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TITLE: Second-Generation Therapeutic DNA Lymphoma Vaccines

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**ABSTRACT**

Lenalidomide is an effective therapeutic agent with direct inhibitory effects on malignant B- and plasma cells and immunomodulatory effects on the T cell activation. The dual function of lenalidomide makes it an appealing candidate for combination with other novel agents for lymphoma and myeloma therapy. In this study, we investigated the immune stimulatory effects of lenalidomide, administrated to mice in doses which provided comparable pharmacokinetics to human patients, on the potency of a novel fusion DNA lymphoma idiotype vaccine. The combination protected mice from lethal challenge with syngeneic A20 murine lymphoma, resulting in significantly improved survival comparing with vaccine or lenalidomide alone and induced immune memory. In vivo depletion experiments demonstrated a requirement for effector CD8+ and CD4+ T cells. Surprisingly, lenalidomide alone was associated with reduced numbers of systemic immune suppressive cells (MDSC/Treg) in tumor-bearing, but not naïve mice, an effect that was independent of tumor burden reduction, suggesting a role of lenalidomide in ameliorating tumor-induced immune suppression. Finally, the combination of lenalidomide and vaccine produced significantly improved survival, compared with controls receiving vaccine or lenalidomide alone in mice with 7 day established tumors. These results demonstrated the dual effect of lenalidomide on enhancing antigen-specific T-cell immunity and a novel mechanism of action reversing tumor immune suppression, which makes it ideal for combination with active specific immunotherapy.

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**Subject Terms**

Lymphoma DNA vaccine Tumor antigen

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Lenalidomide is an effective therapeutic agent with direct inhibitory effects on malignant B- and plasma cells and immunomodulatory effects on the T cell activation. The dual function of lenalidomide makes it an appealing candidate for combination with other novel agents for lymphoma and myeloma therapy. In this study, we investigated the immune stimulatory effects of lenalidomide, administrated to mice in doses which provided comparable pharmacokinetics to human patients, on the potency of a novel fusion DNA lymphoma idiotype vaccine. The combination protected mice from lethal challenge with syngeneic A20 murine lymphoma, resulting in significantly improved survival comparing with vaccine or lenalidomide alone and induced immune memory. In vivo depletion experiments demonstrated a requirement for effector CD8+ and CD4+ T cells. Surprisingly, lenalidomide alone was associated with reduced numbers of systemic immune suppressive cells (MDSC/Treg) in tumor-bearing, but not naïve mice, an effect that was independent of tumor burden reduction, suggesting a role of lenalidomide in ameliorating tumor-induced immune suppression. Finally, the combination of lenalidomide and vaccine produced significantly improved survival, compared with controls receiving vaccine or lenalidomide alone in mice with 7 day established tumors. These results demonstrated the dual effect of lenalidomide on enhancing antigen-specific T-cell immunity and a novel mechanism of action reversing tumor immune suppression, which makes it ideal for combination with active specific immunotherapy.
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1. Introduction

The ultimate goal of a cancer vaccine is to prevent or cure cancer effectively without causing collateral damage. Thus far, current cancer vaccines have not been successful therapeutic agents for cancer patients. Additionally, mechanisms of immunological tolerance to cancer make the generation of immune response to cancer difficult. Therefore, new and safe strategies which can break immune tolerance against tumor antigens are needed in order to improve the efficacy of current cancer vaccines.

Adjuvants, agents which help break immunological tolerance and enhance immune response, are a crucial component of cancer vaccines. In order to enhance the capability of the adjuvant to generate antigen specific immunity, many laboratories have described strategies whereby adjuvants or cytokines are fused to targeted antigen. We previously described the strategy of enhancing the immunogenicity of tumor antigens by fusion of antigens with a chemokine receptor ligand. Thus far, these vaccines have been shown to elicit antigen specific immunity in preclinical lymphoma models.

2. Summary of Progress as related to Project Tasks (SOW)

TASK 1: DESIGN AND CREATE CANDIDATE DNA VACCINE CONSTRUCTS AND
TASK 2: SELECT THE OPTIMAL CHEMOKINE RECEPTOR LIGAND FOR FUSION WITH MODEL ANTIGENS

These tasks have been completed.

TASK 3: ESTABLISH AND CHARACTERIZE AN A20 LYMPHOMA IDIOTYPE-SPECIFIC CD8+ T-CELL LINE.

These studies in the lymphoma model are currently being postponed, as we prioritized the studies below using model systems for which TCR transgenic mice already are available as a source of clonal CD8+ antigen-specific T cells.

TASK 4: INVESTIGATE MECHANISMS BY WHICH ANTIGEN IS CROSS-PRESENTED TO CD8+ T CELLS AND
TASK 5: DETERMINE WHETHER CO-ADMINISTRATION OF CHEMOKINE-ANTIGEN VACCINES WITH IMMUNE ADJUVANTS CAN IMPROVE VACCINE POTENCY

Lenalidomide enhanced lymphoma vaccine-induced prophylactic and memory antitumor immunity

The immunomodulatory activity of lenalidomide provides a potential opportunity to use this anti-myeloma drug as a vaccine adjuvant. To test this hypothesis, we evaluated the antitumor effect of combination therapy with a previously described lymphoma idotype DNA fusion vaccine together with lenalidomide used in various doses and schedules. The most potent protective effect against lethal A20 murine lymphoma challenge was observed in the two groups treated with either a low dose (5 mg/kg) of lenalidomide for 35 consecutive days or a high dose (50 mg/kg) using an intermittent schedule of total 6 injections. The median tumor development time was 33 days for both groups compared with 26.5 days for vaccine alone controls. Interestingly, continuous administration of high dose lenalidomide failed to potentiate the vaccine-induced tumor protection. Lower doses of lenalidomide were not effective. Based on these preliminary results, the treatment schedule using 5 mg/kg for 35 consecutive days was chosen for further development of lenalidomide as a vaccine adjuvant. In addition, pharmacokinetic analysis
showed that following the final dose on Day 35, lenalidomide was rapidly absorbed with \( \tau_{max} \) of 0.5 hour, and the mean \( C_{max} \) and \( AUC_t \) values were 2520 ng/mL and 2940 ng*h/mL, respectively, which was comparable to plasma levels achieved in human patients receiving a daily dose of 25 mg (\( AUC_t \): 3773 ng*h/mL). In the prophylactic experiment, combing lenalidomide with the vaccine resulted in 80% long-term survival in tumor-challenged mice, compared with 30% in the vaccine alone group (P=0.045). Lenalidomide, when used alone, had no protective antitumor effect compared with PBS control. More than 75% of the mice protected from primary tumor challenge in the combination group were resistant to secondary challenge (p<0.01), suggesting the development of antitumor immune memory.

**Antigen-specific antibody responses are not enhanced by lenalidomide**

Antibodies against the antigen (idiotype) were easily detected in 4 out of 5 mice immunized with the vaccine alone. However, adding lenalidomide to the vaccination did not potentiate antigen-specific humoral immunity. The serum titers of antibodies in mice immunized with the combination vaccine + lenalidomide were similar to those found in the mice receiving vaccine alone. The specificity of antibody response was confirmed by showing that the antibodies found in mice receiving vaccine, either alone or combining with lenalidomide, did not bind to an isotype-matched Ig of irrelevant idiotype and by the observation that neither lenalidomide nor PBS treated mice developed idiotype-specific antibody responses. These data suggest that the adjuvant effect of lenalidomide is not due to the enhancement of antibody responses.

**T cell-mediated immunity is involved in the vaccine-potentiating effect of lenalidomide**

We attempted to detect A20 idiotype peptide-specific T cell responses to a reported MHC Class I binding-epitope in the vaccinated mice, but failed to observe splenic CD\(^{8}\) T cells that could specifically recognize the candidate peptide by ELISPOT. Alternatively, we performed \textit{in vivo} T-cell depletion to determine the role of cellular immunity in the protective antitumor effect of the combination therapy. T-cell depletion was achieved by i.p. injection of anti-CD8 (clone 2.43) and/or anti-CD4 (clone GK 1.5) monoclonal antibodies at the effector phase. The results showed that tumor protection elicited by the combination of vaccine + lenalidomide was abrogated partially by CD4\(^{+}\) or CD8\(^{+}\) T cell depletion alone, but completely abrogated by CD8 T-cell depletion in combination with CD4 T-cell depletion. Specifically, without T-cell depletion, 60% of vaccinated mice were alive on Day 60 after tumor challenge, compared with 30% with the treatment of anti-CD4 antibodies, and 10% for CD8 or zero for CD8/CD4 depletion. Taken together, the results suggest that T cells, especially CD8 effector cells are required for the vaccine-potentiating effect of lenalidomide.

**Lenalidomide did not affect MDSC, Treg or NK cell numbers in naïve mice**

To further explore potential cellular mechanisms of the adjuvant effect of lenalidomide, we investigated its effects on other immune cells including myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg) and nature killer cells (NK). In naïve mice, treatment of 5 mg/kg lenalidomide for 35 consecutive days did not alter the numbers of splenic MDSC. In the spleens of lenalidomide-treated mice there were 1.68% ± 0.22 Gr-1\(^{+}\)CD11b\(^{+}\) MDSC, compared with 1.70% ± 0.12 and 1.96% ± 0.27 in PBS-treated and untreated mice, respectively. MDSC slightly increased to 2.38% ± 0.29 when lenalidomide was combined with the idiotype vaccine; however, the change was not statistically significant. Likewise, lenalidomide treatment did not change the proportion of Foxp3\(^{+}\) Treg in CD4\(^{+}\) T cell population. Splenic Treg frequencies were 16.67% ± 0.52, 16.97% ± 0.27 and 19.03% ± 0.51 in the mice receiving lenalidomide, vaccine and the combination, respectively. These frequencies were comparable to those found in untreated (18.27% ± 1.38) and PBS controls (17.57% ± 0.87). The CD49b\(^{+}\)CD3\(^{-}\) NK cell population in CD3- lymphocytes also remained unchanged in mice treated with lenalidomide.
alone (7.78% ± 0.44, compared with 7.38% ± 0.4 in spleens of untreated mice and 7.11% ± 0.69 in PBS controls). Combining lenalidomide with vaccine was associated with a slight decrease of NK cells (6.5% ± 0.23); however, this effect was not lenalidomide-specific since the vaccine alone induced reduction of NK cells to a similar extent (6.59% ± 0.15).

**Lenalidomide effects on MDSC, Treg, and NK cells in tumor-bearing mice**

We then investigated the effects of lenalidomide on MDSC, Treg and NK frequencies in mice bearing established A20 tumors. Tumor challenge dramatically induced splenic MDSC (11.47% ± 1.07, compared with 1.57% ± 0.34 in naive mice) and Treg (31.05% ± 1.49 vs. 18.15% ± 0.58). Treating tumor-bearing with 5 mg/kg lenalidomide for 21 consecutive days was associated with a reduction in splenic MDSC (1.39% ± 0.05), comparable to non-tumor-bearing naïve mice, as well as tumor regression in 60% of mice (not shown). To determine whether the effect of lenalidomide on MDSC numbers could be dissociated from tumor burden reduction, we treated mice with cyclophosphamide, which also induced tumor regression. Although cyclophosphamide induced complete tumor regression in all treated mice, its effect on MDSC was insignificant (8.26% ± 1.35). Unlike their differential roles on MDSC, lenalidomide and cyclophosphamide treatments both were associated with reduction in splenic Treg in tumor-bearing mice.

A slight but statistically significant reduction of splenic NK cell numbers was observed in tumor-bearing compared with naïve mice (6.6% ± 0.51 vs. 8.53% ± 0.4). However, treatment with cyclophosphamide was associated with further reduction in NK cells (4.21% ± 0.58). Lenalidomide treatment restored NK cell numbers (8.54% ± 0.29). Altogether, our data suggest that lenalidomide has the potential to reverse systemic tumor-induced immune suppression by reducing MDSC, Treg and possibly rescuing NK cells, and this effect is independent of its effects on simple tumor burden reduction.

**Combining lenalidomide with lymphoma vaccine demonstrates additive therapeutic antitumor effect against established tumor burdens**

A therapeutic study against established tumors was carried out based on the findings above that lenalidomide both potentiates adaptive T-cell immunity and ameliorates systemic tumor-induced immune suppression. Compared with 15% and 25% survival in lenalidomide (p=0.0052) and vaccine treated mice (p=0.035), respectively, on day 50, the combination was associated with 50% long term survival. Vaccine + lenalidomide combination, lenalidomide alone, and vaccine alone groups were all superior to PBS controls (P<0.0001, 0.0008, and 0.0043, respectively, vs. PBS).

3. **Key Research Accomplishments**

   a. This study is the first report of lenalidomide as an immune adjuvant to enhance the efficacy of cancer vaccine. The fact that lenalidomide is FDA-approved for its antitumor activity in multiple myeloma makes it particularly attractive for consideration as an adjuvant in combination with novel vaccine therapies in clinical trials in this and other B-cell malignancies.

   b. Our data suggest that one of the immune mechanisms of lenalidomide-induced adjuvant effect is to facilitate the development specific T-cell immunity against a model B-cell tumor antigen. The results support the hypothesis that that lenalidomide could serve as an ideal adjuvant in combination with cancer vaccines designed to activate T-cell immunity.

   c. **Publication of results:**

The following manuscript will be submitted for publication:
Potent immunomodulatory effects of Lenalidomide on effector T cells and tumor immune suppression improve the effectiveness of a therapeutic lymphoma vaccine, Ippei Sakamaki, Larry W. Kwak, Soung-Chul Cha, Qing Yi, Beatrisa Lerman, Sekhar Surapaneni, Scott Bateman, Hong Qin

4. Outcomes, Conclusions, and Future Plans
We demonstrate here that lenalidomide has potent adjuvant effects on cancer vaccines, especially those designed to activate anti-tumor cellular immunity. It has dual effects of facilitating effector T-cell immune response and ameliorating tumor-induced immune suppression. Our findings provide the basis for future clinical studies of using lenalidomide as an immune adjuvant for vaccine therapy. We have future plans to apply these results to design a Phase I clinical trial.