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Vaccination with Dendritic Cell Myeloma Fusions in Conjunction With Stem Cell Transplantation and PD1 Blockade

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14. ABSTRACT Most patients with multiple myeloma achieve a complete or near complete response following autologous transplantation. However, patients experience disease relapse from a persistent reservoir of chemotherapy resistant disease. There has been strong interest in developing immunotherapeutic strategies to eradicate residual disease following autologous transplantation. Our group has developed a tumor vaccine model whereby dendritic cells are fused with tumor cells. In clinical trials, vaccination with fusion cell results in anti-tumor immune and disease responses in a subset of patients. However, vaccine efficacy is blunted by tumor mediated immune suppression and the increased presence of regulatory T cells characteristic of patients with malignancy. An important element contributing to tumor mediated immune suppression is the PD-1/PDL-1 pathway. PD-L1 exerts a significant role in promoting T cell tolerance by binding PD-1 on activated T cells and suppressing their capacity to secrete stimulatory cytokines. We have demonstrated that blockade of this pathway results in enhanced immune responses to DC/myeloma fusion cells ex vivo. In the proposed study, we will examine toxicity, immunologic effect and clinical efficacy of CT-011 therapy following stem cell transplantation for patients with myeloma. These endpoints will then be assessed in patients undergoing combined therapy with the vaccine and antibody.					
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Progress Report
P.I.: David Avigan
May 2010

Introduction:

The goal of the project is determine the effect of PD-1 blockade on the capacity of the dendritic cell (DC)/multiple myeloma (MM) fusions to stimulate effective anti-tumor immunity and disease response. We have previously demonstrated that vaccination with DC/ MM fusions potently induces cellular and humoral anti-tumor immune responses¹. However, vaccine efficacy is blunted by the immunosuppressive milieu characteristic of patients with malignancy. A key component of this immunosuppressive environment is the presence of regulatory T cells that are increased in the circulation, tumor bed and draining lymph nodes of patients with malignancy²⁻⁴. We have shown that vaccine responses are augmented following autologous transplantation due to depletion of regulatory T cells and the associated transient reversal of tumor mediated tolerance. Another key component of tumor mediated immune suppression is the increased signaling via the PD-1/PDL-1 pathway^{5,6}. This pathway is upregulated in the setting of chronic viral infection and malignancy and results in an exhausted T cell phenotype^{7,8}. In the present project, we plan to examine the effect of PD-1 blockade on vaccine efficacy. In the present project, we intend to conduct a clinical trial in which patients with multiple myeloma undergoing stem cell transplantation will undergo serial administration of CT-011 antibody post-transplant alone (Cohort 1) or in conjunction with vaccination with DC/MM fusions (cohort 2). In the past year, while meeting regulatory requirements for initiation of the clinical trial, we have performed preclinical studies to characterize the impact of PD-1 blockade on fusion mediated stimulation of T cells.

Body:

Pre-clinical Data

Expression of PDL-1 on plasma cells, DCs, and DC/tumor fusion cells: Expression of PDL-1 on antigen presenting cells plays an important role in blunting activated T cell responses. To assess the role that the PD-1/PDL-1 pathway may play in limiting activated immune responses in our DC/tumor vaccine model, we evaluated expression of PDL-1 on ex-vivo generated DCs, plasma cells, and DC/tumor fusions. DCs were generated by culture of adherent PBMCs for 1 week with GM-CSF and IL-4 to generate immature DCs, followed by maturation in the presence of TNF α for 48 hours. Tumor cells were obtained from bone marrow aspirates of patients with multiple myeloma, and were fused with DCs by coculture in 50% solution of polyethylene glycol. Mean expression of PDL-1 was 97% on DCs (n=5, Figure 1), and 45% on patient derived myeloma cells (n=6, Figure 1). In addition, mean expression of PDL-1 was 90% on DC/myeloma fusions (n=2, Figure 2), suggesting this pathway may provide an inhibitory signal that blunts fusion mediated immunologic response.

Expression of PD-1 on T cells isolated from patients with multiple myeloma:

In infectious disease models, upregulation of T cell expression of PD-1 is associated with an exhausted phenotype facilitating the development of chronic viral infection. We evaluated expression of PD1 on T cells derived from patients with myeloma with active

disease or after cytoreduction following autologous stem cell transplantation. Nonadherent PBMCs obtained from patients with multiple myeloma and normal volunteers were cultured in RPMI supplemented with 10U/ml IL-2, and expression of PD-1 on CD4+ T cells was assessed by flow cytometric analysis. We demonstrate that PD-1 expression is markedly upregulated in patients with advanced multiple myeloma. Compared to a control population of normal volunteers where PD-1 expression on CD4+ T cells was 6% (n=10), a significant increase in PD1 expression was observed on CD4+ T cells isolated from patients with advanced myeloma 20% (n=10, p=0.005, Figure 3A, 3B). Interestingly, PD-1 expression on CD4+ T cells isolated from patients who obtained a complete or very good partial response to transplant returned to levels observed in normal volunteers (mean expression of PD-1 on CD4+ T cells 6%, n=5, Figure 3B). Similarly, increased PD-1 expression was observed on CD8+ T cells isolated from patients with advanced myeloma (5%, n=10) compared to normal controls (1.4%, n=10, p=0.006). These findings suggest that upregulation of PD-1 expression may play a central role in tumor mediated immune suppression.

Effect of antigenic and non-antigenic stimulation on T cell expression of PD-1:

We subsequently assessed the effect of both non-antigenic and antigen mediated stimulation of T cells on PD-1 expression. Following nonspecific T cell activation by exposure to anti-CD3/CD28, mean PD-1 expression on CD4+ T cells increased from 5.1% to 14% (n=6, p=0.03, Figure 4). Similarly, mean PD-1 expression on CD4+ T cells increased from 6% to 23% following stimulation with PHA (n=5, p=0.03, Figure 4). These results suggest that antigen independent stimulation of T cells results in an upregulation of PD-1 expression. We evaluated whether stimulation with DC/tumor fusion cells also results in increased PD-1 expression, and demonstrated that following stimulation with DC/myeloma fusions for 5 days, PD-1 expression on CD4+ T cells increased from 8% to 13% (n=3, p=0.05). In contrast, stimulation with DC/MM fusions in conjunction with PD-1 blockade was not associated with upregulation of PD1 expression (mean CD4+ T cell expression of PD1 9%, n=2).

Effect of PD-1 blockade on T cell polarization following DC/myeloma fusion:

stimulation: We have previously demonstrated that T cell stimulation with DC/myeloma fusion cells results in the concomitant expansion of activated and regulatory T cells. However, fusion mediated stimulation in conjunction with PD-1 blockade resulted in the suppression of regulatory T cell expansion. The percentage of CD4+/CD25+/FOXP3 T cells following stimulation of T cells with DC/MM fusions alone or with CT-011 was 12% and 5%, respectively (n=5, p=0.02, Figure 5).

We further examined the effect of PD-1 blockade on fusion mediated stimulation of activated versus inhibitory T cell populations by quantifying the percentage of cells that expressed interferon gamma (IFN γ) as compared to IL-10. IFN γ secretion by CD4+ T cells in response to stimulation by DC/myeloma fusion cells increased 3 fold in the presence of PD-1 blockade (n=5, p=0.048, figure 6). In addition, a 2.2 fold decrease in IL-10 secretion by CD4+ cells following DC/myeloma fusion stimulation was noted in the presence of PD-1 blockade (n=5, p=0.035, figure 7). These findings demonstrate

that PD-1 blockade enhances vaccine efficacy by biasing toward Th1 cytokines, and limiting the expansion of suppressive T cell populations.

Effect of PD-1 blockade on CTL response following stimulation with DC/myeloma fusions: DC/myeloma fusion cells potently stimulate the expansion of myeloma specific CTLs with the capacity to lyse autologous tumor targets. We demonstrated that CTL activity was further increased in the presence of PD-1 blockade at the time of vaccine mediated T cell activation and coculture of activated effector cells and myeloma targets. Mean CTL lysis was 27% and 31% for T cells stimulated with DC/myeloma cells alone, and in the presence of PD-1 blockade respectively (n=5, p=0.05, Figure 8).

Effect of PD-1 blockade on activation of AKT: We assessed the impact of PD-1 blockade on Akt activation in response to fusion mediated stimulation of T cells. Akt plays an important role in T cell activation and proliferation. T cell expression of the phosphorylated Akt was quantified via flow cytometric analysis. Fusion stimulation resulted in increased CD4+ T cell expression of phosphorylated Akt, from 1.5% to 3.1% (n=7, p=0.034). The presence of CT-011 further increased levels of phosphorylated Akt to 4.2% (N=7, p= 0.04 compared to fusion stimulation alone).

Clinical Trial:

The clinical trial was given initial approval in June 2009. After obtaining SRC and IRB approval, the clinical protocol and informed consent were sent to the DOD for review on June 8 2009. On August 24 2009, we received a response from the DOD with requests for protocol and consent changes. On Sept 24 2009, point by point responses were sent to the DOD along with a revised protocol (9.4.09), revised consent form (8.27.09), International research study form, and all supporting documentation. The protocol underwent HSRRB review on 10/28/09. A conference call was held on 10/28/09 discussing issues raised by the HSRRB review. Final approval was obtained from the Department of Defense in December of 2009. The final version was submitted to the FDA and Institutional Review Board and the study was approved and activated in March 2010. 1 patient has been enrolled on the study, and is undergoing pre-transplant therapy. Another patient is being screened for study enrollment, and the study has been discussed with two patients who are reviewing the consent.

Key Research Accomplishments:

- oral presentation at the American Society of Hematology Annual meeting
- poster presentation at the American Society of Bone Marrow Transplantation Annual Meeting

Reportable Outcomes:

Rosenblatt J, Glotzbecker B, Mills H, Keefe W, Wellenstein K, Vasir B, Wu Z, Zarwan C, Schickler M, Rotem-Yehudar R, Kufe D, Avigan D. CT-011, Anti-PD-1 Antibody, Enhances Ex-Vivo T Cell Responses to Autologous Dendritic/Myeloma Fusion Vaccine Developed for the Treatment of Multiple Myeloma. American Society of Hematology 51st Annual Meeting, New Orleans LA. Oral Presentation, December 2009

Rosenblatt J, Glotzbecker B, Mills H, Keefe W, Wellenstein K, Vasir B, Wu Z, Zarwan C, Schickler M, Rotem-Yehudar R, Kufe D, Avigan D. CT-011, Anti PD-1 Antibody, Enhances Ex-Vivo T Cell Responses to Autologous Dendritic/Myeloma Fusion Vaccine Developed for the Treatment of Multiple Myeloma. ASBMT, Orlando FL. Poster Presentation, February 2010

Jacalyn Rosenblatt¹, Brett Glotzbecker¹, Heidi Mills¹, Baldev Vasir², Dimitrios Tzachanis¹, James D Levine¹, Robin Joyce¹, Kerry Wellenstein¹, Whitney Keefe¹, Michael Schickler³, Rinat Rotem-Yehudar³, Donald Kufe², David Avigan¹. PD-1 blockade enhances ex-vivo T cell responses to autologous dendritic/myeloma fusion vaccine developed for the treatment of multiple myeloma- manuscript in preparation

Conclusion:

Results of our pre-clinical studies demonstrate that T cell expression of PD-1 is upregulated in patients with advanced multiple myeloma, that DC/tumor fusion cells express high levels of PDL-1, and that stimulation with DC/myeloma fusion cells results in a further increase in T cell expression of PD-1. These findings suggest that the PD-1/PDL-1 pathway may play a role in blunting the potency of immunologic response to vaccination. We demonstrate PD-1 blockade in conjunction with DC/tumor fusion cell stimulation results in a skewing toward Th1 rather than Th2 cytokine secretion, decreased regulatory T cell expansion, and enhanced killing of autologous tumor.

After meeting regulatory requirements for the IRB, FDA and DOD, the study is currently activated and open to accrual. We anticipate enrollment to the first cohort to be completed over the next 6 months, and enrollment to the second cohort to be completed over the subsequent 18 months.

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8. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol.* 2007;19:309-314.

Appendices:

Appendix 1: abstracts attached

Appendix 2: Supporting data: figures and figure legends attached

[65] DENDRITIC CELL TUMOR FUSION VACCINATION IN CONJUNCTION WITH AUTOLOGOUS TRANSPLANTATION FOR MULTIPLE MYELOMA

Rosenblatt, J., Avivi, I., Vasir, B., Katz, T., Uhl, L., Wu, Z., Somaiya, P., Mills, H., Joyce, R.M., Levine, J.D., Tzachanis, D., Boussiotis, V., Glotzbecker, B., Francoeur, K., Dombagoda, D., Tsumer, M., Bisharat, L., Giallombardo, N., Conway, K., Fitzgerald, D., Barhad, R., Richardson, P., Anderson, K.C., Munshi, N.C., Rowe, J.M., Kufe, D., Avigan, D. Medicine, Beth Israel Deaconess Medical Center, Boston, MA; Department of Hematology and Bone Marrow Transplantation, Rambam Medical Center, Haifa, Israel; Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

Autologous stem cell transplantation (ASCT) for multiple myeloma (MM) offers a unique setting to explore the role of immunotherapy in eradicating malignancy. Our group has developed a cancer vaccine whereby dendritic cells (DCs) are fused with autologous tumor cells. We are conducting a clinical trial in which MM patients undergo ASCT followed by vaccination with 3 doses of DC/MM fusions (cohort 1). A second cohort receives an additional vaccine prior to stem cell collection (cohort 2). To date, 26 patients have been enrolled in cohort 1 and 9 in cohort 2. Adherent mononuclear cells were isolated from leukapheresis collections and cultured with GM-CSF, IL-4 and TNF α to generate DCs. MM cells were isolated from bone marrow and were identified by expression of CD38 or CD138. DC and MM cells were co-cultured with PEG and fusion cells were quantified by determining the percentage of cells that co-express unique DC and MM antigens. Mean fusion efficiency was 38%, mean dose generated was 3.6×10^6 fusion cells. Fusion cells potently stimulated allogeneic T cell proliferation in vitro (mean stimulation indexes were 13, 60, and 32 for T cells stimulated by MM cells, DCs, and fusion cells respectively). Adverse events were mild, and included injection site reactions, pruritis, myalgias, fever, and tachycardia. ASCT was associated with suppression of measures of cellular immunity. Circulating CD4 cells were depressed post-transplant, and CD4:CD8 ratios remained inverted for >10 months. Following transplant, T cell response to PHA and tetanus toxoid were depressed. In contrast, an increase in circulating tumor reactive lymphocytes was noted, as determined by T cell expression of IFN following ex vivo coculture with autologous myeloma cell lysate (Mean percentage of tumor reactive CD8 cells was 1 and 7.7 pre and post-transplant, respectively; mean percentage of CD4 cells was 0.9 and 3.2). A further amplification of tumor reactive lymphocytes was seen with vaccination (mean percentage of CD4 and CD8 tumor reactive T cells was 6.4 and 13.4, respectively). 24 patients have completed follow up. 3 patients achieved CR at 1 month following ASCT. An additional 7 patients obtained a CR following completion of vaccinations, supporting a role for post-transplant immunotherapy in mediating elimination of disease. In summary, fusion cell vaccination in conjunction with ASCT was well tolerated, induced anti-tumor immunity and induction of post-transplant complete response.

Sunday, February 28, 2010 11:00 AM

Oral Session (10:00 AM - 11:30 AM)

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781 CT-011, Anti-PD-1 Antibody, Enhances Ex-Vivo T Cell Responses to Autologous Dendritic/Myeloma Fusion Vaccine Developed for the Treatment of Multiple Myeloma

Oral and Poster Abstracts

Oral Session: Tumor Immunotherapy: Clinical Cellular Immunotherapies

Monday, December 7, 2009: 4:30 PM

388-390 (Ernest N. Morial Convention Center)

Jacalyn Rosenblatt, M.D.¹, Brett Glotzbecker, MD^{1*}, Heidi Mills^{1*}, Whitney Keefe^{1*}, Kerry Wellenstein^{1*}, Baldev Vasir, PhD^{2*}, Zekui Wu, MD^{2*}, Corrine Zarwan, MD^{1*}, Michael Schickler, PhD^{3*}, Rinat Rotem-Yehudar, PhD^{3*}, Donald Kufe, MD^{2*} and David Avigan, MD¹

¹Beth Israel Deaconess Medical Center, Boston, MA

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³CureTech Ltd., Israel

We have developed a promising cancer vaccine in which autologous tumor cells are fused with dendritic cells (DCs) resulting in the presentation of tumor antigens in the context of DC mediated costimulation. In animal models, vaccination with fusion cells results in eradication of established tumor, and in clinical trials, both immunologic and clinical responses have been observed. However, response to vaccination may be muted by inhibitory pathways that blunt activated T cell responses. The PD-1/PDL-1 pathway is an important element contributing to tumor mediated immune suppression. In infectious disease models, upregulation of T cell expression of PD-1 is associated with an exhausted phenotype facilitating the development of chronic viral infection. In contrast, PD-1 blockade results in the restoration of functionally active T cells and clearance of infection. The PD-1/PDL-1 pathway is also being evaluated as a central mechanism by which tumors escape host immunity. CT-011, is a humanized anti PD-1 antibody that is currently evaluated in Phase II studies for the treatment of hematological malignancies and solid tumors. In this study, we evaluated expression of PD-1 on T cells derived from patients with advanced hematologic malignancies, and PDL-1 expression on primary myeloma cells, ex-vivo generated dendritic cells, and DC/tumor fusion cells. We evaluated the effect of PD-1 blockade with CT-011 on T cell response to DC/tumor fusion cell stimulation in vitro. Tumor cells were obtained from bone marrow aspirates of patients with multiple myeloma. Nonadherent peripheral blood mononuclear cells obtained from patients with multiple myeloma and normal volunteers were cultured in RPMI supplemented with 10U/ml IL-2, and expression of PD-1 on CD4+ T cells was assessed by flow cytometric analysis. DCs were generated from adherent mononuclear cells cultured with rhIL-4, GM-CSF and TNF α and fused with tumor cells by coculture in 50% solution of polyethylene glycol. T cells were stimulated by DC/tumor fusions in the presence or absence of 5ug/ml CT-011. We demonstrate that PD-1 expression is markedly upregulated on T cells in patients with advanced multiple myeloma. As compared to a control population of normal

volunteers in which mean levels of PD-1 expression was 6% (n=7), mean expression in patients with multiple myeloma was 20% (n=9). These findings suggest that upregulation of PD-1 expression may play a central role in tumor mediated immune suppression. Mean expression of PDL-1 was 91% on dendritic cells generated from adherent peripheral blood mononuclear cells obtained from normal volunteers (n=6), and 66% on patient derived myeloma cells (n=3). In addition, PDL-1 expression is found in greater than 90% of DC/tumor fusions (n=2), which potentially provides an inhibitory signal dampening fusion mediated immunologic response. We examined the effect of PD-1 blockade on T cell response to DC/tumor fusions ex vivo with different anti PD-1 antibodies including CT-011. DC/tumor fusions were co-cultured with autologous T cells alone or with antibodies against PD-1. Enhanced fusion mediated stimulation of T cells was noted particularly with CT-011, resulting in a greater than 5 fold increase in T cell proliferation. Interferon gamma secretion by CD4+ T cells in response to stimulation by DC/myeloma fusion cells was increased from 4% to 11% in the presence of CT-011. In addition, IL-10 secretion by CD4+ cells following DC/myeloma fusion stimulation decreased from 6.5% to 3.5% following PD-1 blockade. In summary, we have demonstrated that PD-1 expression is increased in T cells of patients with hematologic malignancy, and CT-011, a PD-1 blocking antibody, enhances activated T cell responses following stimulation with a DC/tumor fusion vaccine. A clinical trial in which patients with multiple myeloma are treated with DC/myeloma fusions in conjunction with CT-011 following autologous transplantation is planned.

Disclosures: **Schickler:** *CureTech, Ltd.:* Employment. **Rotem-Yehudar:** *CureTech, Ltd.:* Employment.

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Figure 1

Appendix 2

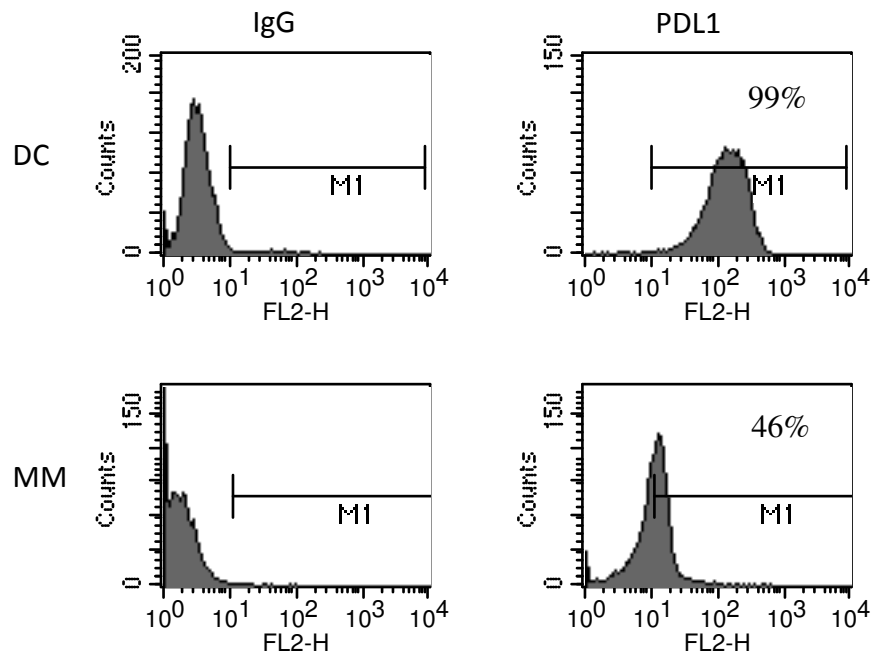


Figure 2

DC/MM Fusions

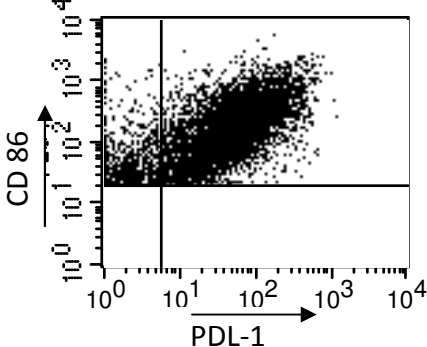
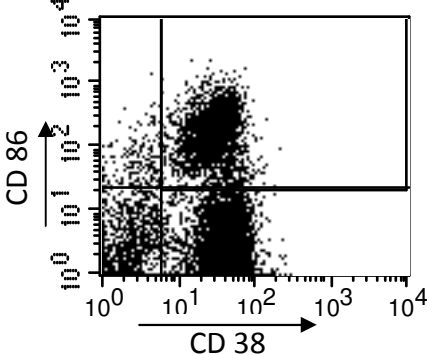


Figure 3

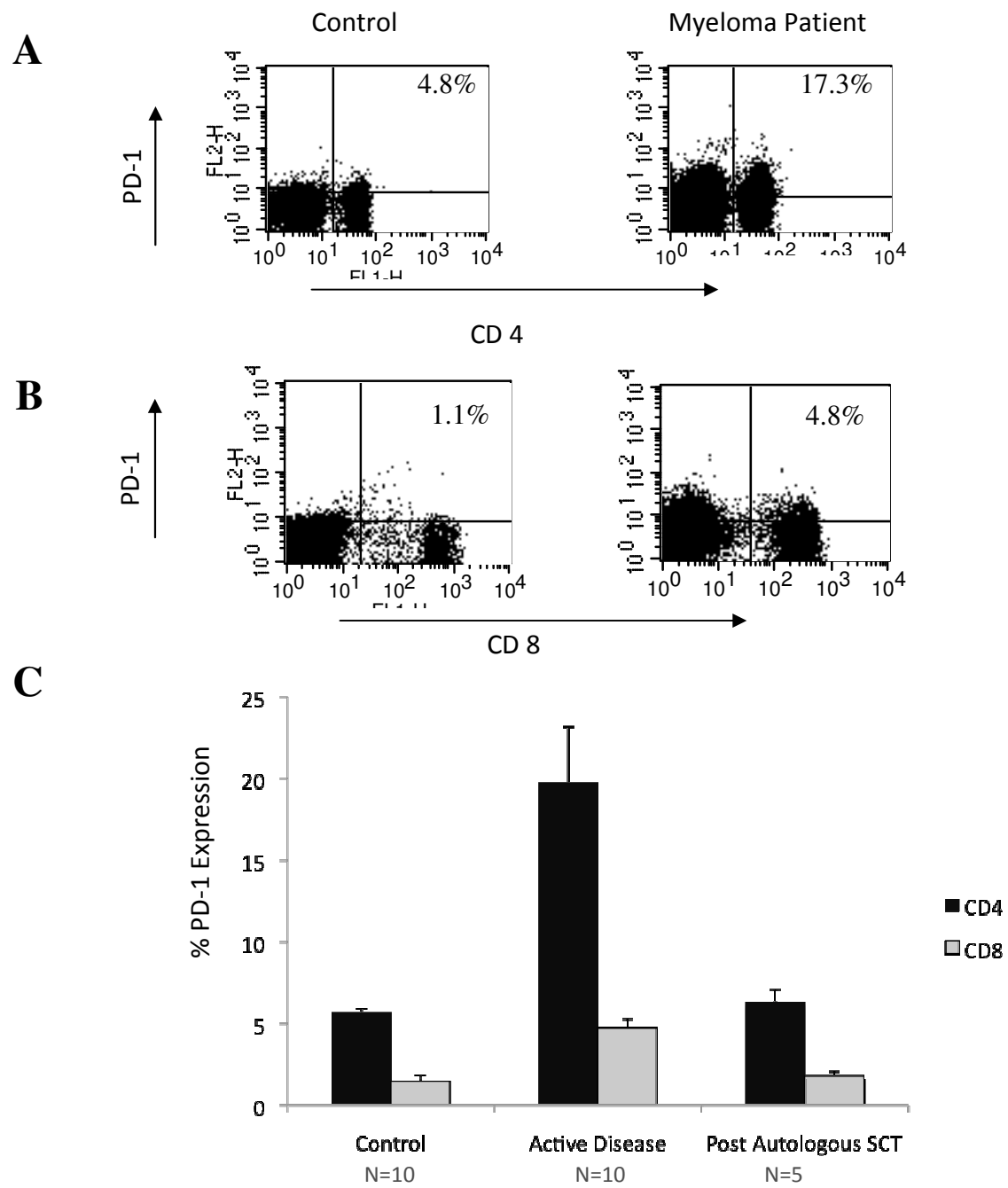


Figure 4

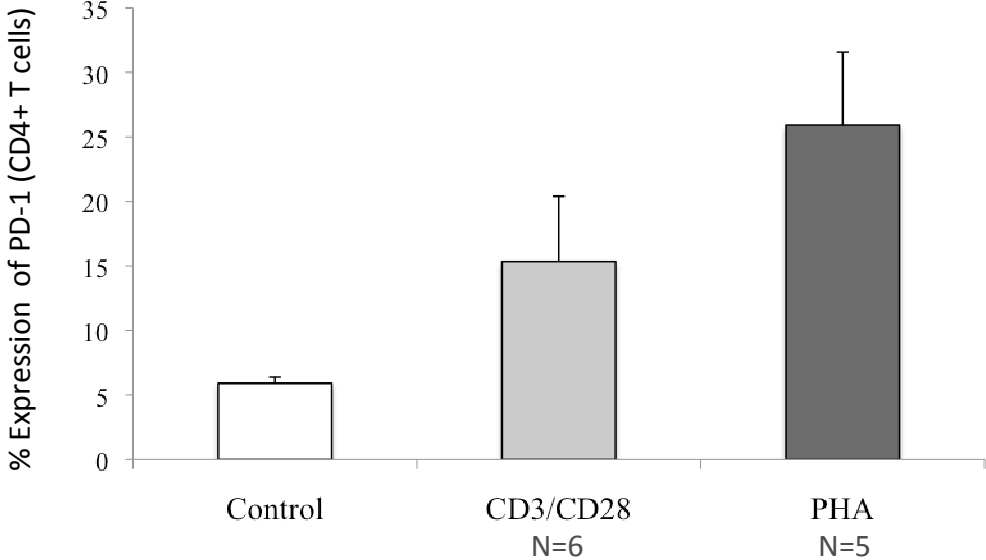


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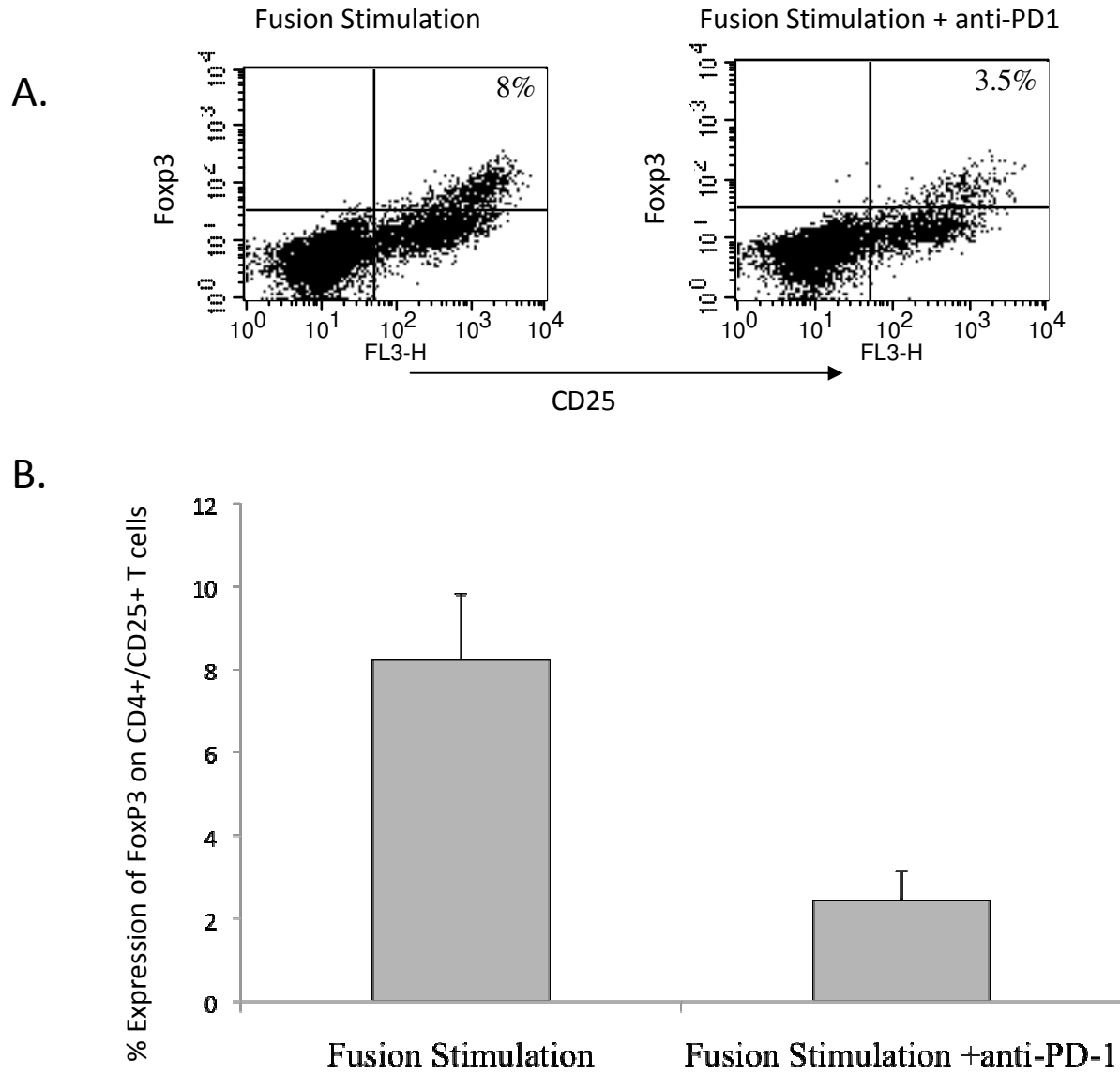
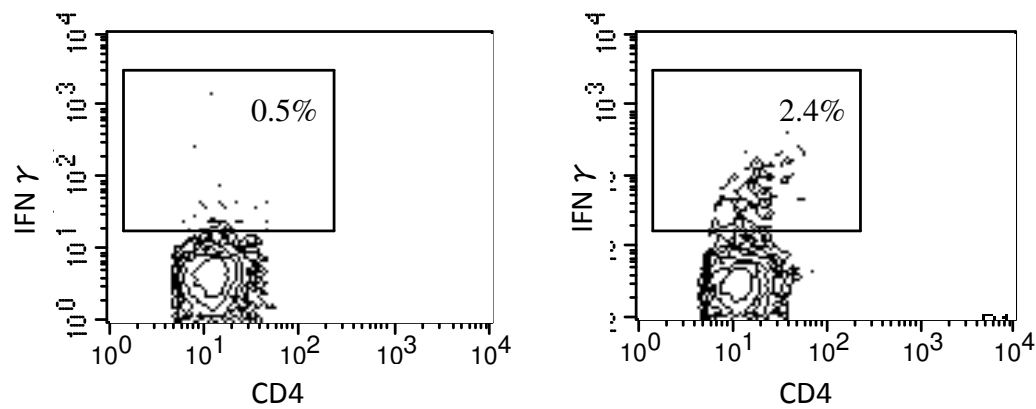


Figure 6

A.



B.

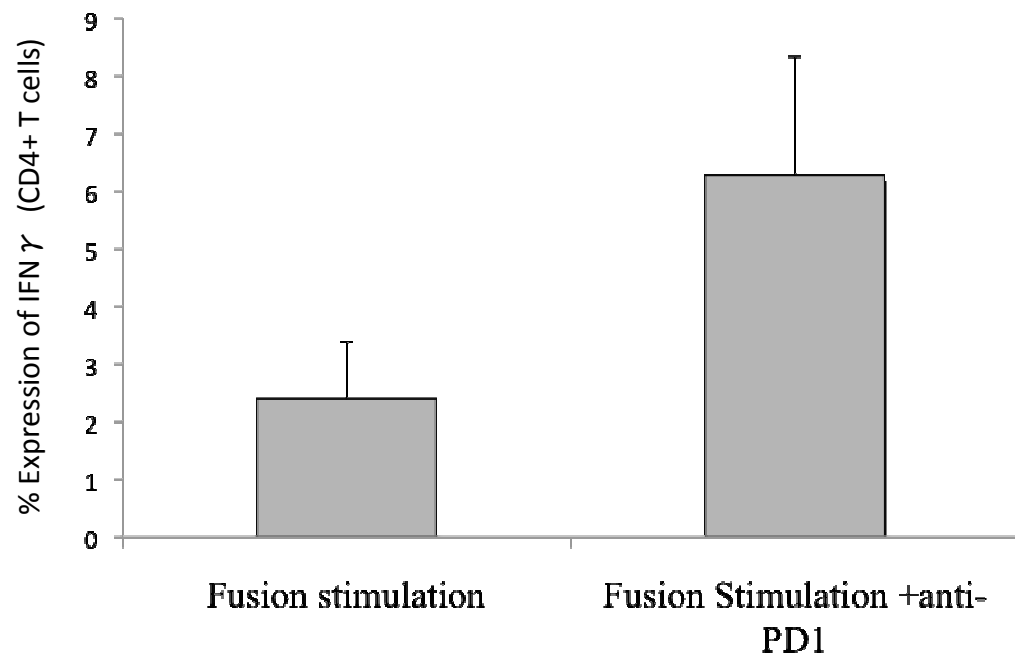


Figure 7

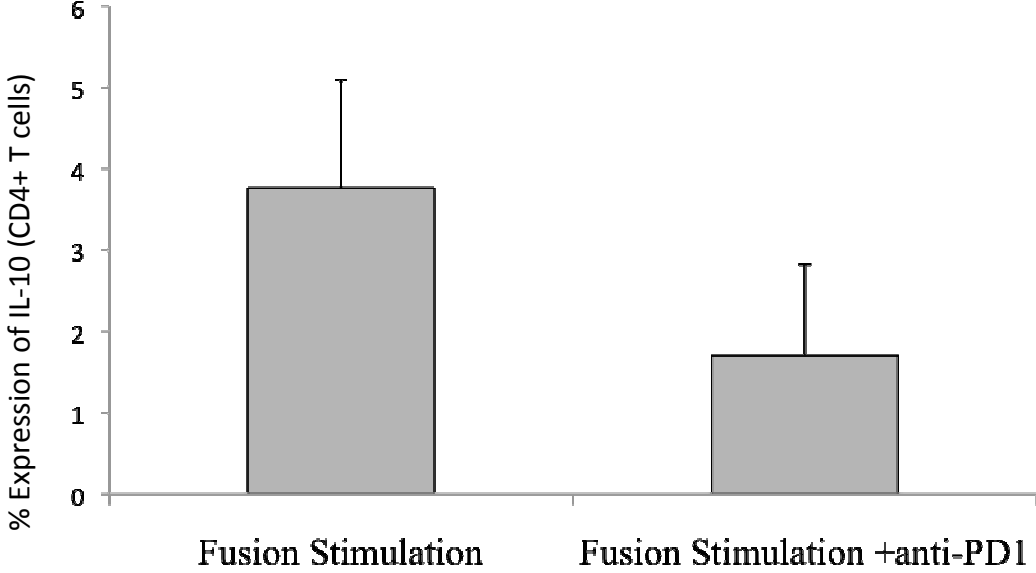
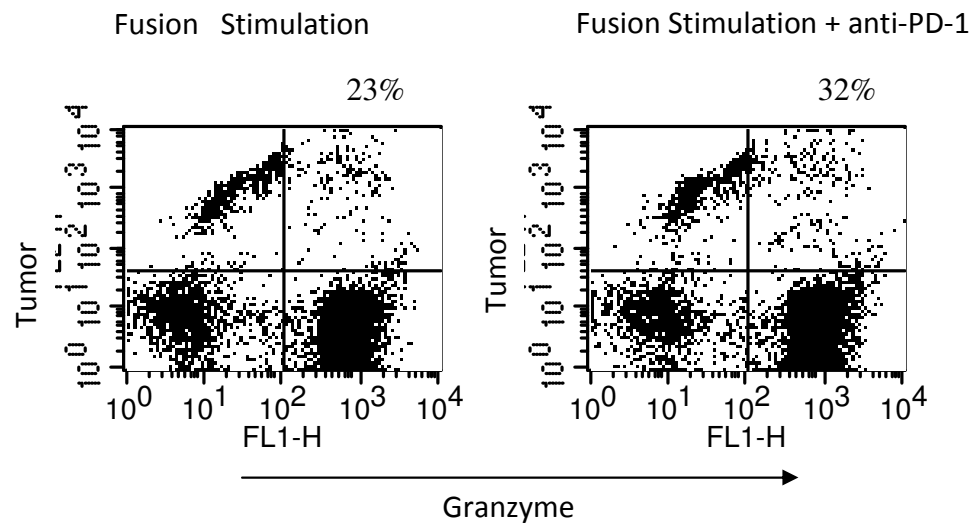


Figure 8



Legends for Figures

Figure 1. Expression of PDL-1 on DCs and plasma cells

- A.** DCs were generated from adherent mononuclear cells isolated from leukopak collections obtained from normal donors. DCs were cultured with GM-CSF and IL-4 for 5 days and then underwent maturation by exposure to TNF α for 48-72 hours. DC preparations were stained with PE conjugated anti-PDL-1 and analyzed by flow cytometric analysis. A representative example is shown.
- B.** Plasma cells were isolated from bone marrow aspirates of patients with active multiple myeloma. Cells were stained with FITC conjugated anti-CD38 and PE conjugated anti-PDL-1. Expression of PDL-1 on gated CD38+ cells was assessed by flow cytometric analysis.

Figure 2. Expression of PDL-1 on DC/MM fusions: Fusion cells were generated by co-culture of DCs and plasma cells in the presence of PEG. Fusion cell preparations were stained with PE-cy5 conjugated CD86, FITC conjugated CD38, and PE conjugated PDL-1. Fusion cells were quantified by gating on cells dually staining for tumor derived CD38 and DC derived CD86 (upper panel). Dual staining cells were gated on, and expression of PDL-1 on the fusion cell population was assessed (lower panel). A representative example is shown.

Figure 3. Expression of PD-1 on T cells isolated from myeloma patients:

- A.** Nonadherent peripheral blood mononuclear cells isolated from normal controls (left) and myeloma patients (right) were incubated with FITC conjugated anti-CD4 and PE conjugated anti-PD1. Expression of PD-1 on CD4+ T cells was assessed by FACS analysis. A representative example is shown.
- B.** Nonadherent peripheral blood mononuclear cells isolated from normal controls (left) and myeloma patients (right) were incubated with FITC conjugated anti-CD8 and PE conjugated anti-PD1. Expression of PD-1 on CD8+ T cells was assessed by FACS analysis. A representative example is shown.

C. Expression of PD-1 was assessed on CD4+ and CD8+ T cell populations isolated from normal controls, patients with active myeloma, and myeloma patients who were cytoreduced following autologous transplant. Nonadherent peripheral blood mononuclear cells were incubated with FITC conjugated anti-CD4 or anti-CD8 and PE conjugated anti-PD1 and assessed by FACS analysis as described above. Mean values with associated standard errors of the mean are shown.

Figure 4. Effect of stimulation on T cell expression of PD-1: T cells were stimulated with anti-CD3/CD28 for 48 hours, or the mitogen PHA (2 ug/ml) for 4 days. Stimulated T cell populations were stained with FITC conjugated anti-CD4 and PE conjugated anti-PD-1. PD-1 expression on T cells before and after stimulation was assessed by flow cytometric analysis. Mean values with associated standard errors of the mean are shown.

Figure 5. Effect of PD-1 blockade in conjunction with DC/myeloma fusion stimulation on regulatory T cell populations: Autologous T cells were co-cultured with DC/myeloma fusions for 5 days at a 10:1 ratio in the presence and absence of 5ug/ml anti-PD1. The cell preparations were incubated with FITC conjugated anti-CD4 and tricolor conjugated anti-CD25. The cells were permeabilized, stained with PE conjugated anti-FoxP3, and analyzed by multichannel flow cytometry. CD4/CD25+ T cells were isolated by FACS gating and expression of Foxp3 was determined. A representative example (A) and mean values of 5 experiments with associated standard errors of the mean (B) are shown.

Figure 6. Interferon gamma production by fusion stimulated T cell populations in the presence and absence of PD-1 blockade: Stimulated T cell preparations were stained for FITC conjugated CD4. Cells were then washed, permeabilized, and incubated with PE conjugated anti-human IFN γ or a matched isotype control antibody. Intracellular expression of IFN γ was determined by flow cytometric analysis. A representative example (A) and mean values of 5 experiments with associated standard error of the means (B) are shown.

Figure 7. Interleukin 10 production by fusion stimulated T cell populations in the presence and absence of PD-1 blockade. Stimulated T cell preparations were stained for FITC conjugated CD4. Cells were then washed, permeabilized, and incubated with PE conjugated anti-human IL-10 or a matched isotype control antibody. Intracellular expression of IL-10 was determined by flow cytometric analysis. Mean values of 5 experiments with associated standard error of the means are shown.

Figure 8. DC/myeloma fusions were cocultured with autologous T cells at a ratio of 1:10 for 5 days in the presence and absence of PD-1 blockade. Autologous tumor cells were used as target cells, labeled with PE labeled Medium T. T cells stimulated by fusions alone (left) or in the presence of PD-1 blockade (right) were co-incubated with labeled target cells in the presence of a fluorogenic granzyme B substrate. Cells were washed and analyzed by flow cytometry. Dead target cells are identified by cells that dually stain for granzyme B and PE label (right upper quadrant). A representative example is shown.