

Accumulation of Memory Precursor CD8 T Cells in Regressing Tumors following Combination Therapy with Vaccine and Anti-PD-1 Antibody

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Abstract

Immunosuppression in the tumor microenvironment blunts vaccine-induced immune effectors. PD-1/B7-H1 is an important inhibitory axis in the tumor microenvironment. Our goal in this study was to determine the effect of blocking this inhibitory axis during and following vaccination against breast cancer. We observed that using anti-PD-1 antibody and a multipeptide vaccine (consisting of immunogenic peptides derived from breast cancer antigens, neu, legumain, and β -catenin) as a combination therapy regimen for the treatment of breast cancer-bearing mice prolonged the vaccine-induced progression-free survival period. This prolonged survival was associated with increase in number of Tc1 and Tc2 CD8 T cells with memory precursor phenotype, CD27⁺IL-7R^{hi}T-bet^{lo}, and decrease in number of PD-1⁺ dendritic cells (DC) in regressing tumors and enhanced antigen reactivity of tumor-infiltrating CD8 T cells. It was also observed that blockade of PD-1 on tumor DCs enhanced IL-7R expression on CD8 T cells. Taken together, our results suggest that PD-1 blockade enhances breast cancer vaccine efficacy by altering both CD8 T cell and DC components of the tumor microenvironment. Given the recent success of anti-PD-1 monotherapy, our results are encouraging for developing combination therapies for the treatment of patients with cancer in which anti-PD-1 monotherapy alone may be ineffective (i.e., PD-L1-negative tumors). *Cancer Res*; 74(11); 2974–85. ©2014 AACR.

Introduction

Vaccination, targeting self-antigens, to treat cancer has been evaluated for several years and the results overwhelmingly show that the approach, used alone, is not effective to any significant extent at mediating tumor regression. Immune regulatory networks, soluble inhibitory factors, and regulatory cells that prevail throughout the body and in the tumor microenvironment (TME) mediate tolerance to self-antigens and inhibit vaccine-induced effector responses (1). Recent research suggests that much of the immune suppression in the TME of several diseases is mediated by the PD-1/PD-L1 axis (2–10). Extensive studies targeting this inhibitory axis using anti-PD-1 and anti-B7-H1 antibodies are being done in pre-clinical models and in the human clinical setting (11, 12). Results obtained from recent trials testing anti-PD-1 and anti-B7-H1 antibodies as monotherapy in patients with differ-

ent types of cancers are encouraging (13, 14). In our recent study, we have shown that PD-1 and B7-H1 both mediate immune suppression in a far more complex manner than engagement of B7-H1 on tumor cells with PD-1 on tumor-infiltrating T cells. For example, we found that both PD-1 and B7-H1 are expressed on infiltrating dendritic cells (DC) during late tumor expansion (15). As emerging data suggest that there is more complexity associated with PD-1/B7-H1 axis (15, 16), additional studies are warranted to understand the unique contributions of PD-1 and B7-H1 to immune suppression in the TME and provide insight into how to target this axis therapeutically.

The generation of memory CD8 T cells is the major goal of active immunotherapy against cancer and blockade of suppressive pathways in the TME along with vaccination has the potential to induce long-lasting protection or result in complete cures (17). In this study, we used a multipeptide antitumor infrastructure vaccine-targeting epithelial tumor cells with neu, breast cancer stem cells (BCSC) and tumor-associated macrophages (TAM) with legumain, and both epithelial tumor cells and BCSCs with β -catenin. BCSCs are resistant to therapy (18) and targeting them is predicted to be associated with significant clinical benefit (19). TAMs (M2 macrophages) exhibit many protumoral functions including promotion of angiogenesis, matrix remodeling, suppression of adaptive immunity (20) and their density in lesions correlates with poor prognosis (21). With an aim to develop a therapeutic strategy that can prevent the recurrence of breast cancer, in

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this study in addition to targeting neu expressing proliferating malignant epithelial cells, we targeted BCSCs and TAMs that are critical to tumor growth. We combined the multi-peptide vaccine with anti-PD-1 antibody as combination therapy against breast cancer in a mouse model observing enhanced survival, increased antigen-specific T-cell responses, a substantial increase in the infiltration of CD8 T cells with a memory precursor effector cell (MPEC) phenotype into regressing tumors and a sharp decrease in the number of tumor-infiltrating immunosuppressive PD-1⁺ DC population. Finally, we showed that blockade of PD-1 on tumor DCs resulted in increase in the expression of memory marker (IL-7R) on CD8 T cells.

Materials and Methods

Mice

Female, 6 to 8 weeks old BALB/c mice were from Jackson Laboratories. Animal care and use were in accordance with institutional guidelines.

Cell lines

TUBO and P815 cell lines were provided by Dr. L. Pease of the Mayo Clinic in 2010 (22). The RAW 264.7 cell line was provided by Dr. A. Schrum of the Mayo Clinic in 2010. All cell lines were authenticated as mouse origin by IDEXX BioResearch.

Epitope prediction

Using epitope prediction programs BIMAS, ProPep-I, and SYFPEITH, sequences of rat neu (neu), legumain, and β -catenin were analyzed and three peptides from each protein with high affinity for MHC class I were identified. The peptide sequences were neu: TYVPANASL (N1), SYGVTVWEL (N2), PYNYLSTEV (N3); legumain: TYEHALRYL (L1), MYQKMFVYI (L2), RYLYV-LANL (L3); β -catenin: SYLDSGIHS (C1), RAIPELTKL (C2), AYGNEQSKL (C3).

Peptides, adjuvants, and antibodies

See Supplementary Materials.

Vaccination

Nontumor-bearing BALB/c mice were given three doses (100 μ g peptides in Complete Freund's adjuvant (CFA) on day 0 and 100 μ g peptides in Incomplete Freund's adjuvant (IFA) on day 3 and day 6) of peptide vaccine subcutaneously at the base of the tail. To determine the effect of PD-1 blockade in enhancing the efficacy of peptide vaccine, mice were given four intraperitoneal (i.p.) injections (on days 0, 3, 6, 9) of PD-1 blocking antibody (200 μ g/mouse/injection) along with the peptide vaccine that was given as per the schedule described above.

In vivo tumor studies

Mice were implanted with 1×10^5 TUBO cells subcutaneously on the right flank. To determine the effect of vaccine, mice were given a total of 3 injections of peptide vaccine in CFA (day 16) and IFA (days 19 and 22) posttumor challenge. For determining the combination effect of PD-1 blockade with peptide vaccine on tumor growth, mice were given 8 i.p.

injections (on days 7, 10, 13, 16, 19, 22, 25, and 28) of PD-1-blocking antibody (200 μ g) and three immunizations of peptide vaccine in CFA/IFA (on days 16, 19, and 22) as described above. The dose of anti-PD-1 was based on our previous studies, which identified 200 μ g as an optimal dose for treating ovarian cancer (15). Anti-PD-1 was started before vaccination to insure steady-state concentrations (23). For *in vivo* cell depletion, anti-CD4 mAb (0.2 mg/dose) or anti-CD8 mAb (0.5 mg/dose) were injected intraperitoneally (days 13–15) posttumor challenge followed by one dose on a weekly basis. For survival experiments, mice with the tumor size 300 mm² were considered moribund.

Measurement of immune responses

T-cell responses and serum MCP-1 levels were measured by enzyme-linked immunosorbent spot assays (ELISpot), multiplexed cytokine assays and enzyme-linked immunosorbent assay (ELISA) respectively as described previously (15, 24). Methods are detailed in the Supplementary Methods.

In vitro PD-1 blockade on tumor-infiltrating DCs

The effect of blockade of PD-1 on tumor DCs in inducing IL-7R and T-bet expression by splenic CD8 T cells was determined using *in vitro* coculture. DCs and CD8 T cells from immunized mice were enriched from tumors and spleen, respectively using CD11c and CD8 microbeads (Miltenyi; ref. 15). Tumor DCs were cultured overnight in the presence of anti-PD-1 or isotype control antibodies (10 μ g/mL). Antibody was then washed from the culture, fresh media was added and the DCs were cocultured with splenic CD8 T cells at 1:4 ratio for 24 hours. Single-cell suspensions obtained from culture wells were stained to determine the expression of IL-7R and T-bet by splenic CD8 T cells.

Flow cytometry

Cell surface molecule staining and flow cytometry were done as previously described (15, 25). Serum antitumor antibody analysis was done using flow cytometry and the methods are detailed in the Supplementary section.

Statistical analysis

Two-tailed Mann-Whitney tests, one-way ANOVAs, or Student *t* tests from GraphPad InStat or GraphPad Prism software were used to analyze the data unless otherwise stated. *P* < 0.05 was considered as significant. For survival analysis, Kaplan-Meier test was used.

Results

A peptide vaccine targeting multiple tumor antigens is immunogenic and induces regression of breast cancer

Immunogenicity of peptides of each of the antigens, identified by epitope prediction programs, was tested by immunizing mice as described in Materials and Methods. Three immunogenic peptides C3, L3, and N1 were identified (Fig. 1A). In the next step, the immunogenicity of these three peptides as a multi-peptide vaccine, C3L3N1, was tested. As shown in Fig. 1B, the multi-peptide vaccine induced IFN- γ responses against individual peptides and antigen-expressing TUBO and legumain⁺ RAW cells (26). IFN- γ responses against the TUBO

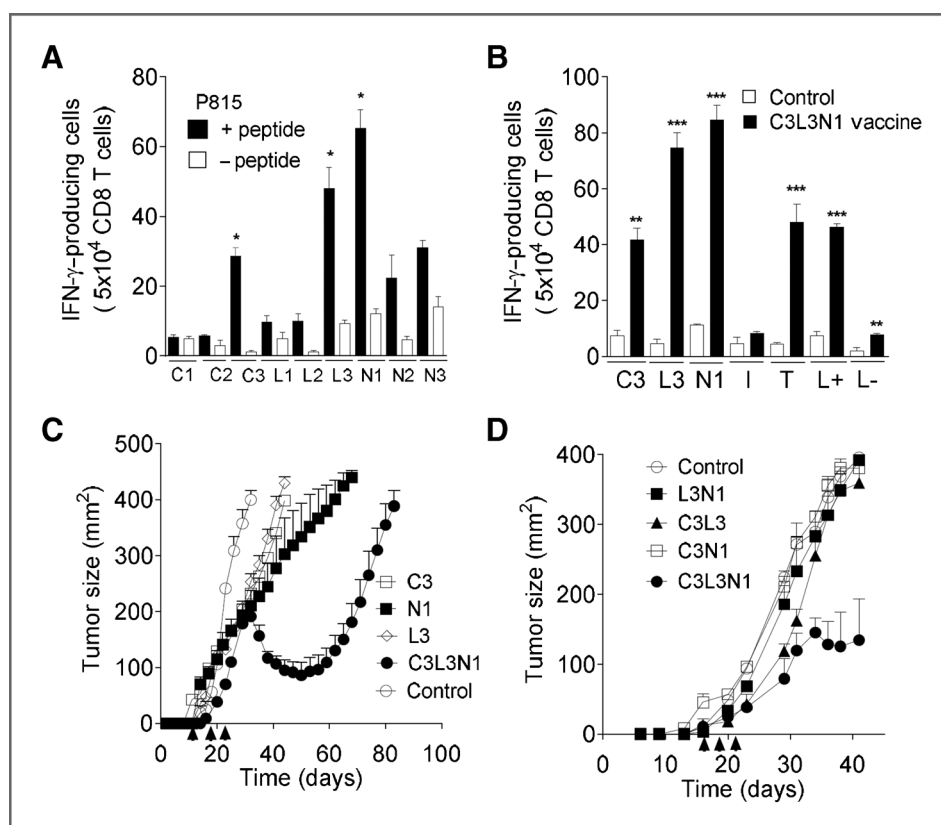


Figure 1. Multi-peptide vaccine induces CD8 T cell responses and reduces tumor burden. A, the mean number (\pm SEM, $n = 3$) of CD8 T cells producing IFN- γ , as assessed by ELISpot, in response to stimulation with P815 cells (A) alone or pulsed with predicted peptides and C3, L3, N1, and control (I), legumain+ (L+) and legumain- (L-; B) RAW cells and neu⁺ and β -catenin⁺ TUBO (T) cells. C and D, tumor growth curves for BALB/c mice vaccinated with individual peptides compared with the C3L3N1 vaccine (C) and combinations of two peptides and C3L3N1 vaccine (D). Symbols show mean tumor size ($n = 4$ per group, \pm SEM). Data are representative of one of three experiments yielding similar results. *, $P < 0.05$; **, $P < 0.005$, ***, $P < 0.0005$.

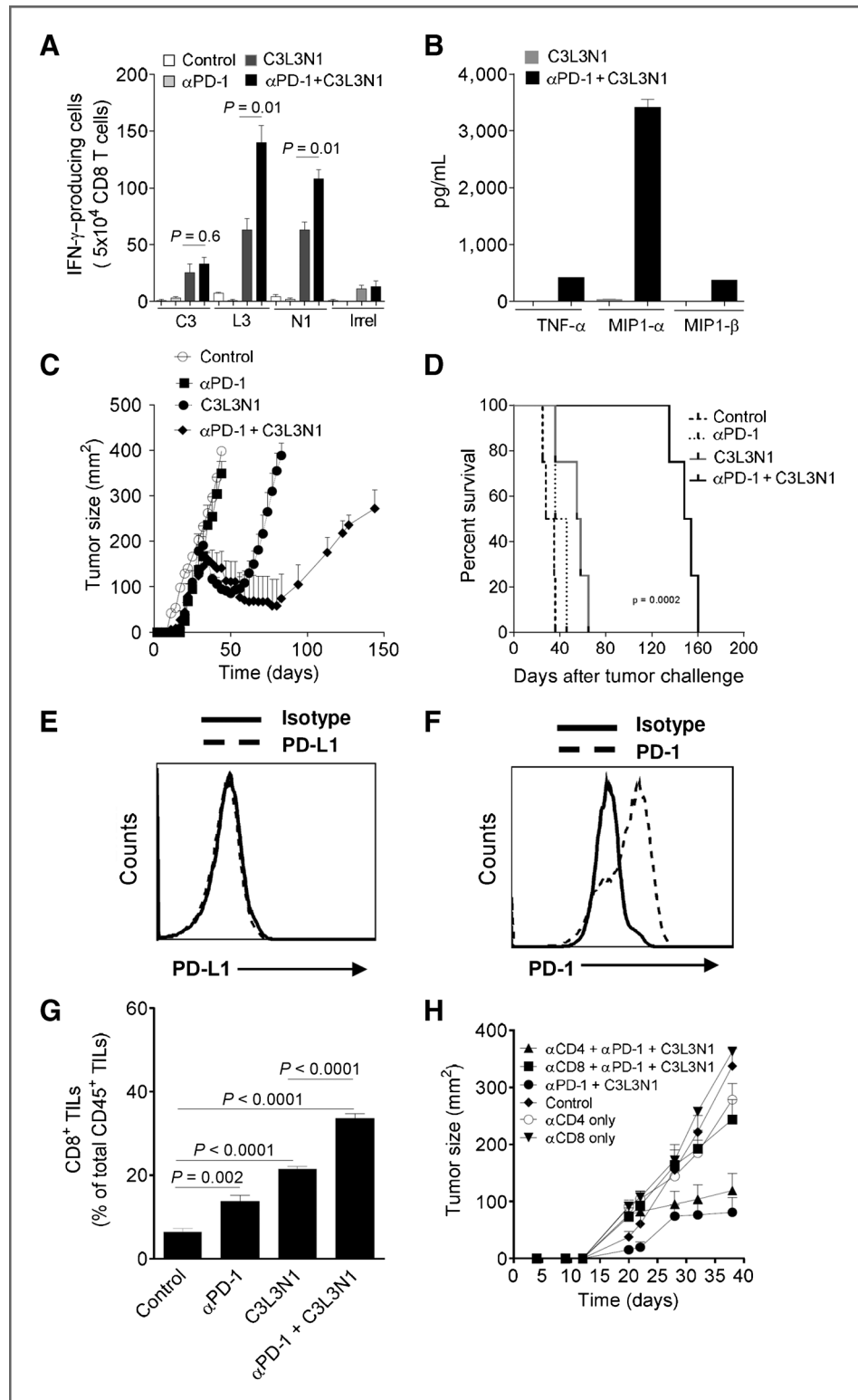
cells (neu⁺ and β -catenin⁺) and legumain⁺ RAW macrophages (L⁺ cells) confirmed that C3, L3, and N1 are naturally processed peptides. The ability of the individual peptides and multi-peptide vaccine to reduce tumor burden in breast tumor (TUBO) bearing BALB/c mice was tested. As shown in Fig. 1C, the multi-peptide vaccine induced tumor regression, whereas individual peptides were ineffective. We then asked whether the combination of any two peptides can induce tumor regression. As shown in Fig. 1D, it was observed that having three peptides as a combination is essential to induce tumor regression. Collectively, these data suggest that the multi-peptide "infrastructure" vaccine is immunogenic and is effective in reducing breast tumor burden in this model.

Combination therapy enhances vaccine efficacy

Even though it was observed that the multi-peptide vaccine induced tumor regression, complete cures were not observed. Given the fact that PD-1 and PD-L1 are reported to be expressed on activated lymphocytes including T cells (27) in addition to being well known that PD-1/PD-L1 is a major immunosuppressive pathway (2), first, we wanted to determine whether blockade of this inhibitory axis during the priming phase would enhance the efficacy of the multi-peptide vaccine. As depicted in Fig. 2A, anti-PD-1 antibody significantly augmented the numbers of antigen-specific effectors when combined with vaccine but had no impact when used alone. In addition, the function of antigen-specific effectors was improved as determined by the enhanced release of cytokines TNF- α , MIP1- α , and MIP1- β (Fig. 2B). Next, we asked whether

using anti-PD-1 therapy could improve the therapeutic efficacy of the multi-peptide vaccine. Tumor-bearing mice were treated with multi-peptide vaccine plus anti-PD-1 antibody as described in Materials and Methods. As shown in Fig. 2C and D, this combination therapy induced regression in all tumors and led to sustained protection from progression, increasing median survival by nearly three-fold when compared with vaccine alone. We also determined the expression of PD-1 and PD-L1 on TUBO tumors, observing that these tumors are PD-1 positive and PD-L1 negative (Figs. 2E and F). Although it could be argued that the effects of anti-PD-1 could be mediated through blocking signaling of PD-1 on tumor cells, two pieces of data rule this out. First, anti-PD-1 alone had no impact on disease outcome as shown in Fig. 2C ($P > 0.05$). Second, *in vitro* studies show that anti-PD-1 treatment of TUBO cells in culture has no impact on tumor cell growth (Supplementary Fig. S1). This suggests that the antibody was not acting by preventing interaction of immune effectors and the tumor. We also observed that combination treatment augmented the numbers of CD8 tumor-infiltrating lymphocytes (TIL) by greater than two-fold over the vaccine only group and five-fold over the control group (Fig. 2G). To determine the role of CD4 and CD8 T cells in inducing sustained protection that was observed in combination therapy group, mice were treated with T-cell-depleting anti-CD8 or anti-CD4 antibodies. Treatment with anti-CD8 mAb (Fig. 2H) reversed the effects of combination therapy and tumor size at Day 39 in the cohort of CD8 T-cell-depleted that received both vaccine and anti-PD-1 as compared with untreated control mice ($P > 0.05$). Overall, there

Figure 2. Anti-PD-1 (α PD-1) treatment with multi-peptide vaccine enhances immune responses during priming phase and delays recurrence of disease. A, mean ELIspot numbers (\pm SEM, $n = 3$) of IFN- γ -producing CD8 T cells, in response to P815 cells pulsed with C3, L3, N1, and control (Irrel) peptides. B, mean (\pm SEM, $n = 2$) cytokine concentrations detected in supernatants of CD8 T cells incubated with P815 cells pulsed with N1 peptide from vaccinated mice. C, tumor growth curves for BALB/c mice ($n = 4$ per group) vaccinated with multi-peptide vaccine and multi-peptide vaccine plus anti-PD-1 antibody. Points, average tumor size; bars, SEM. D, the Kaplan-Meier survival curve representing different groups of TUBO tumor-bearing BALB/c mice. E and F, histograms (gated on R1) representative of PD-L1 and PD-1 expression by TUBO tumor cells. G, mean (\pm SEM, $n = 4$) levels of infiltrating CD8 T cells in treated and control animals. H, tumor growth curves for different groups of TUBO tumor-bearing BALB/c mice ($n = 5$ per group) treated with or without anti-CD4 (α CD4) or anti-CD8 (α CD8) monoclonal antibodies. Points, average tumor size; bars, SD. Data are representative of one of three (A-F, H) experiments with similar results.



were no statistically significant differences in tumors from control mice or those treated with anti-CD4 mAb alone, anti-CD8 mAb alone, or the combination of anti-CD8 mAb, anti-PD-1 mAb, and vaccine ($P > 0.05$). This shows that CD8 T cells are

responsible for tumor regressions and sustained protection that was observed in combination therapy groups. We also observed that combination therapy did not result in significant increases in numbers of CD4 T cells compared with vaccine

group (Supplementary Fig. S2A) and data from the T-cell subset depletion studies shows that depletion of CD4 T cells in tumor-bearing mice treated with combination therapy resulted in protection that was observed in mice without CD4 T-cell depletion. These results suggest that the combination acts independently of helper T cells. Finally, as shown in Supplementary Fig. S2B, we observed that PD-1 blockade alone and combination resulted in two-fold decrease in number of tumor-infiltrating Tregs over control groups (28, 29), but no significant differences were observed when compared with vaccine only group, suggesting that the improved protection observed in combination group, when compared with vaccine group, is not directly dependent on Tregs.

Combination therapy enhances the generation of antigen loss variants

Even though the multi-peptide vaccine with or without anti-PD-1 antibody was able to induce tumor regression, the tumors ultimately recurred. As it was known that immune pressure in tumors results in the development of antigen-negative variants (25, 30), we wanted to determine the cell surface expression of neu antigen on the recurring tumors. As shown in Fig. 3A and B, a significant reduction in neu expression was observed in recurring tumors obtained from vaccinated groups, whereas no loss in neu antigen expression was observed in tumors that were obtained from PBS and anti-PD-1 groups. Importantly, the inclusion of anti-PD-1 further decreased neu expression as compared with the vaccine alone group, suggesting that T cells generated with combination therapy recognized lower levels of peptide:MHC class I complexes.

Combination therapy enhances the numbers of antigen-specific effectors at the effector phase

As we observed that combination therapy resulted in a significant increase in the survival of tumor bearing mice and also given the fact that blockade of tumor-associated immunosuppression is known to enhance function of vaccine-induced effectors (31), next, we wanted to determine whether

combination therapy would increase the effector function of TILs. For this, lymphocytes obtained from tumors on day 35 to 38 (the time point at which tumor regression began in the different groups of vaccinated mice) were analyzed for effector function. As shown in Fig. 4A–F, TILs from combination groups when stimulated with N1 peptide showed a significant increase in IFN- γ^+ , TNF- α^+ , and IL-2 $^+$ CD8 TILs compared with other groups. No significant difference in granzyme B $^+$ CD8 TILs obtained from combination therapy groups and vaccine groups was observed (Fig. 4G and H). Similar results were obtained when evaluating IFN- γ responses to the β -catenin (C3) and legumain peptide (L3), although the overall frequencies of antigen-specific T cells were considerably lower (Supplementary Fig. S3A and S3B). As shown in Fig. 4I and J, combination therapy also resulted in a significant increase in IL-4 $^+$ CD8 T cells as compared with vaccine or anti-PD-1 therapy alone, demonstrating the generation of Tc2 CD8 T cells in addition to Tc1. The generation of Tc2 in combination therapy was also confirmed using multiplexed cytokine assays for IL-4 and IL-5 (Supplementary Fig. S4). Considering the role of IL-4 and IL-5 in driving antibody responses, we evaluated mice for the presence of antitumor antibodies demonstrating that although anti-PD-1 therapy reduces the natural antibody response to tumor, this suppression is mitigated by combining anti-PD-1 with the vaccine (Fig. 4K). Finally, we evaluated the proportional distribution of Tc1 and Tc2 TILs in the tumors (Fig. 4L). As shown, tumors from untreated animals had roughly similar amounts of Tc1 and Tc2 effectors, which were slightly skewed in favor of Tc1 effectors by anti-PD-1 alone. The use of vaccine alone favored a local accumulation of Tc1 over Tc2, whereas the addition of anti-PD-1 with vaccine increased Tc2 effectors, four-fold, from 8% \pm 1.9% (vaccine alone) of the total CD8 TILs to 32% \pm 2.6% in the combination treatment group ($P < 0.0001$). Collectively, these data suggest that PD-1 blockade during vaccination against breast cancer not only enhances effector responses during the priming phase but also at the effector phase.

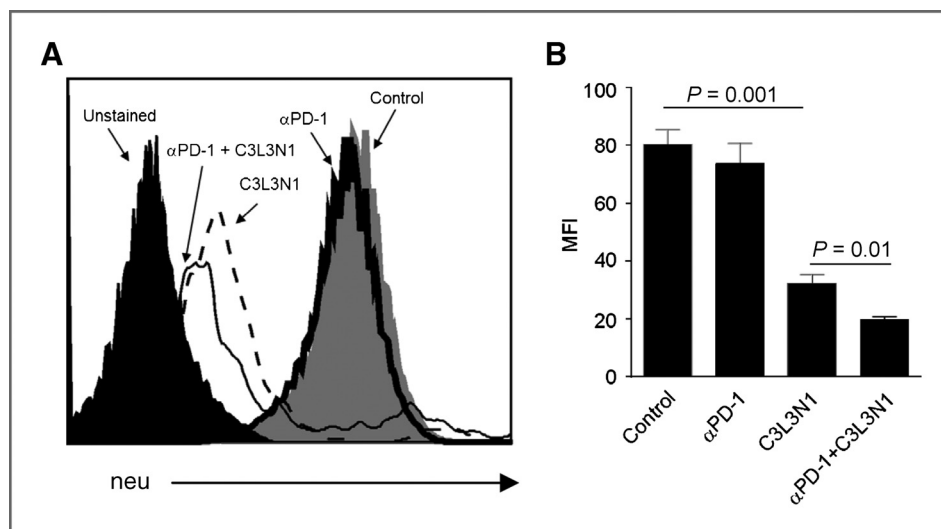


Figure 3. PD-1 blockade enhances the generation of antigen loss variants. A, a histogram representative of neu expression on tumors obtained from different treatment groups. B, the bar graphs representative of mean neu-specific fluorescence intensity (MFI) of the TUBO tumors. Data are shown as mean (\pm SEM) and from three independent experiments.

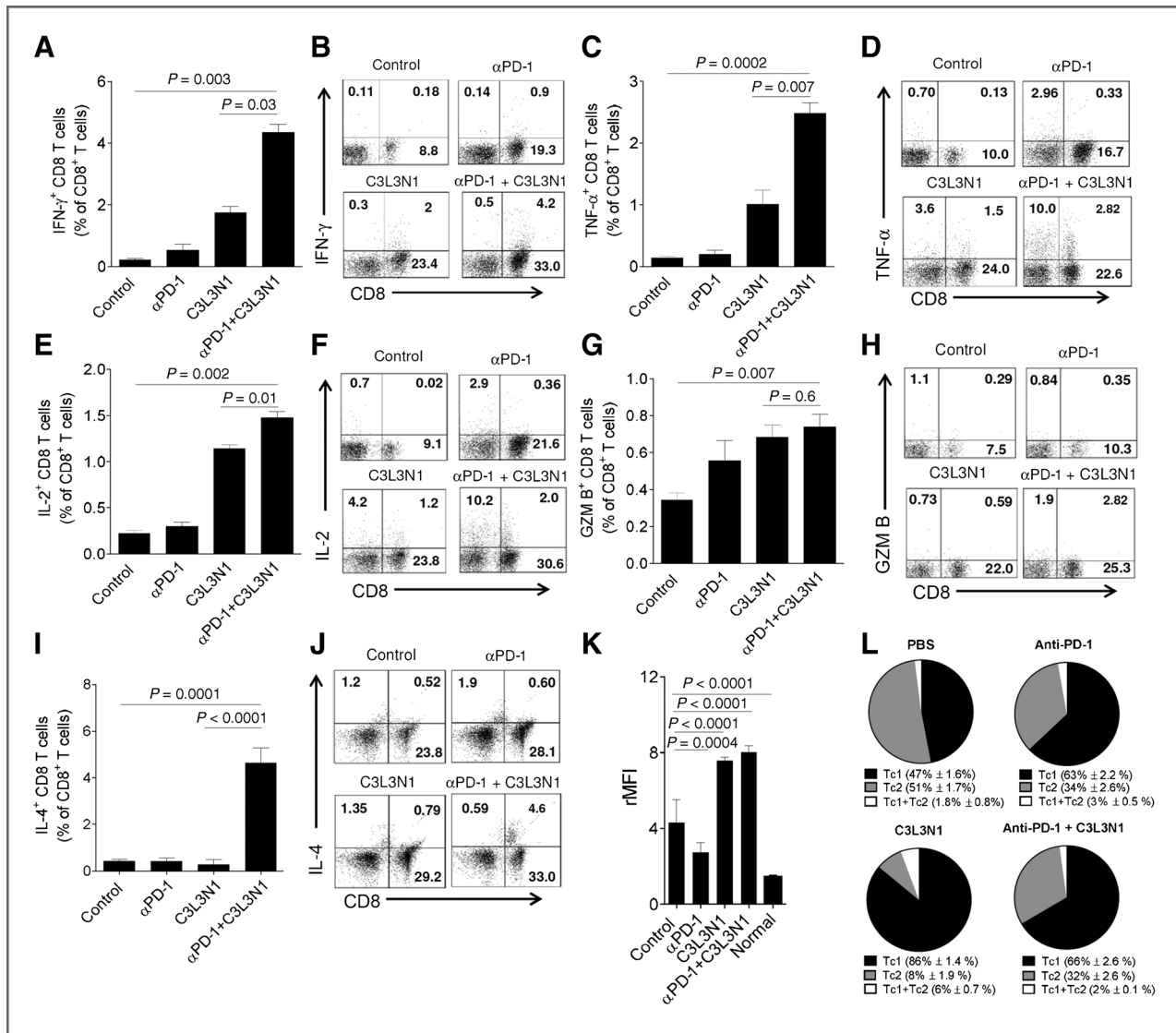


Figure 4. Combination therapy enhances the antigen-specific function of TILs. Shown are intracellular cytokine staining for IFN- γ (A and B), TNF- α (C and D), IL-2 (E and F), granzyme B (G and H), and IL-4 (I and J) in CD8⁺ TIL following peptide stimulation. Shown are means (\pm SEM) of the triplicate samples pooled from three mice (A, C, E, G, and I) and representative flow cytometry dot plots (B, D, F, H, and J). Data are from three experiments with similar results. K, the mean (\pm SEM, $n = 12$) relative mean fluorescent intensity values (rMFI) obtained from antitumor antibody binding experiments. "Normal" serum was obtained from similarly aged but nontumor-bearing mice. L, shown are pie charts representing proportions of Tc1 (IFN- γ ⁺) and Tc2 (IL-4⁺) T cells among total CD8⁺ TIL from mice that received vaccine alone, anti-PD-1 alone, nothing, or the combination. Each slice (shown are means \pm SEM) is calculated from six replicates using flow cytometry.

Combination therapy increases the infiltration of CD8 T cells with MPEC phenotype into regressing tumors

Given that the role of PD-1/B7-H1 axis in regulating the expression of memory markers on CD8 T cells had been demonstrated in recent studies (32–34), we hypothesized that PD-1 blockade during vaccination increases the infiltration of T cells with memory phenotype into regressing tumors. To test this hypothesis, we analyzed the expression of memory markers on CD8 TILs. As shown in Fig. 5A and B, we observed a significant increase in infiltration of CD8 T cells with activated effector memory phenotype, CD44^{hi}CD62L[–]CCR7[–], into regressing tumors of mice treated with combination therapy. As it is widely reported that CD27 is critical for the generation

and long-term maintenance of effector T-cell pool (35, 36) and as our results show that combination therapy prolonged the survival of tumor bearing mice by three-fold, in the next step we analyzed the expression of CD27 on the CD8 TILs in different groups. As shown in Fig. 5C and D, we observed a significant increase in the number CD8 TILs that are CD27⁺ in regressing tumors in combination therapy groups. From these results, it is evident that PD-1 blockade during the vaccination results in increase in infiltration of tumors with CD8 T cells that have memory phenotype and enhanced persistence.

CD8 T cells differentiate into two different cell types upon interaction with target antigen (1) short-lived effector cells (SLEC) and (2) MPECs. SLECs are terminally differentiated

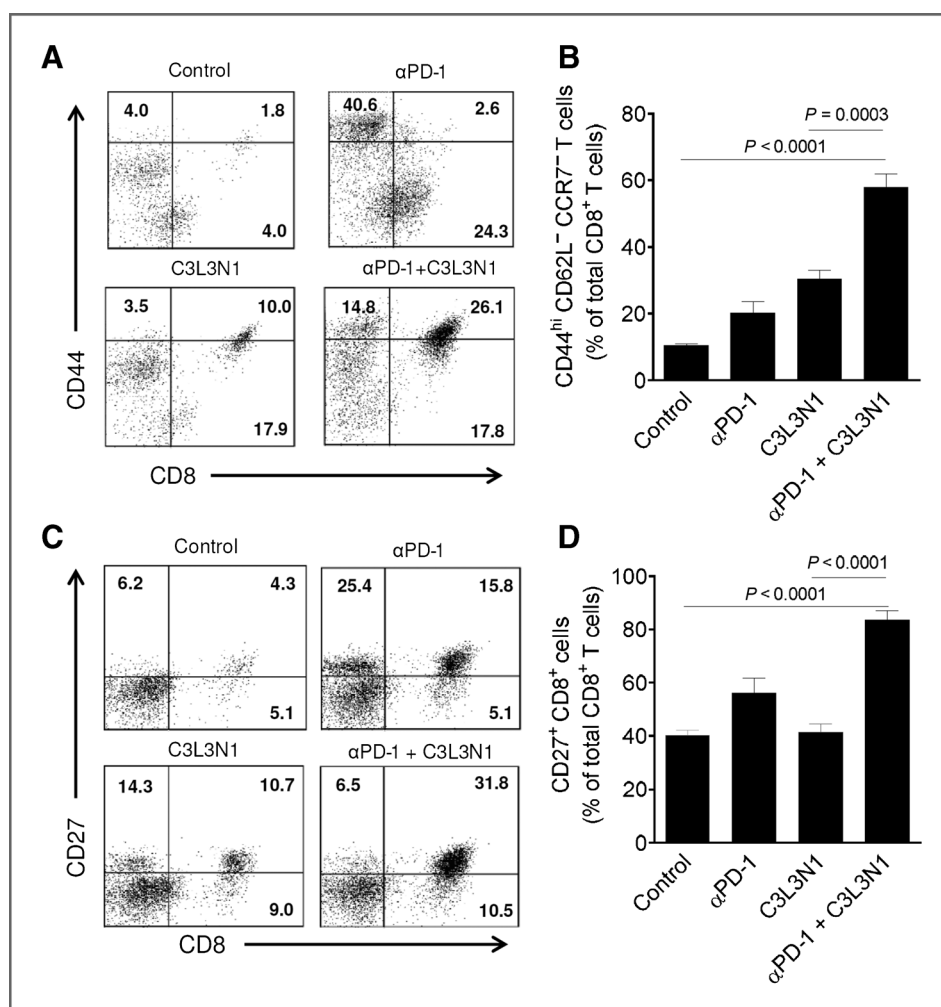


Figure 5. Combination therapy enhanced the infiltration of CD8 T cells with memory phenotype into regressing tumors. TILs were analyzed for the expression of CD44, CD62L, CCR7, and CD27 on CD8 T cells. A, the cytometry dot plots representative of CD8⁺ CD44⁺ lymphocytes in tumors of different treatment groups. All cells were gated on CD62L⁻ and CCR7⁻. Quadrants were established with isotype controls and inset values are the percentage of CD62L⁻ CCR7⁻ lymphocytes that fall in that quadrant. Results shown are representative of one of the three independent experiments with similar results. B, the mean numbers of tumor-infiltrating CD8⁺ CD44^{hi} CD62L⁻ CCR7⁻ T cells in different treatment groups. Data shown are the mean of the (±SEM) six samples pooled from three independent experiments. C, the cytometry dot plots representative of CD27⁺ CD8⁺ TILs from the different treatment groups. Inset values are the percentage of R1-gated lymphocytes that fall in that quadrant. D, the mean numbers of CD27⁺ CD8⁺ T cells in different treatment groups. Data are shown as mean of the (±SEM) six samples pooled from three independent experiments.

effector T cells with little capacity to survive, whereas MPECs are the activated effector T-cell pool that have a greater capacity for survival (37). Thus, in the next step, we asked whether CD8 TILs infiltrating into regressing tumors in combination therapy group have either SLEC or MPEC phenotype. As shown in Fig. 6A–F, combination therapy resulted in increased infiltration of regressing tumors with CD27⁺ CD8⁺ T cells, which express high levels of IL-7R, low levels of T-bet, and high levels of Eomes, suggesting that infiltrating CD8 TILs have MPEC phenotype. We also observed that antigen-specific T cells in the TME of mice, treated with combination therapy, demonstrated highly elevated levels of MCP-1 production (Fig. 6E), and this correlated with a high level of serum MCP-1 in mice that received combination therapy and were actively rejecting tumors, suggesting its potential use as a serum biomarker for antitumor response (Fig. 6H).

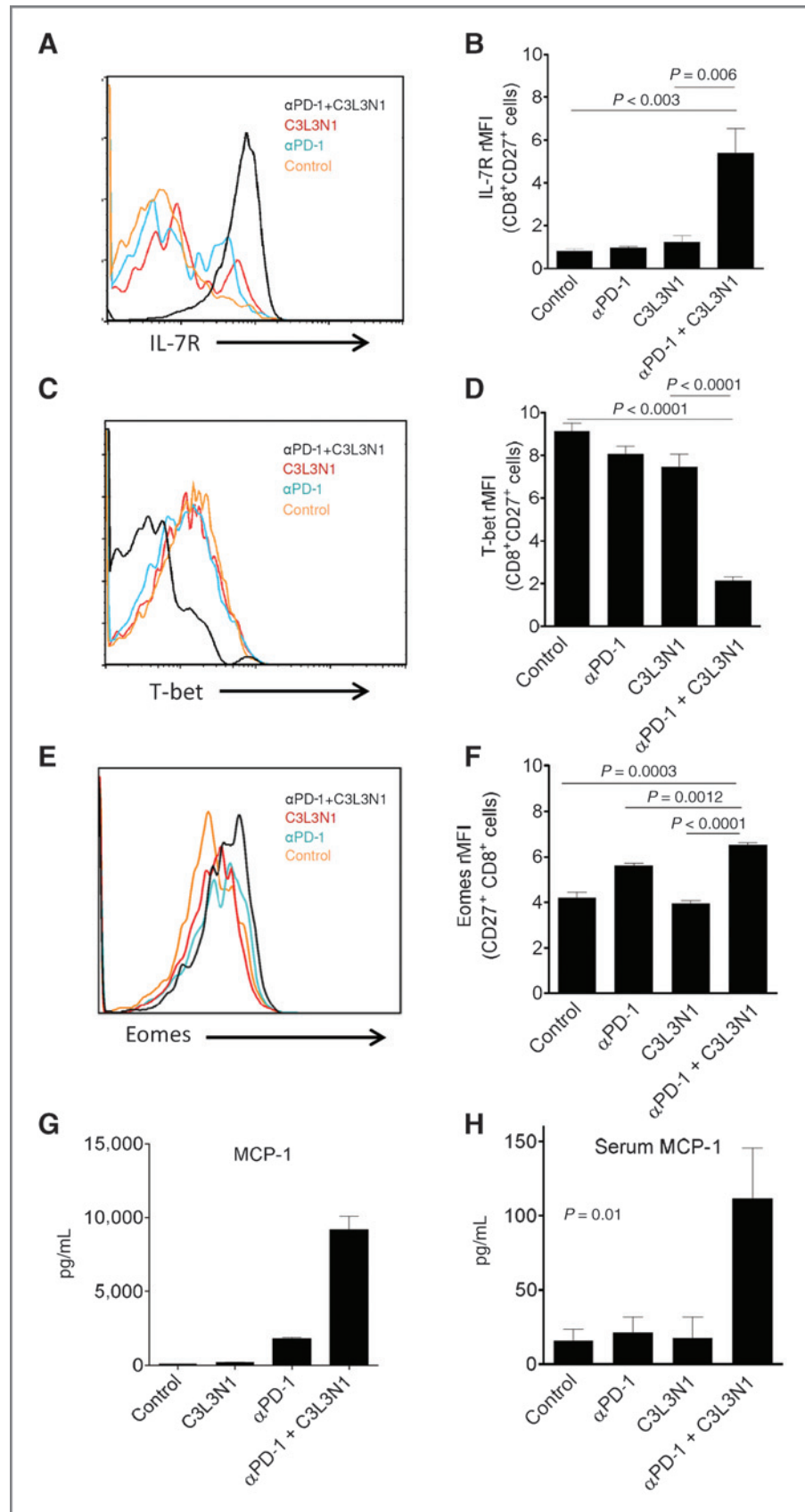
Combination therapy affects PD-1⁺ DC population in the TME and tumor-associated PD-1⁺ DCs regulate IL-7R expression on CD8 T cells

Based on prior results, which suggest that PD-1, PD-L1, and CTLA-4 blockade during vaccination against melanoma

increases PD-1 expression by CD4 and CD8 TILs (28), next, we wanted to determine whether PD-1 blockade during vaccination alters numbers of tumor-infiltrating PD-1⁺ CD8 T cells. We did not observe any effect on the levels of PD-1⁺ CD8 T cells (Fig. 7A), but we observed that breast tumors are infiltrated with PD-1⁺ DCs similar to our prior observations in ovarian tumor model (15). Also, as shown in Fig. 7B and C, we observed that both combination therapy as well as anti-PD-1 antibody treatment alone resulted in significant decrease in the number of PD-1⁺ DCs in the tumors. This suggests that PD-1 blockade during vaccination also modifies the myeloid compartment in the TME and consistent with this, we found that DCs in the TME of mice treated with combination therapy had elevated levels of IL-12 (Fig. 7D). In addition, we also found that the combination treatment resulted in a significant down-regulation of MDSCs (Supplementary Fig. S5). Interestingly, treatment with anti-PD-1, when used alone, led to nearly a doubling of the numbers of the MDSCs (as a % of total leukocytes).

As our results suggest that PD-1 blockade during vaccination alters the expression of IL-7R and T-bet by tumor-infiltrating CD8 T cells, we wanted to determine whether the

Figure 6. Combination therapy enhanced IL-7R expression, Eomes expression and decreased T-bet expression by CD8 TILs. Histograms show expression of IL-7R (A), T-bet (C), and Eomes (E) on/in CD8⁺CD27⁺ TILs from different treatment groups. Shown are the mean rMFI values representing the expression of IL-7R (B), T-bet (D), and Eomes (F) by CD8⁺CD27⁺ TILs from different treatment groups. Data are the mean (±SEM) from three independent experiments. *P*-values in B, D, and F were calculated using a Student *t* test. G, the mean (±SEM) supernatant levels of MCP-1 released by purified CD8 TILs. Data are representative of duplicate samples pooled from three mice and from one of three experiments with similar results. H, the mean (±SEM) serum levels of MCP-1 in treated or untreated mice. Data are representative of duplicate samples pooled from three mice and from one of three experiments with similar results. *P* values in G and H were calculated using a one-way ANOVA.



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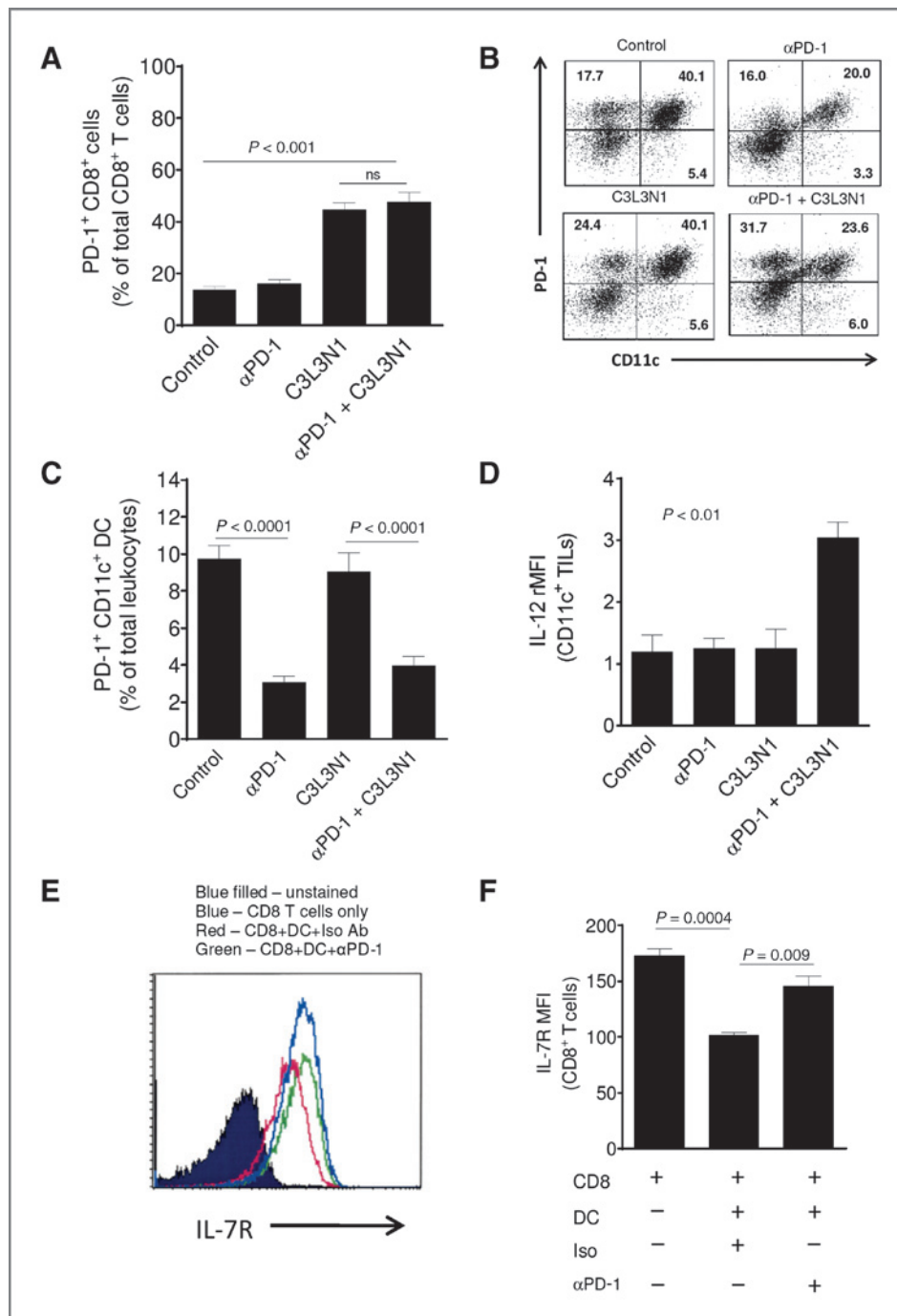


Figure 7. Combination therapy decreased the number of tumor-infiltrating PD-1⁺ CD11c⁺ cells and PD-1 blockade on tumor-infiltrating DC increases IL-7R expression on antigen-primed CD8 T cells. A and B, PD-1⁺ CD8⁺ and PD-1⁺ CD11c⁺ cells in tumors of different treatment groups were analyzed. A, shown are the mean numbers of tumor-infiltrating PD-1⁺ CD8⁺ T cells in different treatment groups. B, the cytometry dot plots (gated on R1) representative of tumor-infiltrating PD-1⁺ CD11c⁺ cells in the different treatment groups. Quadrants were established with isotype controls and the inset values are the percentage of R1-gated lymphocytes that fall in that quadrant. Dot plots shown are the representative of one of three separate experiments with similar results. C, the mean numbers of tumor-infiltrating PD-1⁺ CD11c⁺ cells of total leukocytes in different treatment groups. Data are shown as mean (±SEM) and are from three independent experiments. D, the mean (±SEM, n = 3) rMFI values obtained from staining tumor-infiltrating DCs for IL-12. DCs were obtained from tumors between days 35 and 38. E, the histogram representative of IL-7R expression on splenic CD8 T cells cocultured with tumor-derived PD-1⁺ DCs pretreated with anti-PD-1 antibody or isotype antibody. F, the bar graphs representative of IL-7R expression on splenic CD8 T cells. Data are shown as mean (±SEM) and are from three independent experiments.

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blockade of PD-1 on tumor-infiltrating DCs had any direct effect on CD8 T cells in terms of acquiring the MPEC phenotype. For this, tumor PD-1⁺ DCs were cocultured with splenic CD8 T cells and following coculture CD8 T cells were analyzed for the expression of IL-7R and T-bet as described in the Materials and Methods. Our data show that in this coculture system, PD-1 is expressed only on tumor-derived DCs but not on splenic CD8 T cells (Supplementary Fig. S6). As shown in Fig. 7E and F, it was observed that blockade of PD-1 on tumor-associated DCs rescued IL-7R expression on CD8 T cells. We

did not observe any effect of PD-1 blockade on DCs on T-bet expression by T cells. These results suggest that PD-1 expressed on DCs controls the expression of IL-7R, which is a marker of MPECs (38).

Discussion

In summary, in this study we show that the combination of anti-PD-1 with peptide vaccine greatly enhanced vaccine-induced immune responses and resulted in delayed tumor regrowth following vaccine-induced regression. Key findings

from our study are that PD-1 blockade during cancer vaccination (i) generates CD8 Tc1 and Tc2 TILs that have MPEC phenotype, (ii) decreases the PD-1⁺ DC population (but not the T cells) in tumors, and (iii) regresses B7-H1-negative tumors and greatly enhances progression-free survival.

In addition to confirming previous studies, which show that PD-1 blockade during vaccination results in increased infiltration of effector T cells into tumors and increases their functionality (28, 29), in this study we identified that PD-1 blockade during vaccination induces the tumor-infiltrating CD8 T cells to develop into MPECs, that is IL7R^{hi}T-bet^{lo} CD8 TILs. MPECs are the effector CD8 T cells that serve as precursors to a stable pool of memory T cells (17, 37, 38) and T-bet gradient during inflammation directs the differentiation of effector CD8 T cells into MPECs or SLECs (37). Our observation that combination therapy results in decrease in T-bet expression in CD8 TILs suggests that blockade of PD-1/B7-H1 inhibitory axis not only enhances tumor-infiltrating effector cell functions as reported extensively but also alters (reduces) the T-bet gradient in CD8 TILs. In addition, low levels of T-bet in CD8 TILs suggest that these effector cells are poised to preserve their proliferative capacity and are not directed toward terminal differentiation (39). We also observed that MPECs are CD27⁺, supporting the fact that MPECs induced by our combination strategy have enhanced persistence (35). Furthermore, given the role of MCP-1, a chemokine previously identified to enhance the recruitment of CD8 memory T cells to sites of infection in a paracrine fashion (40, 41), increase in production of MCP-1 by TILs from combination group describes the reason behind increase in the number of MPECs in the regressing tumors. Finally, despite the observation that combination therapy strategy also enhanced Tc2 cytokine production by TILs, depletion studies underscores the fact that MPECs induced sustained protection is independent of CD4 T-cell help. Overall, the ability of combination therapy to enhance the survival of tumor-bearing mice observed in this study can be attributed to the induction of MPECs.

Despite the fact that DCs are the major cell type that generate antitumor immunity in the TME, emerging data show that tumor-associated DCs are immunosuppressive and express PD-1 (15, 42). Similar to our ovarian cancer model (15), tumor-associated PD-1⁺ DCs were also observed in this study. Role of PD-1 blockade in altering the number of tumor-infiltrating regulatory cells, such as Tregs and myeloid-derived suppressor cells (MDSC), was shown in prior studies (28, 29). Recently, Duraiswamy and colleagues showed that these changes (decrease) in the numbers of tumor-infiltrating Tregs are not because of increase in the apoptosis of Tregs (29). Thus, it is plausible to speculate that the decrease in numbers of tumor-infiltrating regulatory cells upon blockade of immune checkpoints could be because of the alteration in their migratory properties. In fact, this speculation can be supported by the recently published results from Peng and colleagues, which show that PD-1 blockade during adoptive T cell transfer increases the infiltration of transferred T cells into the tumor site (43). Thus, in this study, the decrease in numbers of PD-1⁺ DCs observed in tumors of mice treated with anti-PD-1 antibody with or without vaccine could be because of increase in

migration of DCs from tumors probably into the regional lymph nodes. Also, our results from *in vitro* experiments show that PD-1 on tumor-associated DCs regulates the expression of IL-7R, a marker for MPECs, on CD8 T cells. These results provide valuable insight that PD-1 blockade during vaccination against cancer targets the potential antigen-presenting cells of the TME. In addition to prior studies, which show that anti-PD-1 therapy modulates T cells and MDSCs (28, 29), here we show that tumor DCs are also modulated. Studies aimed at understanding the complete mechanics of anti-PD-1 antibody modulation of tumor-associated DC population, which in turn affects the infiltrating CD8 T-cell population seem like a logical next step.

We observed that treatment of tumor-bearing mice with anti-PD-1 antibody alone was not effective and this can be attributed to the lack of PD-L1 expression on the TUBO tumors. Our data are consistent with the recent results from phase I clinical trial, which shows that anti-PD-1 antibody as monotherapy is ineffective in patients with PD-L1-negative tumors (13) and our study shows that combination therapy is an alternative strategy that would be effective against PD-L1-negative tumors. Despite the fact that our combination therapy strategy induced sustained protection against breast cancer, we observed the recurrence of the disease. This may be attributable to the widely accepted cancer immune editing phenomenon (44). It could be possible that in our model, PD-1 blockade during vaccination would have prolonged the equilibrium phase, which is evident from the observed sustained protection (25, 45). However, an alternative explanation is that tolerance mechanisms may have resulted in more rapid decline and elimination of the legumain and β -catenin T cells because these are self-antigens with a more limited T-cell repertoire, which is in contrast to rat neu, which is foreign. Regardless, while developing strategies to vaccinate against cancer with simultaneous blockade of tumor-associated immunosuppression, continued concern should be taken to avoid the recurrence of disease, which might occur because of cancer immunoeediting processes.

Our study does not reveal the antigen-specificity of the majority of the infiltrating CD8 T cells. Although one possibility is that all of the CD8 T cells were responsive to one or more of the antigens included in the vaccine, our flow cytometry studies indicate that only between 5% and 6% of the infiltrating CD8 T cells are specific for the vaccine. Another possibility is that anti-PD-1 treatment may lead to expansion of memory T cells that kill by innate mechanism as recently described (46). In that study, Tietze and colleagues found that cytokine and nonspecific immunotherapy with anti-CD40 led to bystander expansion of memory CD8 T cells that seemed to kill tumor by innate mechanisms including NKG2D ligation. Finally, it is possible that in our model the combination therapy with vaccine and anti-PD-1 would have induced CD8 T cells reactive to tumor antigens other than the ones that are targeted using our vaccine attributable to PD-1 blockade-induced epitope spreading as suggested by Dhodapkar and colleagues (47).

Anti-PD-1 and anti-PD-L1 antibodies are moving toward advance phase clinical trials after being successfully tested in phase I trial (13, 14). We expect that data from this study, which

suggest that combination therapy strategy can provide protection against PD-L1-negative tumors and induce MPECs, can serve to leverage the designing of clinical trials that would use peptide or other vaccines in combination with PD-1 antagonism for the treatment of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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