Vaccination with DC/tumor fusion cells results in cellular and humoral anti-tumor immune responses in patients with multiple myeloma

DC/myeloma fusion vaccine induces immune responses

Jacalyn Rosenblatt, MD¹
Baldev Vasir, PhD²
Lynne Uhl, MD¹
Simona Blotta, MD²,³
Claire MacNamara¹
Poorvi Somaiya¹
Zekui Wu, MD²
Robin Joyce, MD¹
James D. Levine, MD¹
Dilani Dombagoda¹
Yan Emily Yuan¹
Karen Francoeur, RN¹
Donna Fitzgerald, PA¹
Paul Richardson, MD²
Edie Weller, PhD²
Kenneth Anderson, MD²
Donald Kufe, MD²
Nikhil Munshi, MD²
David Avigan, MD¹

¹Beth Israel Deaconess Medical Center       ² Dana-Farber Cancer Institute
³University of Magna Græcia and Cancer Center, Catanzaro, Italy
*Jacalyn Rosenblatt, MD and Baldev Vasir, PhD contributed equally

Corresponding Author:
Jacalyn Rosenblatt, MD
Beth Israel Deaconess Medical Center
330 Brookline Avenue, KS-121
Boston, MA 02215
Phone: (617) 667-9920
Fax: (617) 667-9922
Email: jrosenbl1@bidmc.harvard.edu
Abstract:
We have developed a tumor vaccine in which patient derived myeloma cells are chemically fused with autologous dendritic cells (DCs) such that a broad spectrum of myeloma associated antigens are presented in the context of DC mediated costimulation. We have completed a phase I study in which patients with multiple myeloma (MM) underwent serial vaccination with the DC/MM fusions in conjunction with GM-CSF. DCs were generated from adherent mononuclear cells cultured with GM-CSF, IL-4 and TNFα and fused with myeloma cells obtained from marrow aspirates. Vaccine generation was successful in 17/18 patients. Successive cohorts were treated with 1x10^6, 2x10^6, and 4x10^6 fusion cells, respectively, with 10 patients treated at the highest dose level. Vaccination was well tolerated without evidence of dose limiting toxicity. Vaccination resulted in the expansion of circulating CD4 and CD8 lymphocytes reactive with autologous myeloma cells in 11/15 evaluable patients. Humoral responses were documented by SEREX analysis. A majority of patients with advanced disease demonstrated disease stabilization with 3 patients showing ongoing stable disease at 12, 25, and 41 months, respectively. Vaccination with DC/MM fusions was feasible, well tolerated and resulted in anti-tumor immune responses and disease stabilization in a majority of patients.

Introduction:
While the discovery of novel biological agents has improved therapeutic options for patients with multiple myeloma, curative outcomes remain elusive due to the eventual emergence of resistant disease. Similarly, several studies have demonstrated that autologous stem cell transplantation results in improved disease survival as compared to standard chemotherapy but ultimately does not prevent disease progression\(^1\). In contrast, the unique efficacy of cellular immunotherapy is supported by the observation that allogeneic hematopoietic stem cell transplantation is curative for a subset of patients due to the graft versus disease effect mediated by alloreactive lymphocytes\(^2,3\). However, allogeneic transplantation is associated with significant morbidity and mortality secondary to regimen related toxicity and the lack of specificity of the allo-reactive response, which results in the development of graft versus host disease (GVHD). A major area of investigation is to develop immunotherapeutic strategies to elicit myeloma specific immune responses that will selectively eliminate malignant cells and eradicate residual disease persisting following biologic therapy and autologous stem cell transplantation. A variety of tumor associated antigens have been identified in myeloma cells that may be selectively targeted by host immunity, including the clonal idiotype and the epithelial mucin, MUC1\(^4,6\). However, tumor cells evade immune recognition by presenting antigens in the absence of costimulatory molecules, secreting factors that inhibit antigen presenting and effector cells, and the increased presence of regulatory T cells that inhibit anti-tumor immune responses.

Dendritic Cell (DC)-based tumor vaccines are being explored as a promising strategy to stimulate immune responses that recognize and selectively eliminate malignant cells. DCs represent a complex network of cells characterized by the expression of costimulatory and adhesion molecules necessary to initiate primary immune responses\(^7,8\). DCs in patients with cancer have quantitative and functional deficiencies, which
contribute to tumor associated immune tolerance. In contrast, functionally active DC can be generated ex-vivo by culture of peripheral blood mononuclear cells in the presence of cytokines. DCs loaded with specific tumor antigens by pulsing with peptide or proteins or by the insertion of tumor specific genes elicit antigen specific anti-tumor responses in preclinical experiments, animal models and clinical studies. Single antigen approaches are limited, however, by the small number of known tumor-specific antigens, their variable immunogenicity, and the potential ability of tumor cells to alter expression of individual antigens in order to evade immune recognition. Alternatively, DCs may be loaded with antigens derived from whole tumor cells that potentially stimulate a broader anti-tumor response.

We have developed a promising DC-based cancer vaccine involving the fusion of tumor cells with autologous DCs using polyethylene glycol (PEG). DC/tumor fusions present a broad array of antigens in the context of the potent antigen presenting machinery of the DC fusion partner. DC/tumor fusions uniquely stimulate both helper and cytotoxic T cell responses through the presentation of internalized and newly synthesized antigens, respectively. In diverse animal tumor models including multiple myeloma, vaccination with DC/tumor fusions is protective from an otherwise lethal challenge of tumor cells and, most significantly, results in the eradication of disease in tumor bearing animals. In a MUC1 transgenic mouse model, fusion cells effectively induced anti-tumor immunity against a MUC1 expressing malignancy without the development of autoimmunity. In pre-clinical human studies, fusion cells stimulate autologous antigen specific CD4 and CD8+ responses and effectively stimulate CTL responses that target patient-derived tumor cells. In a hematological malignancy model, fusion cells were more effective in stimulating anti-tumor immunity than DCs pulsed with apoptotic bodies or tumor lysate. In initial clinical studies of patients with solid malignancies, vaccination with DC/tumor fusions was not associated with significant toxicity, stimulated anti-tumor immunity in a majority of patients and induced clinical responses in a subset of patients. A correlation between immunologic and clinical response was observed.

We now report on a phase I study in which patients with multiple myeloma underwent vaccination with autologous DC/myeloma fusion cells. Successive cohorts of patients were treated with escalating doses of fusion cells to define treatment associated toxicity and the maximum tolerated/achievable dose. We demonstrated that vaccination with up to $4 \times 10^6$ DC/myeloma fusion cells was feasible, and well tolerated, without the induction of auto-immunity. Cellular immune responses against autologous myeloma cells and the MUC1 tumor antigen were induced, and humoral immune responses against novel antigens were detected. Disease stabilization was observed in a majority of patients with advanced disease.

**Methods:**

**Patient Characteristics**

Patients eligible for the study included patients with active disease who had received at least 1 prior treatment regimen. In addition, patients with stage 1 myeloma who did not require therapy and were otherwise being observed were eligible. Patients must exhibit at
least 20% involvement of the bone marrow with myeloma cells to facilitate vaccine generation. Patients must not have been treated with chemotherapy, steroids, radiation therapy, or immunotherapy within 4 weeks of study enrollment. Patients with a history of clinically significant autoimmune disease or organ dysfunction as measured by a bilirubin > 2.0 or creatinine > 2.0 were excluded. All patient protocols were approved by the Beth Israel Deaconess Medical Center institutional review board.

**Reagents for Vaccine Characterization and Immunologic Assays**

Purified mouse anti-human monoclonal antibodies (mAbs) against HLA-DR, CD80, CD86, CD40, CD83, CD38, and CD138 were purchased from BD PharMingen (San Diego, CA). Phycoerythrin (PE)-conjugated mouse anti-human mAbs against CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (RPA-T4, IgG1), CD8 (RPA-T8, IgG1) and FITC-, PE-conjugated matching isotype controls IgG1, IgG2a, IgG2b and purified mouse monoclonal IgG1 (MOPC-21) isotype control were also purchased from BD PharMingen. Monoclonal antibody DF3 (anti-MUC1 N-ter) has been described previously. Anti-human CD4 TC-conjugated and matching isotype control (IgG2a) were purchased from Invitrogen. For intracellular cytokine staining, PE-conjugated anti-human mAbs for IFN-γ (mouse IgG1-B27) and the respective PE-conjugated matching isotype controls (rat IgG1-PE and mouse IgG1-PE) were purchased from Invitrogen (Carlsbad, CA). FITC-conjugated goat anti-mouse (IgG1) was purchased from Chemicon International (Temecula, CA).

**Vaccine Generation**

DC/tumor fusions were generated as previously described. Patients underwent aspiration of 20-30 cc of bone marrow, from which mononuclear cells were isolated by ficoll density gradient centrifugation. Autologous plasma was obtained from the leukapheresis product or by harvesting the supernatant following ficoll centrifugation of 50 ml of peripheral blood. Bone marrow mononuclear cells were cultured in RPMI 1640 culture media containing 2 mmol/l l-glutamine (Lonza, Walkersville, MD) and supplemented with heat-inactivated 10% autologous serum, 10 μg/ml of gentamicin (Baxter). In some cases, myeloma cells were cryopreserved in 10% DMSO/90% autologous plasma, and later thawed at the time of fusion generation. Myeloma preparations were characterized by FACS analysis to document expression of tumor associated markers CD38, CD138, and/or MUC1 and the absence of DC associated markers.

Patient-derived DCs were generated from adherent mononuclear cells isolated from a leukapheresis collection. PBMC collections underwent plastic adherence for 1-2 hours in the presence of 1% autologous plasma and nonadherent cells were removed. Adherent cells were cultured with GM-CSF 1000U/mL (Berlex, Wayne/Montville, NJ) and IL-4 500 IU/mL (Cellgenix USA, Antioch, IL) for 5-7 days. Cells were cultured with 2 mmol/l l-glutamine 1% autologous serum and 10 μg/ml of gentamicin. DC then underwent maturation by exposure to TNFα 25 ng/ml (Cellgenix USA, Antioch, IL) for 2-3 days. DC undergo phenotypic analysis with immunohistochemistry and/or flow cytometry analysis to assess expression of MHC class II, costimulatory (CD86, CD40 and/or
CD80), and maturation molecules (CD83) and confirm absence of expression of appropriate tumor associated markers (MUC-1, CD38, and/or CD138).

DC/tumor fusions are generated by mixing tumor cells and dendritic cells at a 1:3-1:10 ratio (dependent on cell yields). The cells were then washed extensively and the cell pellet was re-suspended in a 50% solution of polyethylene glycol (PEG) in phosphate buffered saline (PBS). After a short incubation, the PEG was slowly diluted by the addition of media and after several washing steps the cells are placed in media containing 10% autologous plasma and GM-CSF 500 U/ml, and incubated at 37°C. DC/tumor fusions were quantified by determining the percentage of cells that co-express unique DC and tumor associated antigens by immunohistochemical analysis. A fusion efficiency of at least 10% based on immunohistochemical staining was required as release criteria for vaccine administration. An aliquot of the DC, tumor cells, and fusion cell preparations were sent for microbiological assessment. The fused cells were frozen in autologous plasma (90%) and DMSO (10%) and placed in single use vials. Fusion cell doses were stored frozen in the vapor phase of liquid nitrogen for subsequent use. Prior to patient administration, the sterility of the product was confirmed, including mycoplasma, endotoxin, and sterility assays. At time of vaccine administration, the fused cells were thawed, and viability assay and gram stain were performed. The product was irradiated 30cGy, drawn up into a syringe and delivered to the clinical site.

**Functional Assessment of the DC, MM, and DC/MM Fusion Preparations**

As a measure of immunologic potency, the capacity of the fusion cell preparation to stimulate allogeneic T cell proliferation was assessed and compared to that observed with the unfused DC and myeloma preparations. PBMC were obtained from leukopak preparations undergoing ficoll density centrifugation. Nonadherent PBMC were isolated from a leukopak collection obtained from a normal donor not MHC matched with the patient, and T cells were further enriched by passage through a CD3 column or nylon wool. T cells (1x10^5) were co-cultured for 5 d with MM, DC, and fusion preparations at a ratio of 10:1. T cell proliferation was determined by measuring incorporation of [^3H]thymidine following overnight pulsing (1μCi/well) of triplicate samples.

**Vaccine Administration**

Successive cohorts of at least 3 patients were treated with 1x10^6, 2x10^6, and 4x10^6 fusion cells administered as a subcutaneous injection in the upper thigh at 3 week intervals for a total of 3 doses. The cohort was to be expanded to 6 patients if dose limiting toxicity (DLT) was encountered as defined by vaccine related grade III-IV toxicity. A total of 10 patients were treated at the highest dose level. GM-CSF (100 μg) was administered at the vaccine site on the day of vaccination and for 3 days thereafter. Patients for whom the targeted dose of fusion cells was not achieved were treated at a lower dose level. Patients were seen weekly during the period of vaccination and then monthly for 6 months after the completion of vaccination. Patients underwent serial assessment for evidence of toxicity and auto-immunity by physical exam and laboratory evaluation, including ANA and ESR.

**Vaccine Induction of Tumor Reactive Lymphocytes**

To measure the immunologic response to vaccination, we determined the percentage of
circulating CD4+ and CD8+ T cells that recognize autologous myeloma cells as manifested by the percentage of cells that express IFNγ following ex vivo exposure to autologous tumor lysate. Immunologic assessments were performed prior to each vaccination, and at 1, 3, and 6 months following the last vaccine. The peak response post-vaccination was compared to pre-vaccination levels of tumor reactive T cells, to assess the fold increase in tumor reactive T cells following vaccination. At each time point, mononuclear cells were isolated from peripheral blood by ficoll density centrifugation and cryopreserved. Following completion of the study, PBMC were thawed and 1 x 10^6 cells were cultured with lysate generated by repeated freeze thaw cycles of 1 x 10^5 autologous myeloma cells. As a control, PBMC were cultured with tetanus toxoid (10 μg/ml) or media alone. Following 5 days of coculture, expression of IFNγ by CD4+ and CD8+ populations was determined by intracellular FACS analysis. Cells were restimulated with tumor lysate for 6 h and cultured overnight with 1 μg/ml GolgiStop. The cells were stained with CD4 or CD8 antibodies conjugated to FITC and permeabilized with Cytofix/Cytoperm plusTM. Cells were also stained with PE-conjugated anti-human IFNγ, fixed in 2% paraformaldehyde and analyzed by flow cytometry.

**Vaccine induction of MUC1 Antigen Specific Responses**
In HLA-A2.1 patients, PBMCs were isolated prior to each vaccination and at 1, 3, and 6 months after completion of vaccination as outlined above. The number of CD8+ T cells binding the MUC1 tetramer was determined by bidimensional FACS analysis using CD8-FITC and MUC1 tetramer-PE antibody.

**PHA and Tetanus Induced T Cell Proliferation**
PBMCs were isolated at serial time points (as outlined above) and were cocultured with the PHA mitogen and tetanus toxoid (10 μg/ml) for 3 and 5 days, respectively. T cell proliferation was measured by determining uptake of [3H]thymidine following overnight pulsing (1μCi/well) of triplicate samples.

**Vaccine Site Reactions**
Vaccine site reactions were biopsied in a subset of patients and the presence of CD4 and CD8 infiltrating cells was determined by immunocytochemical analysis. In addition, recruitment of native DCs to the vaccine site was assessed by immunocytochemical staining of the cell infiltrate for CD1a in the vaccine bed.

**SEREX Assessment**
Serological analysis of recombinant cDNA Expression library (SEREX) was performed to assess humoral response to vaccination and to identify targeted novel antigens. The cDNA library was constructed as previously described from total RNA isolated from bone marrow CD138+ cells from a patient with myeloma. The cDNA library was inserted into a recombinant phage vector, transfected into E. Coli, and plated on agar at 5 x 10^4 plaques per 150-mm Petri dish. Expression of recombinant proteins was induced by incubation with IPTG-treated nitrocellulose membranes for 3.5 hours at 37°C. Filters were subsequently washed in TBST to remove excess agar and blocked overnight with 1% w/v nonfat dry milk in TBS. The filters were incubated overnight with pre and post
vaccination patient serum diluted at 1:500 in TBST. The sera were absorbed against phage lysate and the E coli strain to minimize nonspecific antibody binding. Specific binding of antibody to recombinant proteins expressed on the lytic plaques was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG antibody diluted at 1:2000 in TBST. Visualization of the antigen-antibody complexes was accomplished by staining with S-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT). cDNA inserts from clones positive following vaccination were isolated by excision of phagemids and then sequenced with T3 and T7 primers (Dana-Farber Cancer Institute Molecular Biology Core Facility).

Quantification of Regulatory T Cells
As outlined above, PBMCs were isolated at the serial time points and regulatory T cells were quantified by determining the percentage of CD4/CD25^{high} T cells using bidimensional FACS analysis. Expression of FOXP3 by CD4/CD25 cells was measured in selected patients using intracellular FACS analysis.

Clinical Disease Assessment
Patients were required to have measurable disease as manifested by an increased M component in the serum and/or urine or an increase in the serum free light chains. Serial measurements of the M protein were obtained prior to each vaccination and at 1, 3, and 6 months post-vaccination. Bone marrow aspiration and biopsy were performed prior to vaccination and at 1, 3, and 6 months post-vaccination. Skeletal survey and other appropriate radiological evaluations were performed prior to vaccination and at 3 and 6 months post-vaccination.

Results:
Patient Characteristics
Eighteen subjects were enrolled after meeting eligibility criteria. Seventeen subjects have undergone therapy following successful vaccine generation (Table 1). 1 patient was removed from the study due to inadequate cell yields for vaccine generation. Twelve patients were male and 6 female. The mean age was 57 years old. Patients had received a mean of 4 prior treatment regimens and 14 patients had previously undergone high dose chemotherapy with autologous stem cell transplantation. 2 patients had stage 1 disease and had not received prior therapy. Successive cohorts consisting of a minimum of 3 patients were treated with escalating doses of fusion cells. Three, 4, and 10 patients were vaccinated with 1x10^6, 2x10^6, and 4x10^6 fusion cells, respectively. One patient assigned to the 4x10^6 dose level received 2x10^6 fusion cells due to cell yields. One patient received only a single dose of the vaccine due to the development of an unrelated cardiac event for which the patient was taken off study. No dose limiting toxicities were observed and intended dose escalation was completed.

Vaccine Generation
DCs were generated from adherent mononuclear cells obtained from a single leukapheresis collection. The mean yield of mononuclear cells collected was 9.8 x 10^9. DCs were generated from adherent mononuclear cells cultured for 1 week with GM-CSF and IL-4 and then matured for 48 hours with TNFα. The mean yield of DCs was
1.23x10^8 cells with a mean viability of 88%. DCs uniformly expressed HLA-DR, CD11c, CD86, and CD83 consistent with a mature phenotype (Figure 1A). Myeloma specific markers including CD38 and CD138 were absent. Myeloma cells were derived from a single bone marrow aspirate of 20-25 cc and demonstrated a mean yield and viability of 5.7x10^6 cells and 92%, respectively. Myeloma cells uniformly expressed CD38 and/or CD138 (Figure 1B). In contrast, myeloma cells did not express the costimulatory marker CD86 or DC maturation marker, CD83. Fusion cells were quantified by measuring the percentage of cells that co-expressed unique DC (CD86) and myeloma (CD38 or CD138) markers (Figure 1C). The mean fusion percentage was 39% (range 18%-52%) with a mean viability of 84% (range 69%-96%). As a measure of their potency as antigen presenting cells, the capacity of the DC, myeloma, and fusion cells to stimulate proliferation of allogeneic T cells was measured. Mean stimulation indices were 9 (SEM 3), 43 (SEM 7), and 29 (SEM 5) for the myeloma, DC, and fusion cell preparations (Figure 2).

**Toxicity**

Vaccination was well tolerated without significant evidence of autoimmunity (Table 2). All vaccine associated events were of mild to moderate intensity (grade I/II) and of transient duration. The most common event was injection site reactions consisting of erythema and/or pain lasting a few days. Other manifestations of vaccine-induced toxicity included transient fever, myalgias, pruritis and rash. One patient with a history of a prior deep vein thrombosis (DVT) developed a DVT and pulmonary embolus while on study. This event was unlikely to be related to vaccination, but given that GM-CSF may be associated with an increased risk of thrombosis, patients with a history of clinically significant thromboembolic disease (unrelated to myeloma therapy) were subsequently excluded from the trial.

**Vaccine Site Reactions**

13 patients exhibited injection site reactions following vaccination. Biopsy of these areas demonstrated a dense mononuclear cell infiltrate (Figure 3A). A majority of cells expressed CD8 (Figure 3B, left), suggesting recruitment and education by the DC/MM fusions at the site of vaccination. In addition, presence of CD1a cells was observed in the vaccine bed (Figure 3B, right) suggesting that native Langerhans cells recruited by the presence of GM-CSF may participate in the vaccine response.

**Cellular Immunologic Response to Vaccination**

We assessed the impact of vaccination on circulating tumor reactive lymphocytes by determining the percentage of CD4+ and CD8+ T cells that express IFNγ after ex vivo culture with autologous PBMCs pulsed with autologous tumor lysate (Figure 4A). Of 15 evaluable patients, 11 patients demonstrated at least a 2 fold increase in the percentage of CD4 and/or CD8+ tumor reactive T cells (Figures 4B, 4C). Mean baseline and peak post-vaccine levels of tumor reactive CD8+ T cells were 0.6% and 2.4%, respectively (p=0.01). A rise of mean CD4+ tumor reactive T cells was also observed with baseline levels of 1.5% and peak levels of 3.2% (p=0.02). Among responders mean baseline and peak CD8+ tumor reactive T cells were 0.68 and 7.14 (p=0.01), respectively (p=0.04). Mean baseline and peak CD4+ levels were 1.2 and 6.5, respectively (p=0.04).
To assess the impact of vaccination on immunologic response directed against MUC1, tetramer analysis was performed on samples derived from 5 patients that were HLA-A2.1 positive. All patients demonstrated at least a 2 fold increase in tetramer+ cells after vaccination with mean levels of 0.98 and 4.0 at baseline and peak post-vaccination, respectively (Figure 5). To determine if vaccination resulted in nonspecific immune activation and T cell expansion, we quantified T cell response to mitogenic stimulation with PHA and to the tetanus toxoid recall antigen. Response to PHA did not change during the period of vaccination (mean SI 47 and 46 prior to vaccination and 1 month following completion of vaccination respectively). Similarly, no increase in tetanus specific responses was observed following vaccination. In fact, a decrease in tetanus induced T cell proliferation was observed, with a mean SI that decreased from 4.3 prior to vaccination, to 1.7 at 1 month following completion of vaccinations (Figure 6). These data suggest that vaccination induced anti-tumor immunity through the selective expansion of tumor specific T cells rather than non-specific T cell stimulation.

**Impact of Vaccination on Circulating Regulatory T cells**
Regulatory T cells play a critical role in supporting tumor mediated immune suppression. Consequently, we determined the levels of circulating regulatory T cells prior to and following vaccination by quantifying CD4/CD25\textsuperscript{high} cells by bidimensional FACS analysis. No significant change in levels of CD4/CD25\textsuperscript{high} cells was observed with these cells representing 0.5, 0.6, 0.4, and 0.4% of the mononuclear cell population prior to vaccine 1, vaccine 2, vaccine 3, and 1 month following the last vaccination, respectively.

**Humoral response to Vaccination**
SEREX analysis was performed to assess humoral response in patients undergoing vaccination with DC/MM fusions and identify potential novel myeloma targets. A cDNA expression library was constructed from CD138+ myeloma cells obtained from a patient with an IgG paraprotein. The library was screened with pre and post- vaccination serum from 12 patients. Library screening identified 55 clones. After restriction enzyme digestion and DNA sequencing, 10 of the 55 clones were found to correspond to 3 different gene products that have been previously described (Table 3). Humoral responses directed against *Regulators of G protein Signaling 19* (RGS19) was detected in 2/12 patients post-vaccination (Figure 7). The antibody response remained strongly positive at 3 months post-vaccination in both patients. Antibody response against \(\beta\) subunit of Heat Shock Protein 90 (HSP90\(\beta\)) was detected one month post-vaccination in 2/12 patients. Antibodies against these targets were not present prior to vaccination. Antibody response against *BRCA1-associated protein* (BRAP) was detected in pre- and post vaccination serum of 1/12 patients. Importantly no antibody response against any of these antigens has been identified in 5 age-matched healthy persons (Table 3).

**Clinical Response**
Disease response was assessed by serial measurement of the serum and urine paraprotein levels and quantification of the level of bone marrow involvement. Of 16 evaluable patients, 11 patients demonstrated stable disease following vaccination. Three patients have ongoing stable disease following vaccination without evidence of progression at 12,
25, and 41 months. An additional 8 patients exhibited disease stabilization for 2.5 (4 patients) 3, 4, 4.5, and 5 months following vaccination.

**Discussion:**
Multiple myeloma demonstrates resistance to standard biological and chemotherapy but may be sensitive to cellular immunotherapy. Allogeneic transplantation results in targeting of myeloma cells by alloreactive lymphocytes and is associated with durable remissions in a subset of patients. However, the lack of tumor specificity results in significant morbidity and mortality. Investigators have pursued tumor vaccine models in an effort to elicit myeloma specific immunity. Tumor associated antigens that are selectively expressed on myeloma cells include MUC1 and the idiotype protein. Vaccination with idiotype protein in conjunction with GM-CSF has been associated with cellular and humoral immune responses with uncertain clinical effects. Expression of cancer testis antigens such as NY-ESO has been shown to correlate with disease progression and antigen specific CTL responses are induced following repetitive stimulation in vitro with peptide pulsed DCs. Humoral responses directed against NY-ESO have been documented following allogeneic transplantation suggesting that targeting of this antigen may be associated with the graft versus disease response.

In an effort to stimulate a broader anti-tumor immunologic response, investigators have explored the use of whole tumor cells as a source of multiple antigens for vaccination. Patient derived DCs loaded with autologous tumor lysate induced anti-tumor immunity following repetitive stimulation in vitro. In another approach, DCs were loaded with heat shock protein as a chaperone molecule containing cell derived proteins. DCs loaded with autologous or pooled allogeneic HSP 96 elicited anti-myeloma responses in vitro and in vivo models.

We have examined a whole cell based vaccine approach in which patient derived myeloma cells are fused with autologous DCs. In the present study, we examined the feasibility, toxicity, immunologic effects and clinical response of fusion cell vaccination in patients with multiple myeloma. For a majority of patients, vaccine production was accomplished using a single leukapheresis collection for DC generation and an aspirate of bone marrow as a source of autologous myeloma cells. The DC/MM fusions expressed the DC derived costimulatory and maturation markers as well as the tumor associated antigens, CD38, CD138, and/or CD138. In contrast to myeloma cells, fusion cells potently stimulated allogeneic T cell proliferation consistent with their role as potent antigen presenting cells.

In this phase I trial, a majority of the patients had advanced disease and had been treated with a mean of 4 prior regimens. Vaccination was well tolerated without significant toxicity or evidence of autoimmunity. One patient developed a pulmonary embolus which was thought to be unrelated to the vaccine given a prior history of DVTs. Dose escalation to the target of 4 x 10^6 fusion cells was found to be both feasible and safe. The primary toxicity was transient vaccine site reactions, consistent with recruitment of CD8+ T cells into the vaccine bed. This observation suggests that vaccine mediated education of T cells did not necessarily require migration to the site of the draining lymph node.
note, increased levels of Langerhans cells were also observed consistent with their potential contribution in modulating the immune response.

Immunologic responses were measured by determining the effect of vaccination on the levels of circulating tumor reactive lymphocytes as measured by the percentage of circulating T cells expressing IFNγ in response to autologous tumor lysate. Vaccination resulted in at least a 2-fold increase in the percentage of tumor reactive CD4 and/or CD8 T cells in 11 of 15 evaluable patients. Autologous tumor lysate was generated from marrow derived malignant plasma cells. It is possible that an element of the immunologic response was directed against antigens found in normal plasma cells or other marrow elements. As a control to further define the tumor specificity of the immunologic response, it would have been optimal to measure T cell response to antigens derived from isolated normal hematopoietic elements such as dendritic cells or B cells. However, due to limitations with cell yields, these studies were not performed. Of note, vaccination did not result in signs of autoimmunity or suppression of blood counts. Notable, a rise in T cells specific to the MUC1 tumor antigen was detected following vaccination, demonstrating the generation of an immune response to a tumor associated antigen.

Humoral responses were detected using SEREX analysis targeting the product of the RGS19 and HSP90 genes. RGS proteins are a family of proteins involved in the inhibition of G protein-coupled receptor signaling. Following in vivo activation by antigen, B cells rapidly regulate the expression of RGS molecules suggesting that Ag-mediated changes of RGS proteins are important for B cell activation, tolerance, and migration within lymphoid tissues. RGS19 has been shown to play a role in lymphoproliferative disease; however, it has not been well described in multiple myeloma. HSP90, also known as gp96, functions as a molecular chaperone, coupling with a variety of antigenic peptides. Surface expression of gp96 on tumor cells has been shown to result in DC activation and anti-tumor immunity. Moreover, DC pulsed with myeloma-derived gp96 induce myeloma specific CTLs able to lyse myeloma cells. In one patient, an antibody response against BRAP was identified in both pre and post-vaccination serum. Interestingly, antibody responses to BRAP have been previously identified by SEREX in patients with a variety of malignancies (http://www2.licr.org/CancerImmunomeDB).

While the majority of patients in this study had been treated with multiple prior regimens, most exhibited stabilization of disease, with several patients ongoing between 12 and 41 months following vaccination. Several factors likely modified the clinical effects of the vaccine in patients who demonstrated immunologic response. Tumor-mediated immune suppression remains a major challenge for cellular immunotherapy, particularly in those patients with bulky disease. Of note, a majority of patients in this trial exhibited a dampening of the immunologic response by 6 months post-vaccination, suggesting the downmodulation of anti-tumor immunity. Vaccination of patients with a lower disease state, such as following cytoreduction with biological therapy or transplant, will likely increase vaccine response by limiting tumor-mediated immune suppression and may promote longer term responses. In addition, boosting vaccinations at later time points
may result in more durable responses. Heterogeneity of patients in the study may limit interpretation of immunologic and clinical responses. However, all but two patients had advanced and heavily pre-treated myeloma. This study lays the foundation for subsequent studies that evaluate the use of the fusion vaccine in a more homogeneous group of patients who undergo vaccination following cytoreduction in the early post-autologous transplant setting.

Increased levels of regulatory T cells have been observed in patients with malignancy and are associated with worse outcomes. Regulatory T cells inhibit primary T cell activation and are paradoxically expanded by tumor vaccines. In pre-clinical models, we have demonstrated that DC/tumor fusions stimulate the expansion of both activated and suppressor cell populations. In animal models and a clinical trial, depletion of regulatory T cells enhanced vaccine response and tumor eradication\(^{46,47}\). In the present study, mean levels of regulatory T cells remained stable over the period of vaccination.

Recent phase III studies of tumor vaccines have demonstrated a survival benefit in patients with advanced prostate and renal carcinoma without clear evidence of disease regression\(^{48}\). Of note, in a recently reported study, 27 patients with multiple myeloma undergoing vaccination post-autologous transplant with antigen presenting cells pulsed with autologous plasma as a source of idioype protein (Mylolvenge), demonstrated improved survival as compared to 124 sequential patients who had undergone transplant alone\(^{49}\). Modulation of anti-tumor immunity may play a role in determining the pattern of tumor growth and interaction with the host in a manner distinct from that observed with traditional chemotherapeutic agents. In this regard, studies to examine the impact of immunologic response and long term survival are needed for patients with multiple myeloma. Areas of further investigation include vaccination in conjunction with regulatory T cell depletion in the post-autologous transplant setting, and modulation of inhibitory pathways, including CTLA-4 and the PD-1/PDL-1 pathway, as a means of augmenting vaccine response.

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**Authorship Contributions:**
Dr. Jacalyn Rosenblatt was involved in research design, patient accrual and assessments, analyzing data, and manuscript preparation. Dr. Baldev Vasir was involved in research design, running experiments, data analysis, and manuscript preparation. Dr. Lynne Uhl supervised vaccine preparation. Dr. Simona Blotta performed SEREX analysis. Claire MacNamara and Poorvi Somaiya were responsible for vaccine generation. Dr. Zekui Wu was responsible for vaccine characterization. Drs. Robin Joyce, James D. Levine, Nikhil Munshi and Paul Richardson were involved in patient accrual and assessments. Dilani Dombagoda and Yan Emily Yuan were involved in data collection. Karen Francoeur and Donna Fitzgerald were involved in patient assessments. Dr. Kenneth Anderson was involved in patient accrual and study design. Dr. Edie Weller was involved in data analysis. Dr. Donald Kufe was involved in study design, data analysis and manuscript...
preparation. Dr. David Avigan is the principal investigator of the clinical trial, and was involved in study design, data analysis, and manuscript preparation.

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in multiple myeloma: generation of myeloma-specific CTLs using dendritic cells pulsed


Tables:

Table 1: Patient Characteristics

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<tr>
<th>Characteristics</th>
<th>Values</th>
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<tr>
<td>Male, female</td>
<td>12, 6</td>
</tr>
<tr>
<td>Median age years (range)</td>
<td>57 years (29-77 years)</td>
</tr>
<tr>
<td>Median # prior regimens (range)</td>
<td>4 (0-6)</td>
</tr>
<tr>
<td>0 prior regimens</td>
<td>2 patients</td>
</tr>
<tr>
<td>3 prior regimens</td>
<td>4 patients</td>
</tr>
<tr>
<td>&gt; 4 prior regimens</td>
<td>12 patients</td>
</tr>
<tr>
<td>Prior autologous transplantation</td>
<td>14</td>
</tr>
<tr>
<td>Dose level</td>
<td></td>
</tr>
<tr>
<td>1x10^6</td>
<td>3 patients</td>
</tr>
<tr>
<td>2x10^6</td>
<td>4 patients</td>
</tr>
<tr>
<td>4x10^6</td>
<td>10 patients</td>
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Table 2: Related Adverse events

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<tr>
<th>Adverse event</th>
<th>Number of episodes</th>
<th>Grade</th>
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<tbody>
<tr>
<td>Vaccine site reaction (erythema, itching)</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Edema</td>
<td>3</td>
<td>1</td>
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<tr>
<td>Myalgia</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Arthralgia</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Pruritis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>Diarrhea</td>
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<td>Stiffness</td>
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<td>Fatigue</td>
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<td>Eye Swelling</td>
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<td>Decreased appetite</td>
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<tr>
<td>Rash</td>
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<td>Abdominal pain</td>
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<td>Lightheadedness</td>
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<td>Night sweats</td>
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<tr>
<td>Dyspnea</td>
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<td>1</td>
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<tr>
<td>Serious adverse event, possibly related:</td>
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<td></td>
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<tr>
<td>Pulmonary Embolus</td>
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Table 3: Gene products identified by SEREX analysis

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<tr>
<th>Gene product</th>
<th>Gene ID</th>
<th>Gene chromosomal locus</th>
<th>Healthy donors serum (n=5)</th>
<th>Pre-vaccination MM serum (n=12)</th>
<th>1 month Post-vaccination MM serum (n=12)</th>
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<tbody>
<tr>
<td>RGS19</td>
<td>10287</td>
<td>20q13.33</td>
<td>antibody response negative (-)</td>
<td>antibody response negative (-)</td>
<td>2 patients (patient #10,11) antibody response strongly positive (++++)</td>
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<tr>
<td>HSP90β</td>
<td>7184</td>
<td>12q24.2-24.3</td>
<td>antibody response negative (-)</td>
<td>antibody response negative (-)</td>
<td>2 patients (patient #10,11) antibody response positive (+++ )</td>
</tr>
<tr>
<td>BRAP</td>
<td>8315</td>
<td>12q24</td>
<td>antibody response negative (-)</td>
<td>1 patient (patient #2) antibody response positive (+)</td>
<td>1 patient (patient #2) antibody response positive (+)</td>
</tr>
</tbody>
</table>
Figure Legends:

Figure 1. Immunohistochemical staining of DC, tumor, and fusion cells.

A. Autologous DCs were generated from adherent mononuclear cells isolated from a leukapheresis collection. DCs were cultured with GM-CSF and IL-4 for 5 days and then TNFα for 48-72 hours. DC preparations were analyzed for expression of costimulatory molecules. DC expression of CD86 (red) is shown (60X).

B. Patient derived myeloma cells were cultured in RPMI 1640 complete medium and were analyzed for expression of the tumor associated antigens CD138 and CD38. Tumor expression of CD38 (red) is shown (60X).

C. Fusion cells were generated by co-culture of DCs and myeloma cells in the presence of PEG. Fusion cell preparations were analyzed for co-expression of the DC derived costimulatory molecule CD86 (blue) and tumor associated antigen CD38 (red).

Figure 2. Potency of fusion cells in the stimulation of allogeneic T cell proliferation.

Patient derived DC, myeloma cells and fusions were co-cultured with T cells from a healthy donor at a T cell to target ratio of 1:10, 1:30, 1:100, 1:300, and 1:1000. Cells were co-cultured for 5 days and T-cell proliferation was determined by incorporation of $[^3H]$-Thymidine (1 μCi/well) following overnight pulsing. Stimulation index represents CPM of sample/CPM of unstimulated T cells. The results are presented as mean values +/- SEM from 16 samples at an APC:T cell ratio of 1:10.

Figure 3. Biopsy of a vaccine site reaction.

A biopsy of vaccine site reactions was obtained, fixed in formalin, and stained with hematoxylin and eosin. To further characterize the nature of the cellular infiltrate, immunohistochemical staining for CD8+ and CD1a was performed.
A. A representative example of a vaccine site reaction biopsy is shown, demonstrating a dense mononuclear cell infiltrate is at the vaccine site.

B. A representative example of immunohistochemical staining is shown, demonstrating an infiltrate of CD8+ T cells (left) and CD1a+ immature DCs (right) at the biopsy site.

Figure 4.

A. **Intracellular expression of IFNγ by CD4 and CD8 populations.** PBMCs isolated prior to each vaccination, and at serial time points post vaccination, were cocultured with autologous tumor lysate, pulsed with Golgi Stop, labeled with FITC-conjugated CD4 or CD8 antibodies, and then permeabilized by incubation in Cytofix/Cytoperm plus™ (PharMingen). Cells were then incubated with PE-conjugated anti-interferon gamma or a matched isotype control antibody, and fixed in 2% paraformaldehyde. Labeled cells were analyzed by flow cytometry.

B. **Vaccine induction of Tumor reactive CD4+ T cells.** Percentage of CD4+ T cells expressing interferon gamma following ex-vivo exposure to autologous tumor lysate is shown. Percentage of tumor reactive CD4+ T cells is shown prior to the initial vaccination and at the peak time post-vaccination.

C. **Vaccine induction of Tumor reactive CD8+ T cells.** Percentage of CD8+ T cells expressing interferon gamma following ex-vivo exposure to autologous tumor lysate is shown. Percentage of tumor reactive CD8+ T cells is shown prior to the initial vaccination and at the peak time post-vaccination.

Figure 5. Expansion of MUC1 tetramer positive cells following vaccination.
CD8+ T cells binding the MUC1 tetramer were quantified at serial time points (1: prior to first vaccination; 2: prior to second vaccine; 3: prior to third vaccination) in patients who are HLA-A2.1. Binding to a control tetramer was quantified in parallel and the control value was subtracted from that obtained for the MUC1 tetramer. Mean values of 5 patients are presented, with associated SEM. An incremental, though not statistically significant, increase in MUC1 tetramer+ cells is observed following vaccination.

Figure 6. T cell response to PHA and tetanus toxoid prior to and following vaccination.

A. PBMC were collected at the indicated time points and incubated with 2 ug/ml of PHA for 3 days. Proliferation was measured by incorporation of tritiated thymidine. Values are presented as mean stimulation index: proliferation of stimulated/unstimulated cells +/- SEM.

B. PBMC were collected at the indicated time points and incubated with tetanus toxoid at 10 ug/ml for 5 days. Proliferation was measured by incorporation of tritiated thymidine. Values are presented as mean stimulation index: proliferation of stimulated/unstimulated cells +/- SEM.

Figure 7. A representative example demonstrating a humoral immune response against RGS 19 in response to vaccination. Sera from patient number 10 was obtained prior to vaccination (left panel) and one month post-vaccination (right panel) and incubated with E.coli transfected with phage expressing myeloma-derived cDNA. Specific binding of antibody to recombinant proteins expressed on the lytic plaques was
detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG antibody. Antigen-antibody complexes were visualized by staining with S-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT). Vaccination induced an antibody response directed against RGS 19.
Figure 1.
Figure 2.
Figure 3.

A.

B.
Figure 4A.

Figure 4B.
Figure 4C.
Figure 5.
Figure 6.

A

PHA

SI

1 2 3 1 3 6
Vaccination period Months post 3rd Vaccination

B

Tetanus toxoid

SI

1 2 3 1 3 6
Vaccination period Months post 3rd Vaccination
Figure 7.
Vaccination with DC/tumor fusion cells results in cellular and humoral anti-tumor immune responses in patients with multiple myeloma

Jacalyn Rosenblatt, Baldev Vasir, Lynne Uhl, Simona Blotta, Claire MacNamara, Poorvi Somaiya, Zekui Wu, Robin Joyce, James D. Levine, Dilani Dombagoda, Yan Emily Yuan, Karen Francoeur, Donna Fitzgerald, Paul Richardson, Edie Weller, Kenneth Anderson, Donald Kufe, Nikhil Munshi and David Avigan