Lenalidomide-Induced Immunomodulation in Multiple Myeloma: Impact on Vaccines and Antitumor Responses

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Statement of Translational Relevance

Lenalidomide has been developed as an immunomodulatory derivative of the parent compound, thalidomide. Despite its significant clinical efficacy and presumed immune effect, no human studies to date have documented these properties. In this study, we demonstrate these immune outcomes in two ways. Lenalidomide elicits a direct immune-mediated anti-myeloma effect and augments non-specific immunity to increase vaccine efficacy of the pneumococcal 7-valent conjugate vaccine (PCV). This study thus establishes the rationale for utilizing lenalidomide as an adjuvant to augment vaccine efficacy in both malignant and non-malignant individuals.

ABSTRACT

Purpose: To demonstrate that the immunomodulatory drug, lenalidomide, can be utilized in patients with relapsed multiple myeloma to augment vaccine responses.

Experimental Design: Early phase clinical trial of multiple myeloma patients that received at least one prior therapy. Patients were treated with single agent lenalidomide and randomized to receive two vaccinations with pneumococcal 7-valent conjugate vaccine (PCV) on different schedules. Cohort A received the first PCV vaccination prior to the initiation of lenalidomide and the second vaccination while on lenalidomide. Cohort B received both vaccinations while on lenalidomide.
**Results:** PCV-specific humoral and cellular responses were greater in Cohort B than A, and were more pronounced in the bone marrow than the blood suggesting that maximal vaccine efficacy was achieved when both vaccines were administered concomitantly with lenalidomide. Patients with a clinical myeloma response showed evidence of a tumor-specific immune response with increases in myeloma-specific interferon-γ⁺ T-cells and reductions in Th17 cells.

**Conclusions:** This is the first clinical evidence demonstrating that lenalidomide augments vaccine responses and endogenous antitumor immunity in patients and as such may serve as an adjuvant for cancer and possibly infectious vaccines.

**Clinical Trial Registration:** NCT00445484
INTRODUCTION

Thalidomide was the first "novel" drug introduced for the treatment of multiple myeloma and has demonstrated considerable antitumor activity through multiple mechanisms, including via the tumor microenvironment through inhibition of angiogenesis and tumor necrosis factor (TNF)-α. Lenalidomide, an IMiD® immunomodulatory agent, inhibits myeloid cell-mediated inflammatory immune function through inhibition of pro-inflammatory cytokines TNF-α and interleukin (IL)-6. It also increases lymphoid immune function by increasing natural killer (NK) cell numbers and antibody-dependent cell-mediated cytotoxicity, and augments NK T-cell numbers and function through increases in CD1d-mediated presentation of glycolipids. Lenalidomide enhances T-cell cytokine production and proliferation by augmenting activator protein (AP)-1 transcriptional activity, reducing the inhibitory effect of cytotoxic T-lymphocyte antigen (CTLA)-4, and possibly reducing the generation of regulatory T-cells (Tregs). This activity suggests that a major mechanism of lenalidomide clinical activity is through its immunomodulatory role within the tumor microenvironment.

Although utilized in myeloma, the impact of single-agent lenalidomide on antigen-specific immune responses in myeloma patients has not been formally examined. Previous studies have indicated that lenalidomide has the potential to enhance immune responses both in vitro and in patients with advanced tumors. In addition, while vaccines can induce immune-responses...
in myeloma patients, the lack of a measurable clinical benefit is largely due to the profound tumor-associated immune tolerance of patients. Thus, current strategies to improve myeloma vaccines must emphasize modulation of the immune system. This study was designed to determine whether lenalidomide could augment vaccine responses and elicit myeloma-specific immune responses when used in combination with the pneumococcal 7-valent conjugate vaccine (PCV; Prevnar®, Wyeth Pharmaceuticals Inc., Philadelphia, PA), a vaccine conjugated to the modified diphtheria toxin (CRM197).

PATIENTS AND METHODS

Patient Eligibility

This was an open-label, two-cohort study in which all patients received lenalidomide in combination with two PCV vaccinations in one of two randomly assigned vaccine schedules. PCV was chosen because of its ability to invoke both T-cell dependent anti-pneumococcal antibody responses and anti-CRM197 T-cell responses. Patients with relapsed myeloma following 1 to 3 prior therapies were included in this study. The study was approved by the institutional review board at the Johns Hopkins Medical Institutions and all patients provided written informed consent.

Patients were enrolled after 1-month of no myeloma treatment. Patients in both cohorts received lenalidomide at a starting dose of 25 mg/day on days 1 to
21 of each 28-day cycle, for a total of 6 cycles. Cohort A received their first vaccination 2 weeks prior to starting lenalidomide and their second on day 14 of cycle 2 (Fig 1). Cohort B received their first vaccination on day 14 of cycle 2 and their second on day 14 of cycle 4. Steroids were prohibited to avoid immunosuppression. Lenalidomide dose reductions were based on standard clinical practice: 20 mg (dose level −1); 15 mg (level −2); 10 mg (level −3); and 5 mg (level −4). Candida-specific, delayed type hypersensitivity (DTH) was administered at enrollment, prior to each vaccination, and 6 weeks after the last vaccination. Erythema and induration to Candida were recorded at 48 hours by measuring the widest diameters in two perpendicular directions. For purposes of immune monitoring, blood and bone marrow samples were obtained as indicated in the study schema. Samples were obtained at baseline in both cohorts: prior to the first Prevnar administration in Cohort A or prior to initiation of lenalidomide in Cohort B. Subsequent sample time points were prior to the second vaccine and 6 weeks after the second vaccine.

Response Assessment

The clinical response to lenalidomide was assessed after each cycle. Patients with a ≥50% decrease in the monoclonal paraprotein levels were defined as responders (R). Patients whose myeloma progressed by an increase in monoclonal paraprotein levels of ≥25% were defined as progressors (PD). Stable disease (SD) was defined as a less than 50% decrease in their monoclonal protein levels.
Immune Analyses

Serological Responses to PCV

Serum IgG levels against 4 (6B, 14F, 19F, and 23F) of the 7 PCV serotypes were measured by enzyme-linked immunosorbent assay as previously described.\(^{18,19}\) Titers were reported in µg/mL by interpolating Abs\(_{450}\) values in the dose-response curve of the pneumococcal reference standard serum 89SF.

Antigen-Specific T-Cell Responses

Peripheral blood lymphocytes (PBL) and bone marrow (BM) cells were thawed in AIM-V media (Invitrogen, Carlsbad, CA), labeled with carboxyfluorescein succinimidyld ester (CFSE; Invitrogen) and incubated for 10 minutes at 37°C. CRM-197 responses were determined by adding the diphtheria-toxin, CRM197 (Sigma, St. Louis, MO) (10 µg/mL) for 5 days at 37°C, and staining with anti-CD3 (BD-Biosciences, San Jose, CA) and anti-interferon (IFN)-\(\gamma\) (e-Biosciences, San Diego, CA) prior to analysis by flow cytometry. Data were acquired on a FACS Calibur (BD-Biosciences) and analyzed using CellQuest software. Antigen-specific T cells were identified as CFSE\(^{\text{low}}\), γIFN\(^+\) CD3\(^+\) T cells. To identify myeloma specific T cells, BM cells were labeled with CFSE (as above) and incubated in either AIM-V alone, SW780 (non-specific bladder carcinoma cell line) lysate or H929 + U266 (myeloma cell line) lysates each. These cell lines were obtained from the ATCC. BM cells were incubated for 5 days in the
presence or absence of the cell lysates, harvested, and stained with anti-CD3 (BD-Biosciences) and IFN-γ (e-Biosciences) prior to analysis by flow cytometry.

**Flow Cytometry**

Cells were stained for cell surface expression of CD3, CD4, CD8, CD40L, CTLA4, CD14, CD19, CD26, CD56, and CD11c (BD-Biosciences). Cells were enumerated utilizing a FACS Calibur and analyzed utilizing CellQuest Pro software. Intracellular staining for FOXP3 (e-Biosciences), IFN-γ, and IL-17 was performed by adding GolgiPlug (BD-Biosciences) per manufacturer’s recommendations. Extracellular staining was performed as described above.

**Statistics**

\( P \)-values were determined utilizing the Graph-Pad \( t \)-test online software.

**RESULTS**

A total of 22 patients were enrolled, 11 in each cohort. Patients were deemed evaluable if they received both PCV vaccinations; 1 patient in Cohort A and 4 in Cohort B showed evidence of disease progression while on study and were not included in this analysis. Characteristics of the patients are summarized in Table 1.

One reliable measure of systemic immunity is the ability of an individual to generate a DTH reaction to the antigen of interest. As such, Candida DTH
reactions were measured in patients at baseline and upon completion of the study. At baseline, patients in Cohort B were more anergic than those in Cohort A (mean areas of induration 6.29 mm$^2$ vs 51.38 mm$^2$, respectively) (Fig 2A). DTH reactivity increased 9.8-fold in Cohort B whereas Cohort A actually showed a decrease (51.38 mm$^2$ to 27.75 mm$^2$) in the DTH response.

**PCV-Specific Immune Responses**

One of the benefits of utilizing the PCV vaccine lies in the ability to measure both humoral responses to pneumococcal antigens as well as the cellular immune response to the carrier molecule, the diphtheria-derived protein, CRM-197. To examine whether PCV-specific responses could be generated and maintained, or augmented by lenalidomide, antibody titers were examined for 4 of the 7 serotypes present in PCV (Fig 2B and 2C). In Cohort A, antibody titers were stable or decreased in both blood and BM across the vaccination schedule. In contrast, antibody titers in Cohort B showed a continuous rise across the vaccination schedule.

To determine the potential of lenalidomide in augmenting antigen-specific T-cell immunity, CRM197 responses were determined at baseline and after each vaccination. In the peripheral blood, Cohort A showed no increase in CRM197-specific T-cell responses in the blood (1.2 fold above baseline)(Fig 2D). In contrast, Cohort B displayed increases at both time points, with a maximal 4.7-fold increase observed after the first vaccination (Fig 2D).
Overall measures of antigen-specific T-cell responses were significantly greater in the BM than in the blood (Fig 2E). This likely reflects the ability of the BM to serve as a reservoir of antigen experienced T cells. After the first vaccination, CRM-197 specific T cell responses were greater in the BM than the blood (Cohort A 7.5% v 2.9%, respectively, $P = .001$; and Cohort B 11.1% v 5.2%, $P = .002$) (Fig 2D,E). Consistent with the data obtained in the blood, PCV-specific T-cell responses were greater in Cohort B than Cohort A. Interestingly, the antigen-specific response to the second vaccination was not blunted in Cohort A but remained stable in Cohort B. To investigate this, we examined the CRM197-specific T-cell responses based on the patients’ clinical responses. Patients with progressive disease (PD) showed a blunted antigen-specific response to the first vaccination when compared with responders (blood 2.6% v 4.5%, respectively, $P = .32$; and BM 5.8% v 9.6%, $P = .006$). As expected, responses to the second vaccination was further decreased in progressors, stable in patients with stable disease, and increased in responders. Cohort A had 30% progressors and 60% with stable disease. Cohort B had 14% progressors and 28% with stable disease. This marked difference in clinical response rates to lenalidomide appears to be associated with corresponding differences in antigen specific immune reactivity where disease progression significantly blunted the CRM-197 immune response. These results are in keeping with previously published work demonstrating the ability of a growing tumor burden to blunt antigen specific T cell responses²⁰.
**T cell Function**

As an immunomodulatory agent with effects on T cell function, we sought to examine the effect of lenalidomide on various immune parameters in both peripheral blood and BM. Flow cytometric analyses were performed for all evaluable patients. At baseline, the only significant differences observed in T cell parameters was a greater percentage of central memory T cells (T<sub>CM</sub>) characterized as CD45RO<sup>+</sup>/CD62L<sup>+</sup> (Fig 3B and C) and fewer regulatory T cells (Tregs) in Cohort B in the BM. All other parameters were similar in both compartments (blood and BM) for both cohorts. Lenalidomide treatment increased the percentage of T<sub>CM</sub> in both compartments whereas no changes were noted in the effector memory T cell population (T<sub>EM</sub>). It also increased the Treg population in Cohort B in the BM, whereas no significant changes to Tregs were appreciable in the blood in either group. Additional statistically significant changes in immune parameters were observed primarily in Cohort B and were most evident in the BM. Specifically, we observed an increase in IFNγ and in CD40L expression on the CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> (Fig 3B,F, H). These changes suggest that antigen specific T cell activation correlates with overall disease response which was greater in Cohort B. Th17 cells were also reduced in the BM of Cohort B while their levels in Cohort A remained unchanged (Fig 3B).
Other immune parameters such as dendritic cell populations and NK populations did not appear affected by treatment. However, increases in NK-mediated cell lysis were observed in both Cohorts A and B (Supplemental Fig 1).

**Myeloma-Specific Immunity**

We examined whether tumor specific immunity could be detected in our patients. Due to the paucity of autologous tumor available in this study and the abundance of antigen-presenting cells (APCs) in the BM capable of capturing, processing and presenting antigen, myeloma-specific immunity was determined utilizing APCs pulsed with allogeneic myeloma cell lysates and the specificity of this response was assessed by comparing the T cell reactivity towards APCs pulsed with the irrelevant bladder cancer cell line (SW780). Absence of non-specific IFN-γ production in the presence of SW780 confirms the absence of non-specific allo-reactivity and the utility of this assay. The tumor-specific immune response increased in Cohort B upon completion of the study with an average antigen-specific CD3 cell percentage of 7.7% up from a baseline of 2.25% (P = .003) (Fig 4A-C). In contrast, Cohort A showed no significant induction of a tumor specific response.

**DISCUSSION**

This is the first study in humans to examine both the general and antigen-specific immunomodulatory properties of lenalidomide. Vaccine-specific humoral and cellular responses were greater in the cohort receiving both vaccinations concomitantly with lenalidomide (Cohort B), thus supporting the
immunostimulatory role of lenalidomide. These data show the multifaceted mechanisms of lenalidomide. It augments global systemic immunity as demonstrated by increases in Candida DTH reactions, and augments NK cell activity (although not necessarily NK cell numbers). In addition, we demonstrate increases in IFN-γ producing T-cells, decreases Th-17 and increases in antigen specific T cell responsiveness which correlate with clinical responses. Taken together, these data strongly support an immune-mediated antitumor effect of lenalidomide.

This study was designed to demonstrate whether vaccine responses could be augmented through the addition of the immunomodulatory drug, lenalidomide. The study utilized the polyvalent pneumococcal vaccine, Prevnar®, because of our ability to measure both humoral and cellular responses. In this study we were able to confirm this synergy by demonstrating increases in the antibody titers and higher antigen specific T cell responses with simultaneous administration of vaccine and lenalidomide. These in vivo findings confirm the numerous reports describing the immunomodulatory effects of lenalidomide.8,22,23 Considering the profound immune dysfunction associated with myeloma,24 strategies to overcome these obstacles should increase immune responsiveness to infectious vaccines. This could reduce infectious complications which currently represent a major morbidity in myeloma.25 In addition, the addition of lenalidomide to immune-based anti-myeloma strategies could augment their efficacy.
Unlike most other studies published to date, our immune analysis primarily focused on the BM for two major reasons. First, this represents the tumor microenvironment and as such the site in which changes in immune function will have the most significant biologic and clinical effects. Second, the BM is a unique site that enriches for antigen-specific T cell responses. This is confirmed in our study by the greater percentage of antigen-specific T cells in both cohorts in the BM compared to blood (Fig 2D and E) and by the greater changes in overall T cell function seen in the BM in response to lenalidomide (Fig 3B).

Vaccine-specific immune responses appeared greater when vaccine was administered concomitantly with lenalidomide (Cohort B). The potential explanation for this lies in the ability of lenalidomide to augment global immune responsiveness. One parameter critical to the successful maintenance of immune response is the ability of T cells to persist long-term in vivo. Central memory T cells (T_{CM}) have been shown to possess the ability to rapidly proliferate upon antigen rechallenge and to migrate to peripheral tissues as compared to effector memory T cells (T_{EM}). Lenalidomide increased the T_{CM} population in both groups although in the BM the effect was more dramatic in Cohort B. Still unclear is why differences in global immune responsiveness are observed in both groups considering that the extent of lenalidomide therapy was the same and, if anything, Cohort B had slightly more aggressive disease going
into the study. The presence of a significant T\textsubscript{CM} population could prove critical to generating effective vaccine responses.

Vaccine-specific responses were primed with PCV to a greater extent when administered concomitantly with lenalidomide (Cohort B) (Fig 2C and D). Interestingly, the T cell responses to the second vaccine administered 8 weeks later was reduced in Cohort A and stable in Cohort B. This is likely explained by accompanying clinical response. More patients showed stable or progressive disease in Cohort A compared to Cohort B (90% vs 43%). In fact, an analysis of the patients based on response rates showed a reduction in T cell responses to PCV in progressors and an increase in PCV T cell responses in patients achieving at least a partial response (data not shown). Similarly, IFN\textsubscript{γ} and CD40L expression was also increased in Cohort B as would be expected with priming of an antigen specific T cell response.

The role of Th17 cells within the BM microenvironment also warrants discussion. Cohort B showed a significant decline in Th17 in the BM whereas these cells initially decreased and then increased in Cohort A. Myeloma-induced production of IL-6 in the presence of transforming growth factor (TGF)-β skews naïve CD4 cells away from T\textsubscript{regs} towards a Th17 phenotype. As pro-inflammatory agents, Th17 cells facilitate the establishment of a chronic inflammatory state that enhances tumor growth and activated osteoclasts leading to worsening of bone disease. We, thus, conclude that an increase in Th17 cells in the BM likely contributes to disease progression in myeloma. Less clear
is whether lenalidomide itself can directly reduce the generation of Th17 cells through the alteration of cytokine expression by the tumor and/or T cells or whether the reduction is the result of a negative feedback loop simply due to a diminishing tumor size.

The role of T\textsubscript{regs} in hematologic malignancies, and specifically myeloma, is less clear. Decreased numbers of presumptive T\textsubscript{regs} have been reported in myeloma patients compared with normal individuals\textsuperscript{30}. Beyer et al. showed a direct correlation between T\textsubscript{regs} in the blood and disease status\textsuperscript{31} which is consistent with our data in the blood. The role of T\textsubscript{regs} in antitumor immunity likely depends upon their role and function within the tumor microenvironment. In colorectal\textsuperscript{32} and nasopharyngeal cancers\textsuperscript{33} high levels of tumor infiltrating T\textsubscript{regs} were associated with improved survival. These studies underscore the importance of examining the immune response within the tumor microenvironment—which in myeloma is the BM. Specifically, Cohort B which had a greater number of responders, we observed an expected increase in IFN-γ\textsuperscript{+} producing Th1 cells and T\textsubscript{regs} with an associated decrease in Th17 cells in the BM. Although seemingly at odds with previously published clinical data, the increase in T\textsubscript{regs} in patients with clinical responses suggests a potential beneficial role of T\textsubscript{regs} in myeloma.

Extensive pre-clinical data suggests that a major component of lenalidomide’s activity is in augmenting immune responsiveness. Because of the
intrinsic immune-mediated anti-myeloma activity of lenalidomide, it is difficult to separate the antitumor effects from its effect as a vaccine immune-modulator. However, we showed that increased immune responsiveness correlated with both increased vaccine-specific immunity (Fig 2D). This strongly implies an immunomodulatory effect of lenalidomide and not just a tumor-specific cytotoxic effect of the drug. We also observed other immunomodulatory aspects of lenalidomide that underscore its positive immune effects, including an increase in NK cytolytic activity and T_{CM} in both the blood and BM.

PCV in combination with lenalidomide generated interesting and unexpected results. This vaccine was chosen because of its ability to prime both humoral and cellular responses that would enable us to examine both arms of the immune response in patients treated with lenalidomide. We have shown greater increases in both pneumococcal antibody titers as well as CRM197-specific T-cell responses in Cohort B, which received both vaccinations while on lenalidomide. However, other findings warrant discussion. First, the T-cell-specific responses were greater in the BM than in the blood with more than double the percentage of antigen-specific T-cells. This finding significantly underscores the uniqueness of the BM as an immune niche of antigen-specific T-cells.\textsuperscript{34,35} Considering that the operative immunosuppressive mechanisms are likely to be greatest within the tumor microenvironment, the augmented immune responses in the BM compared with the blood of myeloma patients are even more significant. Second, the generation of myeloma-specific immunity upon
completion of vaccination was statistically significant in Cohort B whereas it was negligible in Cohort A. The average daily dose of lenalidomide was equivalent in both groups (Cohort A 21.6 mg/day v Cohort B 19.2 mg/day) as was the overall tumor burden. The only appreciable differences between these two groups were the vaccination schedules and clinical responses to lenalidomide treatment.

In summary, several conclusions can be made from this initial pilot study. This study is the first to show the in vivo immunomodulatory properties of lenalidomide in patients manifest as increases in both global and vaccine-specific immunity as well as provide evidence of myeloma-specific immunity. To further expand and confirm these observations, a clinical trial is open utilizing the lenalidomide platform upon which cancer vaccines will be integrated. We will also determine whether lenalidomide could be employed as an adjuvant for the administration of infectious vaccines in a non-cancer patient population—a use that could increase vaccine efficacy especially in situations of limited vaccine supply.
**Authorship**

KN: designed, conducted and analyzed immune monitoring experiments and contributed to writing of the manuscript. LR: performed the experiments and analyzed the data. AF: research nurse for the trial. AE: contributed patients to the study. MP: conducted and analyzed laboratory data. CAH: contributed patients to the study, reviewed the manuscript. IB: designed the clinical trial, analyzed the data and wrote the manuscript. Funding for the study was provided by Celgene and IB is also a paid consultant for Celgene.

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FIGURE LEGENDS

Fig 1. Trial schema. Patients were assigned to either Cohort A which received the first PCV 14 days prior to initiation of lenalidomide and the second on day 14 of cycle 2 of lenalidomide or Cohort B in which the first PCV was administered day 14 of cycle 2 and the second day 14 of cycle 4. Blood and bone marrow were obtained at the indicated time points for immune monitoring assays.

Fig 2. PCV-specific responses. (A) DTH responses to Candida administered at baseline and 6 weeks after the last vaccine. (B) Cohort A and (C) Cohort B pneumococcal antibody response averages to 4 subtypes (6B, 14F, 19F and 23F) in PBL and BM plasma obtained prior to initiation of the first intervention (PCV vaccination for Cohort A or lenalidomide for Cohort B), 8 weeks after the first vaccine (Post Vac 1) or 8 weeks after the second vaccine (Post Vac 2). Data are graphed as fold difference compared to screen sample. (D) PBL and (E) BM T-cell responses to CRM197; CFSE-labeled PBLs or BM cells were incubated with CRM197 for 3 days after which antigen-specific T-cells were analyzed by flow cytometry as CD3⁺/CFSE<sup>low</sup>/IFN-γ⁺. Data shown are for CD3⁺/CFSE<sup>low</sup> averages from Cohort A and B pre- and post-PCV vaccination 1 and 2. Comparisons in which the p value is <0.05 is indicated by (*).

Fig 3. Flow Cytometric Analysis. (A) Peripheral blood lymphocytes (PBLs) or (B) bone marrow obtained at the indicated time points were labeled with the
indicated antibodies and analyzed by flow cytometry. Comparisons in which the p value is <0.05 is indicated by (+). (C)(D) Representative flow cytometric analysis of BM T cells utilizing CD45RO vs CD62L staining of a representative patient in Cohort A and B, respectively. (E)(F) Representative flow cytometric analysis for CD4+CD40L+ of BM T cells for a patient in Cohort A and B, respectively. (G)(H) Representative staining of CD4+IFNγ OF BM T cells for representative patients in Cohort A and B.

**Fig 4. Myeloma-specific responses.** (A) CFSE-labeled BM cells in either media alone, pulsed with SW780 (non-specific bladder carcinoma cell line) lysate (as negative control) or with H929 + U266 (myeloma cell line) lysate were incubated for 5 days. Cells were stained for CD3+ and IFN-γ. Averages of CD3+/CFSElow/IFN-γ producing cells were analyzed for Cohort A and Cohort B patients pre-treatment and 6 weeks following their last vaccination. (B) FACs example of a post-vaccine response to media alone, SW780 lysate, and H929 + U266 lysate of a patient with progressive disease or (C) responsive disease. Comparisons in which the p value is <0.05 is indicated by (*).

**Fig 4. Impact of lenalidomide on CD4 immunity:** Lymphocytes were analyzed by intracellular staining for CD4+/CD25+ that express FOXP3 cells (Tregs) and CD3+/IFN-γ+ cells (Th1) in the peripheral blood (A) or bone marrow for Tregs, Th1 and Th17 (B). The data was also graphed on the basis of the clinical response to treatment for the peripheral blood (C) or BM (D). (E) Representative flow
cytometric analyses of CD3⁺/IFN-γ⁺ staining of PBL and BM samples of a patient with progressive disease and one with a complete response to lenalidomide treatment. Isotype staining, screen sample, and C6D1 of lenalidomide treatment samples are shown. Comparisons in which the p value is <0.05 is indicated by (*).
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Supplemental Fig 1. NK Cytolytic Activity. (A) Cohort A and (B) Cohort B

natural killer functional assays: K562 cells were labeled with Mito-tracker green, a fluorescent dye, and admixed with PBLs (peripheral blood lymphocytes) at varying ratios (10 PBL:1 K562 – 1 PBL:1 K562). PI (propidium iodide), a measurement of cell death, was also added. Lysis of K562 cells was analyzed by examining the double-positive population of cells and was measured on day 3 in the presence or absence of PBLs.
Figure 1

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# Immune monitoring time-points
Figure 3

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%Cells

Cohort A  Cohort B

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Cohort A BM

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<th>Screen</th>
<th>Post Vac1</th>
<th>Post Vac2</th>
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</thead>
<tbody>
<tr>
<td>CD62L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^0</td>
<td>27.4%</td>
<td>34.2%</td>
<td>23.2%</td>
</tr>
<tr>
<td>10^1</td>
<td>32.5%</td>
<td>36.4%</td>
<td>26.4%</td>
</tr>
<tr>
<td>10^2</td>
<td></td>
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</tbody>
</table>

Cohort B BM

<table>
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<tbody>
<tr>
<td>CD62L</td>
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</tr>
<tr>
<td>10^0</td>
<td>22.1%</td>
<td>34.2%</td>
<td>48.5%</td>
</tr>
<tr>
<td>10^1</td>
<td>21%</td>
<td>29.1%</td>
<td>34.4%</td>
</tr>
<tr>
<td>10^2</td>
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</tbody>
</table>

CD45RO

<table>
<thead>
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<th></th>
<th>Screen</th>
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<th>Post Vac2</th>
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<tbody>
<tr>
<td>CD40L</td>
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<td></td>
</tr>
<tr>
<td>10^0</td>
<td>2.5%</td>
<td>3.2%</td>
<td>2.3%</td>
</tr>
<tr>
<td>10^1</td>
<td>1%</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>10^2</td>
<td></td>
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</tbody>
</table>

CD4

<table>
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<th></th>
<th>Screen</th>
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<th>Post Vac2</th>
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<tbody>
<tr>
<td>IFNγ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^0</td>
<td>1.6%</td>
<td>4.7%</td>
<td>1.9%</td>
</tr>
<tr>
<td>10^1</td>
<td>0.9%</td>
<td>2.1%</td>
<td>13.3%</td>
</tr>
<tr>
<td>10^2</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure 4

A

Percent CD3+CFSE low/IFNγ

No lysate SW780 H929+U266

- Cohort A Pre
- Cohort A Post
- Cohort B Pre
- Cohort B Post

B

CD3 APC

CFSE

Counts

IFN-γ

No lysate +SW780 +H929/U266

0.67% 0.71% 1.25%

C

CD3 APC

CFSE

Counts

IFN-γ

No lysate + SW780 +H929/U266

1.59% 1.88% 5.83%
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cohort A</th>
<th>Cohort B</th>
</tr>
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<tbody>
<tr>
<td>Total Number</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Median Age</td>
<td>67 (54-80)</td>
<td>65 (53-77)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>40%</td>
<td>71%</td>
</tr>
<tr>
<td>Female</td>
<td>60%</td>
<td>28%</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African Americans</td>
<td>30%</td>
<td>28%</td>
</tr>
<tr>
<td>Caucasian</td>
<td>70%</td>
<td>57%</td>
</tr>
<tr>
<td>Other</td>
<td>0%</td>
<td>14%</td>
</tr>
<tr>
<td>Prior Therapies (1-3)</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Myeloma Subtypes</td>
<td></td>
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</tr>
<tr>
<td>IgA</td>
<td>50%</td>
<td>14%</td>
</tr>
<tr>
<td>IgG</td>
<td>40%</td>
<td>57%</td>
</tr>
<tr>
<td>Light Chain</td>
<td>10%</td>
<td>28%</td>
</tr>
<tr>
<td>Percentage Plasma Cells</td>
<td>26%</td>
<td>35.20%</td>
</tr>
<tr>
<td>Overall Response Rate</td>
<td>10%</td>
<td>57%</td>
</tr>
</tbody>
</table>
Lenalidomide-Induced Immunomodulation in Multiple Myeloma: Impact on Vaccines and Antitumor Responses

Kimberly Noonan, Lakshmi Rudraraju, Anna Ferguson, et al.

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