Cytotoxic T Lymphocyte Antigen-4 Blockade Enhances Antitumor Immunity by Stimulating Melanoma-Specific T-cell Motility

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Abstract

It is now clear that anti-CTLA-4 (α-CTLA-4) antibodies stimulate tumor immunity either by relieving inhibition of effector T-cell function or by depletion of regulatory T cells (Treg). Several recent reports, however, have suggested that these antibodies may deliver a “go” signal to effector T cells, thus interrupting T-cell receptor signaling and subsequent T-cell activation. We examined the behavior of melanoma-specific CD8+ pmel-1 T cells in the B16/BL6 mouse model using intravitral microscopy. Pmel-1 velocities in progressively growing tumors were lower than their velocities in tumors given a therapeutic combination that included α-CTLA-4 antibodies, suggesting that successful immunotherapy correlates with greater T-cell motility. When α-CTLA-4 antibodies were injected during imaging, the velocities of pmel-1 T cells in tumor-draining lymph nodes also increased. Because α-CTLA-4 Fab fragments had the same effect as the intact antibody, the higher T-cell motility does not seem to be due to CTLA-4 inhibitory signaling but rather to the release of nonproductive stable interactions between tumor-infiltrating T cells and tumor targets or antigen-presenting cells subsequent to CTLA-4 blockade.

This phenomenon resembles the recently described reversal of the antiviral T-cell motility paralysis by programmed death 1 (PD-1)–specific antibodies during T-cell exhaustion in persistent viral infections. Cancer Immunol Res; 2(10); 970–80. ©2014 AACR.

Introduction

The idea that the immune system can fight cancer has been around for decades, but only recently has it become possible to harness its power for the treatment of patients with cancer in the clinic. FDA approval of Provenge (sipuleucel-T; Dendreon) as a cell-based vaccine for castration-resistant prostate cancer (1) and ipilimumab, a monoclonal antibody against CTLA-4, for the treatment of metastatic melanoma (2), are signs of the growing recognition for the clinical importance of cancer immunotherapy.

CTLA-4 is a protein expressed on the surface of activated T cells subsequent to T-cell receptor (TCR) and CD28 engagement. It interacts with two ligands on antigen-presenting cells subsequent to CTLA-4 blockade. At the amino acid level, CTLA-4 is homologous to the prototypical costimulatory receptor CD28, which also binds to B7-1 and B7-2, and enhances T-cell proliferation and effector function. Multiple molecular mechanisms are proposed for the way in which CTLA-4 inhibits T-cell activation, including successful competition with CD28 for ligand binding or ligand removal by transendocytosis (reviewed in ref. 3).

Despite extensive literature on the efficacy of α-CTLA-4 antibodies in preclinical animal models, as well as in human patients with cancer (recently reviewed in ref. 4), some controversy still exists about the mechanism by which these antibodies mediate tumor regression. Before the identification of regulatory T cells (Treg), multiple studies showing the efficacy of α-CTLA-4 antibodies in the treatment of several tumor models in mice assumed that the effect was due to the blockade of α-CTLA-4 inhibitory signaling in effector T cells (5, 6). The discovery of CD4+ Foxp3+ Tregs that express high levels of CTLA-4 on their surface, however, suggested a different cellular target for the α-CTLA-4 antibodies—the Tregs themselves (7, 8). More recent studies have shown that the blockade of CTLA-4 on both T-effector cells and Tregs is required for optimal tumor immunity (9), and at least in cases in which tumors contain large numbers of macrophages that express Fcγ receptors, α-CTLA-4 antibodies seem to mediate the specific depletion of tumor-infiltrating Tregs via an Fc-dependent mechanism (10–12).

Real-time intravital imaging of tumor-specific immune responses can provide information about the cellular dynamics in lymph nodes and tumors, as well as how cell behaviors change as a result of various immunotherapies. Initial studies of tumor-specific immune responses used transplantable tumors expressing the foreign antigen ovalbumin (OVA) and adoptively transferred OVA-specific OT-1 CD8+ T cells...
(reviewed in ref. 13)). More recent work defined the steady-state dynamics of melanoma-specific CD8\(^+\) pmel-1 T cells, which recognize the self-antigen gp100 in the context of H2-D\(^b\) (14), and compared them with OT-I T cells in the absence of OVA (15). None of these early reports, however, examined whether the T-cell dynamics in tumors might change in the course of successful immunotherapy. Most recently, a study investigated the effects of CTLA-4 blockade alone or in combination with radiotherapy on the dynamics of a polyclonal population of CXC\(R\)6\(^+\) tumor-infiltrating lymphocytes (TIL; ref. 16). Because checkpoint blockade is known to affect TIL composition and tumors have been shown to contain both tumor-specific and nontumor-specific T cells (13), the direct effects of \(\alpha\)-CTLA-4 antibodies on tumor-specific immune responses have yet to be determined.

We have used two-photon microscopy (TPM) to examine the behavior of melanoma-specific CD8\(^+\) pmel-1 T cells in a poorly immunogenic mouse model, B16/BL6. Here, we report the differences in the cellular dynamics in tumors relative to tumor-draining lymph nodes (TDLN) and the changes brought about by GVax, a GM-CSF-secreting irradiated B16/BL6 cellular vaccine, alone or in combination with \(\alpha\)-CTLA-4 antibody blockade. The average velocities of pmel-1 T cells in the tumors of mice treated with GVax alone were lower than their velocities in animals treated with the combination of GVax and \(\alpha\)-CTLA-4 antibodies, suggesting that successful immunotherapy correlates with increased T-cell motility. When \(\alpha\)-CTLA-4 antibodies were injected i.p. in live animals, the velocities of pmel-1 T cells in TDLNs, but not in tumors, increased, regardless of the chronic treatment. Because \(\alpha\)-CTLA-4 Fab fragments had the same effect as the intact antibody, the increased T-cell motility does not seem to be due to CTLA-4 inhibitory signaling but rather due to the physical disruption of the stable interactions between T cells and their tumor targets or APCs, which seem to be detrimental to tumor immunity. This conclusion is in direct opposition to the current model of CTLA-4-mediated inhibition via the reversal of the TCR-induced stop signal (17). Instead, our observations resemble the recently described reversal of the antiviral T-cell motility paralysis by PD-1-specific antibodies during T-cell exhaustion in persistent viral infections (18).

**Materials and Methods**

**Mice**

Six- to 8-week-old C57BL/6 mice were purchased from The Jackson Laboratory. Pmel-1 mice were obtained from N. Restifo (National Cancer Institute, Bethesda, MD) and were crossed to ubiquitin-GFP mice from The Jackson Laboratory. Pmel-1 mice were obtained from N. Restifo (National Cancer Institute, Bethesda, MD) and were crossed to ubiquitin-GFP mice from The Jackson Laboratory. Pmel-1-GFP T-cell transfer, tumor challenge, and Pmel-1-GFP T-cell transfer, tumor challenge, and treatment CD8\(^+\) T cells were purified from lymph nodes of pme-1-GFP mice via negative selection (Miltenyi Biotec). C57/BL6 mice were injected i.p. in live animals, the velocities of pmel-1 T cells in TDLNs, but not in tumors, increased, regardless of the chronic treatment. Because \(\alpha\)-CTLA-4 Fab fragments had the same effect as the intact antibody, the increased T-cell motility does not seem to be due to CTLA-4 inhibitory signaling but rather due to the physical disruption of the stable interactions between T cells and their tumor targets or APCs, which seem to be detrimental to tumor immunity. This conclusion is in direct opposition to the current model of CTLA-4-mediated inhibition via the reversal of the TCR-induced stop signal (17). Instead, our observations resemble the recently described reversal of the antiviral T-cell motility paralysis by PD-1-specific antibodies during T-cell exhaustion in persistent viral infections (18).

**Cell lines**

The B16/BL6 melanoma was originally obtained from J. Fiddler (University of Texas MD Anderson Cancer Center, Houston, TX) and was validated as free from *Mycoplasma* and rodent infectious agents; no other validations were performed.

The B16/BL6 line secreting GM-CSF (GVax) was derived from the parental line and has been described earlier (19). It was also validated as free from *Mycoplasma* and rodent infectious agents. The plasmid pRSET-tdTomato was obtained from R. Tsien (University of California, San Diego, San Diego, CA) and used to replace the GFP in the lentiviral vector pRRLSIN. The B16/BL6 line secreting GM-CSF (GVax) was derived from the parental line and has been described earlier (19). It was also validated as free from *Mycoplasma* and rodent infectious agents. The plasmid pRSET-tdTomato was obtained from R. Tsien (University of California, San Diego, San Diego, CA) and used to replace the GFP in the lentiviral vector pRRLSIN. cPPT.PKG-GFP.WPRE (from Addgene) with tdTomato. Lenti-virus was packaged using 293T cells transfected with the tdTomato-expressing plasmid, as well as pU6-CDG (producing VSV-G envelope) and \(\Delta R B 9.1\) (producing gag-pol), using Fugene 6 (Roche). The parental B16/BL6 cells were infected by centrifugation at 1,200 \(\times g\) using filtered viral supernatants, supplemented with 4 \(\mu \)g/mL polybrene and 25 mmol/L HEPES (Sigma). The resulting B16-Tomato line was validated as free from *Myeloma* and rodent infectious agents. No other validations were performed.

**In vivo antibodies**

\(\alpha\)-CTLA-4 (clone 9H10), \(\alpha\)-B7-1 (clone 16-10A1), \(\alpha\)-B7-2 (clone GL-1), and hamster Ig were purchased from Bio-XCell. \(\alpha\)-Mouse H2-\(D\(^{\text{b}}\) (clone 28-14-8) was purchased from SouthernBiotec. \(\alpha\)-CD28 (clone 37N) was purified by the monoclonal facility at Memorial Sloan Kettering Cancer Center (New York, NY). The F(ab\(_2\)) fragments of 9H10 were generated and purified by Bio-Synthesis, Inc.

**Pmel-1-GFP T-cell transfer, tumor challenge, and treatment**

CD8\(^+\) T cells were purified from lymph nodes of pme-1-GFP mice via negative selection (Miltenyi Biotec). C57/BL6 mice received an adoptive transfer of 200,000 pme-1-GFP T cells and an intradermal (i.d.) challenge of 75,000 B16-Tomato cells on day 0. They were untreated or treated with GVax, \(\alpha\)-CTLA-4, or GVax + \(\alpha\)-CTLA-4 on days 3, 6, and 9. One million irradiated (15,000 rad) GVax cells in 100 \(\mu \)L of PBS were injected i.d. in the contralateral flank at the same time as 200 \(\mu \)g, 100 \(\mu \)g, and 100 \(\mu \)g of \(\alpha\)-CTLA-4 antibodies were injected i.p. in 200 \(\mu \)L of PBS. Mice were monitored for tumor growth or used for TPM experiments. In some cases, the tumor challenge was increased to 150,000 B16-Tomato cells, to ensure adequate sample for flow cytometric analysis.

**Tissue processing for flow cytometry**

Mice were sacrificed on day 13 or 14 after tumor implantation. Inguinal, axillary, and brachial lymph nodes were dispersed through a 70-\(\mu\)m filter. Tumors were digested for 30 minutes at 37°C with a mixture of 0.32 mg/mL Liberase TL (Roche) and 0.2 mg/mL DNase I (Roche) in serum-free RPMI, then dispersed through a 70-\(\mu\)m filter. To analyze T-cell populations, samples were stained with \(\alpha\)-CD45.2 Pacific Blue, \(\alpha\)-CD3 PE-Cy7, \(\alpha\)-CD4 APC-e780, \(\alpha\)-CD8 PerCP-e710, \(\alpha\)-Foxp3-Alexa700 (all from eBioscience), \(\alpha\)-CD4 Qdot605 (Life Technologies), and \(\alpha\)-Ki67-PE (BD Biosciences). Pmel-1 T cells were identified by GFP fluorescence. To assay cytokine secretion, T cells were restimulated with a mixture of purified dendritic cells (DC) and B16/BL6 lysates plus 1 \(\mu\)mol/L of gp100 peptide for 4 hours at 37°C in the presence of brefeldin A (BD Biosciences). Cytokines were detected using \(\alpha\)-IL2 APC and \(\alpha\)-IFN\(\gamma\) PE (eBioscience).
Intravital microscopy

Tumor-bearing mice were anesthetized with isoflurane and immobilized on a custom-built heated stage. Tumors or TDLNs were surgically exposed and placed under a cover slip mounted on a nylon washer. Mouse tissues were kept hydrated using warm PBS. Imaging was performed on an Olympus BX51WI upright microscope using a 20×/0.95 W XUMPlanFL objective. Fluorescence was excited by a 3W Mai Tai HP Ti: Sapphire laser (Spectra-Physics) tuned to 910 nm. For the detection of second harmonic generation, a 493-nm LP dichroic mirror and a 460- to 490-nm bandpass emission filter were used. For GFP fluorescence, a 520-nm LP dichroic mirror and a 500- to 525-nm bandpass filter were used. For tdTomato fluorescence, a 560-nm LP dichroic mirror and a 570- to 640-nm bandpass filter were used. All filters were purchased from Semrock. Images were recorded using Video Savant software (IO Industries). Volumes of approximately 30 xy planes, each spanning 320 × 250 μm, separated by 10-μm z steps, were acquired every minute for 20 minutes before antibody injection and for 1 hour after that. Of note, 250 μg of the indicated antibodies were injected i.p. in the mouse under observation in 0.5 mL of PBS.

Data analysis

Cell movement was analyzed using Imaris (Bitplane AG). Individual cells were identified and tracked by the software after removal of sample drift. The arrest coefficient was defined as the percentage of time a cell spent at instantaneous speeds under 2.5 μm/min. The confinement index was defined as the ratio between displacement and total track length. Statistical analysis was performed using Prism (GraphPad Software, Inc.). Statistical significance was determined by the Student t test (between two groups or conditions) or ANOVA with a post hoc test (three or more groups or conditions). P values of <0.05 were considered statistically significant.

Results

Pmel-1-GFP T cells are reporters for endogenous CD8+ T-cell responses

To determine whether pmel-1 T cells can affect tumor progression subsequent to immunotherapy, we compared the growth of tdTomato-expressing B16/BL6 melanoma (B16-Tomato) in the presence or absence of adoptively transferred GFP-positive pmel-1 T cells. Similar to published results for unmodified B16/BL6 tumors (19), the combination of GVax and α-CTLA-4 significantly delayed tumor progression, whereas each component alone had little effect (Fig. 1A). The adoptive transfer of relatively small numbers of pmel-1 T cells did not significantly affect tumor progression, except in the mice that received no other immunotherapy, in which tumor growth was somewhat delayed (Fig. 1A).

To compare the activation status of the transferred pmel-1 T cells and the endogenous polyclonal CD8+ population, we analyzed TIL and lymph node cells by flow cytometry. As expected, successful immunotherapy significantly increased the number of pmel-1 and polyclonal CD8+ T cells per gram of tumor (Fig. 1B). In addition, the majority of the tumor-infiltrating pmel-1 and polyclonal CD8+ T cells were proliferating (based on Ki67 expression) and were able to produce IFNγ upon restimulation with antigen-pulsed APCs (Fig. 1B). These results suggest that in tumors, pmel-1 T cells mirror the proliferative and cytokine response of the endogenous polyclonal CD8+ population.

The equivalent analysis of lymph node cells led to slightly different conclusions. Whereas the combination therapy significantly increased the proliferating fraction of both pmel-1 and polyclonal CD8+ T cells, considerably more pmel-1 T cells were proliferating without any treatment and that percentage more than doubled after therapy (Fig. 1C). Similarly, a lot more pmel-1 T cells were capable of making IFNγ upon restimulation in the untreated mice, relative to 1% of the polyclonal CD8+ population. Finally, the fraction of pmel-1 T cells that could make IL2 increased from 10% in the untreated controls to 20% in the combination-treated animals, whereas the IL2-secreting portion of polyclonal CD8+ T cells remained less than 1% (Fig. 1C). These results suggest that the pmel-1 T cells in lymph nodes are more activated than the endogenous polyclonal CD8+ population, probably because they are responding to tumor antigens, whereas the vast majority of lymph node CD8+ T cells are not reactive with tumor antigens.

Pmel-1 dynamics in tumors are affected by chronic CTLA-4 blockade

To investigate the response of tumor-infiltrating T cells to chronic CTLA-4 blockade, we compared the intratumoral pmel-1 dynamics of mice treated with GVax alone or GVax + α-CTLA-4 by TPM. After 20 minutes of imaging, we injected 250 μg of α-CTLA-4 antibodies in the peritoneal cavity of the mouse on the microscope stage and continued data acquisition for 1 hour to determine whether this acute antibody blockade affects pmel-1 mobility (Fig. 2A and Supplementary Movies S1 and S2).

The average initial pmel-1 velocities in tumors of mice treated with GVax alone were lower than their velocities in combination-treated mice, suggesting that chronic CTLA-4 blockade increases T-cell mobility. The blockade also decreased the arrest coefficients, but did not affect the displacements or confinement indexes of pmel-1 T cells in tumors (Fig. 2B–E). These results are consistent with published data on the effects of α-CTLA-4 antibodies on T-cell mobility (16, 17).

The bolus injection of α-CTLA-4 antibodies in the mouse under observation did not change pmel-1 velocities, arrest coefficients, or displacements in either group (Fig. 2B–D), but...
it decreased the confinement indexes in both cases (Fig. 2E). The latter outcome is probably due to the unaltered displacements and the significantly longer track lengths that resulted from the longer imaging periods after antibody injection. Overall, the data suggest that the acute blockade of CTLA-4 does not immediately affect pmel-1 mobility in tumors, even though chronic CTLA-4 blockade increases their intratumoral mobility.

**Pmel-1 dynamics in TDLNs are affected by acute CTLA-4 blockade**

To determine whether CTLA-4 blockade changes the behavior of tumor-specific T cells in TDLNs, we performed similar analyses of pmel-1 T cells in TDLNs of untreated mice, as well as in animals that had received α-CTLA-4 antibodies alone, GVax alone, or the combination of GVax + α-CTLA-4. After 20 minutes of imaging, we again injected α-CTLA-4 antibodies into the mouse under observation and continued data acquisition for 1 hour to determine whether the acute antibody blockade affects pmel-1 mobility in TDLN (Fig. 3A and Supplementary Movies S3 and S4).

Unlike the results in tumors, the on-stage injection of α-CTLA-4 antibodies significantly increased the average velocity of pmel-1 cells in TDLNs under all conditions (Fig. 3B). The acute CTLA-4 blockade also significantly decreased the arrest coefficients (Fig. 3C) and increased the average displacement of pmel-1 T cells (Fig. 3D), without affecting their confinement indexes (Fig. 3E). These data are consistent with published reports (16, 17) and suggest that large amounts of α-CTLA-4 antibodies can relatively quickly stimulate tumor-specific T-cell mobility in TDLNs, but not in tumors.

In contrast with the tumor data, the initial velocities, arrest coefficients, displacements, and confinement indexes of pmel-1 T cells in TDLNs were the same, regardless of treatment (Fig. 4A). A comparison of pmel-1 motility in tumors and TDLNs (Fig. 4B) revealed that in GVax-treated mice, the average velocities were lower and the arrest coefficients were higher in tumors relative to TDLNs. In contrast, pmel-1 velocities and arrest coefficients in combination-treated mice were not significantly different between tumors and TDLNs. Despite the similarity of pmel-1 velocities and arrest coefficients in tumors and TDLNs of combination-treated mice, pmel-1 mobility in tumors appeared more restricted than their mobility in TDLNs, as evidenced by the lower average displacements and the lower confinement indexes of the pmel-1 T cells in tumors (Fig. 4B).

**Effects of other antibodies on pmel-1 dynamics in TDLNs**

To determine whether the increase in pmel-1 mobility in TDLNs subsequent to acute CTLA-4 blockade is specific, we also injected the antibodies shown in Fig. 5. Similar to the effects of α-CTLA-4 antibodies, the injection of 250 μg each of α-B7-1 and α-B7-2 antibodies, known to block the ligands for CTLA-4 and CD28 (20, 21), resulted in higher average pmel-1
velocities and longer displacements in TDLNs, without significant changes in their arrest coefficients and confinement indexes (Fig. 5A, 5E–H and Supplementary Movies S5 and S6). In agreement with published reports (16), the antibody blockade of the MHC class I molecule H2-Db, which presents the gp100 peptide to the pmel-1 T cells, led to the interruption of the stop signal mediated by the pmel-1 TCR and resulted in significantly higher average velocities and displacements, lower arrest coefficients, and unaltered confinement indexes of the pmel-1 T cells (Fig. 5B, 5E–H and Supplementary Movies S7 and S8). These results are similar to those obtained with α-CTLA-4 antibodies. In contrast, the injection of α-CD28 antibodies or hamster Ig, a control for the hamster α-CTLA-4 antibody, did not alter the pmel-1 velocities, arrest coefficients, or confinement indexes, although it did increase the average displacements (Fig. 5C–H and Supplementary Movies S9–S12). The latter outcome may be partially due to the longer imaging period after antibody injection relative to the interval before it. Overall, the data suggest that the increased mobility of pmel-1 T cells in TDLNs subsequent to the acute CTLA-4 blockade is specific and can be caused by the blockade of either CTLA-4 or its ligands on the APCs.

Similar effects of α-CTLA-4 Fab fragments and intact antibodies on pmel-1 dynamics in TDLNs

To determine whether the increased pmel-1 motility after the acute CTLA-4 treatment was due to inhibitory signaling or signaling blockade, we also injected 250 µg of α-CTLA-4 Fab fragments in GVax-treated animals and continued imaging for another hour (Fig. 6A and Supplementary Movies S13 and S14). The bolus injection of α-CTLA-4 Fab fragments significantly increased pmel-1 velocities and displacements (Fig. 6B and 6D) and significantly decreased arrest coefficients (Fig. 6C), without affecting confinement indexes (Fig. 6E). The average changes in all values closely resembled those mediated by the intact α-CTLA-4 antibody (Fig. 6B–E), suggesting a similar mechanism of action.

Discussion

Overcoming immune suppression in tumors is the main focus of cancer immunotherapy—the promising “new” weapon in the cancer-fighting arsenal that was recently named “breakthrough” of the year by Science magazine (22). Improvement of therapeutic regimens requires detailed knowledge of T-cell behavior in tumors before and after effective immunotherapy. Our study used melanoma-specific pmel-1 T cells as reporters for the endogenous tumor-specific immune responses and investigated how successful immunotherapy that included α-CTLA-4 antibodies altered their dynamics in tumors and TDLNs. The addition of pmel-1 T cells to the intact immune system of tumor-bearing animals did not significantly affect the growth of B16-Tomato melanoma, and pmel-1 proliferation and cytokine secretion mirrored those of the polyclonal
CD8\(^+\) T cells (Fig. 1), justifying their use as readout of the endogenous response against tumors.

We evaluated the effect of \(\alpha\)-CTLA-4 antibodies on the dynamics of pmel-1 T cells in tumors and TDLNs in two settings—chronic blockade (where animals were treated with either GVax alone or the therapeutic combination of GVax + \(\alpha\)-CTLA-4) and acute blockade (where animals received an i.p. injection of \(\alpha\)-CTLA-4 antibodies during the course of imaging). The results from both lines of experiments showed that \(\alpha\)-CTLA-4 antibodies seem to increase T-cell motility and are consistent with the results of several published reports (16, 17, 23).

Despite the similarities, several differences were also found between the pmel-1 dynamics in tumors and TDLNs. In the setting of chronic blockade, the steady-state pmel-1 velocities in progressively growing GVax-treated B16 melanoma were lower than their velocities in combination-treated tumors (Fig. 2). In contrast, no difference was found in the steady-state velocities of pmel-1 T cells in TDLNs in the presence or absence of immunotherapy (Fig. 4). Furthermore, the mobility of pmel-1 T cells in the tumors of combination-treated mice was comparable with their greater mobility in TDLNs (Fig. 4). These results suggest that pmel-1 T cells in the tumors of GVax-treated mice are mostly immobile and are engaged in stable, nonproductive interactions with target APCs or tumor cells. In contrast, pmel-1 T cells in the combination-treated tumors are more motile and potentially better able to move onto new targets as part of the successful immunotherapy regimen.

In the acute blockade setting, we observed that the i.p. injection of \(\alpha\)-CTLA-4 antibodies during the course of imaging did not affect pmel-1 T-cell velocities in tumors but increased their mobility in TDLNs, regardless of the chronic treatment (Figs. 2 and 3). The differential effects of the acute antibody blockade on pmel-1 velocities in tumors versus TDLNs could be potentially due to permeability differences in the two tissues. To determine whether this outcome was specific to CTLA-4, we also injected antibodies blocking the CTLA-4 ligands, B7-1 and B7-2, and observed a similar phenomenon (Fig. 5). These results suggest that the disruption of the inhibitory interaction between CTLA-4 and its ligands from either direction leads to improved pmel-1 motility. Importantly, \(\alpha\)-CD28 antibodies and hamster Ig did not alter pmel-1 motility (Fig. 5), indicating that the effect is specific to CTLA-4.

Despite the general agreement in the field of cancer immunotherapy that \(\alpha\)-CTLA-4 antibodies mediate their antitumor effects either via blockade of inhibitory signaling on effector T cells or by depletion of Tregs, several recent reports have suggested that the antibodies actually deliver an inhibitory signal to effector T cells (16, 17). The first study to propose this model of CTLA-4-mediated inhibition, known as the reversal of the TCR stop signal by CTLA-4, used microscopy to show that administration of \(\alpha\)-CTLA-4 antibodies increases T-cell motility (17). A more recent report on the effects of CTLA-4 blockade alone or in combination with radiotherapy on the mobility of a polyclonal population of CXCR6\(^+\) TILs reached similar conclusions (16). Although both radiotherapy and \(\alpha\)-CTLA-4 antibodies, which fail to cure the tumors as single agents, increased TIL mobility relative to untreated mice, the successful combination therapy decreased TIL motility to levels observed in control animals (16). It was proposed that whereas \(\alpha\)-CTLA-4 antibodies by themselves are
ineffective as a therapy, because they deliver inhibitory “go” signals via CTLA-4 and subsequently interfere with T-cell responses, radiotherapy induces NKG2D ligands and can restore T-cell stopping to allow for successful tumor treatment (16). Although scientifically important, the report did not address how the same relative motility in untreated controls and combination-treated animals has completely opposite effects on the outcome of tumor therapy.

To discriminate between the two contrasting mechanisms of action for the α-CTLA-4 antibodies (i.e., inhibitory signaling vs. blockade), after the initial observation of pmel-1 motility in TDLNs of GVax-treated mice, we also injected α-CTLA-4 Fab fragments instead of whole Ig (Fig. 6). Fab fragments have been shown to enhance effector T-cell activation, presumably by blocking CTLA-4–mediated inhibition, as they cannot deliver signals to the cells expressing their target (24). The bolus injection of α-CTLA-4 Fab fragments significantly increased pmel-1 motility, and the average changes in all experimental parameters closely resembled those mediated by the intact α-CTLA-4 antibody (Fig. 6), suggesting a similar mechanism of action. This interpretation is consistent with the widely documented positive effects of α-CTLA-4 antibodies on tumor immunity across multiple animal models and human patients with cancer (4).

In addition to the two studies discussed above, several groups have analyzed the effects of α-CTLA-4 antibodies on T-cell motility in mouse models of autoimmune diabetes and delayed-type hypersensitivity (DTH; refs. 23, 25, 26). One study reported no changes in the fast motility of tolerized diabeticogenic BDC2.5 CD4⁺ T cells after treatment with α-CTLA-4 antibodies in lymph nodes or pancreatic islets, unlike the T-cell stopping and subsequent T-cell activation triggered by α-PD-1 or α-PD-L1 antibody blockade (25). Of note, the authors described this antibody-induced T-cell stopping phenomenon as blockade of the tolerance mediated by the PD-1 and PD-L1 reversal of the TCR-induced stop signal (25).

A more recent study of the cell interactions during islet graft tolerance versus immune-mediated islet destruction reported that α-CTLA-4 antibodies had differential effects on different T-cell subsets (23). Although the antibodies increased the velocities of effector and regulatory BDC2.5 T cells in protected grafts, they decreased the velocities of OT-I T cells (23).
Interestingly, RNAi-mediated CTLA-4 reduction in OT-I T cells led to a similar decrease in their velocities under these conditions, a finding consistent with the notion that the antibodies are removing an inhibitory signal, rather than delivering one (23). Similar to the effects of α-CTLA-4 antibodies on the effector BDC2.5 population, the acute depletion of regulatory BDC2.5 T cells following graft establishment led to increased motility of effector BDC2.5 T cells. This increase correlated with greater tissue damage, suggesting that improved T-cell motility in this model correlates with enhanced immune function, similar to our results from combination-treated tumors. The authors concluded that the effects of α-CTLA-4 antibodies on T-cell motility might depend on the cell type and the immune setting (23). Interestingly, the enhanced motility of effector BDC2.5 T cells subsequent to acute Treg depletion suggests an alternative explanation for the increased motility of pmel-1 T cells in our combination-treated tumors. It was recently reported that α-CTLA-4 antibodies seem to cause the specific depletion of tumor-infiltrating Tregs via an Fc-dependent mechanism in several models, including B16/BL6 melanoma (10–12). It is, therefore, possible that the enhanced motility of pmel-1 T cells in combination-treated tumors is, in part, due to the α-CTLA-4 antibody–mediated depletion of Tregs. However, because we observe similar increases in pmel-1 T-cell motility in TDLNs subsequent to acute CTLA-4 blockade, in which the antibodies do not lead to Treg depletion (10), at least some of the motility increase is due to direct effects of the antibody on the pmel-1 effectors. It will be interesting to determine whether α-CTLA-4 antibodies that do not deplete Tregs have similar effects on T-cell motility.

Another recent report on the dynamics of antigen-specific effector T-cell responses in peripheral tissues during DTH supports the idea that the effects of CTLA-4 blockade on T-cell motility may be immune-context dependent (26). The study showed that antigen recognition in inflamed tissues triggered rapid effector T-cell stopping, followed by gradual recovery of motility over the course of 6 hours. This increased motility correlated with the upregulation of CTLA-4 and PD-1 on effector T cells and could be reversed by antibodies to PD-L1 and CTLA-4 (even though in the case of CTLA-4, the velocity decrease did not reach statistical significance; ref. 26). These data suggest that in the case of inflammatory responses, the blockade of CTLA-4 and PD-1 may lead to prolonged T-cell arrest and cause host tissue damage (26).

The idea that antibodies blocking inhibitory molecules can have differential effects on T-cell motility, depending on the immune context, is supported by a study showing that α-PD-1 and α-PD-L1 antibodies improve antiviral T-cell motility and lead to viral clearance in a mouse model of persistent viral infection (18). Inoculation of mice with the clone 13 variant of lymphocytic choriomeningitis virus (LCMV), which establishes a chronic viral infection due to immune-cell exhaustion, causes "motility paralysis" of virus-specific CD4+ and CD8+ T cells. This paralysis is manifested by decreased T-cell velocities in

Figure 6. α-CTLA-4 Fab fragments and intact antibodies have similar effects on pmel-1 dynamics in TDLNs. C57/BL6 mice received an adoptive transfer of pmel-1-GFP T cells and a challenge of B16-Tomato cells. A, top, following GVax treatment, mice were anesthetized, and pmel-1 motility in TDLNs was visualized by TPM. Bottom, after 20 minutes, α-CTLA-4 Fab fragments were injected i.p. and imaging continued for 1 hour. Scale bar, 30 μm. Mean track velocities (B), arrest coefficients (C), displacements (D), and confinement indexes (E) for pmel-1 T cells before and after the injection of α-CTLA-4 Fab or intact Ig are shown. Data are pooled from at least three independent experiments. P values for each antibody group (pre-Ab vs. post 9H10 Fab or Ig) were calculated using a Student t test.
clone 13-infected mice relative to mice infected with the Armstrong strain of LCMV, which is cleared by virus-specific T cells (18). This phenomenon resembles the lower motility of pmel-1 T cells in progressively growing GVAX-treated tumors relative to their velocities in tumors treated with the effective combination therapy in our system. Similar to the effects of α-CTLA-4 antibodies in our protective immunotherapy regimen, the antiviral T-cell paralysis can be reversed by blocking antibodies to PD-1 and PD-L1, both of which have been previously shown to treat persistent viral infections in mice and humans by restoring function to exhausted T cells (18).

While consistent with earlier work showing increased T-cell motility subsequent to the administration of α-CTLA-4 antibodies (16, 17, 23), our study is the first one to report the direct effects of CTLA-4 blockade on tumor-specific immune responses in live animals. Furthermore, our interpretation of the biologic significance of this phenomenon differs from that expressed in the first two publications (16, 17). The original report (17) of the CTLA-4-mediated reversal of the TCR-induced stop signal suggested that, although generally detrimental for T-cell activation, the shorter dwell times may be beneficial for CTL killing, which occurs relatively quickly. In contrast, Ruocco and colleagues (16) concluded that α-CTLA-4 antibodies fail to generate protective tumor immunity and must be combined with radiation because anti-CTLA-4 treatment increases T-cell motility. Unlike these earlier reports, our findings are more consistent with the conclusion that the antibodies mediate their positive effects on tumor immunity by blocking CTLA-4–mediated inhibition and enhancing T-cell motility in tumors, similar to the reversal of the exhausted T-cell motility paralysis during chronic viral infections (18).

Disclosure of Potential Conflicts of Interest
J.P. Allison has ownership interest (including patents) in Jounce Therapeutics and Bristol-Myers Squibb and is a consultant/advisory board member for Jounce Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References


**Cancer Immunology Research**

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Tsvetelina Pentcheva-Hoang, Tyler R. Simpson, Welby Montalvo-Ortiz, et al.


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