Combination of Alphavirus Replicon Particle–Based Vaccination with Immunomodulatory Antibodies: Therapeutic Activity in the B16 Melanoma Mouse Model and Immune Correlates

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Abstract

Induction of potent immune responses to self-antigens remains a major challenge in tumor immunology. We have shown that a vaccine based on alphavirus replicon particles (VRP) activates strong cellular and humoral immunity to tyrosinase-related protein-2 (TRP2) melanoma antigen, providing prophylactic and therapeutic effects in stringent mouse models. Here, we report that the immunogenicity and efficacy of this vaccine is increased in combination with either antagonist anti-CTLA-4 (CTLA-4) or agonist anti-glucocorticoid-induced TNF family–related gene (GITR) immunomodulatory monoclonal antibodies (mAb). In the challenging therapeutic setting, VRP–TRP2 plus anti-GITR or anti–CTLA-4 mAb induced complete tumor regression in 90% and 50% of mice, respectively. These mAbs had similar adjuvant effects in priming an adaptive immune response against the vaccine-encoded antigen, augmenting, respectively, approximately 4- and 2-fold the TRP2-specific CD8+ T-cell response and circulating Abs, compared with the vaccine alone. Furthermore, while both mAbs increased the frequency of tumor-infiltrating CD8+ T cells, anti–CTLA-4 mAb also increased the quantity of intratumor CD4+ Foxp3+ T cells expressing the negative costimulatory molecule programmed death-1 (PD-1). Concurrent GITR expression on these cells suggests that they might be controlled by anti-GITR mAbs, thus potentially explaining their differential accumulation under the two treatment conditions. These findings indicate that combining immunomodulatory mAbs with alphavirus-based anticancer vaccines can provide therapeutic antitumor immune responses in a stringent mouse model, suggesting potential utility in clinical trials. They also indicate that tumor-infiltrating CD4+ Foxp3+ PD-1+ T cells may affect the outcome of immunomodulatory treatments. Cancer Immunol Res; 2(5); 448–58. ©2014 AACR.

Introduction

Malignant melanoma is the deadliest form of skin cancer and is relatively refractory to conventional chemotherapy and radiotherapy. Recent clinical studies have shown that potentiating the immune system with immunomodulatory monoclonal antibodies (mAb) can be successful in treating metastatic melanoma (1). Immunomodulatory mAbs that counteract inhibitory immune receptors, such as CTL antigen-4 (CTLA-4) and programmed death-1 (PD-1), and/or activate costimulatory molecules, such as the glucocorticoid-induced TNF family–related gene (GITR), have shown promising preclinical (2–5) and clinical results (6–8). However, these mAbs, when given as monotherapies, achieve a clinical benefit only in a subset of patients. More effective approaches or combination with other therapies are thus required to improve the management and clinical outcome of the many patients who do not respond or those whose disease eventually progresses (9).

A reasonable setting in which to exploit the immunomodulatory functions of immune checkpoint blocking and costimulatory mAbs is in combination with anticancer vaccines. This is substantiated by the widely accepted concept that, to increase the likelihood of a therapeutic effect, a vaccine needs to be combined with agents able to promote the proper priming and/or effector functions of an immune response. The advantage of costimulation with mAbs, such as anti-GITR and anti–CTLA-4 mAbs, is that they can concurrently provide both effects, by directly or indirectly favoring T-cell activation and impairing the immunosuppressive network via regulatory T cell (Treg) depletion/modulation (10–12). In vivo preclinical...
studies have shown that anticancer vaccines and costimulatory mAbs can provide synergistic antitumor activity (4, 13, 14). However, the experience accumulated so far with clinically available cancer vaccines demonstrated that immune responses induced in patients with melanoma are typically weak and do not correlate with clinical benefit. Indeed, a large phase III trial of gp100 peptides with the CTLA-4–blocking mAb ipilimumab showed no improvement in survival and lower response rates compared with patients treated with ipilimumab alone (6). Identification of new vaccines suitable for combination with immunomodulatory mAbs is thus warranted.

We have reported previously that the alphavirus replicon particles (VRP) encoding melanoma differentiation antigens offer a novel and potent approach to vaccination strategies against melanoma in preclinical settings (15, 16). VRPs are propagation-defective virus-like particles derived from an attenuated variant of the Venezuelan equine encephalitis virus (VEE). VEE-based VRPs have been shown to induce high titers of Abs and robust antigen-specific T-cell responses against the encoded antigens in mice (17–23) and, more recently, in human subjects (24, 25). At the same time, neutralizing antivector immunity does not seem to preclude benefit from repetitive booster vaccinations as opposed to other viral vectors (24, 26–29). In particular, we and others reported that VRP vaccines have the unique capacity to activate both cellular and humoral immunity against melanoma antigens in mice (15, 16). Among the different melanoma antigens systematically evaluated, tyrosinase-related protein-2 (TRP2) provided the most potent antitumor effect when administered using VRPs (15). TRP2 is a melanosomal membrane glycoprotein required for melanin biosynthesis in melanocytes (30–34). TRP2 is expressed in the melanosomes of the melanocytes; the polypeptide matures in the endoplasmic reticulum, and a small proportion of TRP2 is expressed on the plasma membrane, providing a target for Abs (35, 36).

Given the promising activity of VRP–TRP2, we sought to test the therapeutic efficacy of this vaccine in combination with immunomodulatory mAbs targeting coinhibitory or costimulatory receptors in the B16F10 melanoma mouse model. In view of the clinical benefit achieved in patients with melanoma by the anti-CTLA-4 mAb ipilimumab (6), and the wide spectrum of immunomodulatory functions as well as the excellent safety profile demonstrated by anti-GITR mAbs in preclinical settings (4, 12), we evaluated the therapeutic advantages of the VRP–TRP2 vaccine plus either anti–CTLA-4 (9D9) or anti-GITR (DTA-1) mAbs. We found that the combination of VRP–TRP2 with either mAb significantly enhanced the therapeutic activity of each agent alone. This was associated with a substantial increase in the frequency and cytotoxic function of TRP2–specific CD8+ T cells, and an enhanced anti-TRP2 Ab response. Despite the similar immunologic efficacy of the two combinations, the use of anti-GITR mAb + VRP–TRP2 resulted in a significantly augmented therapeutic efficacy when compared with anti–CTLA-4 mAb + VRP–TRP2. This was associated with (i) the ability of anti-GITR mAb to broaden the reactivity of vaccine-induced anti-TRP2 Abs and to facilitate the recruitment of phagocytes to the tumor site, and (ii) the selective intratumor accumulation in anti–CTLA-4 mAb-treated mice of noncytotoxic CD4+ Foxp3+ T cells expressing the negative costimulatory molecule PD-1 and common Treg-associated markers. These findings support the clinical evaluation of immunomodulatory mAbs in combination with alphavirus-based vaccines. They also underscore the importance of further characterizing CD4+ Foxp3+ PD-1+ T cells to clarify whether and how they can affect the outcome of costimulatory mAbs.

Materials and Methods

Mice, cell lines, and vaccine

All mouse procedures were performed in accordance with the institutional protocol guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY). C57BL/6j mice (8–10-week-old females) were obtained from The Jackson Laboratory. Foxp3-GFP mice were generously provided by Dr. Alexander Rudensky and backcrossed to C57BL/6j at MSKCC. Mice were maintained according to NIH Animal Care guidelines, under a protocol approved by the MSKCC Institutional Animal Care Committee. The B16F10 mouse melanoma line was originally obtained from I. Fidler (MD Anderson Cancer Center, Houston, TX). The B78H1 cell line, a B16 variant that does not express TRP2, was obtained from A. Albino (Sloan-Kettering Institute, New York, NY). Cell lines were maintained in RPMI-1640 medium supplemented with 7.5% inactivated FBS in a humidified chamber with 5% CO2 at 37°C for up to 2 weeks after thawing. Both the B16F10 and B78H1 cell lines were checked for Mycoplasma and for rodent infectious agents: the absence of TRP2 expression in B78H1 cell line was confirmed by Western blot analysis. No additional authentication was performed. Mouse TRP2 cDNAs were cloned into the pEBK replicon vector, and VRPs were generated as previously described (37).

VRP immunization and tumor challenge

C57BL/6j mice were vaccinated with 1 × 106 VRP–TRP2, or VRP–GFP as a negative control, via subcutaneous injection into the plantar surface of each footpad, before (prophylactic setting) or after (therapeutic setting) intradermal challenge with 7.5 × 106 B16F10 tumor cells. In the prophylactic setting, mice were vaccinated three times 2 weeks apart, and tumor cells were implanted 2 weeks after the last vaccination. In the therapeutic setting, mice were vaccinated three times 1 week apart and the tumor cells were implanted 3 days before the first immunization. Anti–CTLA-4 (clone 9D9; 100 μg/administration; generously provided by J.P. Allison, University of Texas MD Anderson Cancer Center, Houston, TX) or anti-GITR mAb (clone DTA-1; 500 μg/administration; generously provided by S. Sakaguchi, Osaka University, Osaka, Japan) or control isotype immunoglobulin G (IgG) were administered intraperitoneally following the schedules shown in Fig. 1. Animals were monitored for tumor growth every 2 to 3 days. Mice were considered tumor-free until intradermal lesions were visible or palpable. For analysis of tumor immune-cell infiltrate, B16F10 cells were injected subcutaneously in Matrigel (Matrigel Matrix Growth Factor Reduced; Becton Dickinson).

**ELISPOT assay**

Spleens were harvested 5 to 7 days after the last VRP immunization, CD8⁺ T cells were positively selected using CD8 microbeads (Miltenyi Biotech), and IFN-γ production was determined by standard enzyme-linked immunosorbent spot (ELISPOT) assay (Mabtech) after stimulation with TRP2₁₈₁–₁₈₉ or OVA₂₅₇–₂₆₄ (SIINFEKL) peptide (>80% purity; Genemed Synthesis Inc.) as described previously (38).

**Ex vivo killing assays**

Two types of assays were used to assess tumor killing by tumor-infiltrating lymphocytes (TIL). One is a liquid phase assay in which a 1:1 ratio of carboxyfluorescein succinimidyl ester (CFSE; Invitrogen)-labeled B16F10:B78H1 cells were cocultured with effector T cells at different ratios. B78H1 and B16F10 cells were labeled with 1 and 0.1 μg/mL CFSE, respectively. Seven days after the last VRP immunization, TILs were enriched on a Percoll gradient, and then the CD8⁺ and CD4⁺ Foxp3⁻ T cells were fluorescence-activated cell sorted (FACS) from Foxp3-GFP mice. After overnight coculture, effector cells were washed out, while the adherent target cells were detached and analyzed by flow cytometry.

The second killing assay was performed using a three-dimensional collagen–fibrin gel culture system, as described previously (39). Briefly, 10⁴ viable B16F10 cells were coembedded into collagen–fibrin gels with 10⁵ CD8⁺ or CD4⁺ Foxp3⁻ T cells isolated from tumors or spleens as negative control. Triplicate gels were lysed 48 hours later, and the tumor cells were diluted and plated in 6-well plates for colony formation. After 7 days, plates were fixed with 3.7% formaldehyde and stained with 2%
methylen blue before counting colonies as described previously (39).

**Analysis of circulating IgG**

Serum samples were collected 7 days after the last VRP immunization, and analyzed by ELISA using plates coated with a purified recombinant TRP2 protein (kind gift of Y. Chen, Weill Cornell Medical College, New York, NY) or a TRP2-overlapping peptide library (synthesized by Genemed Synthesis at >80% purity). Goat anti-mouse alkaline phosphatase−conjugated IgG, or biotinylated-IgG1, -IgG2a, -IgG2c followed by incubation with alkaline phosphatase−conjugated streptavidin (Southern Biotech) was used as a detection system with the AttoPhos alkaline phosphatase substrate (Promega). Plates were read with a Perceptive Biosystems, Cytoflour Series 4000 analyzer at excitation 450/50 and emission 580/50 with gain of 25.

**Analysis of tumor-infiltrating immune cells**

Sixteen days after injection, Matrigel plugs were resected, incubated for 1 hour at 37°C with 1 mg/mL Collagenase D (Sigma), and dissociated to obtain a single-cell suspension. Cells were then stained with the following anti-mouse mAbs: anti-CD45.2-APCCY7, anti-CD3-FITC, anti-NK1.1-APC, anti-CD8-PE–Texas Red, anti-CD4-PE, anti-Foxp3-APC, anti–PD-1-APC, anti–OX40-PE, anti-GITR–PECY7, anti–CD27-PE, anti–ICOS-PE (BD Biosciences), and LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Vidé, eBiosciences) or 4-ß-diamidino-2-phenylindole (DAPI) before acquisition. Data were acquired on a BD LSR II cytometer (Becton Dickinson) and analyzed with Flowjo (TreeStar) software version 9.4.2 for Macintosh.

**Cell sorting**

CD4 + Foxp3 + T cells and PD-1 + and PD-1 − CD4 + Foxp3 − T cells were sorted from tumors and/or spleens of Foxp3-GFP mice treated as indicated. TILs were first enriched on a Percoll gradient and then labeled with the following anti-mouse mAbs: anti-CD3-PerCP, anti-CD4-PECY7, and anti–PD-1-APC (BD Biosciences). After staining with DAPI, the entire CD3 − CD4 + GFP − Foxp3 − population or its PD-1 + and PD-1 − fractions were sorted on a BD FACS Aria Cell Sorter (Becton Dickinson) in the MSKCC Flow Cytometry Core Facility.

**Statistical analyses**

A two-sided Student t test was used to detect statistically significant differences. P values for tumor-free survival analyses were calculated with the log-rank (Mantel–Cox) test. Statistical analyses were performed on the Prism 5.0a software (GraphPad Software) version for Macintosh Pro personal computer.

**Results**

**Anti–CTLA-4 and anti-GITR mAbs enhance the therapeutic activity of VRP−TRP2 vaccine**

We have demonstrated that single-agent VRP−TRP2 vaccination has potent antitumor effects in a prophylactic setting in the B16F10 melanoma mouse model (Fig. 1A, left; ref. 15). However, this activity is significantly reduced in a therapeutic setting, where the vaccine is administered 3 days after tumor challenge (Fig. 1A, right). To enhance the efficacy of VRP−TRP2 in therapeutic and therefore clinically relevant conditions, we studied its combination with immunomodulatory mAbs targeting either the coinhibitory receptor CTLA-4 or the costimulatory receptor GITR. Both anti–CTLA-4 and anti-GITR mAbs were used in conditions in which they provided minimal protection as single agents (Fig. 1B, filled circles). Nevertheless, both treatments enhanced significantly the therapeutic activity of VRP−TRP2 (Fig. 1B, filled squares). GITR stimulation during VRP−TRP2 vaccination was particularly effective, achieving tumor regression in 90% of mice (Fig. 1B, i), whereas anti–CTLA-4 mAb plus VRP−TRP2 benefited 50% of the animals, thus doubling tumor-free survival induced by the vaccine alone (Fig. 1B, ii). All of the mice that benefited from the treatment survived a second tumor challenge 3 months later, suggesting the establishment of a long-lasting antitumor immunity via the induction of a memory response (data not shown). In line with the different antitumor activity of the two combinations, only the addition of anti-GITR mAb to the VRP−TRP2 vaccine induced a mild form of autoimmune hypopigmentation of the coat (Supplementary Fig. S1).

**Anti–CTLA-4 and anti-GITR mAbs augment CTL and Ab responses to VRP−TRP2 vaccine**

To investigate the mechanism underlying the differing potency of the two combinations, we first analyzed the adjuvant effect of the mAbs on vaccine-induced TRP2-specific CD8 + T-cell responses in naive (non−tumor-bearing) mice. Both anti-GITR and anti–CTLA-4 mAbs increased the frequency of TRP2-specific CD8 + T cells in the spleens of vaccinated mice by approximately 4-fold, as measured by IFN-γ ELISPOT, 5 to 7 days after the last immunization (Fig. 2A). However, no significant difference was detected between anti-GITR and anti–CTLA-4 mAbs (Fig. 2A). Consistently, when treatments were performed in B16F10 melanoma-bearing mice, the tumor-killing capacity of tumor-infiltrating CD8 + T cells was significantly enhanced by both mAbs in a similar manner (Fig. 2B, i–ii, left). The same results were achieved when CD8 + T-cell cytotoxicity was tested in a more sensitive three-dimensional collagen–fibrin gel culture system (Fig. 2C, left), which mimics a tissue-like environment more faithfully (39). Tumor-infiltrating effector CD4 + T cells from mice treated with either combination or vaccine alone did not show any significant cytotoxic activity ex vivo (Fig. 2B and C, right).

Next, we investigated whether anti-GITR and anti–CTLA-4 mAbs could differentially affect the development of a TRP2-specific Ab response. We have demonstrated previously that VRP−TRP2 has the unique ability to stimulate the production of specific serum IgGs, which are required for optimal antitumor efficacy (15). We thus analyzed the quantity and quality of vaccine-induced anti-TRP2 Abs when anti–CTLA-4 or anti-GITR mAb were coadministered in non−tumor-bearing mice. Seven days after the last vaccination, the serum level of TRP2-specific IgGs was significantly higher in animals cotreated with either mAb, with the IgG2b and IgG2c species being the most prevalent isotypes (Fig. 3A). Interestingly, while VRP−TRP2 alone induced IgG primarily directed to a specific TRP2 N-terminal.
epitope, the addition of either immunomodulatory mAb favored the development of Abs with a broader TRP2 specificity, including reactivity to more central regions of the TRP2 protein in at least 30% of the mice (Fig. 3B). This effect was particularly evident when anti-GITR mAb was combined with the vaccine (Fig. 3B).

These results indicate that, despite differing therapeutic activities of the two combinations, anti-GITR and anti–CTLA-4 mAbs can comparably augment the development of both T- and B-cell responses to the VRP–TRP2 vaccine.

Modulation of the tumor immune infiltrate after treatment with immunomodulatory mAbs

We then investigated whether anti–CTLA-4 and anti-GITR mAbs could differently alter the composition of the immune infiltrate at the tumor site. As the size of the tumors significantly differed among treatment groups (Fig. 4A), the relative proportions, as opposed to absolute numbers of cells, are reported. Addition of either immunomodulatory mAbs to the VRP–TRP2 vaccine doubled the percentage of total CD45+ immune infiltrate, when compared with VRP–TRP2 alone.
(Fig. 4B). CD8$^+$ T cells were the most abundant population in tumors treated with VRP–TRP2 combined with either anti-GITR or anti–CTLA-4 mAb, reaching approximately 40% of CD4$^+$ cells. In contrast, in tumors treated with the vaccine alone, CD8$^+$ T cells constituted less than 20% of the immune infiltrate (Fig. 4C). The comparison between the two combinations highlighted a trend toward an increase in tumor-infiltrating CD11c$^+$ cells in anti-GITR mAb-treated mice, and a significantly higher percentage of CD4$^+$ TILs only when VRP–TRP2 was given in combination with the anti–CTLA-4 mAb (Fig. 4C and Supplementary Fig. S2A). In our previous work, we demonstrated that signaling through activating Fc receptors is important for the antitumor efficacy of VRP–TRP2-induced Abs response (15). Thus, even if limited, the anti-GITR mAb-mediated increase in tumor-associated myeloid cells could contribute to the enhanced therapeutic efficacy of the combination with the vaccine. On the other hand, the significant intratumor accumulation of CD4$^+$ T cells observed with anti–CTLA-4 mAb + VRP–TRP2 led us to hypothesize that this cell population could include CD4$^+$Foxp3$^+$ Tregs, thus explaining the reduced therapeutic advantage of this combination. However, further phenotypic analysis of the CD4$^+$ TIL subsets revealed that the frequency of CD4$^+$Foxp3$^+$ Tregs was lowered to a similar extent by either combination, indicating that the CD4$^+$ TILs expanded with anti–CTLA-4 mAb did not include conventional Tregs (Fig. 4D). The net result at the tumor site was an increase in the CD8$^+$ T-cell/CD4$^+$Foxp3$^+$ Treg ratio when either anti–CTLA-4 or anti-GITR mAb was added (Fig. 4E, i), and in the CD4$^+$Foxp3$^+$ T-cell/CD4$^+$Foxp3$^+$ Treg ratio specifically with the anti–CTLA-4 mAb combination (Fig. 4E, ii). This is consistent with the idea that blocking CTLA-4 during VRP–TRP2 vaccination favors the recruitment of both CD4$^+$Foxp3$^+$ and CD8$^+$ T cells into the tumor microenvironment, while triggering GITR increases primarily CD8$^+$ T-cell frequency. Of note, the effect of anti–CTLA-4 mAb on the accumulation of CD4$^+$ TILs was also evident when mice were treated in combination with a control VRP–GFP vaccine (Supplementary Fig. S2B), indicating that it is not dependent on the presence of the immunizing antigen.

**Treatment with anti–CTLA-4 mAb specifically enhances the accumulation of CD4$^+$Foxp3$^+$ PD-1$^+$ T cells within the tumor**

We next compared the phenotype of CD4$^+$ TILs among the different treatment groups and found that a higher percentage of these cells expressed the surface marker PD-1 in tumors treated with the vaccine + anti–CTLA-4 mAb, compared with the combination with anti-GITR mAb or the isotype control (Fig. 5A). This effect occurred specifically at the tumor site and in the CD4$^+$ T-cell compartment. The percentage of CD4$^+$ T cells expressing PD-1 in the spleen remained unchanged (Supplementary Fig. S3A), and the percentage of PD-1$^+$CD8$^+$ TILs was not affected by the treatment (Supplementary Fig. S3B). Of note, the increase in PD-1$^+$ CD4$^+$ TILs in anti–CTLA-4 mAb-treated mice was only evident when the Foxp3$^+$ population was examined, indicating that these cells did not include...
Further immunophenotypic characterization revealed that these intratumor CD4+Foxp3+ PD-1+ T cells expressed the activation markers CD27 and ICOS at similar levels, but higher amounts of the Treg-associated receptors OX40 and GITR compared with their PD-1− counterparts (Fig. 6A). Standard in vitro cytotoxicity assays showed that neither PD-1− nor PD-1+ CD4+ TILs could directly kill B16F10 tumor cells ex vivo (Fig. 6B). These results exclude the possibility that intratumor CD4+ Foxp3+ PD-1+ T cells induced by anti–CTLA-4 mAb are functional effector T cells.

Discussion

This study demonstrates the therapeutic advantage of combining the VRP–TRP2 vaccine with either antagonist anti–CTLA-4 or agonist anti-GITR mAb in the B16F10 melanoma mouse model. Although the combination with anti-GITR mAb achieved a superior level of antitumor activity than that with the anti–CTLA-4 mAb, both treatments enhanced the development of TRP2-specific cytotoxic CD8+ TILs and circulating Abs significantly above the levels induced by the vaccine alone. However, relative to CTLA-4 blockade, the agonist anti-GITR mAb favored the induction of Abs with a slightly broader anti-TRP2 reactivity, and demonstrated a tendency to facilitate the recruitment of phagocytes at the tumor site, thus pointing to the establishment of a potentially more effective antitumor humoral immunity. On the other hand, treatment with anti–CTLA-4 but not anti-GITR mAb was associated with a selective intratumor increase of CD4+ Foxp3+ PD-1+ T cells lacking cytotoxic functions and expressing common Treg-associated markers. Their high surface expression of GITR indicates that they can be controlled by anti-GITR mAbs, thus potentially explaining the differential accumulation of these cells in the two treatment conditions. This suggests that melanoma-associated CD4+ Foxp3+ PD-1+ T cells may limit the efficacy of immunomodulatory treatments.

Our findings clearly demonstrate that targeting either coinhibitory (CTLA-4) or costimulatory (GITR) receptors may serve to shape the immune response induced by a vaccine with significant antitumor efficacy, yielding a combination with enhanced therapeutic activity. Given that CTLA-4, a major negative regulator of T cells, is rapidly induced in the early phases of immune responses (40), its immediate and persistent inhibition during vaccination may promote T-cell activation during priming. On the other hand, GITR stimulation on vaccine-primed T cells can sustain their expansion (4, 12). Either mechanism can explain the enhanced frequency of TRP2-specific CD8+ T cells in the periphery and cytotoxic activity of CD8+ TILs in mice treated with the combinations versus with the VRP–TRP2 vaccine alone. Aside from the direct positive effects of anti–CTLA-4 and anti-GITR mAbs on effector T cells, increasing evidence is underscoring their ability to counteract tumor-induced Treg expansion and/or suppressive activity (10–12, 41). This dual effect seems to be crucial for the efficacy of immunomodulatory mAbs (10–12), as counteracting immunosuppressive factors is important in allowing the execution of a primed immune response. In view of the bidirectional interactions between the two major immunosuppressive

conventional Foxp3+ Tregs (Fig. 5B). The percentage of PD-1-expressing cells in the CD4+Foxp3+ Treg compartment was decreased to a similar extent by both mAbs (Fig. 5B), and, as a result, the intratumor CD4+Foxp3+ PD-1+ T-cell/Treg ratio was significantly increased only by the anti–CTLA-4 mAb combination (Supplementary Fig. 3C). Interestingly, tumor-selective accumulation of CD4+ PD-1+ T cells occurred to the same degree when mice were treated with anti–CTLA-4 mAb in combination with a control VRP–GFP vaccine (Supplementary Fig. S4), indicating that this effect was dependent on the blockade of CTLA-4 and independent from the antigen.

Figure 4. Anti–CTLA-4 and anti-GITR mAbs differently affect the accumulation of intratumor CD4+ T cells. B16F10 melanoma-bearing mice were treated with VRP–TRP2 in combinations with anti–CTLA-4 (αCTLA-4) or anti-GITR (αGITR) mAb or isotype control as shown in Fig. 1. A, weight of Matrigel plugs when explanted 16 days after tumor injection. B, percentage of live CD45+ immune cells infiltrating the tumor. C, flow cytometry analysis of CD3+CD8+ (CD8), CD3+CD4+ (CD4), CD11c+CD4+ (CD11c), Foxp3+ (CD3), CD103+ (CD103), NK1.1+ (NK), CD3−NK1.1−CD11c−CD11c+ (CD11c), CD3−NK1.1−CD11c− (other) cells infiltrating B16F10-Matrigel plugs. D, CD4+Foxp3+ Treg/CD45+ cell frequency in tumors from mice treated as indicated. E, ratio between frequencies of CD8+ (i) or CD4+Foxp3+ T cells (ii) and CD4+Foxp3− Tregs in tumors from mice treated as indicated. Data represent mean ratios ± SEM of four to seven tumors analyzed individually per group. **, P < 0.01; *, P < 0.05; ns, not statistically significant.
cell subsets, namely Tregs and myeloid-derived suppressor cells (MDSC; ref. 42), interfering with one of them may be sufficient to disinhibit tolerogenic influences in the tumor microenvironment. Along these lines, depletion/inactivation of Tregs has been shown to concurrently reduce MDSC-suppressive functions and counteract the immunosuppressive tumor microenvironment leading to tumor rejection (42). Here, we show that anti-GITR and anti–CTLA-4 mAbs are also able to reduce intratumor Treg frequency when they are combined with the VRP–TRP2 vaccine, thus providing another level of explanation for the improved therapeutic activity of the combination treatments. Furthermore, both anti–CTLA-4 and anti-GITR mAbs enhanced the ability of the VRP–TRP2 vaccine to promote the development of antigen-specific humoral immunity. Not only the magnitude but also the quality of vaccine-induced Ab response was favorably affected, with anti-GITR mAb widening the spectrum of TRP2 epitopes recognized by postimmunization serum samples to a greater extent than anti–CTLA-4 mAb. The concurrent trend toward an increased frequency of intratumor CD11c+ cells after treatment with the anti-GITR mAb combination points to the induction of a more efficient TRP2-specific Ab response that might explain its improved outcome.

Furthermore, we found that tumor infiltration of CD4+ Foxp3+ T cells expressing the coinhibitory receptor PD-1 was significantly increased only when anti–CTLA-4 mAb was administered. However, because the majority of these cells express GITR and therefore can be potentially affected by anti-GITR mAb, we cannot rule out that CD4+ Foxp3+ PD-1+ T cells may also develop in the context of GITR stimulation and then be eliminated/modulated.

Persistent antigen exposure and exhaustion are generally associated with PD-1 upregulation on T cells (43). Within the CD4+ T-cell compartment, not only activated cells, but also Tregs, can express PD-1. In addition to limit proximal T-cell receptor signaling and cytokine secretion, as observed in CD8+ T cells, PD-1 ligation on either naive or Th1-polarized CD4+ T cells promotes their conversion into Tregs (44, 45). Interestingly, PD-1 blockade proved sufficient to inhibit the suppressive function of both murine and human CD4+CD25+ T cells (46–48). One potential reason of PD-1+ CD4+ T-cell accumulation during anti–CTLA-4 mAb therapy may be the establishment of a PD-1–mediated feedback loop able to ensure Akt inhibition even in the absence of CTLA-4 signaling. Indeed, even if CTLA-4 and PD-1 have different ligands and cytoplasmic domains, they share the ability to block T-cell activation by inhibiting Akt, a key regulator of T-cell growth and

Figure 5. Treatment with anti–CTLA-4 mAb favors the accumulation of CD4+ Foxp3+ PD-1+ T cells at the tumor site. B16F10 melanoma-bearing mice were treated with VRP–TRP2 plus isotype control, anti–CTLA-4 (αCTLA-4), or anti-GITR (αGITR) mAb, as shown in Fig. 1. A, representative dot plots (left) and percentage of PD-1+/CD4+ TILs (right). B, representative dot plots and percentage of PD-1– expressing CD4+ Foxp3+ (left) and CD4+ Foxp3– (right) TILs. Each dot represents an individual mouse. *, P < 0.05; **, P < 0.01; ns, not statistically significant.
To be elucidated, our recent melanoma cells of PD-1 

ex vivo background level, calculated as the average percentage killing TRP2 in combination with anti

1:1) sorted from tumors grown in Foxp3-GFP mice and treated with VRP

B16F10 killing.

T cells, which can boost MDSC antigen-nonspecific activity (50). Even if the mechanisms through which CD4

tability to favor intratumor accumulation of CD4

þ T cells (Eff:Target =

1þ) mediated

CD8 TILs induced by VRP–TRP2 + anti–CTLA-4 mAb combination. B, ex vivo cytotoxicity assay measuring killing activity against B16F10 melanoma cells of PD-1– and PD-1+ CD4 Foxp3 T cells (Eff:Target = 1:1) sorted from tumors grown in Foxp3-GFP mice and treated with VRP–TRP2 in combination with anti–CTLA-4 mAb. The dotted line indicates the background level, calculated as the average percentage killing + (3×SD) using naïve splenocytes as effectors. CD4+ T cells purified from TYRP1 TCR transgenic mice were used as a positive control for T cell-mediated B16F10 killing.

The association between the more limited therapeutic advantage of anti–CTLA-4 mAb combination and its ability to favor intratumor accumulation of CD4+ Foxp3 PD-1+ T cells suggests that these cells may negatively affect immune potentiation and tumor immunity after immunomodulatory treatments. Lack of antitumor cytotoxic activity and expression of Treg-associated markers in these cells bring forth the possibility that they represent a dysfunctional CD4+ T-cell population potentially en route to be reprogrammed toward a suppressive phenotype. Alternatively, they may indirectly favor the establishment of a tolerogenic tumor microenvironment, as described for antigen-experienced CD4+ T cells, which can boost MDSC antigen-nonspecific suppressive activity (50). Even if the mechanisms through which CD4+ Foxp3 PD-1+ T cells may limit antitumor immunity remain to be elucidated, our recent finding that concurrent blockade of CTLA-4 and PD-1 in patients with advanced melanoma compares favorably with either strategy used alone (9) supports this hypothesis.

Functional characterization of the CD4+ Foxp3+ PD-1+ TILs expanded during treatment with anti–CTLA-4 mAb, and identification of how to modulate or eliminate this population will be crucial to define the optimal context in which to exploit CTLA-4 blockade for cancer immunotherapy. On the other hand, the excellent therapeutic activity and safety profile of the VRP–TRP2 vaccine in combination with anti-GITR mAb in a therapeutically challenging setting opens up the possibility of additional avenues for improving cancer immunotherapy, especially given the safety and immunologic efficacy of VRP-based vaccines demonstrated in humans (24), and the fact that anti-GITR mAbs are already in clinical development (Trial NCT01767454).

Disclosure of Potential Conflicts of Interest

J.D. Wolchok has ownership interest (including patents) in the VRP-TRP2 vaccine. No potential conflicts of interest were disclosed by the other authors. M.F. Maughan and R. Olmsted were employees of AlphaVax, Inc. This does not alter the author’s adherence to all CIR policies on sharing data and materials.

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Acknowledgments

The authors thank S. Terrulli for her assistance in the preparation of this article, current and former Wolchok laboratory members for their helpful critical comments, as well as members of the MSKCC flow cytometry core facility for their technical support.

Grant Support

This study was supported by grants from the National Cancer Institute (R01CA56821), Swim Across America, the Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Cancer Foundation for Research. F. Avogadri was supported by the Lucille Castori Center for Microbes, Infection & Cancer fellowship.

Disclosure of Potential Conflicts of Interest

Information on potential conflicts of interest and financial relationships relevant to this article is included in the Disclosure of Potential Conflicts of Interest section.

Received December 6, 2013; revised January 17, 2014; accepted January 28, 2014; published OnlineFirst February 26, 2014.

References


Figure 6. Phenotypic and functional characterization of intratumor CD4+ Foxp3- PD-1+ T cells. A, histogram plots showing the expression of the indicated surface markers in PD-1+ (filled gray) or PD-1- (black) CD4+ Foxp3 TILs of B16F10 melanoma cells of PD-1- and PD-1+ CD4 Foxp3 T cells (Eff:Target = 1:1) sorted from tumors grown in Foxp3-GFP mice and treated with VRP–TRP2 in combination with anti–CTLA-4 mAb. The dotted line indicates the background level, calculated as the average percentage killing + (3×SD) using naïve splenocytes as effectors. CD4+ T cells purified from TYRP1 TCR transgenic mice were used as a positive control for T cell-mediated B16F10 killing.
Melanoma Vaccine Combined with Immunomodulation


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