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Combination Immunotherapy after ASCT for Multiple Myeloma Using MAGE-A3/Poly-ICLC Immunizations Followed by Adoptive Transfer of Vaccine-Primed and Costimulated Autologous T Cells

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Abstract

Purpose: Myeloma-directed cellular immune responses after autologous stem cell transplantation (ASCT) may reduce relapse rates. We studied whether coinjecting the TLR-3 agonist and vaccine adjuvant Poly-ICLC with a MAGE-A3 peptide vaccine was safe and would elicit a high frequency of vaccine-directed immune responses when combined with vaccine-primed and costimulated autologous T cells.

Experimental Design: In a phase II clinical trial (NCT01245673), we evaluated the safety and activity of *ex vivo* expanded autologous T cells primed *in vivo* using a MAGE-A3 multipeptide vaccine (compound GL-0817) combined with Poly-ICLC (Hiltonol), granulocyte macrophage colony-stimulating factor (GM-CSF) ± montanide. Twenty-seven patients with active and/or high-risk myeloma received autografts followed by anti-CD3/anti-CD28–costimulated autologous T cells, accompanied by MAGE-A3 peptide immunizations before T-cell collection and five times after ASCT. Immune responses to the vaccine were evaluated by cytokine production (all patients), dextramer binding to $CD8^+$ T cells, and ELISA performed serially after transplant.

Results: T-cell infusions were well tolerated, whereas vaccine injection site reactions occurred in >90% of patients. Two of nine patients who received montanide developed sterile abscesses; however, this did not occur in the 18 patients who did not receive montanide. Dextramer staining demonstrated MAGE-A3–specific CD8 T cells in 7 of 8 evaluable $HLA\text{-}A2^+$ patients (88%), whereas vaccine-specific cytokine-producing T cells were generated in 19 of 25 patients (76%). Antibody responses developed in 7 of 9 patients (78%) who received montanide and only weakly in 2 of 18 patients (11%) who did not. The 2-year overall survival was 74% [95% confidence interval (CI), 54%–100%] and 2-year event-free survival was 56% (95% CI, 37%–85%).

Conclusions: A high frequency of vaccine-specific T-cell responses were generated after transplant by combining costimulated autologous T cells with a Poly-ICLC/GM-CSF–primed MAGE-A3 vaccine. *Clin Cancer Res; 20(5); 1355–65. ©2013 AACR.*

Introduction

Allogeneic stem cell transplants can eradicate myeloma through a T-cell–mediated "graft-versus-myeloma" (GVM) effect (1). Autologous stem cell transplantation (ASCT) is rarely curative due partly to the lack of GVM (2). Retrospective studies suggest that better clinical outcomes

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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This article is dedicated to the memory of D. Sewell.

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Translational Relevance

Relapse of myeloma and other hematologic malignancies after autologous stem cell transplantation (ASCT) is frequent. Posttransplant immunotherapy using adoptive T-cell transfers and tumor antigen vaccines may increase the frequency and durability of responses. Earlier studies of posttransplant combination immunotherapy showed that about one third of patients developed tumor antigen vaccine–specific T-cell responses after ASCT. To be clinically effective, a higher frequency of tumor-directed immune responses will be needed. To increase the frequency and magnitude of such responses, we studied whether adding the TLR-3 agonist and novel vaccine adjuvant Poly-ICLC to a multipeptide vaccine based on MAGE-A3, a myeloma-relevant cancer-testis antigen, would elicit a high frequency of vaccine-directed immune responses when combined with vaccine-primed and costimulated autologous T cells. This study showed that about three fourths of patients developed vaccine-specific immune responses by cytokine production assays based on this strategy.

following ASCT for myeloma and other hematologic neoplasms may be associated with rapid posttransplant lymphocyte recovery (3, 4). Myeloma-reactive T cells are present at low frequencies in the marrow and blood of patients with untreated myeloma, suggesting that strategies to augment the recovery and function of autologous T cells posttransplant may be beneficial (5, 6).

Posttransplant immunosuppression including prolonged depletion of $CD4^+$ T cells increases the risk for serious infections with varicella zoster virus, cytomegalovirus, and *Streptococcus pneumoniae* (7). The 23-valent pneumococcal polysaccharide vaccine is not recommended by the American Society for Blood and Marrow Transplantation (ASBMT) until 1 and 2 years after transplant and immunogenicity is limited because of delayed immune reconstitution following ASCT (8).

We performed a series of clinical trials of peritransplant immunotherapy for myeloma patients under the hypothesis that transfers of *ex vivo* costimulated autologous T cells will improve functional T-cell recovery thereby providing a platform for enhanced GVM effect and protection from infections. Autologous T cells are stimulated by coculture with immunomagnetic beads conjugated to anti-CD3 and anti-CD28 monoclonal antibodies to prevent T-cell anergy through combined CD3 and CD28 signaling (9, 10). In a randomized clinical trial, 54 patients with myeloma received infusions of 5 to 10×10^9 costimulated autologous T cells after autotransplantation along with immunizations using the pneumococcal conjugate vaccine (PCV, Prevnar-7; ref. 11). Patients who were assigned to receive pre- and posttransplant PCV immunizations along with an "early" (day +12) infusion of vaccine-primed costimulated T cells, exhibited sustained antibody responses to the pneumococcal antigens and robust T-cell responses to the vaccine carrier protein (diphtheria toxoid, CRM-197). The importance of immunizing patients before steady-state T-cell collections and *ex vivo* expansion was reinforced by a subsequent study of ASCT for myeloma, which showed that posttransplant seroconversion to an influenza vaccine required *in vivo* priming of autologous T cells before collection, expansion, and adoptive transfer (12).

To test whether pre- and post-ASCT immunizations in conjunction with adoptive transfer of vaccine-primed and costimulated autologous T cells could induce early immune responses to a cancer antigen vaccine, 56 patients with advanced myeloma were enrolled in a follow-on study using a multipeptide tumor antigen vaccine composed of HLA-A2–restricted peptides derived from hTERT and survivin. Using a 5-fold higher dose of T cells ($\sim 5 \times 10^{10}$ cells) administered at day +2 along with 1 pretransplant and 3 posttransplant immunizations, robust immune recovery occurred by day +14 posttransplant (13). By tetramer analysis, 36% of the HLA-A2$^+$ patients developed immune responses to the hTERT/survivin vaccine (14). Using dendritic/myeloma cell fusion vaccines as posttransplant immunotherapy, other investigators also reported myeloma-directed T-cell responses and robust clinical responses of which about one fourth were delayed posttransplant indicative of a vaccine-mediated response (15).

To address the limitations of our earlier work including the relatively low frequency of immune responses and the lack of apparent event-free survival (EFS) benefit, we developed a new clinical trial using a MAGE-A3 cancer-testis antigen (CTAg) vaccine. This vaccine was injected with a novel adjuvant, the toll-like receptor 3 (TLR-3) agonist Poly-ICLC (Hiltonol) along with the standard formulation of montanide and granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance T-cell priming and boosting.

MAGE-A3 is a member of the CTAgs whose expression is limited to spermatogenic germs cells and certain tumors. Along with MAGE-A1/A2/A4, CT-7, and NY-ESO-1, MAGE-A3 is detected in about 50% of myeloma tumors overall, at higher frequency in advanced-stage disease and may be associated with inferior EFS (16–19). Naturally occurring T-cell immunity to MAGE-A1/A2/A3 antigens may also correlate to myeloma stage with $CD4^+$ T-cell immunity operating in patients with MGUS but $CD8^+$ T-cell immunity predominating in patients with myeloma (20). Successful induction of cellular and humoral MAGE-A3 immunity in the clinical transplant setting was demonstrated by immunizing a syngeneic donor with MAGE-A3 protein and transferring MAGE-A3–primed donor T cells to the patient (21). Studies in melanoma also suggest that MAGE-A3 may be expressed in cancer stem cells (22).

The MAGE-A3 vaccine used in this study (compound designation: GL-0817) is a large peptide composed of both class I and class II epitopes linked by furin-sensitive linkers (RVKR). GL-0817 was designated as an Orphan drug by the U.S. Food and Drug Administration (FDA). The protein also contains the HIV-1-TAT membrane translocation sequence

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(RKKRRQRRR, the Trojan peptide), which delivers the peptides into the endoplasmic reticulum and facilitates the formation of MHC class I complexes (23). This MAGE-A3 Trojan peptide vaccine was first tested in a phase I dose-escalation trial of patients with unresectable squamous cell head and neck cancers (24).

Hiltonol (Poly-ICLC) is polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose and a dsRNA viral-mimic that augments both innate and adaptive immunity. Compared with other TLR agonists, the TLR-3 ligand polyinosinic-polycytidylic acid, is a strong inducer of Th1 $CD4^+$ T-cell and antibody responses to microbial and tumor antigens in animals (25–27). Hundreds of patients with cancer and normal volunteers have received Hiltonol by injection or intranasally without any serious adverse effects (28, 29). Furthermore, in patients with myeloma, Poly-ICLC has been shown to activate blood-derived dendritic cells (30). We hypothesized that adding this adjuvant to the standard montanide/GM-CSF formulation would enhance MAGE-A3–directed T-cell responses.

Low-dose lenalidomide was started at day +100 for maintenance (31). Recent work also indicates that lenalidomide may be immunostimulatory (32–36). T cells from patients with chronic lymphocytic leukemia exhibit impaired immune synapse formation with antigen-presenting cells, which can be corrected by lenalidomide (37). Furthermore, in patients with myeloma, lenalidomide enhanced both the antibody and cellular immune responses to the Prevnar PCV when these were given concurrently (38). These preclinical and clinical studies suggest that lenalidomide could potentiate the immune responses to microbial vaccines and perhaps cancer vaccines as well.

Herein we report the toxicities, clinical outcomes, and immune responses of 27 patients who received costimulated autologous T cells, which were primed *in vivo* with the MAGE-A3 Trojan peptide vaccine (GL-0187) admixed with Poly-ICLC (Hiltonol), GM-CSF ± montanide following ASCT for myeloma. This strategy led to a high frequency of functional vaccine-directed T-cell responses. B-cell responses also occurred but mainly in the presence of montanide.

Materials and Methods

Patients

Study participants were at least 18 years old with symptomatic multiple myeloma. Patients received first-line therapy using at least 3 cycles of standard regimens (typically bortezomib, thalidomide, or lenalidomide plus dexamethasone) by their referring oncologists. For enrollment, patients were required to have measurable disease (based on serum/urine electrophoresis studies or serum-free light chain studies); patients in complete remission were not eligible unless they had high-risk cytogenetic features (e.g., chromosome 13 or 17 deletions, 4;14 or 14;16 translocations, or complex karyotypes). All patients had adequate organ function as defined by serum creatinine levels ≤3.0 mg/dL, left ventricular ejection fraction ≥45%, and lung function parameters ≥40% predicted. Of note, patients were *not* required to have demonstration of MAGE-A3 expression in the myeloma cells for study eligibility. The rationale was 2-fold: (i) the study was designed primarily to develop a strategy for optimizing post-ASCT immune responses to a tumor antigen vaccine; and (ii) development of an effective MAGE-A3 immune response could conceivably prevent emergence of MAGE-A3$^+$ relapsed disease even in patients with myeloma, which was originally MAGE-A3–negative. All participants gave written informed consent in accordance with the Declaration of Helsinki; study approval was obtained from the Institutional Review Boards of the University of Maryland and the University of Pennsylvania and the FDA.

Trial design

The design of the trial is depicted in Fig. 1. Briefly, after eligibility was confirmed and registration completed, patients received a first pretransplant injection of 300 mcg of the MAGE-A3 Trojan peptide vaccine (GL-0817; >92% purity and good manufacturing grade) mixed with 150 mcg of GM-CSF (clinical grade; Berlex Laboratories, Inc.) and 2 mg of Hiltonol (Poly-ICLC; clinical grade; Oncovir Inc.). The full sequence of GL-0817 is KVAELVHFL/RVKR/FLWGPRALV/RVKR/VIFSKASSSLQL/RKKRRQRRR, which includes 2 HLA-A2–restricted class I epitopes (KVAELVHFL and FLWGPRALV) and a promiscuous class II epitope (VIFSKASSSLQL). For the first 9 patients, the aqueous solutions were also emulsified in 1.2 mL of montanide ISA 51 VG (Seppic Inc.) but after 2 patients developed severe injection-site reactions, which evolved into sterile abscesses, the montanide was eliminated from the vaccine preparation for the remaining 18 patients. The vaccine mixtures were injected into the right or left thigh by deep subcutaneous injection. All patients received an intramuscular injection of

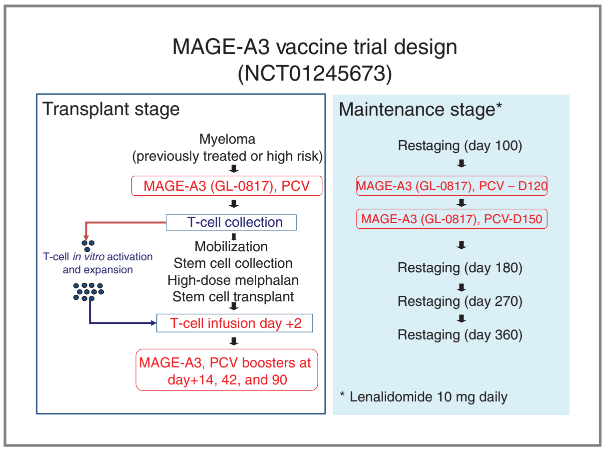

Figure 1. The trial design depicting the transplant and maintenance phases. Note that only one MAGE-A3 vaccination was given before T-cell collection and 5 immunizations were given after transplant at days +14, +42, +90, +120, and +150. About 10 days (7–14) elapsed between the first set of immunizations and the T-cell apheresis. Blood samples for immune monitoring were obtained at enrollment, the time of apheresis (T-cell collection), and days +14, +60, +100, and +180 after transplant.

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Prevnar-13—the PCV—into the nondominant deltoid muscle. About 10 days after the first set of immunizations, all patients had steady-state apheresis to collect approximately 1×10^8 mononuclear cells per kilogram body weight. Patients then proceeded to stem cell mobilization using cyclophosphamide at a dose of 1.5 to 3.0 g/m^2 followed by subcutaneous injections of G-CSF (10 μg/kg). High-dose therapy was melphalan (200 or 140 mg/m^2 if age $\geq$70 years) followed by infusions of autologous stem cells ($\geq 2 \times 10^6$ CD34$^+$ cells/kg body weight) at day 0. Costimulated autologous T cells were infused on day +2. Supportive care measures included antibiotic prophylaxis and administration of G-CSF starting on day +5. Five additional sets of immunizations (MAGE-A3 and PCV) were given at days +14, +42, +90, +120, and +150 using the same procedures that were used for the first immunization. Lenalidomide maintenance at 10 mg per day was started at day +100 after transplant, meaning that the final 2 sets of immunizations occurred while patients were taking lenalidomide.

T-cell expansion and adoptive transfers

The mononuclear cell apheresis product was monocyte depleted by counter flow centrifugal elutriation (CaridianBCT Elutra Cell Separation System) because monocytes may inhibit lymphocyte proliferation. Monocyte-depleted mononuclear cells were cryopreserved until 9 to 11 days before the scheduled reinfusion date (day +2 posttransplant). Cells were thawed and cocultured with Dynal paramagnetic M-450 beads (DynalInvitrogen) coated with anti-CD3 (OKT3; Ortho Biotech) and anti-CD28 (clone 9.3) monoclonal antibodies. CD3/CD28 beads were added at a ratio of 3 beads per cell to a Baxter Lifecell flask, and cultures were subsequently transferred to a WAVE Bioreactor system (GE Healthcare Biosciences; ref. 39). Additional details of T-cell expansion and harvesting are described elsewhere (13, 14). The harvested cells were transported by courier from the cell production facility to the patient and infused on the same day (day +2 of transplant). The cells were infused over 20 to 60 minutes without a leukocyte filter, after premedication with acetaminophen and diphenhydramine. The target number of costimulated T cells for infusion was 5×10^{10}.

Immunoassays

In vitro *peptide stimulation.* Peripheral blood mononuclear cells (PBMC) were obtained from whole blood processed fresh by ficoll gradient and cryopreserved until all time points through day +180 were collected. Upon thawing for analysis, the viability of the cryopreserved cells that were tested ranged from 50% to 90%. *In vitro* peptide stimulation of PBMC to assess immune response was performed as previously described (40). Briefly, PBMC were presensitized with either class I peptides—CTL1 (KVAELVHFL), CTL2 (FLWGPRALV), class II peptide—HTL (VIFSKASSSLQL), or the whole MAGE-A3 vaccine and the cells were analyzed on day 8.

Flow cytometric and MHC class I dextramer analysis. Phenotypic analysis of lymphocyte subsets was done using monoclonal antibody and isotype controls by flow cytometry as previously described (14) using a FACSCanto cytometer and FACSDiva software (BD Biosciences Immunocytochemistry Systems). Data were analyzed using FlowJo software (TreeStar Inc.). Peptide/MHC class I dextramer analysis was performed using soluble peptide/HLA-A2 tetramers purchased from Immudex. The cutoff for an induced vaccine (positive) dextramer response of CD8$^+$ T cells in the peripheral blood was defined as a distinct population of cells constituting greater than 0.05% of at least 7,500 events and at least 3 times the enrollment level (before first immunization) at one or more posttransplant timepoints.

Intracellular cytokine analysis. PBMC were stimulated *in vitro* with peptide as described above. On day 8, PBMC (1×10^6/mL) were washed, then incubated in complete media (RPMI with 10% human AB serum, 2 mmol/L glutamine, 20 mmol/L HEPES, and 15 μg/mL gentamicin) with 2 μg/mL of peptide or phorbol 12-myristate 13-acetate/ionomycin for 5 hours with brefeldin A added for the last 4 hours. Cells were then labeled with fluorochrome-conjugated monoclonal antibody against cell surface molecules at 4°C, and then fixed and permeabilized (Cytofix/Cytoperm Kit; BD Biosciences) before staining with anti-IFN-γ or interleukin (IL)-2 monoclonal antibody or isotype control and analysis by flow cytometry. The cutoff for a positive cytokine response to the vaccine was also defined as a distinct population of cells constituting greater than 0.05% of at least 7,500 events and at least 3 times the enrollment level (before first immunization) at one or more posttransplant timepoints.

ELISA assays. Serum was obtained before and after vaccination by centrifugation and stored at −80°C. Clinical-grade MAGE-A3 vaccine (GL-0817) was used to coat 96-well plates and measure specific antibody levels by ELISA as described (41). A reciprocal titer was estimated from optical density readings of serially diluted plasma samples as described (41). To be considered significant, reciprocal titers had to be more than 100.

qRT-PCR analysis for MAGE-A3 expression. RNA was isolated from frozen PBMC using RNAqueous RNA Isolation Kits (Ambion), and cDNA synthesized using iScript cDNA Synthesis Kits (Bio-Rad). Samples were analyzed for expression of MAGE-A3 and Gus-B (housekeeping gene) transcripts using ABI Taqman-based technologies, a qualified qPCR assay and the following ABI recommended gene-specific primer probe sets: MAGE-A3: HS03985994_uH (specific for 5′-UTR sequences of the MAGE-A3 gene); GUS-B: HS99999908_m1. The melanoma cell line A375 (positive for MAGE-A3) served as the reference sample.

Statistical methods

Data were analyzed by both parametric and nonparametric methods. Wilcoxon rank sum test were used for 2 sample comparison. Fisher exact test was used to test association between 2 categorical variables. Longitudinal data analysis was used to compare immune responses between the current and earlier trials. Log-rank test was used to

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compare survival distributions between 2 groups. Cox proportional hazards regressions were used to analyze EFS and overall survival (OS). All analyses were conducted using Rstudio (42).

Results

Patient characteristics

From October 2010 to July 2012, 27 patients were enrolled. Table 1 shows the principal clinical characteristics of the study patients. This cohort of active disease and/or high-risk patients had a median marrow plasmacytosis of 5% at enrollment (range 0–60%) including 42% of patients with $\geq$10% myeloma cells at enrollment despite a median of 2 prior lines of treatment (range 1–5) with lenalidomide, bortezomib-based therapy, or both. In addition, 41% of patients had cytogenetic abnormalities at diagnosis. The median T-cell dose infused was 4.19×10^{10} CD3$^+$ cells (range 1.42×10^9 to 5.15×10^{10}).

Table 1. Characteristics of patients

	Values
HLA-A2 status, *n* (%)	
Positive	10 (37)
Negative	17 (63)
Number of prior therapies	2 (1–5)
Age, yr, median (range)	57 (42–69)
Prior therapy, *n* (%)	
Lenalidomide only	4 (15)
Bortezomib only	10 (37)
Both bortezomib and lenalidomide	13 (48)
Race, *n* (%)	
White	18 (67)
Asian	3 (11)
Black or African American	6 (22)
Gender, *n* (%)	
M	16 (59)
F	11 (41)
Cytogenetics, *n* (%)	
Normal	15 (56)
Abnormal	11 (41)
NA	1 (4)
Myeloma subtypes, *n* (%)	
Immunoglobulin A	4 (15)
Immunoglobulin G	19 (70)
Light chain	4 (15)
% Marrow plasmacytosis at EN	5 (0–60)
β_2 microglobulin level at EN, mg/L, median (range)	1.88 (1.1–26)
CRP at EN, mg/dL, median (range)	1.2 (0.2–159.6)
Serum-free κ, mg/L, median (range)	11.24 (4.18–267.03)
Serum-free λ, mg/L, median (range)	8.2 (1.27–1,204)
κ/λ ratio, median (range)	1.46 (0.15–109.42)
CD4 cells/μL at EN	552 (0–1,457)
CD8 cells/μL at EN	285 (0–1,084)
CD3 cells/μL at EN	828 (0–2,237)
M-spike at EN, g/dL, median (range)	0.43 (0–2.60)

NOTE: EN, enrollment; CRP, C-reactive protein; 100% of patients received either bortezomib-based or lenalidomide-based induction therapy or both.

Toxicities from T-cell infusions and immunizations

Infusions of costimulated T cells were well tolerated with grade 1–3 chills and rigors in about 20% of patients, grade 1–3 nausea/diarrhea in about 20%, and grade 1–2 fatigue in about 5% of patients. The scope and frequency of these early postinfusion effects were similar to our earlier studies (14). Vaccine reactogenicity to the MAGE-A3 Trojan peptide immunizations containing Montanide, GM-CSF, and Hiltonol was significant. In the current trial, 9 of 15 (60%), 6 of 13 (46%), 6 of 14 (43%), and 11 of 16 (69%) of evaluable patients at one of the study sites (UMD) had injection site redness and/or induration of 50 mm or more during the week following the pretransplant, day +14, day +42, and day +90 immunizations, respectively. We observed that these injection site reactions tended to be prolonged and in 2 patients (of the first 9 enrolled) they evolved into sterile abscesses after the second and fourth immunizations. Because of a concern that a possible "depot" effect of the montanide could be involved in maintaining the local reaction, the montanide was eliminated from the vaccine formulation in the remaining 18 patients. Thereafter, vaccine reactions were more transient and no additional sterile abscesses were observed. Supplementary Fig. S1A shows the >10 cm injection site reaction, which developed in one of the 2 patients who later developed a sterile abscess after receiving the Hiltonol–GM-CSF–montanide triple adjuvant. Another patient who had significant injection site induration after receiving the vaccine with triple adjuvant consented to a skin biopsy, which showed infiltration of predominantly CD3$^+$/CD4$^+$ T lymphocytes (Supplementary Fig. S1B and S1C). The adverse events that were possibly/probably/definitely related to the immunizations or possibly represented delayed effects of the T-cell transfers are summarized in Supplementary Table S1. Specifically, symptoms of delayed rashes and/or diarrhea suggestive of autologous graft-versus-host disease were observed in 52% (grades I–III) and 14% (grades I and II) of patients, respectively, which were similar to the frequencies observed in our earlier studies of day +2 posttransplant T-cell transfers (13, 14).

Clinical outcomes and survival

With a median survival follow-up of 18 months, 4 patients died from myeloma progression yielding an estimated 2-year OS for the entire cohort of patients of 74% [95% confidence interval (CI), 54%–100%]. Five additional patients relapsed at 7, 9, 17, 18, and 18 months posttransplant, yielding an estimated 2-year EFS of 56% (95% CI, 37%–85%); the median EFS has not been reached. The OS and EFS curves are depicted in Fig. 2. The proportion of the 26 evaluable patients with clinical responses (defined as very good partial response, near-complete response, or

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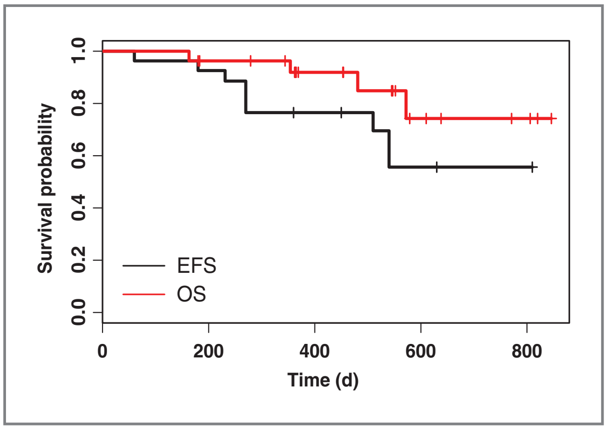

Figure 2. EFS and OS curves.

complete response) at days +100 and +180 were 16 of 26 (62%) whereas 9 of 26 (35%) had partial responses or stable disease at both of these timepoints.

Immune responses to the MAGE-A3 Trojan peptide vaccine + poly-ICLC (Hiltonol)

T-cell responses to the MAGE-A3 Trojan peptide vaccine were evaluated by dextramer analysis (for patients with HLA-A2$^+$) and by cytokine production (for all patients) on both CD4$^+$ and CD8$^+$ T cells. The definition of a positive response was defined as a distinct population of cells constituting greater than 0.05% of at least 7,500 events and at least 3 times the enrollment level (before first immunization) at one or more posttransplant timepoints (day +14, +60, +100, and day +180). The pie charts in Fig. 3A show that dextramer staining was detected in 3 of 6 (50%) and 5 of 6 (83%) of the evaluable A2-positive patients after culture with the CTL-1 and CTL-2 class I peptides and staining using the corresponding CTL-1 and CTL-2 dextramers, respectively. After culture with the whole MAGE-A3 Trojan peptide vaccine, 7 of 8 (88%) of the evaluable A2-positive patients exhibited positive staining with either the CTL-1 or CTL-2 dextramers. Altogether, 7 of 8 (88%) of the evaluable A2-positive patients had a positive response in one or more of the dextramer assays performed on peripheral blood samples collected at posttransplant days +14, +60, +100, and +180. Figure 3B shows the dot plots as a function of time for a representative patient (02710-217). To evaluate whether the T-cell responses were *functional*, mononuclear cells from all the patients were cultured with whole vaccine or with the class II peptide (HTL) followed by restimulation. As shown in Supplementary Fig. S2, IFN-γ cytokine production was detected on CD4$^+$ or CD8$^+$ subsets in 18 of 25 (72%) patients after culture and stimulation with the HTL (class II peptide) or the MAGE-A3 vaccine. One additional patient had a positive IL-2 response on CD4$^+$ T cells (not shown). Figure 4A and B show the % of IFN-γ–producing CD4$^+$ T cells for all 25 evaluable patients at enrollment and at serial timepoints after transplant after culture and restimulation with the whole MAGE-A3 Trojan peptide vaccine whereas Fig. 4C shows the % of IFN-γ–producing CD8$^+$ T cells at these same timepoints (see Supplementary Fig. S2 for expanded bar graph of CD8 responses). These bar graphs highlight the higher frequency and magnitude of the CD4 responses versus the CD8 responses in this system and confirm the expected predominance of cytokine responses for both T-cell subtypes during the day +14 to day +180 time frame. Figure 4D shows the dot plots as a function of time for a representative patient (02710-212) after culture and restimulation using both the MAGE-A3 whole vaccine and the HTL (class II) peptide.

To evaluate the impact of lenalidomide on MAGE-A3 vaccine-specific immune responses, we compared the MAGE-A3 vaccine–specific CD4 and CD8 responses at day +100 (pre-lenalidomide) and day +180 (post-lenalidomide). The median IFN-γ–producing CD4 response for all evaluable patients after culture and restimulation using the MAGE-A3 whole vaccine was 0.052% at day +100 versus 0.126% at day +180 ($P = 0.37$ by Wilcoxon paired rank sum test) whereas the median CD8 response was 0.025% at day +100 versus 0.05% at day +180 ($P = 0.035$).

Sixteen patients had enrollment marrow samples that were tested for MAGE-A3 expression in the myeloma cells by PCR and 4 of 16 (25%) had positive signals. All 4 patients relapsed after transplant in keeping with the adverse prognosis associated with CTAg expression. Two of these patients had positive IFN-γ responses whereas a third patient was negative and the fourth patient was not evaluable. One of the 2 MAGE-A3–positive patients with a positive IFN-γ response had MAGE-A3–negative myeloma at the time of posttransplant relapse. Three additional patients with myeloma progression were tested for MAGE-A3 expression at the time of relapse, including 2 patients who had positive IFN-γ responses during the study and these 2 patients exhibited negative or weak (1 patient each) MAGE-A3 expression in their myeloma cells at the time of relapse. The third patient had a posttransplant relapse with MAGE-A3–positive myeloma despite having developed a positive IFN-γ response during the study.

B-cell responses and effect of montanide on immune responses

Figure 5 shows box and whisker plots of the log-transformed titers of antibody responses over time for the cohort of 9 patients who received montanide with their vaccine formulations versus the cohort of 18 patients who did not. Elimination of the montanide virtually abolished antibody responses to the vaccine ($P < 0.0001$ at day +100 and day +180), suggesting that the "depot" effect of the montanide may be important for successful generation of B-cell responses to this peptide vaccine. Of 7 patients who received montanide and were evaluable for T-cell responses by cytokine production, 6 patients (86%) had positive responses whereas of 18 patients who did not receive montanide 13 patients (76%) had positive responses. Thus, deleting montanide did not significantly diminish the frequency of T-cell responses and in fact the estimated EFS was

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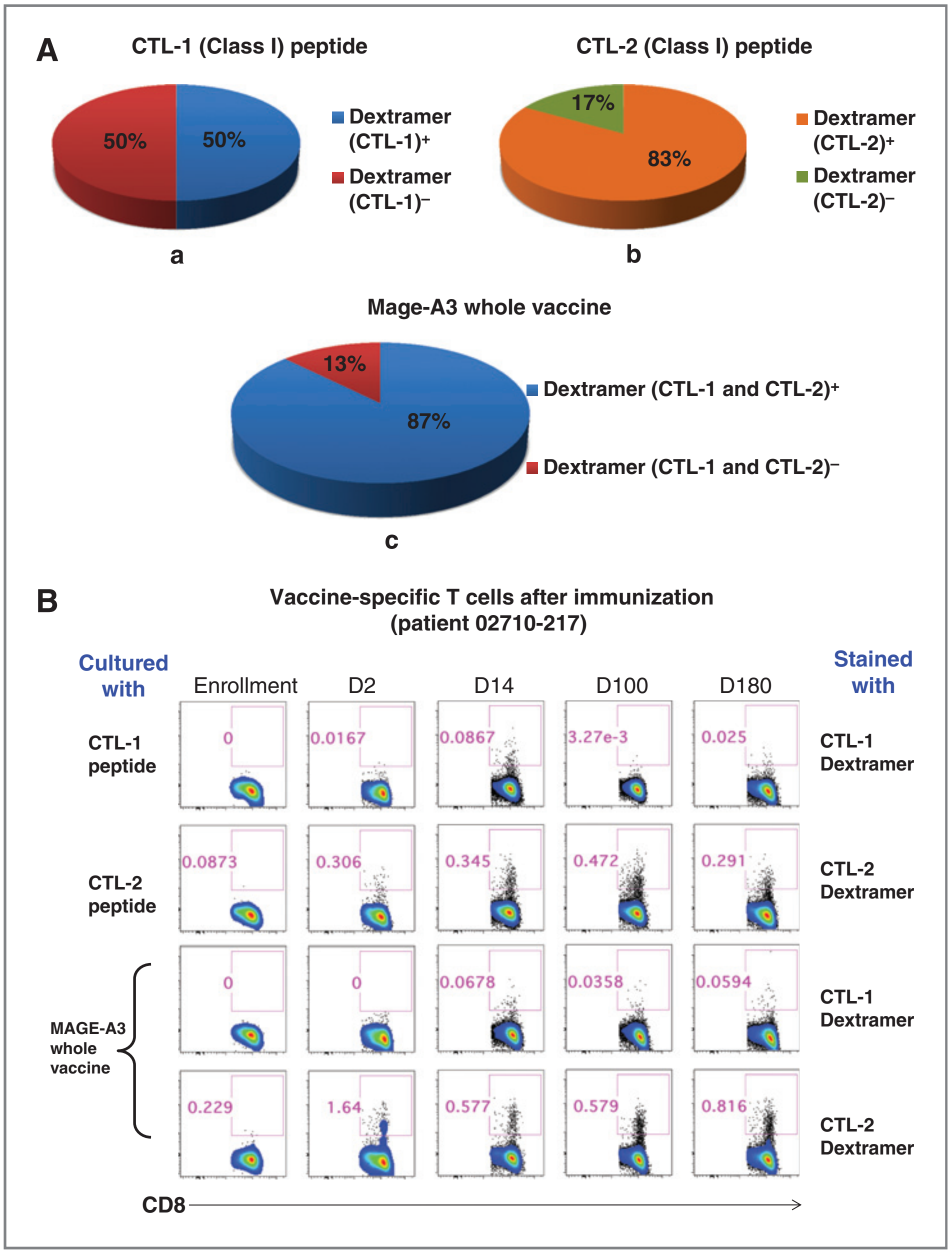

Figure 3. Dextramer studies of T-cell responses against the MAGE-A3 Trojan peptide vaccine in patients with HLA-A2+ ($N = 8$ evaluable patients). A, pie charts show that 3 of 6 (50%) of evaluable patients with HLA-A2+ had positive dextramer staining with CTL-1 (class I) dextramer on CD8+ T cells after culture with the CTL-1 peptide and 5 of 6 (83%) had positive dextramer staining with CTL-2 (class I) peptide after CTL-2 culture, whereas 7 of 8 evaluable patients had positive dextramer staining using either the CTL-1 or CTL-2 peptides after culture of cells with the whole vaccine. Note that peripheral blood samples for *in vitro* culture and dextramer staining were obtained at enrollment, day +2 (optionally), day +14, day +60, day +100, and day +180 after transplant; B, serial dot plots show proportions of dextramer-positive CD8+ T cells after culture and staining for a specific patient at multiple timepoints after ASCT.

better in the non-montanide cohort with a marginal statistical significance ($P = 0.07$).

Immune responses to the PCV vaccine: comparison to earlier trial

Antibody responses to the Prevnar-13 vaccine were assessed by ELISA for each of 4 saccharide antigens including 6B, 14, 19F, and 23F as described (11). The frequency of patients who had responses to ≥2, ≥3, or all 4 serotypes by day +100 was 53.9%, 30.8%, and 23.1%, respectively. By day +180 after transplant, after lenalidomide maintenance was started and patients received 2 additional immunizations at days +120 and +150, the frequencies rose to 69.2%, 50%, and 34.6%, respectively. Supplementary Fig. S3 shows the geometric means of the total antibody responses as a function of time in the earlier trial versus the current trial. At day +180, we observed a marginally statistically significant difference ($P = 0.053$), suggesting that the addition of lenalidomide plus 2 additional booster immunizations may have induced a further increase in the magnitude of the PCV antibody response, which had seemed to plateau after the day +100 timepoint in the earlier trial.

Correlations between immune parameters and clinical outcome

We tested for associations between certain immune cell parameters or vaccine-related immune responses and clinical outcomes including myeloma disease response at day

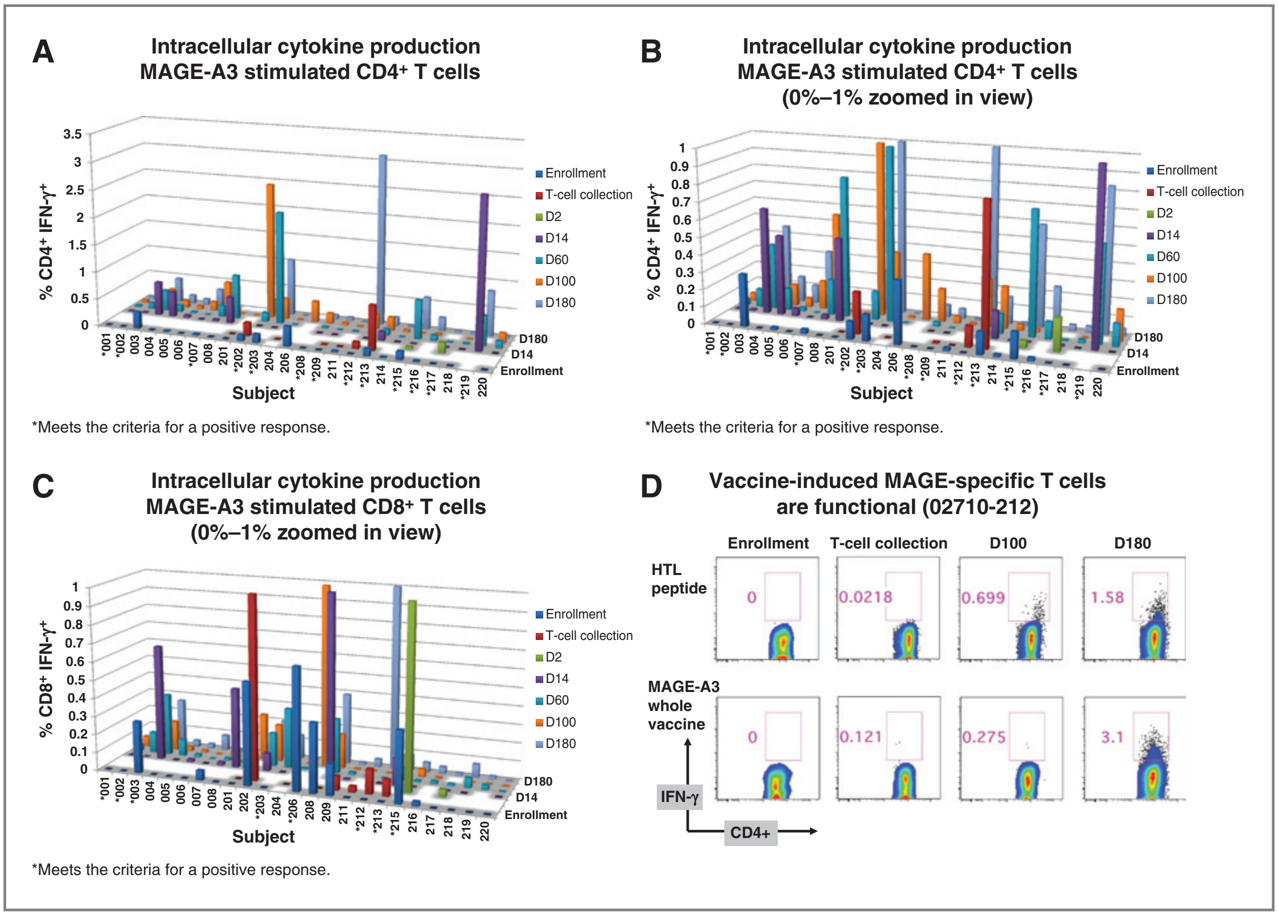

Figure 4. Functional studies of T-cell responses to the MAGE-A3 Trojan peptide vaccine ($N = 25$ evaluable patients). A, bar graph showing the % IFN-γ–producing CD4$^+$ T cells at serial timepoints for all evaluable patients ($N = 25$) after culture and restimulation with the MAGE-A3 whole vaccine; B, zoomed in view of CD4$^+$ responses (0–1% range); C, bar graph showing the % IFN-γ–producing CD8$^+$ T cells at serial timepoints for all evaluable patients ($N = 25$) after culture and restimulation with the MAGE-A3 whole vaccine (0–1% range shown, see Supplementary Fig. S2 for expanded bar graph); D, serial dot plots show the proportions of IFN-γ–positive CD4$^+$ T cells after culture and restimulation with whole MAGE-A3 vaccine (bottom) and the HTL (class II) peptide (top) for a specific patient at multiple timepoints after ASCT.

100 and day 180 and EFS as well as OS. Although the EFS was better for both positive CD4$^+$ IFN-γ responders and positive CD8$^+$ IFN-γ responders, the differences were not statistically significant ($P = 0.27$ and 0.22, respectively). However, a double positive IFN-γ response on both CD4$^+$ and CD8$^+$ T cells was marginally associated with better EFS ($P = 0.059$).

Discussion

High-dose melphalan remains an integral part of the treatment program for myeloma as well as a valuable platform for introducing long-term maintenance strategies, although relapses are common (31, 43). Animal models suggest that the early posttransplant immunologic milieu may be particularly conducive to induction of antitumor immune responses to cancer vaccines and adoptive T-cell immunotherapy (44, 45).

Our earlier studies have shown that about 1 of 3 of patients with myeloma who were immunized before and after transplant with a multipeptide tumor antigen vaccine along with an infusion of vaccine-primed and *ex vivo* anti-CD3/anti-CD28 costimulated autologous T cells at day +2 after transplant developed vaccine-directed T-cell responses by tetramer analysis (14). In an effort to increase the frequency of such vaccine-directed immune responses and their potential clinical impact, we studied a tumor antigen vaccine based on MAGE-A3 (compound GL-0817) and tested whether the TLR-3 agonist Hiltonol (Poly-ICLC) could increase its immunogenicity while maintaining a good safety profile.

We observed an 88% immune response rate by dextramer analysis in the subgroup of patients with HLA-A2$^+$. The use of dextramers rather than tetramers may account in part for this higher frequency, however, a similar magnitude of response (76%) was observed in the entire study cohort when the T cells were analyzed for cytokine production in response to vaccine exposure. Although this increased frequency of vaccine-specific responses was possibly due partly to the unique molecular features of the MAGE-A3 Trojan peptide vaccine

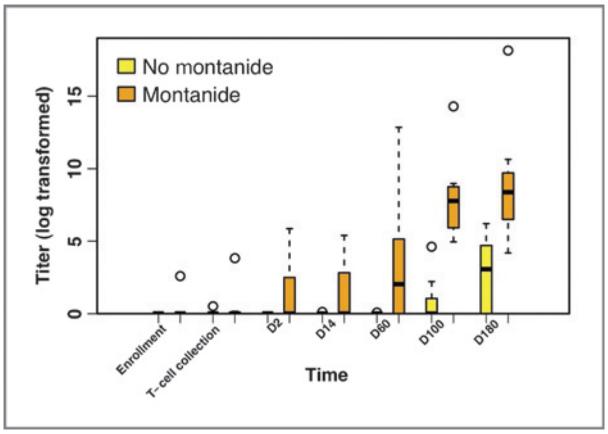

Figure 5. ELISA assay for antibody responses to the MAGE-A3 Trojan peptide vaccine. Shown are box and whisker plots at multiple timepoints of the log transformed antibody titers for the cohort of 9 patients who received montanide in their vaccine formulations (yellow or left-sided boxes) versus the cohort of 18 patients who did not receive montanide (orange or right-sided boxes). Open circles denote outlier responses. The differences at days 100 and 180 were statistically significant with $P < 0.0001$.

including the HIV-1-TAT membrane translocation sequence, it seems likely that the addition of Poly-ICLC (Hiltonol) to the standard adjuvants of montanide and GM-CSF contributed to this response as has been reported using other peptide-based vaccines (46). The triple adjuvant combination also led to severe reactogenicity, necessitating elimination of montanide from the vaccine formulation because the "depot" effect of montanide might have maintained the local reaction. Elimination of the montanide did not affect the T-cell responses and was marginally associated with better EFS, but the vaccine-specific antibody responses markedly decreased. Thus, the "depot" effect of the montanide may be important for induction of B-cell responses. Conversely, a strong "depot" effect of montanide may be deleterious for induction of therapeutic T-cell activity. In a murine model, protracted antigen presentation using oil-based emulsions trapped vaccine-specific $CD8^+$ T cells at vaccination sites thereby preventing migration to tumors and leading to downregulation and apoptosis (47).

A limitation of this study was that MAGE-A3 expression in the myeloma cells was not required for study entry, thus reducing our ability to evaluate the clinical impact of vaccine-specific T- and B-cell responses. In previous studies of adoptive T-cell immunotherapy, higher posttransplant levels of $CD4^+$ T cells and lower percentages of FOXP3+ T cells (Tregs) were associated with improved EFS (14). In this study we found that double positive vaccine-directed IFN-γ responses on $CD4^+$ and $CD8^+$ T cells together was possibly associated with better EFS. This observation may be worthy of further study.

Posttransplant lenalidomide has been shown to improve progression-free survival and may improve overall survival with long follow-up (31). In addition, lenalidomide is immunostimulatory through activation of T cells and NK cells and may repair defective immune synapses in the T cells of patients with hematologic malignancies (32–37). A marginally significant increase in antibody responses to the PCV (Prevnar-13) occurred at day 180 after patients started lenalidomide at day +100 after transplant, supporting the notion that lenalidomide may improve immune responses to microbial vaccines (38). Significantly higher $CD8^+$ T-cell responses to the MAGE-A3 vaccine were also observed at day +180 as compared with day +100 (see Fig. 4B and D), suggesting that lenalidomide (which was started at day +100) may augment MAGE-A3–specific CD8 immune responses.

Enthusiasm for autologous T-cell immunotherapy has grown after reports of successful therapy of leukemia using autologous T cells engineered to express anti-CD19 chimeric antigen receptors (CART-19 cells; refs. 48 and 49). Studies using genetically modified autologous T cells, which are engineered to express affinity-enhanced T-cell receptors (TCR) for myeloma target antigens including CTAgs NY-ESO-1/LAGE-1, are also in progress and show early promise (50). However, toxicities from such gene-modified T cells are considerable and in the case of MAGE-A3 affinity-enhanced TCRs included fatal T-cell–mediated cardiomyopathy (51). Thus, safe and effective targeting of certain tumor antigen targets such as MAGE-A3 may still require specific priming and activation of naturally occurring T cells with tumor antigen vaccines and costimulation. This study shows that a high frequency of functional tumor antigen vaccine–specific T cells can be generated early after autologous stem cell transplantation for myeloma using a MAGE-A3 vaccine (GL-0817) formulated with Poly-ICLC along with vaccine-primed and *ex vivo* costimulated autologous T cells. Further studies on patients with MAGE-A3$^+$ myeloma or other hematologic malignancies should help to better define whether these vaccine-specific T cells exhibit clinical activity.

Disclosure of Potential Conflicts of Interest

S.E. Strome is employed (other than primary affiliation; e.g., consulting) as a cofounder in Gliknik Inc. S.E. Strome has commercial research grant from Gliknik Inc. Also, S.E. Strome has ownership interest (including patents) and is a consultant/advisory board member in Gliknik Inc. A.M. Salazar has ownership interest (including patents) in Oncovir. B.L. Levine has commercial research grant from Novartis. B.L. Levine also has ownership interest (including patents) in Novartis. C.H. June has ownership interest (including patents) in patents on cell therapy that are owned by the U.S. government and licensed to Life Technologies. No potential conflicts of interest were disclosed by the other authors.

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