not predict immune response, though there was a weak (NS) trend toward more FOXP3⁺ cells in those with CD4⁺ T cell response to the vaccine (Supplemental Figure 3i).

Clinical Outcome

Overall patient survival to two years has been about 90% (Supplemental Figure 4).

Discussion

This was a pilot study to evaluate the safety and immunogenicity of administering MAGE-A3 immunotherapeutic in the skin (i.d./s.c.). Vaccine site reactions were limited to grade 1 and 2 and were transient, with grade 2 injection site reactions in 58% after i.d./s.c. injection and 77% after i.m. injection. Thus, there is much less toxicity locally than we have observed when vaccinating with peptides in IFA [32,34]. There were only minor differences in the constitutional/systemic toxicities between groups. These data provide the first human experience with cutaneous (i.d./s.c.) administration of protein Ag plus AS15, and demonstrate that it is well-tolerated. This supports the safety of future studies of cutaneous administration of AS15, or its components (QS-21, MPL, or CpG) with recMAGE-A3 and/or other Ags.

Protein vaccines have typically been effective at inducing Ab responses and CD4⁺ T cell responses, but have been less effective at inducing CD8⁺ T cell responses [35]. Our data are consistent with that prior experience. We found durable Ab responses in all patients. The role of these Abs is not known, but in other studies, Ab responses to vaccination with peptides or protein are associated with T cell responses to those antigens [36,37]. We also detected multifunctional CD4⁺ T cell responses in 64% of patients, and weak CD8⁺ T cell responses in 16%. The primary immunologic endpoint was T cell response in the SIN, which was numerically higher in group B for CD4⁺ T cell response rates (64% (90%CI(35, 86%)), vs 31% (90%CI(11, 57%))). These are not convincingly different in this small pilot

study, but may suggest a trend toward higher induction of CD4⁺ T cell responses with the i.d./s.c. route. Similar trends favored the i.d./s.c. route for alternative criteria for defining CD4⁺ T cell responses (Table 3). None of these rates reached the target of 87% from the study design, but may be compared to the 54% null rate, based on responses to MAGE-A3 peptides using a peptide vaccine [29]. The rate of CD8⁺ T cell responses falls well below the target for both study groups.

In prior work, we developed methods for measuring T cell responses in the SIN [28,38–40]. This approach yields high numbers of T cells, and assessment of T cell responses in the SIN has been more sensitive than assessing those responses in PBMC [28]. There has been limited prior experience mapping lymphatic drainage from muscle [41,42]. The present study is, to our knowledge, the first to test whether i.m. vaccines can be monitored by mapping SIN by i.m. injection of Tc99m-sc. We have found that i.m. injection does map a draining node, and that the resulting node reflects immune responses to the vaccine with greater sensitivity than the PBMC. Anecdotally, because a few patients were injected i.d. overlying the i.m. vaccine sites, it may be that accurate mapping from i.m. sites can also be obtained by i.d. injection, but i.m. injection is straightforward and may be less painful than i.d. injection. In prior experience with monitoring T cells in the SIN, we vaccinated 3 times in the same skin site, one week apart, and harvested a node a week later [28,29,43]. We adjusted the approach for this study to match the schedule used for MAGE-A3 immunotherapeutic in prior studies (every 3 weeks), with only two vaccines administered in the same site. This appears to have been effective for immune monitoring. Thus, the SIN biopsy approach may be adapted to different vaccine schedules and regimens.

Enumeration of T cell and DC populations in the SIN revealed no significant differences between study groups. Numbers of FOXP3⁺ cells in the SIN generally exceeded numbers of T-bet⁺ cells, and were about half the number of CD4⁺ cells. This may reflect transient immune activation and/or a regulatory T cell response to vaccine-induced activation of other cells. The numbers of FOXP3⁺ cells did not differ significantly between groups or between immune responders and non-responders. Especially in settings where the immunotherapy induces larger Ag-specific T cell populations, more significant differences in immune activation may be expected in the nodes. It would be appealing also to evaluate the Ag specificity, activation state and differentiation state of T cells responding to Ag, as we have done in vaccine sites and blood after peptide vaccination [44], and where peptide/MHC tetramers enabled identification of the reactive T cells. It is, however, less straightforward to identify T cells responding to protein in the setting of a wide range of MHC molecules and in a population of patients that was not selected for the expression of a particular HLA allele.

Conclusion

Overall, the data in the present manuscript support i.d./s.c. immunization with MAGE-A3 immunotherapeutic, both as a safe approach and as one that is at least as immunogenic as i.m. injection. These data also demonstrate the feasibility of identifying and analyzing SIN draining i.m. injection sites. The rates of CD4⁺ T cell responses to MAGE-A3 were numerically greater with i.d./s.c. injection than with i.m. injection but were not significantly

different, given the limited sample size for this pilot study. It is possible that CD8⁺ T cell responses may be more frequent than were detected in this study because overlapping 15-mer peptides may be less effective for reconstituting the MHC-class I restricted T cell epitope than the minimal epitope (commonly a 9-mer). Randomized clinical trials of MAGE-A3 immunotherapeutic in melanoma and non-small cell lung cancer have not yet provided convincing clinical benefit with this strategy [24,25]. However, AS15 continues to offer promise as a component of active specific immunotherapy especially for induction of Ab and CD4⁺ T cell responses. Thus, the present manuscript supports administration of AS15 with other Ags, with or without other immune modulators. This study also provides preliminary data about the range of responses to i.d./s.c. vs. i.m. vaccination that may help to design future randomized trials with adequate power to detect significant immunologic effects and whether these may lead to higher clinical benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EBV-B

Epstein-Barr virus-transformed B cells

FFPE

formalin-fixed and paraffin-embedded

GSK

Glaxo Smith Kline

IFA

incomplete Freund's adjuvant

MAGE-A3 immunotherapeutic

recombinant MAGE-A3 combined with AS15 immunostimulant

MPL

monophosphoryl lipid A

recMAGE-A3

recombinant MAGE-A3 protein

SIN

sentinel immunized node

Tc99m-sc

technetium 99m-sulfur colloid

TLR

toll-like receptor

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Figure 1.
Clinical Trial Schema

Figure 2.

Titers of serum Ab to recMAGE-A3 through week 26 are shown for Groups A (**a,c**) and B (**b,d**), without (**a,b**) and with (**c,d**) log transformation. Each line represents a different patient. Vertical dashed lines indicate the dates of serum collection. Vaccinations were completed by week 12.

Figure 3. In vitro multifunctional T cell responses to MAGE-A3 after administration of MAGE-A3 immunotherapeutic

Lymphocytes from PBMC or SIN of vaccinated patients were stimulated in vitro with recMAGE-A3 for 14 days, then intracellular cytokine levels were measured after an additional stimulation with a pool of overlapping MAGE-A3 peptides or negative controls (a pool of peptides from the negative control protein, PRAME, and culture media only); corrected for pre-existing response; and negative controls by 0.2%. High responses were > 5x and > 1% difference. **a)** An example gating strategy is shown. Cultured PBMC were detected using forward and side scatter and the live/dead indicator, Aqua, from which live CD3+ cells were identified. Representative individual patient examples of CD4+ T cell responses include: **b)** High T cell response in the SIN and response in PBMC week 7, patient VMM1109 (Group A - i.m.); **c)** high response in SIN and response in PBMC week 1, patient VMM1077 (Group B - i.d./s.c.); **d)** a CD8+ T cell response is shown for patient VMM1100 (Group A - i.m.)

Table 1

Clinical characteristics

	Arm A (IM)	Arm B (ID/SQ)	Total
N	13	12	25
Gender N male (%)	5 (38%)	7 (58%)	12 (48%)
Age: Mean (range)	56 (39–82)	54 (40–54)	55 (39–82)
Race/Ethnicity			
White, non-Hispanic	13	11	24
American Indian	0	1	1
Stage at registration			
IIIB	3	5	8
IIIC	5	3	8
IV	5	4	9
ECOG Performance status			
0	12 (92%)	10 (83%)	22 (88%)
1	1 (8%)	2 (17%)	3 (12%)
Reason off-treatment			
Treatment completed	13	11	24
Disease progression	0	1	1

Table 2

MEL 55 Maximum Grade Treatment-Related Adverse Events

		Study Groups		Total Study
		Arm A IM N=13	Arm B ID/SC N=12	Both arms N=25
Adverse Event Category	Adverse Event Description	Gr 2	Gr 2	Gr 2
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	CHILLS	1		1
	FATIGUE	4	2	6
	FEVER	1		1
	FLU LIKE SYMPTOMS	1	2	3
	INJECTION SITE REACTION	10	7	17
INJURY, POISONING AND PROCEDURAL COMPLICATIONS	WOUND COMPLICATION		1	1
INVESTIGATIONS	LYMPHOCYTE COUNT DECR	1		1
Total		18	12	30

Table 3

Immune responses by ELISpot and by Flow cytometry

Patient ID VMM	Study Group	ELISpot response	Multifunctional		CD4 ⁺ TNF α ⁺ and/or IFN γ ⁺	CD8 ⁺ TNF α ⁺ and/or IFN γ ⁺	CD8 ⁺ TNF α ⁺ and/or IFN γ ⁺
			CD4 ⁺ TNF α ⁺ IFN γ ⁺	CD8 ⁺ TNF α ⁺ IFN γ ⁺			
1075	A	SIN	SIN	-	SIN	-	-
1079	A	-	-	-	SIN	-	-
1080	A	SIN	SIN	-	SIN	-	13
1082	A	-	1	-	-	-	-
1087	A	-	7	-	-	-	-
1091	A	-	13	-	-	-	-
1094	A	-	(13,26)	-	-	-	-
1100	A	-	-	1, (26)	-	-	-
1103	A	-	-	-	-	-	-
1107	A	-	-	-	-	-	-
1109	A	SIN	7, SIN	-	SIN	-	-
1110	A	-	-	-	-	-	-
1117	A	SIN	SIN	-	SIN	-	-
1073	B	-	1	-	1 (7)	-	-
1076	B	1, SIN	SIN	SIN	-	-	-
1077	B	SIN	1, (7,13), SIN	-	(1, 13), SIN	-	-
1078	B	-	SIN	1	SIN	-	1
1086	B	-	(1) 7, 13, SIN	-	(1,7,13),SIN	-	-
1089	B	-	-	-	26	-	-
1093	B	-	-	-	-	-	-
1095*	B	*	-	-	-	-	-
1098	B	-	SIN	26	SIN	-	26
1106**	B	**	** ; 13	**	**	**	**
1112	B	SIN	7,13, SIN	-	SIN	-	-
1113	B	-	(13), 26, SIN	-	(13,26), SIN	-	-

Patient ID VMM	Study Group	ELISpot response	Multifunctional		CD4 ⁺ TNF α ⁺ and/or IFN γ ⁺	CD8 ⁺ TNF α ⁺ and/or IFN γ ⁺
			CD4 ⁺ TNF α ⁺ IFN γ ⁺	CD8 ⁺ TNF α ⁺ IFN γ ⁺		
T cell response in SIN	A	4/13 (31%)	4/13 (31%)	0/13 (0%)	5/13 (38%)	0/13 (0%)
	B	3/10 (30%)	7/11 (64%)	1/11 (9%)	6/11 (55%)	0/11 (0%)
	All	7/23 (30%)	11/24 (46%)	1/24 (4%)	11/24 (46%)	0/24 (0%)
T cell response in PBMC	A	0/13 (0%)	4/13 (31%)	1/13 (8%)	0/13 (0%)	1/13 (8%)
	B	1/12 (8%)	6/12 (50%)	2/12 (17%)	2/12 (17%)	2/12 (17%)
	All	1/25 (4%)	10/25 (40%)	3/25 (12%)	2/25 (8%)	3/25 (12%)
T cell response in SIN and PBMC	A	0/13 (0%)	1/13 (8%)	0/13 (0%)	0/13 (0%)	0/13 (0%)
	B	1/10 (10%)	4/11 (36%)	0/11 (0%)	0/11 (0%)	0/11 (0%)
	All	1/23 (4%)	5/24 (21%)	0/24 (0%)	0/24 (0%)	0/24 (0%)
T cell response in SIN or PBMC	A	4/13 (31%)	7/13 (54%)	1/13 (8%)	5/13 (38%)	1/13 (8%)
	B	3/12 (25%)	9/12 (75%)	3/12 (25%)	8/12 (67%)	2/12 (17%)
	All	7/25 (28%)	16/25 (64%)	4/25 (16%)	13/25 (52%)	3/25 (12%)

* SIN ELISpot not done for VMM1095;

** SIN not done for VMM1106; (not evaluable, NE)

Responses are denoted by the week in which the response was observed from PBMC, or by the presence of a response in the SIN (eg: 13, SIN = response in PBMC week 13, and in the SIN). Weeks marked in parentheses (eg (13)) represent weeks in which a fold-increase of 1.5 – 2 when at least one other time point met criteria for positivity (2x).