

treatment; however, no change was noted for T-bet. Higher secretion of IFN- γ , granzyme B, and perforin were detected consistently after ipilimumab treatment at weeks 7 and 24 when compared with those present pretreatment. These findings suggest that CTLA-4 blockade may induce the expression of lytic granules on NY-ESO-1-specific cytotoxic CD4⁺ T cells via Eomes, but further detailed mechanistic studies are needed to conclusively show this.

In summary, previous studies from several groups have shown the following important points: (i) CTLA-4 blockade enhances regression of spontaneous mouse tumors by expansion of transferred effector CD4⁺ T cells (12); (ii) adoptive transfer of autologous CD4⁺ T cells directed against NY-ESO-1 has resulted in tumor regression in humans (14); and (iii) ipilimumab promotes the expansion of infused CD8⁺ CTLs as memory CTLs (37). Our data now bring these observations together by revealing that CTLA-4 blockade with ipilimumab induces the expression of Eomes as well as markers of lytic degranulation on high-affinity cytotoxic-CD4⁺ T cells. These cytotoxic NY-ESO-1-specific CD4⁺ T cells are able to lyse autologous tumor cells, which naturally express the cognate NY-ESO-1 antigen. It brings forth a potentially novel therapeutic mechanism in which CTLA-4 blockade or inhibition with other immunomodulatory antibodies might be combined with CD4⁺ T cells to enhance cytolytic function in cancer immunotherapy clinical trials.

Disclosure of Potential Conflicts of Interest

J.P. Allison has ownership interest (including patents) and J.D. Wolchok has a commercial research grant and is a consultant/advisory board member for

Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Teresa Rasalan and Sapna Tandon for their excellent technical assistance. This article is dedicated to the memory of Dr. Lloyd Old, our mentor and friend and one of the true leaders in investigational cancer medicine. His contributions to tumor immunology inspired much of this work.

Grant Support

This work was supported by grants from the NIH (RC2CA148468; to J.D. Wolchok), the Melanoma Research Alliance (to S. Gnjatic and J.D. Wolchok), Swim Across America (to J.D. Wolchok), the Cancer Research Institute (to S. Gnjatic and J.D. Wolchok), the Virginia and D.K. Ludwig Fund for Cancer Research (to J.P. Allison), the Lita Annenberg Hazen Foundation (to J.D. Wolchok), and the Commonwealth Foundation for Cancer Research (to J.D. Wolchok).

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Received May 31, 2013; revised August 2, 2013; accepted August 5, 2013; published OnlineFirst August 12, 2013.

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Cancer Immunol Res 2014;2:70-79. Published OnlineFirst October 14, 2013.

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Research Article

Paradoxical Activation of T Cells via Augmented ERK Signaling Mediated by a RAF Inhibitor

Margaret K. Callahan^{1,6,7}, Gregg Masters⁸, Christine A. Pratilas^{2,4}, Charlotte Ariyan^{3,7}, Jessica Katz⁸, Shigehisa Kitano⁵, Valerie Russell¹, Ruth Ann Gordon¹, Shachi Vyas⁵, Jianda Yuan⁵, Ashok Gupta⁸, Jon M. Wigginton⁸, Neal Rosen^{1,4,7}, Taha Merghoub^{1,6}, Maria Jure-Kunkel⁸, and Jedd D. Wolchok^{1,5,6,7}

Abstract

RAF inhibitors selectively block extracellular signal-regulated kinase (ERK) signaling in BRAF-mutant melanomas and have defined a genotype-guided approach to care for this disease. RAF inhibitors have the opposite effect in BRAF wild-type tumor cells, where they cause hyperactivation of ERK signaling. Here, we predict that RAF inhibitors can enhance T-cell activation, based on the observation that these agents paradoxically activate ERK signaling in BRAF wild-type cells. To test this hypothesis, we have evaluated the effects of the RAF inhibitor BMS908662 on T-cell activation and signaling *in vitro* and *in vivo*. We observe that T-cell activation is enhanced in a concentration-dependent manner and that this effect corresponds with increased ERK signaling, consistent with paradoxical activation of the pathway. Furthermore, we find that the combination of BMS908662 with cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade *in vivo* potentiates T-cell expansion, corresponding with hyperactivation of ERK signaling in T cells detectable *ex vivo*. Finally, this combination demonstrates superior antitumor activity, compared with either agent alone, in two transplantable tumor models. This study provides clear evidence that RAF inhibitors can modulate T-cell function by potentiating T-cell activation *in vitro* and *in vivo*. Paradoxical activation of ERK signaling in T cells offers one mechanism to explain the enhanced antitumor activity seen when RAF inhibitors are combined with CTLA-4 blockade in preclinical models. *Cancer Immunol Res*; 2(1); 70–79. ©2013 AACR.

Introduction

The treatment of advanced melanoma has recently been transformed by two new therapies now incorporated in the standard of care: RAF inhibitors and checkpoint-blocking antibodies. The identification of a novel genetic mutation involved in melanoma transformation, the BRAF^{V600E} mutation, led to the development of RAF inhibitors, including vemurafenib, dabrafenib, and BMS908662 (XL281; refs. 1–5). This activating BRAF mutation leads to increased extracellular signal-regulated kinase (ERK) signaling and promotes tumor cell proliferation. RAF inhibitors block the aberrant ERK signaling in BRAF-mutant melanomas. An overall survival

benefit associated with vemurafenib in BRAF-mutant metastatic melanoma led to its approval by the U.S. Food and Drug Administration (FDA) in August 2011 (3). It is increasingly clear that anticancer therapies, including chemotherapies and targeted inhibitors, can have underappreciated, but clinically relevant, effects on the immune system (6–8). In the clinic, any RAF inhibitor-mediated immunomodulatory activity could affect the antimelanoma immune responses and would be especially relevant in combination with prior or concomitant immunotherapies. In the present study, we have focused on characterizing the effects of the RAF inhibitor, BMS908662, on T-cell activity *in vitro* and *in vivo*.

T cells require two signals for activation: engagement of the T-cell receptor (TCR) with a cognate MHC-peptide complex and engagement of a costimulatory molecule such as CD28. These stimuli activate signal transduction cascades including phosphorylation of ERK via CRAF and/or BRAF (9–14). The magnitude and duration of ERK activation appear to modulate T-cell function. Overexpression of RAS or RAF can enhance T-cell activation and proliferation and conversely inhibition of RAS blocks T-cell activation (10, 15, 16). This pathway is also important in various T-cell functions, including the differentiation of T-cell subtypes, cytokine signaling, chemotaxis, and survival (17, 18).

Accumulating evidence supports the notion that RAF inhibitors have distinct effects on BRAF wild-type cells compared with BRAF-mutant cells. Although RAF inhibitors block ERK signaling in BRAF-mutant cells, RAF inhibitors paradoxically

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

T. Merghoub, M. Jure-Kunkel, and J.D. Wolchok contributed equally to this work.

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doi: 10.1158/2326-6066.CIR-13-0160

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enhance ERK signaling in cells that are wild type for BRAF (19–22). This phenomenon is clinically manifest in the accelerated growth of squamous cell cancers and keratoacanthomas in patients treated with RAF inhibitors (23–25). Paradoxical activation seems to be a class effect of RAF inhibitors mediating enhanced signaling of RAF dimers in the presence of drug (19–22).

The antitumor activity of the checkpoint-blocking antibody ipilimumab is entirely dependent upon T-cell activation (26). The importance of ERK signaling in T-cell activation suggested that RAF inhibitors could alter T-cell function and consequently the activity of checkpoint-blocking antibodies. Because T cells express wild-type BRAF, we reasoned that they would be susceptible to the same RAS-dependent paradoxical ERK hyperactivation observed previously in BRAF wild-type tumor cells.

BMS908662 (XL281; Bristol-Myers Squibb/Exelixis) is a pan-RAF inhibitor with activity against mutant BRAF as well as wild-type A, B, and CRAF. It has been tested as a monotherapy and combined with ipilimumab in a first-in-human phase I study (5, 27). To better understand the immunologic activities of RAF inhibitors and the consequences of their combination with checkpoint-blocking antibodies, we explored the effect(s) of BMS908662 alone or in combination with anti-CTL antigen-4 (anti-CTLA-4) on tumor progression and on T-cell activation *in vitro* and *in vivo*.

Materials and Methods

Cell culture and T-cell activation *in vitro*

T cells were cultured in RPMI supplemented with 10% (v/v) FBS. BMS908662 (provided by Bristol-Myers Squibb/Exelixis) or PD325901 [synthesized at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY)] or PLX4720 (provided by Plexikon) was added to cultures as indicated. In the experiments involving cultured cells (Figs. 1–3), inhibitors were added to the cell culture media at the same time that the cells were added to the culture (concomitant with the initial stimulation). Murine CD4⁺ and CD8⁺ T cells were purified from C57BL/6J (Jax) splenocytes using Miltenyi MACS beads according to the manufacturer's instructions. Murine T cells were activated *in vitro* with antibodies against mouse CD3 (145-2C11) and CD28 (37.58), produced by the Monoclonal Antibody Core Facility at MSKCC. The human Jurkat T-cell line was purchased from American Type Culture Collection. Jurkat cells or human peripheral blood T cells were activated with antibodies specific for human CD3 (UCHT1) and CD28 (CD28.2). Cell culture plates were coated with the CD3 antibody at a concentration of 10 µg/mL and the CD28 antibody was added to media at a concentration of 0.5 µg/mL. The NY-ESO-1-specific T-cell line, developed from a patient diagnosed with stage IV melanoma, was generously provided by S. Kitano. The NY-ESO-1 T-cell line was stimulated with antigen-presenting cells (APC) pulsed with the cognate peptide NY-ESO-1^{94–102} (MPFATPMEA). A cultured B-cell line derived from the same patient was used as an antigen-presenting cell for stimulation of the NY-ESO-1-specific T-cell line. Expression of CD69, an early activation marker, was measured 12 to 24 hours after

T-cell activation by flow cytometry using samples collected on an LSRII (BD) and analyzed using FlowJo software (TreeStar). Proliferation was evaluated 3 to 4 days after stimulation by quantifying the dilution of dye in carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells or by intracellular staining for the proliferation marker Ki67. Production of IFN-γ was measured by intracellular cytokine staining 4 to 6 hours after T-cell activation. Unless indicated otherwise, all antibodies were obtained from BD.

Detection of ERK signaling in T cells

Cultured Jurkat T cells were resuspended in RPMI supplemented with 0.2% (v/v) FBS on ice. Anti-CD3 antibody (OKT3) was added at a concentration of 0.5 µg/mL and incubated for 15 minutes on ice. Anti-mouse secondary antibody (Jackson ImmunoResearch) was added at a concentration of 0.1 µg/mL and incubated for 15 minutes on ice. Cells were then warmed to 37°C for the indicated period of time, ranging from 0 to 240 minutes. Cells were then fixed in 3% paraformaldehyde for 10 minutes at room temperature, centrifuged, and resuspended in ice-cold 95% methanol, and then stored at –80°C for up to 72 hours. Fixed cells were stained using antibodies specific for phosphorylated ERK (pERK) or total ERK (Cell Signaling Technology) and a secondary, allophycocyanin-conjugated anti-rabbit antibody (Jackson ImmunoResearch). ERK signaling was assessed in murine splenocytes after similar stimulation.

Mice

Female BALB/c mice (Harlan Laboratories) or female A/J mice (The Jackson Laboratory) 9 to 11 weeks old were used for antitumor efficacy studies. Female C57BL/6J and C57BL/6-Tg (Tcratcrb)1100Mjb/J (OT-1) mice (The Jackson Laboratory) 9 to 11 weeks old were used in the ovalbumin (OVA)-antigen challenge studies and for T-cell culture experiments. All experiments have been carried out according to the Institutional Animal Care and Use Committee (IACUC) guidelines under approved protocols at MSKCC or Bristol-Myers Squibb.

Antigen-specific T-cell expansion *in vivo*

Spleen cells from OT-1 mice were isolated, suspended in PBS, and injected via the tail vein into C57BL/6J mice (3 × 10⁶ cells/mouse) on day –1. On day 0, 0.25 mg of SIINFEKL peptide diluted in PBS was injected *i.v.* Mice were treated with control vehicles, BMS908662 [30 or 100 mg/kg *per os* (p.o.) on days 1, 3, and 5], or 4F10-11 [20 mg/kg intraperitoneal (i.p.) on days 1 and 4]. On day 5, whole blood was stained with H-2Kb/SIINFEKL tetramer and anti-CD3 antibody, and the number and percentage of tetramer⁺/CD3⁺ cells were quantified by flow cytometry.

Treatment of transplanted tumors

BALB/c mice were injected *s.c.* with 1.0 × 10⁶ CT-26 cells in 0.2 mL Hanks Balanced Salt Solution. Four days after implantation, mice were randomized into groups of 8 or 10 animals. A/J mice were injected *s.c.* with 2.0 × 10⁶ SA1N cells in 0.2 mL Hanks Balanced Salt Solution. Eleven days after implantation, mice were randomized into seven groups of 8 mice. Control vehicles, BMS908662 (100 mg/kg, p.o.), or 4F10-11

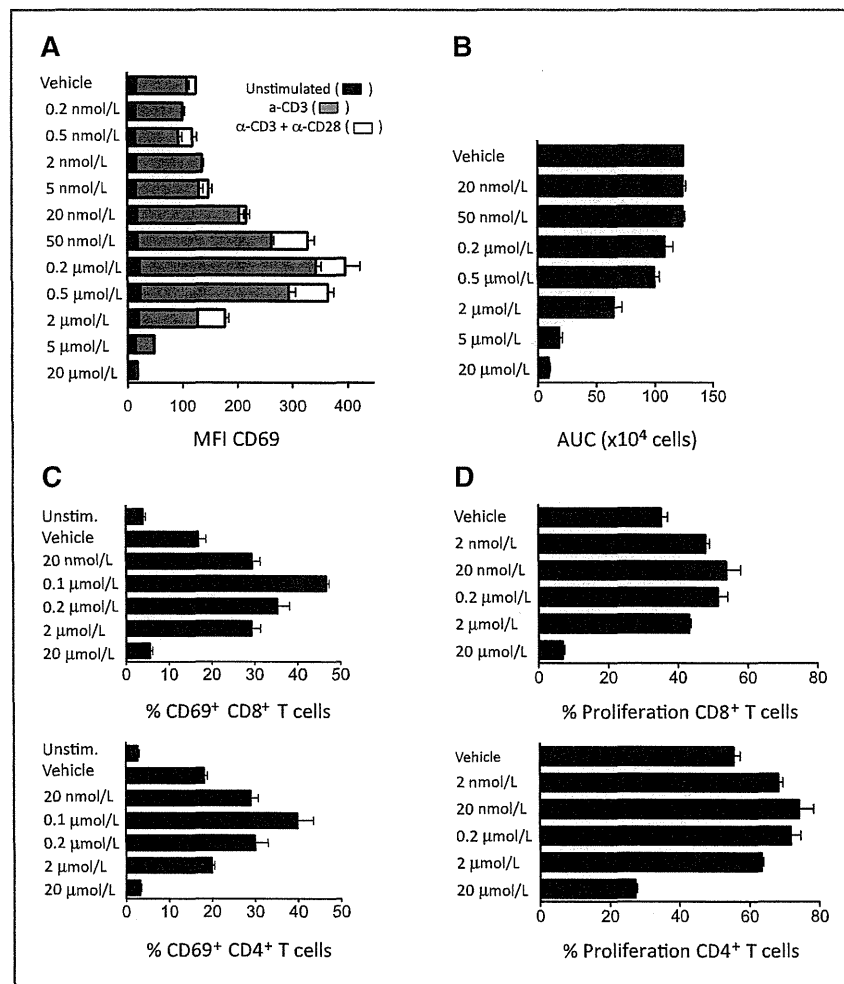


Figure 1. BMS908662 enhances human T-cell activation *in vitro* in a concentration-dependent manner. **A**, Jurkat T cells were activated with anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of BMS908662. Upregulation of the activation marker, CD69, was assessed by flow cytometry. Median fluorescence intensity (MFI) reflects the level of expression of CD69. One experiment representative of three independent experiments is shown here. Representative flow cytometry data are presented in Supplementary Fig. S1. **B**, the human BRAF-mutant tumor cell line, SK-MEL-19, was cultured in the presence of increasing concentrations of BMS908662. The number of cells was quantified daily for 3 days of culture and the growth curve under each condition was used to calculate an area under the curve (AUC) reflecting growth inhibition. Additional details on growth inhibition may be found in Supplementary Fig. S2. **C**, human healthy donor peripheral blood mononuclear cells were activated *in vitro* with anti-CD3 and anti-CD28 antibody in the presence of the indicated concentrations of BMS908662. The upregulation of CD69 as a reflection of T-cell activation was measured in CD8⁺ (top) or CD4⁺ (bottom) T cells. **D**, human healthy donor peripheral blood mononuclear cells were labeled with CFSE and then activated with anti-CD3 and anti-CD28 antibody in the presence of the indicated concentrations of BMS908662. Proliferation was measured by quantifying the percentage of CD4⁺ or CD8⁺ cells with diluted CFSE after activation. In all experiments, samples were treated and analyzed in triplicate and error bars represent SE.

(20 mg/kg, i.p.) were administered as described in the figure legends. Tumors were measured with calipers two-dimensionally twice weekly and tumor volume was calculated as $L \times (W^2/2)$, length (L) being the longer of the two measurements. Body weights were recorded biweekly. In some cases, mice died before the completion of the experiment; if a mouse was sacrificed because of a high tumor burden, it was evaluated as not protected from tumor challenge. If the mouse died for other reasons, the mouse was censored from the experiment and was counted as unevaluable.

Immunoblotting

Cell lysates were prepared and immunoblotting was performed as previously described using antibodies specific for pERK or total ERK (Cell Signaling Technology).

Results

BMS908662 potentiates T-cell activation *in vitro*

To test the immunomodulatory activity of BMS908662, we evaluated its effects on the activation of cultured human and

mouse CD4⁺ and CD8⁺ T cells. Activated T cells may be characterized by the upregulation and cell surface expression of activation markers (such as CD69), production of cytokines (such as IFN- γ), and proliferation. T cells may be activated *in vitro* with anti-CD3 and anti-CD28 antibodies that engage the TCR and the CD28 costimulatory molecule, respectively. First, we tested the impact of BMS908662 on cultured human T cells. Initial experiments were performed using Jurkat cells, a well-characterized human CD4⁺ T-cell line that has been used as a model to investigate TCR signaling (28). Cultured Jurkat cells readily upregulate activation markers, such as CD69, after stimulation with anti-CD3 and anti-CD28 antibodies. Jurkat cells were cultured in the presence of titrated concentrations of the RAF inhibitor BMS908662, or vehicle control, in the presence or absence of stimulating antibodies. The upregulation of CD69 was enhanced up to 3-fold in the presence of BMS908662 at a concentration of 0.2 μ mol/L, compared with cells treated with vehicle alone ($P < 0.001$; Fig. 1A). In contrast, at higher concentrations of BMS908662 (5 μ mol/L and above) activation was attenuated when compared with the vehicle control. Activation was entirely abrogated at a concentration of 20 μ mol/L, the highest concentration tested and a concentration at which the viability of the cells was preserved, as has been previously described (data not shown; refs. 29, 30). As a comparator, BRAF-mutant tumor cells were treated with BMS908662; inhibition of growth was evident at concentrations of 0.2 μ mol/L and above. Notably, concentrations of BMS908662 between 0.2 and 2 μ mol/L enhanced T-cell activation while inhibiting tumor cell proliferation; concentrations of drug >5 μ mol/L inhibit T-cell activation and tumor cell proliferation. A similar pattern of dose-dependent activation was observed in both CD4⁺ and CD8⁺ T cells from healthy human donors activated *in vitro*, in which BMS908662 increased surface CD69 expression at concentrations between 20 nmol/L and 2 μ mol/L, but attenuated activation at concentrations above 2 μ mol/L (Fig. 1C). Likewise, the proliferation of activated CD4⁺ and CD8⁺ T cells could be enhanced by the addition of BMS908662. This effect was evident when T-cell proliferation was measured directly by quantifying the dilution of CFSE in cultured T cells (Fig. 1D) or indirectly by staining for the proliferation marker Ki67 (data not shown). In the dose range of 20 nmol/L to 2 μ mol/L, T-cell proliferation was increased by up to 25% in CD4⁺ T cells and up to 53% in CD8⁺ T cells.

Next, we investigated the effect of BMS908662 on purified murine CD4⁺ or CD8⁺ T cells activated *in vitro* with a combination of anti-CD3 and anti-CD28 antibodies. Proliferation of both CD4⁺ and CD8⁺ T cells could be enhanced by BMS908662 in a dose-dependent fashion (Supplementary Fig. S3A and S3B). For CD8⁺ T cells, at a concentration of 0.5 μ mol/L, BMS908662 increased the percentage of cells that proliferated, as measured by dilution of CFSE, from 31% to 58% ($P < 0.01$). At concentrations above 2 μ mol/L, proliferation was attenuated. For CD4⁺ T cells, the drug seemed to potentiate proliferation over a wider range, perhaps reflecting a heterogeneous response within a diverse population of CD4⁺ T cells. Again, concentrations of BMS908662 above 2 μ mol/L inhibited T-cell proliferation. Likewise, we evaluated upregulation of CD69, an

early activation marker, to assess T-cell activation in the presence of BMS908662. CD4⁺ T cells stimulated in the presence of BMS908662 demonstrated an increase in CD69 expression at a concentration of 0.2 μ mol/L, whereas concentrations above 2 μ mol/L seemed to block upregulation of CD69 (Supplementary Fig. S3C). For murine CD8⁺ T cells, increased CD69 expression was not observed under these conditions, but inhibition was seen with higher concentrations of the drug.

To test T-cell activation under more physiologic stimuli, we evaluated the effect of BMS908662 on antigen-specific T cells stimulated with MHC-peptide complexes. We made use of a human CD4⁺ T-cell line with specificity for a peptide (amino acid 94–102) within the cancer-testis antigen NY-ESO-1. T cells were stimulated with cognate peptide-pulsed (MPFATPMEA) antigen-presenting cells in the presence or absence of titrated concentrations of BMS908662. The production of IFN- γ by T cells was quantified by intracellular cytokine staining and flow-cytometric analysis. For these tumor antigen-specific T cells, cytokine production after antigen-specific activation was enhanced in the presence of BMS908662, with a 35% increase in cytokine staining at a concentration of 0.2 μ mol/L compared with cells treated with a vehicle control ($P \leq 0.05$; Fig. 2). As previously noted, higher concentrations of BMS908662 had the opposite effect and inhibited cytokine production.

BMS908662 potentiates ERK signaling in human T cells *in vitro*

After observing a pattern of dose-dependent potentiation of T-cell activation in cultured cells, we hypothesized that paradoxical activation of ERK signaling could explain this phenomenon. To directly evaluate ERK signaling in T cells treated with BMS908662, levels of pERK and total ERK were measured in activated Jurkat cells using a flow cytometry-based approach. Jurkat cells activated with cross-linked anti-CD3 antibody have a robust increase in pERK within

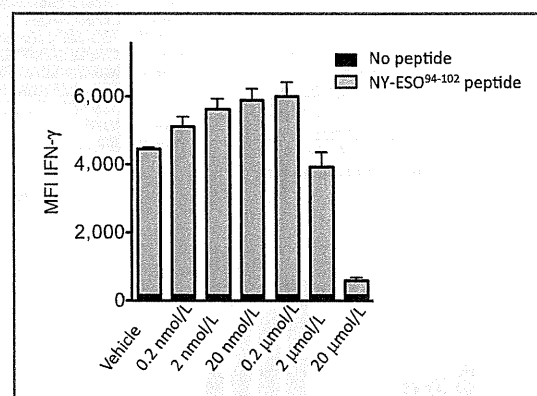


Figure 2. BMS908662 increases antigen-specific T-cell activation *in vitro*. A CD4⁺ NY-ESO-1-specific human T-cell line was activated *in vitro* with NY-ESO-1 peptide-pulsed APCs. The production of IFN- γ was quantified by intracellular cytokine staining followed by flow-cytometric analysis. Cells were treated in triplicate and the median fluorescence intensity (MFI) for IFN- γ staining within the CD8⁺ population is plotted.

minutes of stimulation. This pulse of signaling is transient, and pERK levels return to baseline levels within approximately 1 hour. We tested the effect of BMS908662 on the pattern of T-cell signaling, evaluating Jurkat cells treated with inhibitor at concentrations between 20 pmol/L and 20 μmol/L. Over this wide range of concentrations, two patterns emerge; for clarity, these data are presented in two separate graphs reflecting a single experiment. First, at higher concentrations of inhibitor (0.2–20 μmol/L), ERK activation is clearly attenuated with a diminished peak of pERK and a rapid return to baseline levels; at the highest concentration (20 μmol/L), ERK activation is nearly abrogated (Fig. 3A). In contrast, at lower concentrations of drug (0.2 μmol/L and below), the pattern of ERK activation begins to change. In this range, there is a sustained elevation of pERK after initial activation (Fig. 3B). The effect of BMS908662 on T-cell activation is most apparent at time points 10 to 60 minutes after activation. Evaluating a single time point, 15 minutes after activation, the concentration-dependent effect of the drug on ERK signaling is apparent (Fig. 3C). When compared with cells treated with vehicle control, cells treated with 0.2 nmol/L to 0.5 μmol/L

BMS908662 have higher sustained pERK levels, whereas levels of total ERK are comparable across the titrations of BMS908662; in cells exposed to 20 nmol/L BMS908662, pERK levels are twice as high as the vehicle control-treated cells 15 minutes after activation (Fig. 3C and D). In contrast, at concentrations between 2 and 20 μmol/L, BMS908662-treated cells have lower levels of pERK than the vehicle-treated controls. Finally, hyperactivation of ERK signaling mediated by BMS908662 may be entirely reversed by the addition of a MEK inhibitor, PD325901 (Fig. 3E). For Jurkat cells activated in the presence of 50 nmol/L BMS908662, ERK signaling is attenuated by PD325901. These observations support the notion that BMS908662 paradoxically activates ERK signaling in T cells upon initiation signaling via the TCR. These findings correlate closely with the effects of BMS908662 on upregulation of the activation marker CD69 described in Jurkat cells above (compare Figs. 1A and 3D). In both experiments, concentrations of BMS908662 between 20 nmol/L and 2 μmol/L resulted in hyperactivation of stimulated cells reflected in increased levels of CD69 or pERK; small differences in the concentration in which this effect is maximal may reflect differences in experimental design

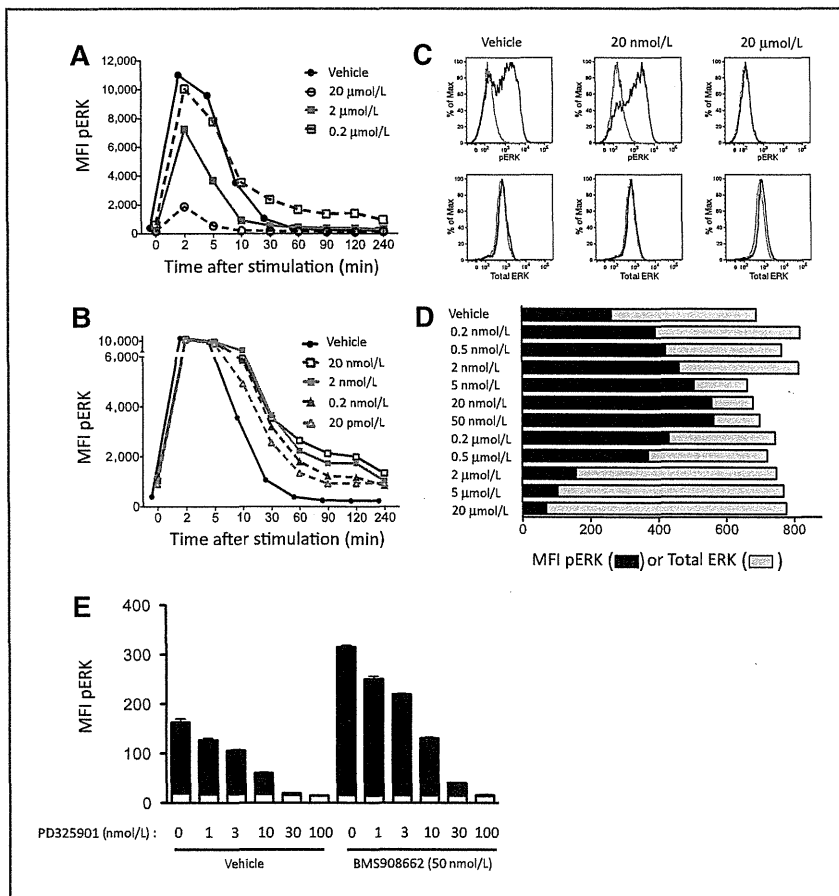


Figure 3. BMS908662 potentiates ERK signaling in human T cells *in vitro*. A and B, Jurkat T cells were activated with anti-CD3 antibody in the presence of titrated concentrations of BMS908662, as described in Supplementary Fig. S4. Levels of pERK, reflected in the median fluorescence intensity (MFI) of staining with a pERK-specific antibody, were measured at the indicated time points after activation. One experiment representative of two similar independent experiments is presented. C, Jurkat T cells were activated as described above. Fifteen minutes after activation, levels of pERK and total ERK were measured by flow-cytometric analysis. Examples of histograms demonstrating staining for pERK (top) and total ERK (bottom) for cells treated with vehicle, 20 nmol/L or 20 μmol/L of BMS908662 are presented. The solid black lines represent cells after 15 minutes of activation, and the solid gray lines represent cells that have not been stimulated. D, Jurkat T cells were activated as above. Fifteen minutes after activation, levels of pERK and total ERK were measured by flow-cytometric analysis. The MFI of staining for pERK or total ERK in cells treated with titrated concentrations of BMS908662 is presented. One experiment representative of three independent experiments is shown.

including the duration of stimulation or the endpoints tested.

BMS908662 enhances antigen-specific T-cell expansion *in vivo*, alone, or in combination with CTLA-4 blockade

The observation that BMS908662 alters T-cell activation *in vitro* prompted us to question how BMS908662 would affect T-cell function *in vivo*, at doses typically used to generate antitumor activity. To further explore this possibility, we tested T-cell activation in a mouse model of antigen-specific T-cell expansion. C57BL/6 recipients of OT-I transgenic T cells were immunized with the OVA²⁵⁷⁻²⁶⁴ peptide (SIINFEKL) followed by systemic treatment with BMS908662, CTLA-4 blockade, or a combination of both (Fig. 4A). After 5 days, the expansion of OT-I T cells was measured in whole blood as a proportion of tetramer⁺ cells compared with CD3⁺ cells. In mice treated with BMS908662 alone, the RAF inhibitor had a dose-dependent effect, maximally enhancing expansion of the antigen-specific T cells at the higher dose of 100 mg/kg (9.8-fold). CTLA-4 blockade also enhanced expansion of the OVA-specific T cells (3.9-fold). However, the combination of both agents has the most robust effect on T-cell expansion. The combination of CTLA-4 blockade and BMS908662 at a dose of 100 mg/kg increased antigen-specific T-cell expansion 24.1-fold. In agreement with the experiments performed on cultured T cells (Fig. 1), systemic treatment with BMS908662 enhances T-cell expansion. Furthermore, in this setting, the combination of BMS908662 and CTLA-4 blockade has clearly superior activity expanding antigen-specific CD8⁺ T cells.

Systemic treatment with BMS908662 enhances potential for T-cell activation

We speculated that the paradoxical activation of the ERK pathway in T cells observed *in vitro* (Fig. 3) would correspond with the enhanced proliferation of T cells observed *in vivo* (Fig. 4A). To test whether these doses of BMS908662 administered systemically would have a positive effect on ERK signaling, we treated mice with BMS908662 at doses of 100 or 30 mg/kg or with a vehicle control. After 5 days of treatment, splenocytes were harvested as a source of T cells and were activated directly *ex vivo*. Fifteen minutes after activation, splenocytes were fixed and stained for pERK or ERK along with surface markers for CD4⁺ and CD8⁺ T cells (Fig. 4B and C). BMS908662 administered systemically had a positive effect on T-cell signaling *ex vivo*. For example, in CD8⁺ T cells stimulation *ex vivo* increased pERK levels 2-fold, whereas CD8⁺ T cells from mice treated with BMS908662 were more sensitive to stimulation *ex vivo* and demonstrated a more robust increase in pERK levels: 3.4-fold (at a dose of 30 mg/kg) and 7.1-fold (at a dose of 100 mg/kg). This trend was similar for both CD4⁺ and CD8⁺ T cells, in which the potential for ERK signaling detected *ex vivo* closely matched the antigen-specific expansion of T cells *in vivo* (compare with Fig. 4A).

Combination of RAF inhibitor and CTLA-4 blockade demonstrates superior antitumor activity

Next, we evaluated the impact of BMS908662 on the antitumor activity of CTLA-4 blockade. We chose two mouse

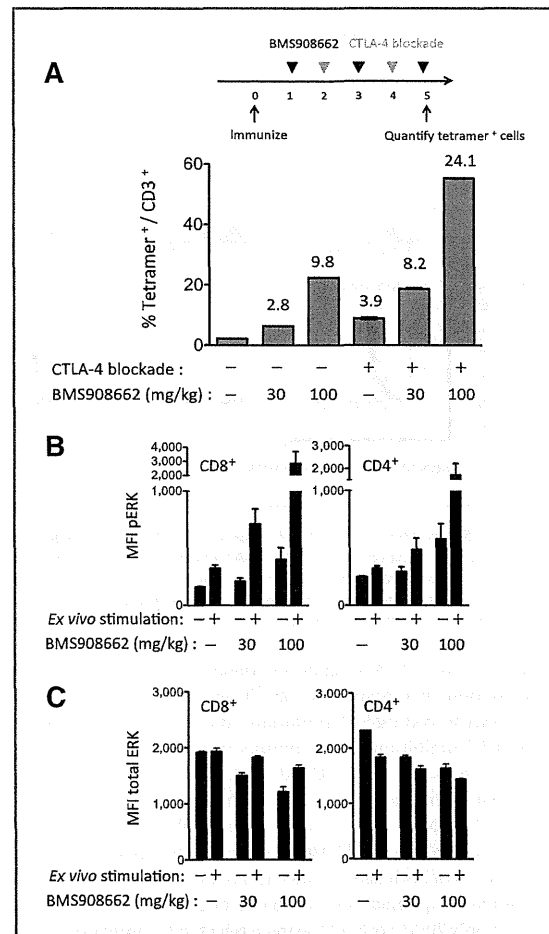


Figure 4. BMS908662 enhances antigen-specific T-cell expansion *in vivo* alone and in combination with CTLA-4 blockade. **A**, B6 mice were adoptively transferred with OT-I TCR transgenic T cells. Antigen-specific expansion of OT-I cells was measured after immunization with OVA. Mice treated systemically with BMS908662, or treated with CTLA-4-blocking antibody or the combination of both were compared with mice treated with vehicle control. Five days after immunization, the percentage of tetramer⁺ CD3⁺ population was measured. In each experiment, 5 mice per group were treated. One experiment, representative of two similar experiments, is shown here. Compared with mice treated with vehicle alone, mice treated with 30 mg/kg BMS908662 or 100 mg/kg BMS908662 had 2.8- or 9.8-fold more peripheral OT-1 cells after 5 days. For mice treated with CTLA-4 blockade in combination with either 30 mg/kg BMS908662 or 100 mg/kg BMS908662 there was an 8.2- or 24.1-fold increase in peripheral OT-1 cells detected at this time point. **B** and **C**, B6 mice were treated systemically with BMS908662. After 5 days of treatment, splenocytes were harvested and ERK signaling was measured after *ex vivo* activation by staining for levels of pERK (**B**) or total ERK (**C**) in CD4⁺ and CD8⁺ T cells. Representative flow cytometry data are presented in Supplementary Fig. S5.

models of syngeneic transplantable tumors: CT-26, a colon carcinoma cell line, and SA1N, a fibrosarcoma cell line, to evaluate the potential of combination therapy. Both tumors have demonstrated sensitivity to CTLA-4 blockade, where the

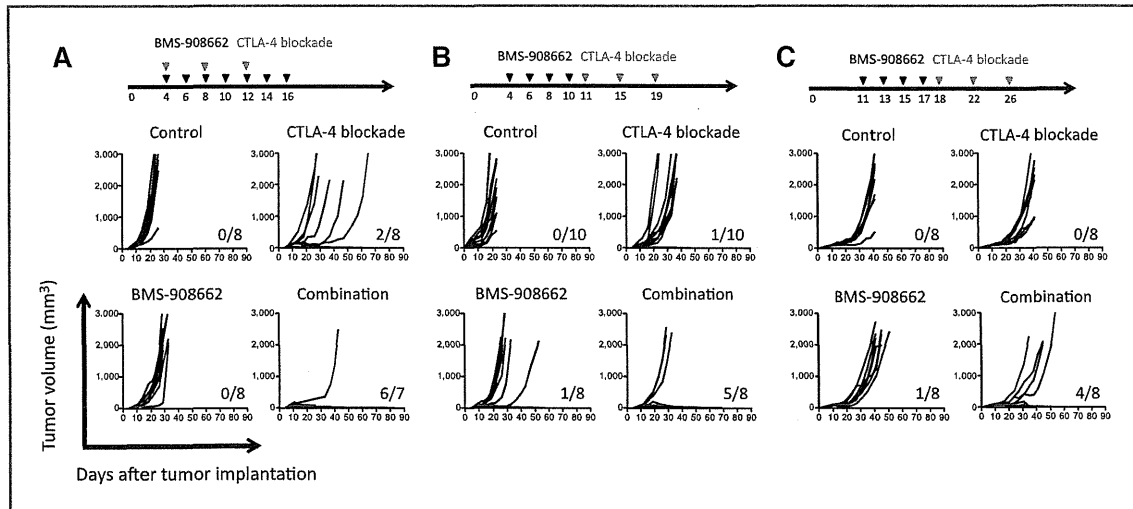


Figure 5. The combination of BMS908662 and CTLA-4 blockade demonstrates synergistic antitumor activity. The syngeneic murine transplantable tumors CT-26 colon carcinoma (A and B) or SA1N fibrosarcoma (C) were used to test the antitumor activity of BMS908662, CTLA-4 blockade, or a combination of both as compared with vehicle control-treated mice. For each experiment, 7 to 10 mice were treated in each experimental group. The sequencing of treatment is indicated for each experiment. Under all conditions tested, combination therapy was superior to monotherapy. For the CT-26 tumor, this effect was additive. For the SA1N tumor, which is insensitive to either monotherapy under these conditions, the combination demonstrates synergy ($P < 0.05$).

activity of CTLA-4 blockade depends upon the timing of initiation of therapy (31, 32). These tumors do not harbor known activating RAF mutations and are relatively insensitive to RAF inhibition as a monotherapy. First, we chose a treatment schedule where CTLA-4 blockade has partial activity, initiating treatment 4 days after implantation of CT-26 tumor (Fig. 5A). Mice were treated with a vehicle control, CTLA-4 blockade alone, BMS908662 alone, or the concomitant combination of both agents. Mice treated with CTLA-4 blockade as monotherapy had clear evidence of antitumor activity; however only 20% (2 of 8) of the mice rejected the implanted tumor. The BMS908662 as monotherapy showed minimal activity with none of the mice achieving tumor regression. In the combination, surprisingly, the treatment conferred complete tumor rejection in 85.7% (6 of 7) of the mice. This finding suggested that the addition of BMS908662 could improve the antitumor activity of CTLA-4 blockade. To test this further, we altered the schedule of treatment to delay the initiation of CTLA-4 blockade until 11 days after transplantation, diminishing the activity of CTLA-4 as a monotherapy (Fig. 5B). In this setting, 10% (1 of 10) of the mice treated with CTLA-4 blockade alone rejected the transplanted tumor. Again, mice treated with BMS908662 alone had minimal antitumor activity (1 of 8 mice rejected tumor). In contrast, the combination of both drugs together controlled tumor growth in 62.5% (5 of 8) of the mice, underscoring the potential of BMS908662 to enhance the antitumor activity of CTLA-4 blockade in which monotherapy is suboptimal. Similar results were observed in two independent experiments. Finally, we tested the activity of this combination for treatment of the SA1N fibrosarcoma (Fig. 5C). Here, we delayed initiation of CTLA-4 blockade until day 18, a time when CTLA-4 blockade had minimal activity as monotherapy in this tumor model. As with the CT-26 tumor line, SA1N was largely

insensitive to BMS908662 as monotherapy, with only 12.5% (1 of 8) of the mice showing tumor regression. However, the combination of both agents showed robust activity, rendering 50% (4 of 8) of the mice tumor free. For the SA1N tumor, the combination of these two drugs demonstrated synergy, as calculated using the EOHSA (excess over highest single agent) method ($P < 0.05$; ref. 33). The superiority of the combination therapy, synergistic for the SA1N tumor, was unexpected, given the low level of direct antitumor activity for BMS908662 alone in these models. These data support a model in which BMS908662 acts outside of the tumor to potentiate T-cell activation and thus enhances the activity of CTLA-4 blockade.

Discussion

These studies were initiated to better understand the immunomodulatory properties of RAF inhibitors. Most studies to date have supported the general view that these agents are unlikely to impair the immune system (30, 34, 35). Several studies have provided evidence that RAF inhibitors may enhance immune activation. Boni and colleagues found that the selective RAF inhibitor PLX4720 increased the expression of several melanocytic differentiation antigens *in vitro* and consequently enhanced the activation of antigen-specific T cells *in vitro* (29). Wilmott and colleagues observed that patients with melanoma treated systemically with vemurafenib or dabrafenib had an increase in intratumoral CD8⁺ T-cell infiltration (36), a finding also observed by Frederick and colleagues, who described changes in immune cytokines and T-cell activation markers in the tumor microenvironment of BRAF-treated patients (37). Vemurafenib-mediated changes in tumor cytokine production were described by Khalili and colleagues and were linked to a reduction in T-cell suppression

by tumor-associated fibroblasts (38). Finally, modulation of MAPK pathway signaling seems to be linked to tumor expression of the T-cell inhibitory molecule, PD-L1 (39). These studies have focused primarily on RAF inhibitor-mediated changes in targeted BRAF-mutant tumor cells that may have immunologically relevant downstream effects on stromal and immune cells in the tumor microenvironment (29, 38, 39). In addition, a recently reported study by Koya and colleagues found evidence of enhanced T-cell antitumor activity for adoptively transferred T cells in the presence of vemurafenib and the authors reported increased IFN- γ expression in antigen-specific T cells in the presence of vemurafenib (40). In contrast, only one group has reported that PLX4720 can have a negative effect on antitumor immune responses (41).

Recent studies have elucidated the effects that selective RAF inhibitors have on cells that do not harbor the BRAF^{V600E} mutation. In cells that are wild-type for BRAF, these inhibitors may paradoxically activate ERK signaling via transactivation of RAF dimers (9–12). This paradoxical activation depends upon signaling upstream of RAF, as in the case of hyperactivating RAS mutations. This seems to be a class effect of ATP-competitive RAF inhibitors. This phenomenon has been suggested as an explanation for the emergence of squamous cell carcinomas and keratoacanthomas in patients treated with RAF inhibitors (13, 14). In addition, we recently reported a case in which the emergence of a RAS-mutant leukemia was dependent upon paradoxical ERK activation in the presence of vemurafenib, demonstrating the generality of this effect in a diversity of cell types (42). In the present article, we extend this phenomenon to include nonneoplastic cells, wild-type T cells that use RAS activation for signaling upon TCR engagement.

In this study, we present evidence that RAF inhibitor-mediated paradoxical activation enhances T-cell activation and function. We have isolated the direct effects that the RAF inhibitor, BMS908662, has on T-cell activation *in vitro* and *in vivo*. BMS908662 (XL281) is an ATP-competitive pan-RAF inhibitor. It is reported to have activity against both mutant and wild-type BRAF as well as CRAF (5). Consistent with what we would expect based on prior studies of paradoxical activation in tumor cells, this phenomenon is dose dependent; in T cells, doses in the range of 20 pmol/L to 0.2 μ mol/L *in vitro* and 30 to 100 mg/kg *in vivo* are activating, whereas higher doses are inhibitory. Evidence for paradoxical pathway activation is seen in cultured T-cell lines and in primary human and murine T cells, in cells exposed to drug *in vitro* and *in vivo*, and in cells activated *in vitro* and *in vivo* via antigen-specific and nonspecific stimuli. Furthermore, the MEK inhibitor, PD325901, which targets the molecule activated downstream of RAF, can block this paradoxical pathway activation in T cells. Finally, this effect is dependent on a T-cell activating stimulus, such as engagement of the TCR that triggers ERK signaling. Koya and colleagues have reported that murine splenocytes treated with vemurafenib in culture express higher levels of pERK after 24 hours, although this experiment did not identify the cell type or types responsible for this observation (40). Although we have used the RAF inhibitor BMS908662 for these studies, paradoxical activation is a class effect of RAF inhibitors and we have found that PLX4720 has a similar effect on T cells *in vitro* albeit

with a unique dose range (Supplementary Fig. S6). This mechanism may help explain some of the positive effects of RAF inhibitors on immune activation that have been reported to date. Additional studies will be necessary to interrogate more closely which T-cell subsets and T-cell functions are most sensitive to RAF and to further explore the similarities and differences between RAF inhibitors with respect to T-cell activation. Notably, a prior study by Comin-Anduix and colleagues evaluating the effect of PLX4032 on cultured T cells did not detect evidence for paradoxical activation (30). This may reflect differences in T-cell culture or activation conditions, or less likely a difference in the drugs tested (PLX4720 and BMS908662 vs. PLX4032).

Several potentially immunomodulatory effects of RAF inhibitors have been described, including changes in tumor cytokine production, tumor antigen expression/presentation, tumor PD-L1 expression, tumor infiltration by T cells, and changes in T-cell effector function (29, 36–38, 40, 43). In the present study, we have used BRAF wild-type tumors to more directly isolate the effects that RAF inhibitors have on immune cells that have antitumor activity. Because the tumors themselves are less sensitive to the RAF inhibitor, it is more likely that BMS908662 is acting outside the tumor to enhance activation of the immune system under the conditions we have tested. The robust effect that BMS908662 has in combination with CTLA-4 blockade on antigen-specific T-cell expansion supports the notion that the RAF inhibitor acts via the T cell to enhance the activity of CTLA-4 blockade. An alternative possibility is that BMS908662 is changing the sensitivity of the tumor to immunotherapy without directly altering tumor growth. The present study does not distinguish between these two possible explanations, nor does it account for the relative impact of the direct antitumor effects in BRAF-mutant melanoma compared with the importance of immunomodulatory activities of RAF inhibitors. Additional studies in mice and in humans are needed to better understand the mechanisms that contribute to favorable outcomes with combination therapy, and clearly identify the drugs, doses, and schedules most likely to translate successfully into the clinic.

Disclosure of Potential Conflicts of Interest

M.K. Callahan has received a commercial research grant from Bristol-Myers Squibb for which she also serves as a consultant/advisory board member. J. Katz has ownership interest (including patents) in Bristol-Myers Squibb. J.M. Wigginton is employed as Senior Vice-President, Clinical Development, for MacroGenics, Inc. and has ownership interest (including patents) in the same. N. Rosen is a consultant/advisory board member for Novartis. M. Jure-Kunkel is employed as Senior Principal Scientist for Bristol-Myers Squibb and has ownership interest (including patents) in the same. J.D. Wolchok has received a commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member of the same. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

This work was supported by grants from the National Cancer Institute (R01 CA56821), Swim Across America, and the Melanoma Research Alliance, the

Goodwin-Commonwealth Fund for Cancer Research and the Virginia and D.K. Ludwig Fund for Cancer Research. M.K. Callahan has received support from an MSKCC training grant from NIH (T32 CA009512-5), from the Cancer Research Institute Irvington Postdoctoral Fellowship Program, and from the Department of Defense (CA110094).

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Received September 20, 2013; accepted September 23, 2013; published OnlineFirst October 14, 2013.

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