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

Aaron P. Rapoport1, Nicole A. Aqui5, Edward A. Stadtmauer6, Dan T. Vogl6, Yin Yan Xu5, Michael Kalos5, Ling Cai1, Hong-Bin Fang1, Brendan M. Weiss6, Ashraf Badros1, Saul Yanovich1, Gorgun Akpek1, Patricia Tsao5, Alan Cross1,2, Dean Mann1, Sunita Philip1, Naseem Kerr6, Andrea Brennan6, Zhaohui Zheng6, Kathleen Ruehle1, Todd Milliron1, Scott E. Strome3, Andres M. Salazar4, Bruce L. Levine6, and Carl H. June6

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-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...
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-variant 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 \textit{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 x 10^6 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 \textit{ex vivo} expansion was reinforced by a subsequent study of ASCT for myeloma, which showed that posttransplant seroconversion to an influenza vaccine required \textit{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 multipepptide tumor antigen vaccine composed of HLA-A2–restricted peptides derived from hTERT and survivin. Using a 5-fold higher dose of T cells (~5 x 10^9 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 CTAs whose expression is limited to spermatogenic germ 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.
(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
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 $1 \times 10^8$ 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/m² followed by subcutaneous injections of G-CSF (10 μg/kg). High-dose therapy was melphalan (200 or 140 mg/m² if age ≥70 years) followed by infusions of autologous stem cells ($≥2 \times 10^6$ 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 (Cari-dianBCT 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 (Dynal/Invitrogen) 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 $5 \times 10^7$.

**Immunooassays**

**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 (ELWGPRALV), class II peptide—HIL (VIIFKASSSLQL), 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 FACSCan to 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 Immunex. 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 ($1 \times 10^7$/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/ Cytoperim 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: HS099999908_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
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 ≥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 $4.19 \times 10^{10}$ CD3$^+$ cells (range $1.42 \times 10^9$ to $5.15 \times 10^{10}$).

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 reactivity 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 “depo” 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 CD3$^+$ 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 post-transplant, 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

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients</th>
<th>Values</th>
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</thead>
<tbody>
<tr>
<td><strong>HLA-A2 status, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (37)</td>
</tr>
<tr>
<td>Negative</td>
<td>17 (63)</td>
</tr>
<tr>
<td><strong>Number of prior therapies</strong></td>
<td></td>
</tr>
<tr>
<td>2 (1–5)</td>
<td></td>
</tr>
<tr>
<td><strong>Age, yr, median (range)</strong></td>
<td>57 (42–69)</td>
</tr>
<tr>
<td><strong>Prior therapy, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Lenalidomide only</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Bortezomib only</td>
<td>10 (37)</td>
</tr>
<tr>
<td>Both bortezomib and lenalidomide</td>
<td>13 (48)</td>
</tr>
<tr>
<td><strong>Race, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>18 (67)</td>
</tr>
<tr>
<td>Asian</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Black or African American</td>
<td>6 (22)</td>
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<tr>
<td><strong>Gender, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>16 (59)</td>
</tr>
<tr>
<td>F</td>
<td>11 (41)</td>
</tr>
<tr>
<td><strong>Cytogenetics, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15 (56)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>11 (41)</td>
</tr>
<tr>
<td>NA</td>
<td>1 (4)</td>
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<tr>
<td><strong>Myeloma subtypes, n (%)</strong></td>
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</tr>
<tr>
<td>Immunoglobulin A</td>
<td>4 (15)</td>
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<tr>
<td>Immunoglobulin G</td>
<td>19 (70)</td>
</tr>
<tr>
<td>Light chain</td>
<td>4 (15)</td>
</tr>
<tr>
<td><strong>% Marrow plasmacytosis at EN</strong></td>
<td>5 (0–60)</td>
</tr>
<tr>
<td><strong>β2 microglobulin level at EN, mg/L, median (range)</strong></td>
<td>1.88 (1.1–26)</td>
</tr>
<tr>
<td><strong>CRP at EN, mg/dL, median (range)</strong></td>
<td>1.2 (0.2–159.6)</td>
</tr>
<tr>
<td><strong>Serum-free κ, mg/L, median (range)</strong></td>
<td>11.24 (4.18–267.03)</td>
</tr>
<tr>
<td><strong>Serum-free λ, mg/L, median (range)</strong></td>
<td>8.2 (1.27–1.204)</td>
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<tr>
<td><strong>κ/λ ratio, median (range)</strong></td>
<td>1.46 (0.15–109.42)</td>
</tr>
<tr>
<td><strong>CD4 cells/μL at EN</strong></td>
<td>552 (0–1,457)</td>
</tr>
<tr>
<td><strong>CD8 cells/μL at EN</strong></td>
<td>285 (0–1,084)</td>
</tr>
<tr>
<td><strong>CD3 cells/μL at EN</strong></td>
<td>828 (0–2,237)</td>
</tr>
<tr>
<td><strong>M-spike at EN, g/dL, median (range)</strong></td>
<td>0.43 (0–2.60)</td>
</tr>
</tbody>
</table>

NOTE: EN, enrollment; CRP, C-reactive protein; 100% of patients received either bortezomib-based or lenalidomide-based induction therapy or both.
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
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 $/C21_2$, $/C21_3$, 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.
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 vaccine-directed immune responses was possibly due partly to the unique molecular features of the MAGE-A3 Trojan peptide vaccine.
responses on CD4\(^+\) study we found that double positive vaccine-directed IFN-
associated with better EFS. This observation may be worthy
immunostimulatory through activation of T cells and NK
with long follow-up (31). In addition, lenalidomide is
progression-free survival and may improve overall survival
levels of CD4\(^+\) sions trapped vaccine-specific CD8\(^+\) protracted antigen presentation using oil-based emul-
ation of therapeutic T-cell activity. In a murine model,
induction of B-cell responses. Conversely, a strong
specific antibody responses markedly decreased. Thus, the
montanide did not affect the T-cell responses and was
have maintained the local reaction. Elimination of the
montanide did not affect the T-cell responses and was
arginated with better EFS, but the vaccine-
specific antibody responses markedly decreased. Thus, 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, yet the vaccine-
specific antibody responses markedly decreased. Thus, the
"depot" effect of montanide may be important for induction of B-cell responses. Conversely, a strong
"depot" effect of montanide may be deleterious for induc-
tion 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 adaptive 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-\(\gamma\) 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 cardiomy-
opathy (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 autol-
ogous 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 Gilknik Inc. S.E. Strome has commercial research grant from
gilknik Inc. Also, S.E. Strome has ownership interest (including patents) and is a consultant/advisory board member in Gilknik Inc. A.M.
Salazar has ownership interest (including patents) in Oncuvir. 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.

Authors' Contributions
Conception and design: A.P. Rapoport. A.N. Aqui. S.E. Strome. A.M.
Salazar. B.L. Levine. C.H. June
Development of methodology: A.P. Rapoport. N.A. Aqui. E.A. Stadtmauer,
D. Mann. Z. Zheng. B.L. Levine. C.H. June
Acquisition of data (provided animals. acquired and managed patients,
provided facilities, etc.): A.P. Rapoport. N.A. Aqui. E.A. Stadtmauer, D.T.
B.L. Levine
Analysis and interpretation of data (e.g., statistical analysis, biosta-
tistics, computational analysis): A.P. Rapoport. A.N. Aqui. E.A. Stadtmauer,
Written, review, and/or revision of the manuscript: A.P. Rapoport. N.A.
Levine. C.H. June

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 signi
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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

Aaron P. Rapoport, Nicole A. Aqui, Edward A. Stadtmauer, et al.


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