Autologous Lymphocyte Infusion Supports Tumor Antigen Vaccine–Induced Immunity in Autologous Stem Cell Transplant for Multiple Myeloma

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Abstract

Autologous stem cell transplant (autoSCT), the standard consolidation therapy for multiple myeloma, improves disease-free survival, but is not curative. This could be an ideal setting for immunologic therapy. However, the immune milieu is impaired after autoSCT. We hypothesized that autologous lymphocyte infusion would restore immune competence, allowing immunotherapies such as cancer vaccines to elicit tumor antigen–specific immunity in the setting of autoSCT. In this pilot study (NCT01380145), we investigated safety, immunologic, and clinical outcomes of autologous lymphocyte infusion combined with peri-autoSCT immunotherapy with recombinant MAGE-A3 (a multiple myeloma–associated antigen) and adjuvant. Thirteen patients with multiple myeloma undergoing autoSCT were enrolled. Autologous lymphocyte infusion and MAGE vaccination were well tolerated. Combination immunotherapy resulted in high-titer humoral immunity and robust, antigen-specific CD4+ T-cell responses in all subjects, and the responses persisted at least one year post-autoSCT. CD4+ T cells were polyfunctional and Th1-biased. CD8+ T-cell responses were elicited in 3 of 13 subjects. These cells recognized naturally processed MAGE-A3 antigen. Median progression-free survival was 27 months, and median overall survival was not reached, suggesting no differences from standard-of-care. In 4 of 8 subjects tested, MAGE-A protein expression was not detected by IHC in multiple myeloma cells at relapse, suggesting therapy-induced immunologic selection against antigen-expressing clones. These results demonstrated that autologous lymphocyte infusion augmentation of autoSCT confers a favorable milieu for immunotherapies such as tumor vaccines. This strategy does not require ex vivo manipulation of autologous lymphocyte products and is an applicable platform for further investigation into combination immunotherapies to treat multiple myeloma.

Introduction

Multiple myeloma, a malignancy of immunoglobulin (Ig)-secreting plasma cells, is the second most common hematologic cancer (1). The introduction of agents such as immunomodulatory drugs (ImmD) and proteasome inhibitors and use of autologous stem cell transplantation (autoSCT) as standard consolidation therapy have improved both disease-free and overall survival. Despite these advances, relapse is the rule and most patients die of their disease. In contrast, allogeneic (alloSCT) studies with long-term follow-up demonstrated a survival plateau in multiple myeloma, indicating a subset of patients who are cured (2). Depletion of T cells from allogeneic grafts increase relapse rates, and objective responses are observed with donor lymphocyte infusion (3), demonstrating that immunologic therapy, in the form of T-cell–mediated "graft-versus-myeloma" effect can confer long-term remission. However, alloSCT is only applicable in a minority of patients with multiple myeloma due to age and health restrictions and to procedure-related morbidity and mortality. Broadly applicable strategies that confer safe and effective "host-versus-myeloma" immune responses are therefore needed.

The period around autoSCT invites exploration of additional multiple myeloma immunotherapies, as autoSCT generally confers the lowest tumor burden in the natural history of the disease and engraftment of the cellular immune compartments provides the opportunity to sculpt antitumor immunity. Challenges to this approach include delayed immune reconstitution, which results in poor responses to infectious disease vaccines (4). Rapoport and colleagues demonstrated that pre-autoSCT vaccination followed by early (day +12) post-autoSCT transfer of vaccine-primed T cells, activated ex vivo with anti-CD3/anti-CD28–coated beads, and posttransplant booster vaccinations restored immune responses for over a year post-autoSCT in some subjects (5). Combination immunotherapy may offer a minimally invasive approach to augment autoSCT and improve antitumor immunity.
competence to a pneumococcal vaccine in patients undergoing autoSCT (5). Patients who did not receive T-cell transfer until day +90 failed to develop durable pneumococcal immunity, demonstrating the need for early T-cell reconstitution during the post-SCT period (5). Follow-up studies have expanded on this use of cellular augmentation modalities for autoSCT as immunotherapy strategies for multiple myeloma, demonstrating generation of immune responses to infectious and tumor antigens, as well as the safety of even earlier adoptive T-cell transfer (day +2 or 3 post-SCT; refs. 6, 7). Several groups have explored analogous adoptive transfer strategies following autoSCT, including the use of activated marrow-infiltrating lymphocytes (8), or T cells genetically modified to express transgenic T-cell (9) or chimeric antigen (10) receptors. Although these studies have demonstrated the feasibility and safety of these approaches, the use of in vivo–activated T cells has some disadvantages, including the risk of autologous graft-versus-host-disease (GVHD; ref. 11) and need for extensive manipulation in a specialized cell-processing facility, which adds expense and complexity and limits these approaches to a few centers. Simpler reconstitution strategies could be applied broadly at any transplant center. Murine studies demonstrated that infusion of unmanipulated, vaccine-primed lymphocytes within a few days of myeloablative therapy followed by posttransfusion vaccination leads to robust in vivo expansion of transferred cells and long-lasting antitumor immunity (12).

On the basis of these results, we hypothesized that augmentation of autoSCT with vaccine-primed autologous lymphocyte infusion would create a favorable milieu for the generation of tumor antigen–specific immune responses. We conducted a pilot study of this strategy with an immunotherapeutic product containing recombinant Melanoma Antigen Gene A3 (recMAGE-A3) protein, a member of the Cancer-Testis Antigen (CTAg) group of cancer-associated genes that has been investigated in tumor immunotherapy (13). MAGE-A3 is expressed in 30%–40% of newly diagnosed patients with multiple myeloma and up to 77% of relapsed patients with multiple myeloma (14–18), is immunogenic in patients with multiple myeloma (19, 20), and is a functional target that inhibits apoptosis in multiple myeloma cells (21). In our study, we administered recMAGE-A3 with AS15 immunostimulant (22) to 13 multiple myeloma subjects pre- and post-autoSCT in conjunction with early post-SCT vaccine-primed autologous lymphocyte infusion. We report here analysis of safety, clinical outcomes, and immune responses. This strategy was safe in the target multiple myeloma population and supported robust MAGE-A3–specific humoral and CD4+ T-cell immune responses. These results demonstrate that autologous lymphocyte infusion-augmented autoSCT broadens the scope of immunotherapeutic strategies to treat multiple myeloma.

Materials and Methods

Subjects

Subjects with symptomatic multiple myeloma, who were within 12 months of starting treatment, had achieved a Very Good Partial Response (VGPR) or better by International Myeloma Working Group (IMWG) criteria after induction therapy (23), had no prior stem cell transplant, and were eligible to receive high-dose chemotherapy and autologous stem cell rescue by standard institutional criteria, were enrolled at three centers (see Supplementary Fig. S1 for full eligibility criteria). Choice of induction therapy was not dictated by the study. Subjects must have had a bone marrow biopsy specimen or plasmacytoma that was positive for MAGE-A antigen by IHC. Written informed consent was obtained from each subject and the study was conducted with approval of each institution’s institutional review board in concordance with the Declaration of Helsinki. The trial was registered in clinicaltrials.gov under NCT01380145.

IHC

IHC was performed on formalin-fixed, paraffin-embedded bone marrow or plasmacytoma biopsy specimens using the M3H67 mAb specific for MAGE-A3, as well as other homologous MAGE-A family members, as described previously (14, 16). The following scoring system was used: negative: focal: <5% multiple myeloma cells positive; ≥ 5%–25%; ≥ 25%–50%; ≥ 50%–75%; ≥ 75%. Subjects with a score of ≥ 75% were deemed “MAGE-A positive” and were eligible for the study. When feasible, IHC for MAGE-A expression was also performed at time of progression.

Vaccine

The vaccine preparation was comprised of 300 μg of the recombinant ProtD-MAGE-A3/His, a 432-aa fusion protein containing 109 amino acids of Haemophilus influenzae B Protein D (ProtD), the full-length MAGE-A3 protein, and a polyhistidine tail (His), and the proprietary immunostimulant AS15. AS15 is a combination of 3-O-desacyl-4’-monophosphoryl lipid A (MPL, 50 μg, produced by GSK), Quillaja saponaria Molina, fraction 21 (QS-21, 50 μg, licensed by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus Inc.), and Cpg 7909 synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (420 μg), in a liposomal formulation (22). Each dose was given intramuscularly.

Treatment regimen

Injection #1 was given 6–7 weeks before autologous stem cell transplantation, at least three weeks after completing induction therapy. Three weeks later, subjects had steady-state leukapheresis/10 post-SCT (i.e. days 10, 31, 52, 73, 94). Two additional immunizations (#2 to 6) were given every three weeks (± 7 weeks before autologous lymphocyte infusion on day +3. Immunizations #2 to 6 were given every three weeks (±3 days) starting on day 10 post-SCT (i.e. days 10, 31, 52, 73, 94). Two additional immunizations (#7 and #8) were given at 3-month intervals (±7 days; i.e., days 180 and 270). Maintenance lenalidomide was allowed at the discretion of the treating physician, starting 3 months or more after autoSCT (Supplementary Fig. S1).

Sample collection

Blood samples for immunologic correlates were obtained pretreatment, 3 weeks after immunization #1 (at time of PBL leukopheresis), at time of stem cell collection, and post-SCT on day 31 (immunization #3), day 73 (immunization #5), day 194 (2 weeks postimmunization #7), day 284 (2 weeks postimmunization #8), and day 365 (1 year post-SCT). Bone marrow aspirate samples were collected pretreatment, and at 3 months and 1-year post-SCT. Plasma was collected by centrifugation of whole blood and cryopreserved. PBMCs were isolated by
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Ficoll-Paque gradient and cryopreserved for immunophenotyping and cellular assays.

**Assessment of humoral immunity**

Antibodies to MAGE-A3 were measured by ELISA, as described previously (24), using two different preparations of MAGE-A3: a baculovirus-produced, histidine-tagged recombinant protein, and a pool of 20-mer overlapping peptides covering the entire sequence of MAGE-A3 (named MAGE-A3 1–30). Briefly, peripheral blood and bone marrow plasma was thawed and tested in duplicate for each patient in 4-fold serial dilutions from 1/100 to 1/100,000 for presence of IgG against the two different preparations of MAGE-A3. These antigen preparations were chosen to confirm specificity to MAGE-A3, by avoiding detection of potential microbial components related to the vaccine product (which was E. coli produced) and by measuring reactivity to synthetic linear epitopes. All patients were tested at selected time points against a "tagless" MAGE-A3 protein produced in E. coli but lacking the histidine tag, and some patients were also tested against a different pool of 57 shorter MAGE-A3 peptides provided by GlaxoSmithKline (named MAGE-A3 1-57). Reactivity to Protein D was assessed using an E. coli-produced protein provided by GlaxoSmithKline. Results are expressed as reciprocal titers based on the predicted dilution at which a linear extrapolation of the titration curve meets a cutoff determined from a healthy donor serum pool. Titers were considered significant if >1:100. Induction of responses was considered significant if going from undetectable (<100) to detectable (>100) or if increasing in titers over time by at least 4×. Titers at individual time points were also confirmed side-by-side for all patients in the same assay, to allow cross-patient comparisons.

**Assessment of cellular immunity**

Monitoring of IFNγ-producing CD4+ and CD8+ T cells specific for MAGE-A3 was performed by ELISPOT and intracellular cytokine staining following in vitro 11-day (for CD8) or 20-day (for CD4) presensitization as described previously (24). Briefly, CD4+ and CD8+ T cells were sequentially purified from PBMCs using magnetic beads (Life Sciences) and stimulated once with remaining irradiated CD8+CD4+ cells pulsed overnight with pools of overlapping 20-mer peptides covering the entire MAGE-A3 sequence (30 peptides in total overlapping by 10AA). As controls, an overlapping peptide pool covering the entire sequence of influenza A nucleoprotein (NP) was used. After a culture period of 11 days for CD8+ T cells and 20 days for CD4+ T cells in RPMI containing 10% SAB supplemented with glutamine, antibiotics, nonessential amino acids, IL2 (10 U/mL; Roche), and IL7 (20 ng/mL, R&D Systems), CD8+ and CD4+ T cells were harvested and tested by IFNγ ELISPOT on autologous EBV-B lymphoblastoid cells (B-EBV) or PHA-activated T cells (T-APC) pulsed with MAGE-A3 peptide pools (1-30 20 mers or 1-57 shorter peptide pool), control NP peptide pool, or irrelevant DMSO. Direct ex vivo responses (using PBMC without presensitization) were also assessed by ELISPOT at selected time points. B-EBV cells were infected overnight with adenovirus or vaccinia virus recombinant for MAGE-A3 as cognate target or NY-ESO-1 as control.

ELISPOT

Flat-bottomed, 96-well nitrocellulose plates (MultiScreen-HA) were coated with IFNγ mAb (4 µg/mL, 1-D1K, Mabtech) and incubated overnight at 4°C. After washing with RPMI, plates were blocked with 10% human AB-type serum for 2 hours at 37°C. Presensitized CD8 or CD4 T cells (5 x 10^5 and 1 x 10^6) and 5 x 10^5 target EBV-B or T-APC cells pulsed overnight with 1 µmol/L peptides from MAGE-A3 or control peptides were added to each well and incubated for 20 hours in RPMI medium 1640 without serum. PMA/ionomycin was used for quality control of CD8 and CD4 T cells (1 x 10^4 without targets). Plates were then washed thoroughly with water containing 0.05% Tween 20 to remove cells, and IFNγ mAb (0.2 µg/mL, 7-B6-1-biotin; Mabtech) was added to each well. After incubation for 2 hours at 37°C, plates were washed and developed with streptavidin-alkaline phosphatase (1 µg/mL, Roche) for 1 hour at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium; Sigma) was added and incubated for 10 minutes. After final washes, plate membranes display dark-violet spots representing IFNγ secreting CD8+ or CD4+ T cells that were counted with the CTL Immunospot analyzer and software (Cellular Technologies). The average from duplicates was calculated, and a positive response was determined as >50 spots and >2 x number of spots for control targets pulsed with DMSO. Results are shown after subtraction of spots with DMSO control targets and elevated spot counts to cognate antigen-pulsed targets not reaching the threshold for positivity due to high reactivity with DMSO-pulsed targets were assigned a value ≤50. An immunologic response was defined as MAGE-A3 CD4+ and CD8+ T cells going from undetectable at baseline (<50 spots) to detectable postimmunization (see above) against MAGE-A3 peptides compared with negative control peptides. In case of preexisting MAGE-A3 CD4+ or CD8+ T cells, an immunologic response was defined as at least 4× increase in number of specific spots over baseline.

**Ex vivo responses**

Briefly, ex vivo responses were measured at selected time points using a 48-hour IFNγ ELISpot after thawing PBMCs and overnight rest, using direct pulsing of 1 x 10^6 PBMCs (no separate antigen-presenting cells) with a pool of MAGE-A3 overlapping 20-mer peptides (1-30) or with control DMSO or Influenza NP peptide pool. PMA/ionomycin was used as a positive control. Ex vivo responses to MAGE-A3 were considered positive if >25 spots and at least 2× the levels observed to negative control DMSO.

**Intracellular staining of cytokines**

B-EBV target cells pulsed with shorter MAGE-A3 peptide pool 1-57, NP pool, or DMSO were washed and incubated with MAGE-A3 1-30 or NP presensitized effector T cells at a 1:2 ratio in 200 µL X-VIVO-15 at 37°C for 6 hours. Brefeldin-A (Sigma-Aldrich) at 10 µg/mL was added after the first two hours of culture. Cells were then fixed and permeabilized (BD Biosciences), and stained with appropriate antibodies against CD4, CD8, IFNγ, TNFα, IL4, or IL5 (BD Biosciences). PMA/ionomycin was used as a positive control. Cells were subsequently analyzed by flow cytometry with gating on morphologically defined lymphocytes, CD4-, or CD8-positive cells. A positive response was determined as >0.5% and 2× the percentage for DMSO control target. An immunologic response was defined as MAGE-A3 CD4+ or CD8+ T cells going from undetectable at baseline (<0.5%) to detectable postimmunization (see above) against MAGE-A3 peptides compared with negative control peptides. In case of preexisting MAGE-A3 CD4+ or CD8+ T cells, an immunologic response is defined as
at least 4 \times increase in percentage of MAGE-A3–specific T cells over baseline. Results are shown after subtraction of percentage with DMSO control targets and elevated percentages to cognate antigen-pulsed targets not reaching the threshold for positivity due to high reactivity with DMSO-pulsed targets were assigned a value ≤0.5%.

Generation of CD4+ T-cell lines

Presensitized CD4+ cells were restimulated with MAGE-A3 overlapping peptide-pulsed autologous antigen-presenting cells (APC), and then CD154+,CD4+ cells (representing the activated MAGE-A3–specific fraction) were sorted by flow cytometry. This CD154+ fraction then underwent serial nonspecific expansions over 3–5 weeks using PHA, IL2, IL7, and irradiated feeder cells (allo-PBMCs), as described in ref. 25, to generate CD4+ T-cell lines. Lines were then tested for MAGE-A3 specificity and IFNγ production via ELISpot. Cytotoxicity against MAGE-A3–pulsed or control autologous target cells was assessed using europium-release assay according to the manufacturer’s instructions (Delfia, Perkin-Elmer).

Clinical outcomes

Adverse events were graded according to NCI CTCAE v4. Hematologic responses were determined according to IMWG criteria. Progression-free survival (PFS) and overall survival (OS) was calculated from time of enrollment and estimated using the Kaplan–Meier method (GraphPad Prism 5.0). Data cutoff date for PFS and OS was June 30, 2015.

Statistical analyses

The primary study endpoint was safety and tolerability; secondary endpoints were immune responses and clinical outcomes. Frequency of adverse events and hematologic responses were analyzed descriptively. Changes in antibody titers and ELISpot over time from baseline were assessed using linear mixed-effects models with random subject intercept (26). An immune response was defined as (i) increase in antibody titers in at least two posttreatment samples of at least 5-fold over baseline, or (ii) increase in T-cell frequency in at least two posttreatment samples of at least 5-fold over baseline and 3-fold over background. This was designed as a pilot study to enroll up to 16 subjects to obtain at least 12 “evaluable” subjects for the planned post-SCT vaccinations. At the time this “evaluable” threshold was met for the 12th subject, a 13th subject had already been enrolled, so a total of 13 subjects were treated on study.

Results

Subject characteristics

Ninety-three subjects were prescreened over 18 months. Thirty (32%) were MAGE-A3 positive, in agreement with previously reported frequencies of expression in newly diagnosed subjects. Thirteen subjects were enrolled (see Supplementary Fig. S1 for subject flow chart). Subject characteristics are listed in Table 1. Median age was 56, and 5 of 11 tested had at least 1 high-risk cytogenetic abnormality, which is higher than expected for the general multiple myeloma population. Twelve of 13 subjects had lenalidomide and bortezomib during induction, and 9 of 13 had a change in induction therapy prior to enrollment due to suboptimal response, indicative of high-risk clinical behavior regardless of cytogenetics/FSHl. Twelve subjects were in VGPR entering the study, with 1 in complete response (CR).

Feasibility and safety

PBL collection for autologous lymphocyte infusion yielded a median of 1.4 \times 10^6 CD3+ cells/kg (range 1.0–2.6), with all subjects reaching the targeted goal. Pre-SCT immunization did not impact stem cell yield, with a median of 11.4 \times 10^6 CD34+ cells/kg (range 8.3–37.2) collected. Autologous lymphocyte infusions were well tolerated, and no engraftment-like syndrome or autologous GVHD was noted. Engraftment to an absolute neutrophil count (ANC) >500/μL occurred at a median of 11 days. Protocol treatment was feasible with 12 of 13 subjects completing the planned treatment, with only one missed vaccination, and one subject withdrawing early due to progression. There were no unexpected autoSCT-related toxicities and no treatment-related deaths. Treatment-related adverse events were primarily grade I and self-limited, with the most common being injection site reaction/pain reported in 54% of subjects (Supplementary Table S1). There were eight serious adverse events (SAE) reported in four subjects (Supplementary Table S2), three of which were possibly related to protocol treatment. One subject developed self-limited grade 2 myalgia and grade 1 injection-site reaction shortly after vaccination, which led to a 24-hour hospitalization. One subject developed a skin disorder 14 months after the final vaccination, initially diagnosed as psoriasis, later evolving to grade 3 mixed connective tissue disorder. No other autoimmune toxicities were noted. Lenalidomide maintenance was discontinued in one of five subjects due to grade 1 rash, which is a
common AE for this agent, and there were no unexpected toxicities associated with lenalidomide.

Clinical outcomes
At enrollment, 12 subjects were in VGPR and one in CR after induction therapy. Restaging at three months post-SCT showed 6 subjects in CR [three in stringent CR (sCR)] and 7 subjects in VGPR. Lenalidomide maintenance was started in five subjects, although discontinued in 2 subjects within 2 months of initiation (one for progression, one for rash). At the 1-year post-SCT restaging, there were four VGPR and five CR (four in sCR), including two subjects whose hematologic response improved between three and 12 months post-autoSCT, neither of whom received lenalidomide maintenance. Four subjects had progression at or before the one-year restaging visit. With a median follow-up of 31 months, three subjects remain progression-free, and median PFS is 27 months. Three subjects have died, two from myeloma and one from complications of salvage allogeneic transplant, with median OS not reached (Fig. 1). Understanding the limited statistical power of study, we observed no trend suggesting association between PFS and degree of MAGE-A expression at diagnosis or lenalidomide maintenance versus no maintenance. No association was observed between PFS and the immune response data described in the following sections, including spreading of humoral immune response, MAGE-A3–specific CD8+ T-cell response, or loss of MAGE-A3 expression.

Humoral immune responses
All subjects developed robust MAGE-A3–specific antibody titers in response to protocol therapy. High antibody titers (1.104–109) against the immunized antigen were detected as early as three weeks after the first postpriming vaccine and persisted to at least 1-year post-ASCT (Fig. 2A). Titers from peripheral blood and bone marrow plasma were concordant (Supplementary Fig. S2). Kinetics of antibody induction differed from subject to subject. Three subjects seroconverted after a single immunization (pre-SCT; M15, M27, and S25). Another eight subjects seroconverted by day 31 after autoSCT, following two immunizations (pre-SCT and day 10 post-SCT; F06, F10, M08, M26, S02, S10, S13, S36). The remaining two subjects seroconverted by day 73, after 4 immunizations (F02, S33). All antibody responses were significantly higher than baseline \((P < 0.001 \text{ at each time point})\). Specificity of antibody responses to MAGE-A3 was confirmed in all subjects using a pool of overlapping 20-mer peptides covering the full-length protein (Fig. 2B) or tagless MAGE-A3 protein (Supplementary Table S3), although recognition of linear epitopes (peptide pool) was weaker and sometimes delayed compared with full-length protein recognition. Epitope mapping demonstrated that vaccination induced a polyclonal response against at least 7 distinct epitope regions of the MAGE-A3 protein (Fig. 2C). The diversity of responses agreed with observations from a prior study (LUD99-010) in non–small cell lung cancer (NSCLC) using recombinant MAGE-A3 with an immunostimulant composed of MPL and QS21 in oil/water emulsion that did not contain CpG 7909 (27). However, average titers in this study were 1–2 logs higher than those in the previous study, illustrating the potency of the CpG-containing adjuvant (24, 27). Immunoglobulin isotype and IgG subclass analysis analyses showed that IgG was the dominant isotype, with IgG1 and IgG3 being the dominant subclasses (Fig. 2D). High-titer antibody responses to the H. influenzae Protein D component of the vaccine were also seen in all subjects after treatment (Fig. 2E). Antibody responses against a panel of control proteins not included in the vaccine did not change significantly in the majority of subjects, although three subjects demonstrated sustained titers to four nonimmunized, cancer-associated antigens after vaccination, raising the possibility of antigen spreading in a minority of cases (Supplementary Fig. S3).

Cellular immune responses
All subjects demonstrated induction and/or expansion of CD4+ T-cell responses against MAGE-A3, which persisted to at least 1-year post-SCT as assessed by in vitro restimulation assay (Fig. 3A). Seven subjects had no baseline reactivity, but developed MAGE-A3–specific CD4+ T cells after the first (F06, M26, S02), or second vaccination (F02, F10, M08, M27), mirroring what was observed with antibody titers. Six subjects (M15, S10, S13, S25, S33, S36) had baseline CD4+ T-cell responses that were detected by coinoculation with peptide-loaded antigen-presenting cells (APC), either autologous T-cell APC (T-APC) or B-cell EBV blasts (B-EBV). All of these subjects showed significant increases in CD4+ T-cell responses after vaccination plus autologous lymphocyte infusion (Fig. 3B, mean at baseline: 229 spots, mean from d31 onward: 1,155 to 2,295 spots, \(P < 0.001 \text{ at each time point})\). Given the expansion of MAGE-A3–specific CD4+ T cells after in vitro restimulation, we also examined direct ex vivo cellular responses without in vitro restimulation and found that 5 of 13 subjects had detectable ex vivo ELISpot responses against MAGE-A3, typically at later time points after receiving the final 2 booster vaccinations (Fig. 3C). Once detected, CD4+ T-cell responses were consistent over time and were generally of similar magnitude regardless of APC or peptide pool used (20mers vs. shorter peptides, Supplementary Fig. S4). As a control, influenza nucleoprotein (NP)-reactive CD4+ T cells were detected throughout treatment in most subjects and on average did not significantly change over time (Fig. 3B).

Cytokine concentrations were quantified in the supernatants of ELISpot cocultures, demonstrating Ag-specific secretion of GM-CSF, TNFα, MIP-1β, IL-5, and IL-8, whereas IL-2, IL-19, and IL-10 were not detected (Fig. 3D). Intracellular cytokine staining showed that vaccine-elicited, MAGE-A3–specific CD4+ T cells...
produced both IFNγ and TNFα, but not IL4, indicating polyfunctional, Th1-biased immune responses (Fig. 3E).

To further characterize the functional capacity of these vaccine-induced CD4+ T cells, we sorted and enriched MAGE-A3–specific CD4+ cells from PBMCs of 3 subjects obtained at day +194 post-autoSCT, based on CD154 (CD40L) expression following antigen stimulation (Fig. 3F). All of these T-cell populations showed specific IFNγ production upon reexposure to autologous APC pulsed with MAGE-A3 overlapping peptides and full-length protein compared with negative control stimulation (Fig. 3G), and CD4+ cells from subjects S13 and S25 also recognized autologous target cells transduced by viral vectors expressing full-length MAGE-A3. This demonstrates the capacity of the CD4+ cells to recognize endogenously processed and presented epitopes that mimicked the natural state of a tumor expressing intracellular MAGE-A3 and HLA class II. We did not observe direct cytolytic activity by these CD4+ T cells against MAGE-A3–expressing targets.

In contrast to the activation of Ag-specific CD4+ T cells, CD8+ T-cell responses were noted in only three subjects (F06, M27, S25; Fig. 4A), in agreement with results from previous vaccines with full-length recombinant proteins favoring CD4+ over CD8+ T-cell induction (28, 29). These CD8+ cells were capable of recognizing naturally processed MAGE-A3, as demonstrated by IFNγ production against targets transduced by viral vectors expressing full-length MAGE-A3 (Fig. 3C). All three of these subjects had detectable ex vivo CD4+ responses (Fig. 4B). Post-autoSCT CD8+ responses against influenza NP were seen in 10 of 13 subjects, demonstrating that CD8+ function was not globally impaired in most subjects (Fig. 3B). Eight subjects who relapsed had bone marrow or plasma cell biopsy available, in contrast to their original tumor (Fig. 4C and D). 4 (50%) no longer had detectable MAGE-A3+ multiple myeloma cells by IHC (Fig. 4E and F).

MAGE expression has not been reported in longitudinal studies in multiple myeloma, suggesting that the experimental therapy exerted selective pressure against MAGE-A3+ clones. However, there was no correlation between CD8+ T-cell activity and loss of MAGE-A3 expression at progression.

Immunophenotyping of peripheral blood mononuclear cells by time-of-flight mass cytometry (CyTOF) demonstrated a significant increase in effector and central memory CD4+ T cells (CD45RA–, CCR7+; Fig. 5A), together with significantly higher expression of costimulatory molecules 4-1BB (Fig. 5B) and ICOS (Fig. 5C), the activation marker CD38 (Fig. 5D), the immune checkpoint receptors PD-1 (Fig. 5E), but not LAG-3 (Fig. 5F) on CD4+ T cells in the post-autoSCT period. Cells were sustained through the study period, indicating a higher state of activation in the post-ASCT period compared with baseline.

In summary, autologous lymphocyte infusion in combination with autoSCT is a safe and feasible platform for immunotherapy, including therapeutic tumor vaccines, for multiple myeloma. Twelve of 13 subjects completed the entire protocol, with one coming off early for progression. This strategy provided a favorable immune milieu for antigen-specific humoral and CD4+ T-cell immune responses in all subjects. CD8+ immunity was observed in three subjects. Loss of MAGE-A3 expression at progression in 4 of 8 of evaluated subjects indicates immunologic efficacy of this approach. MAGE-A3–specific humoral and cellular responses are summarized in Supplementary Fig. S5.

Discussion

The results reported here provide proof-of-principle that autologous lymphocyte infusion augmentation of autoSCT is a promising component for multiple myeloma combination.
immunotherapy. This strategy is safe, feasible, and did not adversely impact the delivery of standard-of-care autoSCT. Preclinical studies demonstrate that the lymphopenic period after myeloablative chemotherapy and autoSCT is a favorable environment for generating antitumor immunity through vaccination. Several mechanisms have been identified, including (i) tumor antigen release due to cell death; (ii) enhanced dendritic cell activation due to greater availability of Toll-like receptor agonists from translocation of gut flora and other microbiota; (iii) increased availability of T-cell survival factors (e.g., IL7 and IL15); and (iv) elimination of suppressor populations (e.g., MDSCs and Tregs; refs. 30–33). The addition of autologous lymphocyte infusion, in this case in conjunction with therapeutic tumor vaccinations, resulted in robust, humoral, and Th1-biased CD4\(^+\) T-cell immunity in all subjects, and CD8\(^+\) T-cell activity in three subjects. These results support further investigation into combined immunotherapy building upon the platform of autologous lymphocyte infusion-augmented autoSCT, because evidence is accumulating for a critical role for the immune system in controlling this disease. Both adaptive and innate immune responses against autologous multiple myeloma cells were detected in MGUS and smoldering multiple myeloma subjects but were lost with progression to active disease. These responses could be restored \textit{ex vivo} with appropriate stimulation (34, 35).

**Figure 3.** Autologous lymphocyte infusion + MAGE-A3 vaccine elicits Th1-biased CD4\(^+\) T-cell immunity. Immune monitoring of peripheral CD4\(^+\) (A) T-cell responses to MAGE-A3 by ELISPOT after \textit{in vitro} sensitization (IVS), using autologous EBV-B cells pulsed with MAGE-A3 OLP. Spots indicate average counts per 50,000 cells after subtraction of spots against EBV-B target pulsed with irrelevant DMSO target. B, Statistical analyses of median (red bars) and mean (black bars) IFN\(\gamma\) ELISPOT numbers for CD4\(^+\) and CD8\(^+\) T-cell responses to MAGE-A3 or control NP after IVS with respective cognate peptide pool, pooling all results from B-EBV and T-APC targets. Wilcoxon paired t tests indicate significant differences from baseline (\(P < 0.05\); \(P < 0.01\); \(P < 0.001\)). Yellow circles: pre-autoSCT specimens (d-45 screening and d-24 leukopheresis); blue circles, post-autoSCT time points. C, \textit{Ex vivo} immune monitoring of T-cell responses from PBMCs show significant increase from baseline one year after treatment. D, Assessing polyfunctionality of CD4\(^+\) T cells against MAGE-A3 using cytokine secreted from supernatants of ELISPOT cultures shown in A, after subtraction of values against DMSO target. Results indicate significant induction of GM-CSF, TNFa, MIP-1\(\beta\), IL5, and IL8 (Wilcoxon t test). E, Polyfunctionality of CD4\(^+\) T-cell responses to MAGE-A3 by intracellular staining for indicated cytokines was confirmed by flow cytometry, as shown in example on top and by significant IFNy and TNFa presence (paired t test). F, Quality of T-cell responses to MAGE-A3 was assessed by their capacity to recognize naturally processed MAGE-A3 full-length antigen either in the form of recombinant viral vector (vaccinia or adenovirus) encoding MAGE-A3 after APC infection (endogenous processing) or recombinant protein exogenously loaded to APCs. To characterize CD4\(^+\) T-cell quality, MAGE-A3-specific cells were enriched from IVS cultures using CD154-guided cell sorting. G, MAGE-A3-specific CD4\(^+\) T-cell lines obtained after enrichment in three subjects shown were able to recognize MAGE-A3 naturally processed protein, as well as protein from viral vectors in S13 and S25. \(P < 0.05\), Mann-Whitney t-test.
Cellular and humoral immune responses against myeloma-associated antigens were detected in multiple myeloma subjects after undergoing alloSCT, and were correlated with clinical outcome (20). In the same study, 22 subjects who underwent autoSCT were screened for humoral immune responses to CTAs, and only one subject developed an antibody response against MAGE-A3 after transplant, suggesting that autoSCT alone is not sufficient to induce specific immune responses against MAGE-A3 in the majority of patients. The strategy of pre-SCT immunization and lymphocyte collection, early post-SCT autologous lymphocyte infusion, and booster immunizations was designed to leverage the favorable immunologic milieu following myeloablative therapy and the diversity of mature, competent lymphocytes provided by autologous lymphocyte infusion, without incurring the time, resources, and expense of \textit{ex vivo} engineering or manipulation of \textit{T} cells. These strategies may be broadly applicable, as autoSCT is already standard-of-care management in eligible multiple myeloma subjects and the methods and resources described are available at most transplant centers.

The combined vaccine and autologous lymphocyte infusion augmentation strategy was effective in stimulating humoral and Th1-biased CD4\(^+\) T-cell responses to the immunized antigen, with 100\% of subjects responding. These results indicate that the vaccine antigen was efficiently taken up and processed by antigen-presenting cells and presented to CD4\(^+\) T cells on MHC class II, and that the AS15 immunostimulant formulation promoted Th1-biased priming/boosting. This occurred despite the immune compromise exhibited by myeloma subjects in general and especially after autoSCT, indicating the power of this strategy to generate antigen-specific immunity in the autoSCT setting.

Analysis of MAGE-A3 expression in multiple myeloma cells at diagnosis and relapse demonstrated that 4 of 8 subjects did not express MAGE-A3 by IHC in their multiple myeloma cells at the time of progression. MAGE-A3 expression is typically more frequent in relapsed multiple myeloma compared with newly diagnosed subjects (14). In a prior longitudinal analysis, MAGE-A3 expression was sustained over time (18), and there...
are no published examples of MAGE-A3 expression being lost from diagnosis to relapse. These results suggest that our treatment strategy exerted immunologic pressure on multiple myeloma cells, either by eliminating MAGE-A3–expressing clones and/or inducing loss of MAGE-A3 expression as a means of escape despite the absence of broad CD8+ cell activity, reminiscent of clinical reports describing adoptive transfer of tumor Ag-specific CD4+ clones inducing regression of tumors even in the absence of cytocytic activity (36–38). This may occur through both direct killing of targets, or through indirect mechanisms such as cytokine production that recruits macrophages, NK cells, or other effector cells into the microenvironment (19, 37, 39, 40). In addition, downregulation of tumor antigen expression has been described following T-cell therapy targeting CTAg (9, 41). The four subjects who progressed with continued MAGE-A3 expression may have had inadequate CD8+ T-cell responses, downregulation of MHC class I and/or II on tumor cells, inhibitory Treg activity, and/or engagement of inhibitory immune coreceptors such as PD-L1, which is commonly expressed on multiple myeloma cells and in the tumor microenvironment (42, 43). In our study, CD4+ T cells post-SCT demonstrated elevated PD-1 expression, raising the intriguing possibility that addition of an immune checkpoint inhibitor targeting the PD-1/PD-L1 axis may increase the clinical efficacy of this strategy, as has been demonstrated in preclinical and ex vivo studies (44, 45). This study was not powered for assessment of clinical efficacy and we did not observe a trend toward benefit over standard-of-care autoSCT in this treatment group despite the induction of humoral and CD4+ cellular immunity. Several factors may have impacted the clinical outcome. First, there was little induction of T-cell responses in only 3% (46). This may have also contributed to the limited clinical impact of this vaccine formulation in melanoma subjects, where CD4+ T-cell responses were seen in 76% of subjects and CD8+ T-cell responses in only 3% (46). This may have also impacted the clinical outcome. First, there was little induction of T-cell responses in only 3% (46). This may have also contributed to the limited clinical impact of this vaccine formulation in melanoma subjects, where CD4+ T-cell responses were seen in 76% of subjects and CD8+ T-cell responses in only 3% (46). This may have also contributed to the limited clinical impact of this vaccine formulation in melanoma subjects, where CD4+ T-cell responses were seen in 76% of subjects and CD8+ T-cell responses in only 3% (46).
Expression of a cluster of CTAg, including MAGE-A3, was associated with resistance to CTLA-4 blockade in melanoma; the authors speculated that the inhibition of immunogenic cell death mediated by these genes may negatively impact T-cell priming to tumor-associated antigens (53). Similar mechanistic effects have been demonstrated to contribute to poor CD8+ T-cell priming observed in this study. Therefore, a median PFS of 27 months in this MAGE-A3+ cohort, 45% of which had high-risk cytogenetics and most of whom received no maintenance therapy, is consistent with PFS observed in other post-SCT cellular therapy trials in the high-risk multiple myeloma population (6, 8, 9).

In conclusion, we have demonstrated that autologous lymphocyte infusion and therapeutic tumor vaccine augmentation of autoSCT is safe, feasible, and stimulates tumor Ag-specific B and Th1-biased CD4+ T-cell immunity. Clinical outcome and modest CD8+ T-cell activity suggests that the vaccine formulation used here was not sufficient to confer long-term disease control. However, the immunologic efficacy of this strategy supports further investigation of other combinations of immunotherapy built upon this platform. Future clinical trials may test vaccines that incorporate a diversity of myeloma-associated antigens utilizing synthetic long peptides to better promote CD8+ T-cell priming (54), along with potent immunostimulants such as AS15 to ensure integrated CD4+ and B-cell immunity. Another promising approach under investigation uses individualized neoantigen vaccines composed of peptides containing nonsynonymous, immunogenic epitopes derived from whole-exome sequencing of tumor specimens (55). Immunomodulatory drugs (ImiD), such as lenalidomide, are approved for multiple myeloma and have a plethora of favorable immunologic activities, including T, B, and NK-cell activation, dendritic cell maturation, and promotion of antigen presentation (56). Immune checkpoint inhibitors targeting the B7/CTLA-4 and PD-1/PD-L1 axes are approved for solid tumor indications and are under investigation in multiple myeloma. The combination of these strategies with vaccine and autologous lymphocyte infusion augmentation of autoSCT in rational sequence and composition may realize the goal of “host versus myeloma” immunity that recapitulates the alloSCT experience in a therapy that is feasible for the majority of patients with multiple myeloma. These strategies may also be applicable to other hematologic malignancies where autoSCT is a common salvage therapy, such as Hodgkin and non-Hodgkin lymphomas.

Disclosure of Potential Conflicts of Interest
A.D. Cohen reports receiving commercial research funding from Bristol-Myers Squibb and Novartis; is a consultant/advisory board member for GlaxoSmithKline, Bristol-Myers Squibb, Celgene, Takeda, Kite Pharma, Oncopeptides. Jansen, Seattle Genetics, and Aray Biopharma; and has received additional remuneration from Janssen. N. Lendvai reports receiving commercial research funding from GlaxoSmithKline, Takeda, Karyopharm, Sanofi, Amgen, Pharmacycics, and Aray Biopharma, and is a consultant/advisory board member for Karyopharm and Amgen. S. Gnjatic reports receiving commercial research funding from Immune Design, Pfizer, Janssen R&D, and Bristol-Myers Squibb; has ownership interest in OncoMed and NY-ESO-1 peptides; and is a consultant/advisory board for Merck, OncoMed, Neon Therapeutics, and B4CC. H.J. Cho reports receiving commercial research funding from Agensus Inc. and Genentech Roche, and is a consultant/advisory board member for Genentech Roche, Celgene, Bristol-Myers Squibb, and GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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References


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