Durable Suppression of Acquired MEK Inhibitor Resistance in Cancer by Sequestering MEK from ERK and Promoting Antitumor T-cell Immunity

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**ABSTRACT**

MAPK targeting in cancer often fails due to MAPK reactivation. MEK inhibitor (MEKi) monotherapy provides limited clinical benefits but may serve as a foundation for combination therapies. Here, we showed that combining a type II RAF inhibitor (RAFi) with an allosteric MEKi durably prevents and overcomes acquired resistance among cancers with KRAS, NRAS, NF1, BRAF<sup>non-V600</sup>, and BRAF<sup>V600</sup> mutations. Tumor cell–intrinsicly, type I RAFi plus MEKi sequester MEK in RAF complexes, reduce MEK/MEK dimerization, and uncouple MEK from ERK in acquired-resistant tumor subpopulations. Immunologically, this combination expands memory and activated/exhausted CD8<sup>+</sup> T cells, and durable tumor regression elicited by this combination requires CD8<sup>+</sup> T cells, which can be reinvigorated by anti–PD-L1 therapy. Whereas MEKi reduces dominant intratumoral T-cell clones, type II RAFi cotreatment reverses this effect and promotes T-cell clonotypic expansion. These findings rationalize the clinical development of type II RAFi plus MEKi and their further combination with PD-1/L1-targeted therapy.

**SIGNIFICANCE:** Type I RAFi + MEKi are indicated only in certain BRAF<sup>V600MUT</sup> cancers. In contrast, type II RAFi + MEKi are durably active against acquired MEKi resistance across broad cancer indications, which reveals exquisite MAPK addiction. Allosteric modulation of MAPK protein/protein interactions and temporal preservation of intratumoral CD8<sup>+</sup> T cells are mechanisms that may be further exploited.

**INTRODUCTION**

RAS–RAF–MEK–ERK signaling is hyperactivated in more than 30% to 40% of human cancers (1), including approximately 70% of advanced melanoma driven by BRAF<sup>V600</sup>, non-BRAF<sup>V600</sup> (atypical BRAF), NRAS, and NF1 mutants. Type I RAF inhibitors (RAFi), such as vemurafenib, dabrafenib, and encorafenib, were first developed successfully against advanced BRAF<sup>V600MUT</sup> melanoma, but acquired resistance is almost universal. As a single agent, allosteric MEK1/2 inhibitors (MEKi), such as cobimetinib, trametinib, binimetinib, and selumetinib, elicit limited clinical response rates in a wide range of malignancies that harbor RAF and RAS mutations, for example BRAF<sup>V600MUT</sup> and NRAS<sup>MUT</sup> melanoma (2, 3). However, the combination of type I RAFi + MEKi suppresses acquired resistance in tumors driven by BRAF<sup>V600</sup> mutants and increases the therapeutic window in patients (4, 5), although acquired resistance caused by MAPK pathway reactivation is still commonplace (6–8). In human cancers where RAS–RAF–MEK–ERK signaling is not hyperactivated by BRAF<sup>V600</sup> mutants, a MEKi-based combination that can sustainably block the MAPK pathway and resistance-associated reactivation has not been developed successfully.

A durably effective MEKi-based combination should prevent MAPK reactivation (9, 10) and preserve and/or promote antitumor T-cell immunity. MAPK-targeted therapy combined with immune-checkpoint blockade is undergoing clinical testing (11, 12), and antitumor T-cell immunity has been proposed to contribute to the durability of MAPK-targeted therapy (13). MEKi may be deleterious to tumor antigen–specific T cells (14, 15), which may be ameliorated by pulsatile MEKi dosing (16).

Next-generation MAPK pathway inhibitors may help overcome MEKi resistance commonly caused by MAPK reactivation, which is in turn caused by upregulation of “back-to-back” RAF/RAF and/or “face-to-face” RAF/MEK dimerization. For instance, BRAF<sup>V600</sup> mutants, which signal as monomers but form dimers due to resistance-associated alterations, and non-V600, BRAF-activating mutants, which signal as RAS-independent dimers, can both be targeted by a novel dimer breaker, PLX8394 (17). These types of compounds “break” the paradox of type I RAFi, which is BRAF monomer–specific but has the liability of inducing RAF dimers and causing paradoxical MAPK hyperactivation in RAS-activated cancer cells. However, paradox breakers are not effective against CRAF wild-type (CRAF<sup>WT</sup>) or BRAF<sup>WT</sup> homodimers or...
heterodimers, which drive MAPK hyperactivation in the majority of cancers. Type II RAFi (aka dimeric or omni-RAFi) has antitumor activity in \textit{RAS}^{MUT} or \textit{BRAF}^{MUT} cancers (18–22) but, as a single agent, does not appear to be highly active clinically (23, 24).

To evaluate the clinical potential and mechanisms underlying the combination of type II RAFi and allosteric MEKi, we investigated: (i) durability in preventing as well as overcoming acquired MEKi resistance across potentially MAPK-addicted cancer lineages, (ii) combinatorial mechanistic action consistent with preventing MAPK reactivation, and (iii) \textit{in vitro} T-cell impacts.

### RESULTS

#### Type II RAFi Combination Prevents and Overcomes Acquired MEKi Resistance

We tested type II RAFi (BGB-283 or RAF-709) at a submicromolar (0.5 \textmu mol/L) concentration and/or allosteric MEKi (trametinib or binimetinib) at a nanomolar (20 \textmu mol/L) concentration against a panel (\textit{n} = 22) of human melanoma, colorectal carcinoma, pancreatic ductal adenocarcinoma (PDAC), and non–small cell lung carcinoma (NSCLC) cell lines driven by \textit{BRAF}^{V600E}, \textit{NF1}^{−/−}, \textit{NRAS}^{MUT}, or \textit{KRAS}^{MUT} in short-term (14-day) clonogenic assays (Fig. 1A; Supplementary Fig. S1A; Supplementary Table S1 for a list of cell lines and \textit{in vitro} models used in this study). In general, although type II RAFi or MEKi individually were ineffective at preventing short-term growth, type II RAFi + MEKi prevented macroscopic growth over 14 days. Moreover, using a panel of \textit{BRAF}^{V600E}, \textit{NF1}^{−/−}, \textit{NRAS}^{MUT}, or \textit{KRAS}^{MUT} melanoma sublines (\textit{n} = 4) with acquired resistance to type I RAFi + MEKi (vemurafenib + selumetinib/AZD-6244; ref. 7), we tested whether switching from type I RAFi to type II RAFi would overcome resistance (Fig. 1B). In one set of cultures, we maintained these sublines with both type I RAFi and MEKi or withdrew one or both inhibitors. As expected, withdrawal of both reduced growth fitness due to drug addiction (7, 25). In two additional sets of cultures, we introduced (or switched to) a type II RAFi (BGB-283 or RAF-709). Importantly, switching from type I to type II RAFi (over 10–15 days) overcame growth of resistant sublines, but this occurred only when an allosteric MEKi was present.

In patient-derived xenograft (PDX) models of PDAC (\textit{n} = 2, \textit{KRAS}^{MUT}) or of NSCLC (\textit{n} = 1, \textit{BRAF}^{V600E,\textit{V600M}}; \textit{n} = 1, \textit{KRAS}^{MUT}), daily treatment with BGB-283 (20 mg/kg, orally) or trametinib (3 mg/kg, orally) alone led to minimal or transient tumor growth deceleration (Fig. 1C and D). However, in all four PDXs, combination treatment achieved durable tumor regression (86–104-day follow-ups), with complete responses (CR) noted in both NSCLC PDXs. Because the combination of type II RAFi + MEKi was highly effective in preventing MEKi resistance in short-term cultures of \textit{NRAS}^{MUT} melanoma cell lines (Fig. 1E; Supplementary Fig. S1A), we also tested the combination against PDXs of \textit{NRAS}^{MUT} melanoma (\textit{n} = 3). \textit{In vivo}, responses to daily treatment with BGB-283 (20 \textmu g/kg, orally) or trametinib (3 mg/kg, orally) alone ranged from none to tumor stabilization (without regression; Fig. 1E). In all three \textit{NRAS}^{MUT} melanoma PDXs, the combination achieved highly durable tumor regression in all tumors, resulting in 2/5 to 4/5 CRs (113–141-day follow-ups; Fig. 1E). CRs were confirmed in one PDX model by stopping treatment with both type II RAFi + MEKi at day 125, with no relapse during a 109-day follow-up. Tumors that relapsed in two mice were sensitive to retreatment on day 162 with both inhibitors (secondary CRs; Supplementary Fig. S1B).

#### Differences of Type II RAFi + MEKi (vs. Type II RAFi + ERKi) in Antagonizing Acquired MEKi Resistance

We then tested whether the durability of type II RAFi + MEKi in preventing acquired MEKi resistance is specific to this combination when compared with type II RAFi + ERKi. To compare the relative combinatorial potencies in preventing or overcoming acquired MEKi resistance, we first identified concentrations of MEKi (trametinib) versus ERKi (SCH772984 or BVD-523) with functionally equivalent impacts on the short-term (10 days) growth of MEKi-naive, parental \textit{NRAS}^{MUT} melanoma cell lines (M207, M245, and M296) or the short-term (8–11 days) growth of isogenic \textit{NRAS}^{MUT} melanoma sublines with acquired MEKi (trametinib, 0.1 \textmu mol/L) resistance (M207 SDR1, M245 SDR5, M296 SDR3; SDR, single-drug resistant; ref. 25). In MEKi-naive, parental cells, 0.01 \textmu mol/L trametinib and 0.1 \textmu mol/L SCH772984 were approximately equivalent in suppressing short-term (10 days) growth or short-term (2 hours) pERK levels (Fig. 1F). In isogenic sublines with acquired MEKi resistance, switching from trametinib at 0.1 \textmu mol/L to SCH772984 at 0.1 \textmu mol/L or BVD-523 at 1 \textmu mol/L had no or minimal impact on short-term (8–11 days) growth, as measured by the MTT assay, viable cell counting, or clonogenic assay (Supplementary Fig. S1C and S1D). We also evaluated the temporal impact of single-agent MEKi versus ERKi doses on the MAPK pathway in \textit{NRAS}^{MUT} melanoma sublines with acquired MEKi resistance. We measured pERK and p-p90RSK levels after treatment with MEKi or ERKi for 1 hour or 48 hours, with or without prior treatments with the same inhibitor for 0, 2, 4, or 6 days (Supplementary Fig. S1E–S1G). Within this time span, treatment(s) with trametinib at 0.1 \textmu mol/L, SCH772984 at 0.1 \textmu mol/L, or BVD-523 at 1 \textmu mol/L resulted in similar p-p90RSK levels, which are consistent with similar effective ERK activities and growth rates. However, in all acquired-resistant sublines, the highly similar effective ERK activities over time (as measured by p-p90RSK levels and cellular growth rates) between MEKi versus ERKi treatments contrasted glaringly with pERK levels. Only with ERKi (but not MEKi) treatment did we observe high rebinding levels of pERK 1 hour and 48 hours after the last fresh dose of treatment, regardless of the duration of prior ERKi treatment. This pERK rebound was stronger with BVD-523 and MK-8353 than with SCH772984. Unlike BVD-523 and MK-8353, SCH772984 is known to inhibit both the kinase activity of ERK and MEK-mediated phosphorylation of the ERK activation loop. Rebound pERK levels in response to BVD-523 (1 \textmu mol/L) and MK-8353 (1 \textmu mol/L) treatment was abundant within 1 hour after treatment but were also readily detectable within 2 days of SCH772984 at 0.4 \textmu mol/L and within 4 to 6 days of SCH772984 at 0.1 \textmu mol/L. To our surprise, ERKi-induced pERK rebound levels did not accelerate growth rates of \textit{NRAS}^{MUT} melanoma sublines with acquired MAPKi resistance.

Having established growth rate–equivalent concentrations of MEKi versus ERKi, we compared the relative potencies of combination with type II RAFi in preventing acquired MEKi resistance in drug-naïve cells. In cancer cell lines, acquired resistance to targeted therapy develops through temporal
Type II RAFi and MEKi to Treat MAPK-Addicted Cancers

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Figure 1. Type II RAFi + MEKi forestall and overcome acquired resistance in BRAF\textsuperscript{V600E}, NF1\textsuperscript{MUT}, KRAS\textsuperscript{MUT}, and NRAS\textsuperscript{MUT} cancers. A, Clonogenic growth (14 days) of indicated inhibitor-naive BRAF\textsuperscript{V600} or NF1-mutant melanoma, BRAF\textsuperscript{V600MUT} or KRAS\textsuperscript{MUT} colorectal carcinoma, KRAS\textsuperscript{MUT} NSCLC, and KRAS\textsuperscript{MUT} PDAC cell lines after treatment with vehicle (DMSO), BGB-283 (0.5 \(\mu\)mol/L), trametinib (0.02 \(\mu\)mol/L), or trametinib (0.02 \(\mu\)mol/L) plus BGB-283 (0.5 \(\mu\)mol/L) or alternatively with RAF-709 (0.5 \(\mu\)mol/L), binimetinib (0.02 \(\mu\)mol/L), or binimetinib (0.02 \(\mu\)mol/L) plus RAF-709 (0.5 \(\mu\)mol/L). Data representative of two replicates. B, Clonogenic growth (15 days, left two sublines; 10 days, right two sublines) of indicated type I RAFi + MEKi double-drug resistant (DDR; hereafter all names of sublines with acquired drug resistance shown in red text) BRAF\textsuperscript{V600E} melanoma sublines. DDR sublines were maintained on both PLX4032 (1 \(\mu\)mol/L) and AZD6244 (1 \(\mu\)mol/L), either PLX4032 or AZD6244, or withdrawn from both inhibitors. On top of these four conditions, a type II RAFi (BGB-283 at 1 \(\mu\)mol/L or RAF-709 at 1 \(\mu\)mol/L) was added. Data are representative of two replicates. (continued on next page)

Figure 1. Type II RAFi + MEKi forestall and overcome acquired resistance in BRAF\textsuperscript{V600E}, NF1\textsuperscript{MUT}, KRAS\textsuperscript{MUT}, and NRAS\textsuperscript{MUT} cancers. A, Clonogenic growth (14 days) of indicated inhibitor-naive BRAF\textsuperscript{V600E} or NF1-mutant melanoma, BRAF\textsuperscript{V600MUT} or KRAS\textsuperscript{MUT} colorectal carcinoma, KRAS\textsuperscript{MUT} NSCLC, and KRAS\textsuperscript{MUT} PDAC cell lines after treatment with vehicle (DMSO), BGB-283 (0.5 \(\mu\)mol/L), trametinib (0.02 \(\mu\)mol/L), or trametinib (0.02 \(\mu\)mol/L) plus BGB-283 (0.5 \(\mu\)mol/L) or alternatively with RAF-709 (0.5 \(\mu\)mol/L), binimetinib (0.02 \(\mu\)mol/L), or binimetinib (0.02 \(\mu\)mol/L) plus RAF-709 (0.5 \(\mu\)mol/L). Data representative of two replicates. B, Clonogenic growth (15 days, left two sublines; 10 days, right two sublines) of indicated type I RAFi + MEKi double-drug resistant (DDR; hereafter all names of sublines with acquired drug resistance shown in red text) BRAF\textsuperscript{V600E} melanoma sublines. DDR sublines were maintained on both PLX4032 (1 \(\mu\)mol/L) and AZD6244 (1 \(\mu\)mol/L), either PLX4032 or AZD6244, or withdrawn from both inhibitors. On top of these four conditions, a type II RAFi (BGB-283 at 1 \(\mu\)mol/L or RAF-709 at 1 \(\mu\)mol/L) was added. Data are representative of two replicates. (continued on next page)

Figure 1. Type II RAFi + MEKi forestall and overcome acquired resistance in BRAF\textsuperscript{V600E}, NF1\textsuperscript{MUT}, KRAS\textsuperscript{MUT}, and NRAS\textsuperscript{MUT} cancers. A, Clonogenic growth (14 days) of indicated inhibitor-naive BRAF\textsuperscript{V600E} or NF1-mutant melanoma, BRAF\textsuperscript{V600MUT} or KRAS\textsuperscript{MUT} colorectal carcinoma, KRAS\textsuperscript{MUT} NSCLC, and KRAS\textsuperscript{MUT} PDAC cell lines after treatment with vehicle (DMSO), BGB-283 (0.5 \(\mu\)mol/L), trametinib (0.02 \(\mu\)mol/L), or trametinib (0.02 \(\mu\)mol/L) plus BGB-283 (0.5 \(\mu\)mol/L) or alternatively with RAF-709 (0.5 \(\mu\)mol/L), binimetinib (0.02 \(\mu\)mol/L), or binimetinib (0.02 \(\mu\)mol/L) plus RAF-709 (0.5 \(\mu\)mol/L). Data representative of two replicates. B, Clonogenic growth (15 days, left two sublines; 10 days, right two sublines) of indicated type I RAFi + MEKi double-drug resistant (DDR; hereafter all names of sublines with acquired drug resistance shown in red text) BRAF\textsuperscript{V600E} melanoma sublines. DDR sublines were maintained on both PLX4032 (1 \(\mu\)mol/L) and AZD6244 (1 \(\mu\)mol/L), either PLX4032 or AZD6244, or withdrawn from both inhibitors. On top of these four conditions, a type II RAFi (BGB-283 at 1 \(\mu\)mol/L or RAF-709 at 1 \(\mu\)mol/L) was added. Data are representative of two replicates. (continued on next page)

stages: early slow-cycling or drug-tolerant persistence (pseudosenescence) and later fast-cycling or proliferative growth (26). Accordingly, using parental NRAS\textsuperscript{MUT} melanoma cell lines (n = 3) in short-term (15 days) or long-term (30 days) cultures (Fig. 1G), we compared the efficacies of type II RAFi (BGB-283 at 0.5 \(\mu\)mol/L) in preventing growth when combined with functionally equivalent doses of MEKi (trametinib) versus ERKi (SCH772984; Fig. 1F). Although BGB-283 + trametinib and BGB-283 + SCH772984 were similarly effective at preventing 15-day growth, BGB-283 + trametinib was far more effective than BGB-283 + SCH772984 at preventing 30-day growth. Because proliferative MEKi-resistant clones are expected to
arise with longer-term culture, type II RAFi + MEKi may be more active than type II RAFi + ERKi in overcoming the growth of proliferative MEKi-resistant clones. To test this hypothesis, we compared the relative potencies of type II RAFi + MEKi versus type II RAFi + ERKi in overcoming acquired MAPKi resistance in three melanoma subsets: NRAS\textsuperscript{MUT} melanoma sublines (n = 3) with acquired MEKi resistance (Fig. 1H; Supplementary Fig. S1H), BRAF\textsuperscript{V600MUT} melanoma sublines (n = 4) with acquired resistance to type I RAFi + MEKi (Supplementary Fig. S1I and S1J), and NF1\textsuperscript{−/−} melanoma..
sublines with acquired MEKi resistance (*n* = 4; Supplementary Fig. S1K and S1L). Using functionally equivalent doses of MEKi (trametinib) versus ERKi (SCH772984, BVD-523, and MK-8353; Fig. 1H; Supplementary Fig. S1C–S1G), we observed consistently (in 11 of 11 acquired MAPKi-resistant melanoma sublines) that BGB-283 + trametinib was more effective than BGB-283 + SCH772984 (or BVD-523, MK-8353) at overcoming the growth of established acquired-resistant clones.

**NRASMUT Melanoma Acquires MEKi Resistance via ERK Reactivation**

Given the durability of type II RAFi + MEKi in preventing and overcoming MEKi resistance, we tested the hypothesis that NRASMUT melanoma acquires MEKi resistance predominantly through ERK reactivation. We treated NRASMUT PDXs (*n* = 5) with trametinib (5 mg/kg/day) until acquired-resistant tumors (*n* = 16) emerged (Fig. 2A). The durability of response

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**Figure 1. (Continued)** F, Top, clonogenic growth (10 days) of indicated inhibitor-naïve NRASMUT melanoma cell lines after treatment with vehicle (DMSO) or indicated concentration of MEKi (trametinib) or ERKi (SCH772984). Data are representative of two replicates. Bottom, Western blots with indicated antibodies of lysates from NRASMUT melanoma cell lines treated (2 hours) with vehicle (DMSO) or the same concentrations of MEKi or ERKi (top). G, Clonogenic growth (15 or 30 days) of indicated inhibitor-naïve NRASMUT melanoma cell lines after treatment with vehicle (DMSO) or trametinib, SCH772984, or the type I RAFi PLX-4032 (15 days only) at the indicated concentrations, ±BGB-283. Overconfluent cultures without BGB-283 cotreatment were terminated early and not shown for day 30. Data are representative of two replicates. H, As in G, except NRASMUT melanoma sublines (single-drug resistant or SDR, indicated in red) with acquired MEKi (trametinib) resistance were used. Day 15 cultures are shown except those marked with * (which indicates day 30 cultures). Cultures marked with # display the drug addiction phenotype.
was highly variable across distinct and within each model(s). Together with aforementioned NRAS\textsuperscript{Q61K} melanoma parental (P; n = 3) and isogenic, acquired trametinib-resistant cell lines (n = 5), we extracted genomic DNAs (gDNA) and total RNAs from vehicle-treated tumors and isogenic resistant (R) tumors for whole-exome sequencing (WES; along with patient-matched normal gDNAs) and RNA sequencing (RNA-seq). We then integrated WES/RNA-seq analysis to evaluate the recurrence of gain-of-function (GOF) or loss-of-function (LOF) gene-based events of 723 cancer-related genes (COSMIC v.88; ref. 27; Supplementary Table S2). We ranked-ordered the recurrence of somatic, resistance-associated alterations based primarily on sample counts and secondarily on patient frequencies (Supplementary Fig. S2A). Among the top 20 GOF genes were overlapping cMET and ERBB2 mRNA upregulation (≥2-fold). However, the most recurrent GOF genes at the genomic level were NRAS [7/21 samples with copy-number gain or mutant allele-specific loss-of-heterozygosity (LOH) events] and RAF1 [5/21; Supplementary Fig. S2A; Supplementary Tables S3 and S4]. We have previously shown (4, 7, 13, 26, 28, 29) that BRAF\textsuperscript{V600E/M} melanoma acquires resistance to type I RAFi + MEKi by omic...
alterations (e.g., BRAFV600E/K amplification–driven overexpression and RAF-regulated MEK1/2 mutants; refs. 7, 30) that enhance RAF/RAF and/or RAF/MEK interactions. Although RAFI/CRAF genomic alterations are not known to cause MAPKi resistance in BRAFV600MUT melanoma, we observed highly recurrent (and largely mutually exclusive) GOF alterations in NRAS, RAF1/CRAF, BRAF, and MAP2K1/2 (Fig. 2B and C). MAP2K2 harbored resistance-causative somatic mutations (F57L, V64F, and F133L; Supplementary Fig. S2B), as equivalent positions in MAP2K1 (F53, V60, and F129) have been shown to confer MAPKi resistance in BRAFV600MUT melanoma (7). Phylogenetically, resistant tumors or sublines are sometimes distantly related to common ancestral clones (Fig. 2D; Supplementary Fig. S2C), indicating that tumor heterogeneity (preexisting or induced by therapy) contributes to MEKi resistance.

To evaluate the functional roles of NRASMUT and RAF1/CRAF upregulation, we first overexpressed NRASMUT (vs. NRASWT) in the M245 P line to a level similar to NRASMUT upregulation observed in M245 SDR4 or SDR5 (Fig. 2E, left). We also knocked down NRAS overexpression in M245 SDR4 and SDR5 using two independent shRNAs (Fig. 2E, right). We observed in clonogenic assays that NRASMUT overexpression conferred resistance to trametinib relative to M245 P or M245 P overexpressing NRASWT (Fig. 2F, left). We also observed that NRAS knockdown in either M245 SDR4 or SDR5 sensitized these sublines to 0.1 μmol/L of trametinib (Fig. 2F, right). As we have reported (25), MEKi-resistant NRASMUT melanoma sublines are highly addicted to MEKi withdrawal. NRAS knockdown in the single-drug resistant (SDR) sublines abolished MEKi addiction, likely because NRASMUT overexpression drives pERK rebound and hence cell death after MEKi withdrawal. Moreover, M245 SDR3 upregulates CRAF expression; M245 SDR5, both CRAF and BRAF; and M207 SDR1, BRAF (Supplementary Tables S1, S3, and S4). Thus, we knocked down CAF alone, both CRAF/BRAF, or BRAF alone in the respective SDR sublines using previously validated shRNAs (refs. 7, 29; Fig. 2G). Whereas P lines were highly sensitive to 0.1 μmol/L of trametinib relative to M245 P lines (Fig. 2G). Whereas P lines were highly sensitive to 0.1 μmol/L of trametinib, CRAF and/or BRAF knockdown abolished the efficacy of MEKi (Fig. 3C); (iii) retard pMEK release from BRAF/CRAF (which promotes pMEK levels; Fig. 3D). Thus, type II RAFi cotreatment of MEKi-treated/resistant sublines led to pERK loss but curiously pMEK persistence.

Type II RAFi RAF709 as a single agent has been reported to induce RAF dimerization (19). In MEKi-naïve/nontreated P cells, type I RAFi (vemurafenib, 0.5 μmol/L) as a single agent induced both BRAF/CRAF interaction and pERK levels. In contrast, type II RAFi (BGB-283 or RAF709) as a single agent at 10 μmol/L induced equivalent levels of BRAF/CRAF interaction but suppressed pERK levels (Fig. 3E; Supplementary Fig. S3E). In MEKi-treated/resistant SDR cells, type I RAFi cotreatment also induced both BRAF/CRAF interaction and pERK levels. Importantly, type II RAFi cotreatment induced BRAF/CRAF interaction (on top of higher preexisting levels compared with P cells) at 0.1 μmol/L, leading to pERK suppression at submicromolar concentrations of type II RAFi. These findings suggest that the effective concentration of type II RAFi to induce signal-incompetent (pERK-suppressed) BRAF/CRAF complexes depends on MEKi cotreatment and/or upregulated levels of BRAF/CRAF complexes (both conditions present in SDR sublines compared with P lines). The upregulated levels of BRAF/CRAF complexes are apparently still capable of phosphorylating MEK (Fig. 3C) but somehow incapable of productively signaling to ERK.

We then investigated whether the high abundance of BRAF/CRAF complexes, which confers MEKi resistance (Fig. 2), also confers sensitivity of SDR sublines to type II RAFi cotreatment. In support of this hypothesis, individual or combined BRAF and/or MEKi knockdown abolished the efficacy of BGB-283 combination treatment (Fig. 3F). Using SDR sublines, we then investigated whether MEKi cotreatment enhances the ability of type II RAFi to induce signal-incompetent BRAF/CRAF complexes, resulting in pERK suppression. In this hypothetical model, type II RAFi + MEKi (i) foster BRAF/CRAF interaction (since BRAF and/or CRAF protein upregulation fosters the combinatorial efficacy of type II RAFi + MEKi; Fig. 3F); (ii) sequester or stabilize MEK/pMEK within the BRAF/CRAF scaffold (which promotes pMEK levels; Fig. 3C); (iii) retard pMEK release from BRAF/CRAF (which would in turn reduce MEK dimerization and thereby reduce the pool of active MEK accessible to ERK); and (iv) retard MEK/ERK binding (which is required for ERK activation). To test this model, we used MEKi-resistant NRASMUT melanoma sublines and measured the effects of single (type II RAFi or MEKi) versus double (type II RAFi + MEKi) inhibitor treatments on the in situ levels of protein/protein complexes within the MAPK pathway by proximity ligation assay (PLA). Consistent with prediction (i) of the model, acute (2-hour) treatment with type II RAFi + MEKi (BGB-283 + trametinib, LXH-254 + trametinib, or RAF-709 + binimetinib) upregulated BRAF/CRAF complexes compared with single-agent treatment (Supplementary Fig. S4A–S4C). After 12 days of treatments with inhibitor(s) (treatment refreshed every two
days with additional last dose 12 hours prior to analysis), we observed that type II RAFi alone had little effect on CRAF/MEK or MEK/ERK levels. In contrast, type II RAFi + MEKi significantly induced CRAF/MEK and reduced MEK/ERK levels (Fig. 4A and B), consistent with predictions (ii) and (iv). Induction of CRAF/MEK and reduction of MEK/ERK were also observed as early as 2 hours after treatment with the aforementioned three type II RAFi + MEKi plus two additional combinations (BGB-283 + binimetinib; BGB-283 + cobimetinib; Supplementary Fig. S4A–S4D).

We had shown earlier (Fig. 1H; Supplementary Fig. S1C–S1G) that BGB-283 + trametinib was more effective than BGB-283 + SCH772984 (or BVD-523, MK-8353) at overcoming the growth of established acquired MEKi-resistant melanoma subclones. SCH772984, compared with trametinib, acutely reduced p-p90RSK level (Fig. 4C). As observed previously, single-agent ERKi treatment led to rebound pERK levels (BVD-523 > SCH772984), which curiously failed to increase the growth rate (Supplementary Fig. S1C–S1G). BGB-283 cotreatment with BVD-523 did not reduce the rebounding pERK level and with SCH772984, which unlike BVD-253 inhibits ERK phosphorylation by MEK,
Figure 4. Type II RAFi and MEKi coordinate RAF/MEK stabilization, sequestering MEK from ERK. A, PLAs detecting CRAF/MEK and MEK/ERK proximity complexes in NRAS\textsuperscript{MUT} melanoma SDR sublines withdrawn from trametinib for 12 days or treated for 12 days with trametinib (0.1 μmol/L), SCH-772984 (0.1 μmol/L), or BVD-523 (1.0 μmol/L), ± BGB-283 cotreatment (1 μmol/L). Last fresh dose of inhibitor(s), 12 hours before analysis. DAPI, nuclear stain. Scale bars, 20 μm. B, Quantifications of PLA signals in A expressed as the fold change (FC) of PLA dots (per nucleus) of BGB-283 cotreatment over DMSO or single inhibitor treatment. N = 5 fields; mean ± SDs; all comparisons with respect to FC (BGB-283 + DMSO/DMSO); *, P < 0.1; **, P < 0.05; ***, P < 0.01 based on the t test. C, Western blots (WB) of lysates from culture conditions in A using indicated antibodies. D, PLA detecting CRAF/CRAF, BRAF/BRAF, BRAF/MEK, and MEK1/MEK1 proximity complexes in two NRAS\textsuperscript{MUT} melanoma SDR sublines treated with vehicle or trametinib (0.1 μmol/L) for 48 hours, ± BGB-283 (1 μmol/L, last 2 hours). DAPI, nuclear stain. Scale bars, 20 μm. E, Quantification of PLA data in D. N = 5 fields; mean ± SDs. FCs: black, BGB-283 + DMSO/DMSO; gray, BGB-283 + tram/tram. Latter FC is compared with the former FC. ***, P < 0.05; ***, P < 0.01 based on the t test. (continued on next page)
**Figure 4. (Continued)**  
**F.** Tumor volumes of NRAS_PDX1 R2 (with acquired trametinib resistance) in mice that were (starting on day 78, indicated by an arrow) maintained on trametinib (5 mg/kg/day, orally), switched to BGB-283 (20 mg/kg/day, orally), or treated with BGB-283 (20 mg/kg/day, orally) on top of trametinib (5 mg/kg/day, orally). N = 5 tumors per group; means ± SEMs.  
**G.** IF of pERK levels (top row) and PLA of indicated protein/protein proximity complexes (bottom three rows) for three groups of PDX tumors in **F.** Tumors were collected on day 3 or 5 (from day 78). Images are representative of five tumors per group.  
**H.** Quantifications of PLA signals in **G** of CRAF/BRAF, CRAF/MEK, and MEK/ERK complexes expressed as FC of PLA dots per nucleus of each indicated treatment condition versus continuous trametinib monotherapy. Mean ± SDs. **,** P < 0.05; **,** P < 0.01 based on pairwise t test.  
**I.** IP-WBs or direct WBs of WCLs after treatment of indicated trametinib-resistant sublines with vehicle (DMSO) or indicated inhibitor(s) as in **A.** IgG, isotype control for IP.  
**J.** Predicted conformational rearrangements of MEK1 and BRAF in complex upon binding to trametinib and BGB-283, respectively. Light brown, BRAF in its MEK1-bound tetrameric conformation; dark brown, BRAF in its BGB-283-bound conformation (BRAF P-loop in orange). Dark blue, MEK1 in BRAF-bound tetrameric conformation; light blue, MEK1 in its Tak-733-bound conformation (MEK1 activation loop in green). Red arrows, predicted and actual rearrangements of MEK1 and BRAF upon trametinib and BGB-283 binding, respectively.
did reduce the pERK rebound level but only to a level comparable to that with trametinib alone (Fig. 4C).

To validate our model further, we performed additional PLA assays in NRAS^MUT melanoma sublines with acquired MEKi resistance. Although BGB-283 + trametinib induced BRAF/CRAF complexes, this combination reduced BRAF or CRAF homo-complexes (Fig. 4D and E). BRAF/MEK complexes, just like CRAF/MEK complexes, were induced by BGB-283 + trametinib. Importantly, BGB-283 + trametinib reduced MEK1 homo-complexes, suggesting that RAF-phosphorylated/sequestered MEK could not be released from BRAF/CRAF scaffolds and thereby could not homodimerize and become activated. We also performed PLA assays in KRAS^MUT non-melanoma tumor cell lines. First, we derived sublines (HCT116-R, Su86.86-R, and H2122-R) from KRAS^MUT colorectal carcinoma, PDAC, and NSCLC cell lines (Fig. 1A) that had adapted to increasing doses of trametinib (up to 0.05 μmol/L) with proliferative resistance. In short-term (7 days) clonogenic assays, adding type II RAFi (BGB-283 or RAF-709 at 0.5 μmol/L) to MEKi (trametinib or binimetinib, respectively, at 0.05 μmol/L) strongly reduced clonogenic growth (Supplementary Fig. S4E). With 6 hours of treatment with inhibitor(s), we found that BGB-283 addition to trametinib (vs. BGB-283 alone) induced BRAF/CRAF and CRAF/MEK protein complex levels and reduced MEK/ERK and pERK levels (Supplementary Fig. S4F and S4G) in all three non-melanoma cancer cell lines with acquired MEKi resistance. To validate key mechanistic features of our model in vivo, we retransplanted the NRAS^PDX1 R2 (Fig. 2) acquired trametinib-resistant tumor into mice that were treated daily with trametinib. Trametinib-resistant tumors; starting volume at 500 mm^3) were assigned into three groups: trametinib (5 mg/kg/day, orally), BGB-283 (20 mg/kg/day, orally), or trametinib (5 mg/kg/day, orally) plus BGB-283 (20 mg/kg/day, orally). Switching from trametinib to BGB-283 did not induce tumor regression, whereas the combination of trametinib and BGB-283 rapidly induced tumor regression (CRs in 2 of 5 mice or tumors; Fig. 4F). We then analyzed NRAS^PDX1 R2 tumors early (days 3 and 5) on each of the three treatments for BRAF/CRAF, BRAF/MEK, and MEK/ERK in situ complexes by PLA and for pERK levels by immunofluorescence (IF; Fig. 4G and H). Consistent with cell line observations, type II RAFi + MEKi induced BRAF/CRAF and CRAF/MEK complexes and their combinations. To corroborate PLA findings, we performed co-IP and observed that type II RAFi + MEKi treatment preferentially (vs. type II RAFi + ERKi) induced endogenous CRAF/MEK and CRAF/pMEK complexes (Fig. 4I; Supplementary Fig. S4H and S4I).

To predict how RAF binding to BGB-283 and MEK binding to trametinib might enhance RAF/MEK complexes, we modeled the structure of the BRAF/MEK1 dimer bound to these inhibitors and calculated the change in buried solvent accessible surface area (SASA) as a result of dual inhibitor binding (see details in Methods). We superimposed the individual experimental or predicted structures of trametinib-bound MEK1 and BGB-283-bound BRAF (PDB ID 3PPI and 4RSY) on a dimer of the BRAF/MEK1 tetramer (PDB 4MNE; Supplementary Fig. S5). It is thought that BRAF and MEK1 form a face-to-face dimer (Supplementary Fig. SSA), with contribution to binding from the activation loop of both kinases (Supplementary Fig. SS5–SS7). When this model is compared with the BRAF and MEK1 conformations in the BRAF/MEK1 tetramer (Fig. 4J), the conformational changes of the activation loop of MEK1 upon trametinib binding and of the P-loop of BRAF upon BGB-283 binding could increase the contact surface between the two proteins and thereby the binding affinity. To estimate the effect of the latter conformational change, we calculated variation of the buried SASA upon binding of BRAF and MEK1, for both the apo conformation of BRAF (PDB ID 4MNE) and its BGB-283–bound conformation. Because the P-loop of BRAF was not resolved in the X-ray crystal structure of the apo form, we modeled it ab initio. For the residues of the BRAF P-loop (465–469) and for the MEK1 residues in the vicinity (residues 73–82 and 97–101), we calculated that the total buried SASA increased from 78 ± 5 Å^2 for the apo form of BRAF to 98 Å^2 for the BGB-283–bound BRAF, which suggests stronger binding.

### Type II RAFi + MEKi Elicit CD8^+ T Cell–Mediated Tumor Regression

Beyond tumor cell–intrinsic mechanisms, we evaluated the contribution of CD8^+ T cells to tumor regression. First, we introduced mutational burden into a syngeneic model of murine NraS^Ger melanoma (called NIL) we recently reported (25) by exposing it to radiation (UV), thereby deriving a subline called NILER1–4 and generating a relative increase of 25.3 mutations per megabase of gDNA. As subcutaneous allografts, NILER1–4 tumors displayed increased durability of MEKi (trametinib 3 mg/kg/day) response (vs. NIL tumors; starting volume at ~100 mm^3; Fig. 5A). However, systemic CD8^+ T-cell neutralization abolished this gain in durability, while having no effect on MEKi-resistance development in NIL tumors. These findings suggest that CD8^+ T cells suppress acquired MEKi resistance by recognizing neoantigen(s). Using larger (~200 mm^3) NILER tumors, we tested a non–tumor-regressive dose of trametinib (1 mg/kg/day, orally), two doses of BGB-283 (10 or 20 mg/kg/day, orally), and their combinations. Comparable to the studies using PDXs, BGB-283 at these doses did not induce tumor regression. Combination with trametinib at the higher dose (20 mg/kg/day) of BGB-283 induced durable tumor regression beyond 35 days and 7 of 7 CRs on day 52 (with 3 of 7 confirmed CRs after treatment withdrawal on day 52; Fig. 5B). The average weekly body weights of mice in all treatment groups increased commensurate with the Jackson Laboratory reference weights. However, NILER tumor–bearing mice treated with trametinib (1 mg/kg/day) + BGB-283 (20 mg/kg/day) began to lose weight after 40 days of continuous treatments.

We also tested trametinib and BGB-283 at these dosages in syngeneic tumor models of other cancer lineages. In Kras^Mut colorectal carcinoma (CT-26) and PDAC (KPC) syngeneic models, neither trametinib (1 mg/kg/day, orally) nor BGB-283 (20 mg/kg/day, orally) alone induced regression of established (~200 mm^3) tumors, but the combination elicited durable tumor regression in both colorectal carcinoma and PDAC models, resulting in 6/8 and 2/8 CRs, respectively, on day 42. Body weight loss was noted only in mice treated with the combination, beginning around day 24 (Fig. 5C and D). To determine whether the addition of BGB-283 can overcome well-established
Figure 5. Tumor regression in response to type II RAFi plus MEKi in syngenic models requires CD8+ T cells. A, Volumes of NIL versus NILER1-4 tumors in response to trametinib (Tam) treatment (3 mg/kg/day, orally; starting day 0) ± anti-CD8 neutralization (starting –1 day). Inset, CD8+ T-cell levels as a percentage of CD45+ splenocytes at the end of experiments. N = 8 tumors/group; means ± SEMs. P value, Student t test. B, Volumes of NILER1-4 tumors with indicated treatments starting at 200 mm³. Trametinib at 1 mg/kg/day, orally, n = 7 tumors/group; means ± SEMs. CRs confirmed by withdrawal of trametinib + BGB-283 (20 mg/kg/day, orally) without tumor recurrence for 35 days of observation. Right, average body weights of mice in each group measured twice a week. C and D, Volumes of CT-26 (C) or KPC (D) tumors with indicated treatments starting on days 12 and 13, respectively (at 200 mm³ tumor volume). Trametinib at 1 mg/kg/day, orally. Anti-CD8 treatment started on days 11 and 12, respectively. N = 8 tumors/group; means ± SEMs. Unconfirmed CRs, 0/8 (C) or 2/8 (D) in the trametinib (tram) + BGB-283 (20 mg/kg/day, orally) groups on day 45 or 46. P value, Student t test. Right, average body weights of mice in each group measured twice a week. CRC, colorectal carcinoma. E and F, Volumes of individual CT-26 tumors relative to tumor-matched baseline volumes (400–450 mm³) on day 0, when tumor-bearing mice were assigned to each of the three indicated groups. Trametinib 2 (E) or 3 (F) mg/kg/day, orally. Tumors/group [trametinib (tram), BGB-283, trametinib + BGB-283] B, B, 6 (E) or B, B, 8 (F). Unconfirmed CRs, 0/6 (E) or 1/8 (F) in the trametinib + BGB-283 (20 mg/kg/day, orally) groups on day 23. Right, average body weights of mice in each group measured twice a week. (continued on following page)
Type II RAFi and MEKi to Treat MAPK-Addicted Cancers

Figure 5. (Continued) G, Volumes of NILER1-4 tumors with indicated daily single-agent treatments starting at 200 mm³. N = 8 tumors/group; means ± SEMs. Functionally equivalent doses of trametinib (orally) versus SCH772984 (intraperitoneally) were selected for use in H. H, Volumes of NILER1-4 tumors with indicated treatment starting at 200 mm³. Trametinib at 0.1 mg/kg/day, orally, SCH772984 at 25 mg/kg/day, i.p. N = 8 tumors/group; means ± SEMs. Right, average body weights of mice in each group measured twice a week. I, Volumes of NILER1-4 tumors with indicated treatment starting at 200 mm³. Trametinib at 0.1 mg/kg/day plus BGB-283 at 10 or 20 mg/kg/day ± anti-CD8 neutralization (starting day 8). N = 8 tumors/group; means ± SEMs. J, Volumes of NILER1-4 tumors with indicated treatment starting at 200 mm³ and day 10. Trametinib at 0.1 mg/kg/day plus BGB-283 at 10 or 20 mg/kg/day ± anti-CD-L1 treatment. N = 8 tumors/group; means ± SEMs. P value, Student t test: Right, average body weights of mice in each group measured twice a week.

Type II RAFi + MEKi Expand T Naïve/T Central Memory and Shrink T Regulatory Cell Compartments Systemically

We evaluated NILER tumor histology and T-cell infiltration levels and patterns, on days 4 and 11, in response to treatment with vehicle, BGB-283 (20 mg/kg/day, orally), trametinib (1 mg/kg/day, orally), or both (Fig. 5B). Vehicle-treated tumors displayed sheets of spindled tumor cells, immature blood vessels, and scattered necrosis (Fig. 6A and B) as well as tumor-infiltrating lymphocytes (TIL; Supplementary Fig. S6A), consistent with findings of CD4+ and CD8+ immunohistochemistry (Fig. 6B). Tumors treated with BGB-283 or trametinib alone, which displayed only growth deceleration but not regression (Fig. 5B), showed small foci of tumor regression on days 4 and 11 and large clefts on day 11 and increased TILs in both the invasive margins and tumor centers (Fig. 6A; Supplementary Fig. S6A). In contrast, durable tumor regression induced by BGB-283 + trametinib was associated with a marked histologic response characterized by large foci of tumor regression and necrosis, tumor cell balloon degeneration, apoptosis, and melanosis (Fig. 6B). Maximal TIL scores were reached by day 4 in both tumor centers/margins, along with extensive infiltration by histiocytes and melanophages. In vehicle- or trametinib-treated tumors, the CD8∶CD4+ ratios were 1 or less. In BGB-283–treated tumors, this ratio was consistently 1. However, by day 11 on BGB-283 + trametinib treatment, this ratio was 2–3 (Supplementary Fig. S6A), consistent with CD8+ T-cell expansion.

To assess systemic T-cell impacts of non-tumor-regressive (single-agent) versus tumor-regressive (combination) versus vehicle treatments (Fig. 5B) in NILER tumor-bearing mice, we performed mass cytometry (CyTOF) on peripheral blood mononuclear cells (PBMC) and dissociated secondary lymphoid organs (tumor-draining lymph nodes and spleens) on days 4 and 11 (n = 3 mice/group). In all T-cell compartments, single and combined inhibitor treatment, short- and long-term, induced CD4+ and CD8+ T cells as percentages of CD45+ cells (Fig. 6C; Supplementary Fig. S6B). We analyzed further T-cell subpopulations by t-distribution stochastic neighbor embedding (t-SNE; Fig. 6D; Supplementary Fig. S6C and S6D). Short- and long-term, single and combined inhibitor treatments tended to induce the proportion of CD4+ and CD8+ T-naïve cells (Tsc, CD62L+CD44+) and T central memory cells (Tcm, CD62L+CD44+) but reduce CD8+ T effector memory cells (Tem, CD62L+CD44-; Fig. 6E and F; Supplementary Fig. S6E–S6H). This pattern was more consistent on day 4 (during maximal tumor volume reduction by combination treatment) in response to type II RAFi + MEKi, but not to type II RAFi or MEKi alone. Consistently, only among mice cotreated with type II RAFi + MEKi, the proportions of CD8+ Tcm and Tem cells were significantly anticorrelated (Fig. 6G; vehicle, R = −0.35, P = 0.14; type II RAFi, R = −0.43, P = 0.078; MEKi, R = −0.058, P = 0.82; type II RAFi + MEKi, R = −0.6, P = 0.0091). Moreover, combination type II RAFi + MEKi treatment reduced the levels of CD4+ T regulatory cells (Treg, CD4+FOXP3+) and their proliferation (Supplementary Fig. S6I and S6J). We also analyzed the profiles of splenic T cells (collected on day 4) after ex vivo cultures for 3 or 4 days with or without anti-CD3 + anti-CD28 stimulation. We found that CD4+ and CD8+ T cells alike from the spleens of mice that were treated in vivo with single-agent or combined inhibitors were more capable of activation (Supplementary Fig. S7A and S7B). Moreover, splenic CD4+ Treg cells from inhibitor-treated mice (especially type II...
Figure 6. Type II RAFi + MEKi elicits systemic expansion of central memory CD8\(^{+}\) T cells in mice bearing Nrat\(^{+/+}\) melanoma. A, Hematoxylin and eosin (H&E) stains of NILER1-4 tumors (days 4 and 11) on vehicle, BGB-283 (20 mg/kg/days), trametinib (1 mg/kg/day), or both. Representative of three tumors per group. Ruler = 1 mm. Colored boxes delineate magnified areas in B. B, Magnified areas of NILER1-4 tumors analyzed by H&E and representative CD8 and CD4 IHC. Scale bar, 100 μm. Highlighted H&E images of BGB + trametinib-treated tumors show areas of inflammation (short arrows), necrosis (long arrows), apoptotic bodies (day 11, top) and tumor regression with melanosis (day 11, bottom). C, Indicated tissue samples (PBMC, spleen, draining lymph nodes or LNs; n = 3 per site) were obtained for CyTOF analysis on days 4 and 11 after starting each treatment group. Frequencies of CD4\(^{+}\) or CD8\(^{+}\) T cells. Mean ± SEMs. Pairwise group comparisons with respect to the vehicle-treated group. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. D, t-SNE maps (left) of CD8\(^{+}\) T-cell subsets (within CD45\(^{+}\) cells) in the PBMC, spleen, and draining LNs analyzed by CyTOF (pooled data from days 4 and 11). Heat maps (right) showing the expression values of immune phenotypic protein markers normalized to the maximum mean value across subsets. E and F, As in C, except CyTOF analysis is for indicated CD8\(^{+}\) TEM (E) or TTD (F) subsets. G, Pearson correlation between CD8\(^{+}\) TCM versus TEM subset frequencies in BGB-283 + tram-treated mice on (pooled data from days 4 and 11).

RAFi + MEKi-treated mice on \(ex vivo\) day 3) were less capable of \(ex vivo\) expansion (Supplementary Fig. S7C). \(Ex vivo\) stimulation-induced T-cell proliferation was more robust among mice treated with inhibitors (Supplementary Fig. S7D and S7E). Consistent with prior analysis (Fig. 6), CD4\(^{+}\) and CD8\(^{+}\) T-cell compartments from the spleens of inhibitor-treated tumor-bearing mice, with and without \(ex vivo\) stimulation, expanded the TEM and TCM subpopulations at the expense of the TTD subpopulation (Supplementary Fig. S7F).

**Type II RAFi + MEKi Expand Intratumoral CD8\(^{+}\) TEM and Activated T Cells**

To assess intratumoral T-cell impacts, we performed CyTOF on dissociated vehicle- versus type II RAFi + MEKi-treated tumors (n = 3 tumors/group) on day 5 and observed that combination treatment increased CD8\(^{+}\) T cells (% of CD45\(^{+}\) cells) by \(\sim 7\)-fold, based on t-SNE analysis (Fig. 7A; Supplementary Fig. S8A). This finding indicates an even higher increase in the CD8\(^{+}\) T cell-to-tumor cell ratio, given induction of the CD45\(^{+}\) and reduction of the tumor cell compartments elicited by combination therapy. We analyzed further CD8\(^{+}\) T-cell subpopulations by t-SNE (Fig. 7B; Supplementary Fig. S8B). Importantly, type II RAFi + MEKi elevated the levels of intratumoral CD8\(^{+}\) TEM (\(\sim 3\)-fold), T cytotoxic (Tc; \(\sim 3\)-fold), and T terminally differentiated (TTD; \(\sim 5\)-fold) cells (% CD45\(^{+}\) cells; Fig. 7C). PD-1\(^{+}\) expression expanded from <0.5% (vehicle) to \(\sim 5\%\) and \(\sim 7\%\) (type II RAFi + MEKi) in, respectively, the CD8\(^{+}\) TEM and TTD subpopulations (Fig. 7D). This was accompanied by an increase in Ki-67 positivity among the CD8\(^{+}\) TTD cells. We corroborated these CyTOF findings with single-cell RNA-seq (scRNA-seq) analysis of combined CD4\(^{+}\) and CD8\(^{+}\) T cells sorted from vehicle- versus type II
Figure 7. Cotreatment with type II RAFi + MEKi heightens tumor infiltration by effector memory and activated/exhausted CD8+ T cells and T-cell clonal expansion. A, t-SNE map (left) of tumor-infiltrating CD45+ cells analyzed by CyTOF [pooled data from vehicle- and trametinib (Tram) + BGB-283-treated groups]. Frequencies of each cluster (right). N = 3 tumors per group; mean ± SEMs. Pairwise group comparisons with respect to the vehicle-treated group. P value, paired Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, t-SNE map (left) of tumor-infiltrating CD8+ T-cell subsets analyzed by CyTOF [pooled data from vehicle- and trametinib + BGB-283-treated groups]. Heat map (right) showing the expression values of immune phenotypic protein markers normalized to the maximum mean value across meta-clusters. C and D, As in A, except CyTOF analysis is shown for indicated CD8+ T-cell subsets (C) or the percentages of PD-1 or Ki-67 positivity within indicated subsets (D). E, UMAP of tumor-infiltrating CD4+ and CD8+ T cells (n = 14,199) analyzed by scRNA-seq (pooled data from vehicle- and trametinib + BGB-283-treated groups), n = 4 tumors per group by combining FACS-sorted CD4+ and CD8+ T cells. Clusters denoted by distinct colors are labeled with inferred cell types (left). Heat map (right) showing differentially expressed genes (rows) among different T-cell subsets (columns). Specific genes that are associated with different T-cell clusters are highlighted along the right. F, Frequencies of indicated CD8+ T-cell subsets analyzed by scRNA-seq based on treatment status. G, Box plot of terminally exhausted gene signature scores (scRNA-seq) for all CD8+ T cells in each treatment group. P value, Wilcoxon rank-sum test; ****, P < 0.0001. H, Violin plot showing Tcx expression levels (scRNA-seq) for all CD8+ T cells in each treatment group. P value, Wilcoxon rank-sum test; ****, P < 0.0001. I, Vehicle-, BGB-283-, trametinib-, and trametinib + BGB-283-treated tumors on days 4 and 11 (n = 3 per group, except one outlier in vehicle-treated tumor; day 11) were analyzed by TCR-seq. Dot plot showing the number of TCR clones for α or β chain (red dots, average values). P value, paired Student t test; ****, P < 0.0001. J, As in I, except TCR-seq analysis is shown for sizes of large TCR clones (frequency ≥ 0.05). Pairwise comparison between days 4 and 11 was performed in each treatment group with a paired Student t test;*, P < 0.05; **, P < 0.01; ***, P < 0.001. K, Clustering of TCR repertoires of tumor-infiltrating T cells by the levels of shared CD3ε sequences (using the Jaccard index). L, Spearman correlations between frequencies on days 4 and 11 of shared α chain CD3ε sequences in each condition. Mean frequency on day 4 or 11 of each shared CD3ε sequence (at least in two samples of each condition) was calculated.
RAFi + MEKi–treated tumors on day 5 (sorted cells from four independent tumors were combined per condition). T-cell sub-populations visualized by t-SNE were analyzed for differential gene expression (Fig. 7E). Importantly, CD8+ T cells (as a percentage of all CD8+ T cells) that coexpress activation- and exhaustion-associated genes increased from ~20% to ~65% in vehicle- to type II RAFi + MEKi–treated tumors (Fig. 7F). An increase in the activated/exhausted CD8+ T cell-to-tumor cell ratio is expected to be even greater, given expansion in both the CD45+ and CD8+ T-cell compartments elicited by combination therapy. Consistently, type II RAFi + MEKi–treated tumors harbored CD8+ T cells with higher activation/exhaustion score and expression of the master regulator of exhaustion, Tox (Fig. 7G and H; refs. 33, 34).

We then evaluated whether intratumoral expansion of activated CD8+ T cells induced by type II RAFi + MEKi is clonal by performing T-cell receptor sequencing (TCR-seq) analysis of vehicle-, BGB-283-, trametinib-, or trametinib + BGB-283–treated NILER1-4 tumors (n = 3/group) on days 4 and 11. By analyzing the CDR3 clonotypes of both TCRα and β chains, we observed that trametinib + BGB-283 increased the number of T-cell clones (Fig. 7I). Moreover, whereas trametinib temporally reduced the sizes of the top or all dominant (>5%) T-cell clones, BGB-283 cotreatment erased and enhanced the sizes of top or dominant clones (Fig. 7J; Supplementary Fig. S8C and S8D). We also calculated the diversity and Gini (clonality) indices, which showed that trametinib increased but trametinib + BGB-283 reduced the diversity of T-cell clonotypes. Accordingly, trametinib reduced, whereas trametinib + BGB-283 maintained, T-cell clonality (Supplementary Fig. S8E and S8F). To evaluate the overlap of T-cell clones across treatment conditions and time points, we calculated the Jaccard indices and overlap coefficients (Fig. 7K; Supplementary Fig. S8G). Importantly, sample-to-sample comparisons among combination-treated tumors generally exhibited the highest percentages of intersecting clones (up to 8%). Group-to-group comparisons consistently showed the highest levels of overlap when combination-treated tumors as a group were compared against any other group, including itself. We observed within the combination treatment group the most significant positive correlation between d4 and d11 TCR clonotype frequencies (Fig. 7L; Supplementary Fig. S8H), suggesting that type II RAFi + MEKi elicits persistent expansion of tumor antigen–specific TCR clones. These findings suggest that type II RAFi + MEKi elicited the most robust tumor-specific T-cell reactivity, as trametinib + BGB-283 cotreatment increased TCR clonality as well as the number of distinct clones and converged T-cell clonotypes. Taken together with prior findings (Figs. 5 and 6), we conclude that type II RAFi + MEKi induces durable tumor regression by recruiting PD-L1 therapy–responsive, tumor antigen–specific CD8+ T cells.

**DISCUSSION**

MAPK-targeted therapy elicits clinically meaningful activity in only a handful of BRAFV600E cancers. From recent BRAFV600E–focused basket trials, it remains unknown whether lack of addiction to the MAPK pathway explains poor clinical efficacy in some BRAFV600E cancer histologies. In RASMut or BRAFV600E cancers, addiction to the MAPK pathway has also been cast into doubt by trials testing single-agent MEK or ERK inhibitors. Our study showing the highly durable efficacy of a specific pair of MAPK-targeted agents (type II RAFi + allosteric MEKi) across multiple driver mutations and cancer histologies (BRAFV600E, BRAFV599E, KRAS, NRAS, and NF1-mutant melanoma and colorectal, pancreatic, and lung cancers) strongly supports MAPK pathway addiction.

The first combinatorial therapy developed successfully against the MAPK pathway in the clinic (for patients with advanced BRAFV600E melanoma) consists of type I RAFi plus an allosteric MEKi. However, despite high response rates, acquired resistance is commonplace and frequently due to MAPK pathway reactivation. Our data suggest that a specific combination of type II RAFi + MEKi could help overcome acquired resistance to the current standard of care (type I RAFi + MEKi).

Clinical development of MAPK-targeted combinatorial agents in oncology has centered on direct impacts on tumor cells (vs. immune cells) via inhibition of the activity of kinase(s). Insights from this study shine a light on the importance of indirect impacts on T cells and allosteric dysregulation of protein/protein interactions. This study also provides foundational knowledge for clinical development vis-à-vis identifying susceptible cancer histologies, biomarkers, and rational combinations with immunotherapies.

Previously, the allosteric action of dual MAPK inhibitors has not been examined at the level of protein/protein interactions in the MAPK pathway. Oncogenic Ras (which forms dimers) recruits cytosolic Raf/Mek heterodimers to the cell surface through the Ras-binding domain of Rafs. Face-to-face Raf/Mek heterodimers are then brought together by the back-to-back dimerization of Rafs, which facilitates autocatalytic phosphorylation of the Raf activation loop. Loosening of the Raf/Mek heterodimers is thought to facilitate the assembly of Raf homodimers at the Raf surface. Phosphorylation of Raf dimers from Raf dimers, Raf/MEK interaction, and ERK phosphorylation. Here we provided evidence that type II Rafi and allosteric MEKi act in concert to stabilize and sequester pMEK in Raf complexes (specifically BRAF/CRAF complexes, which is considered the most active complex among all Raf dimers). This action, which is likely facilitated by the high abundance of Raf/Raf and/or Raf/MEK complexes in MEKI-resistant tumor clones, may therefore be selectively robust against acquired resistance. The end result of MEK sequestration by Raf is reduced MEK dimerization and uncoupling of MEK or pMEK interaction with ERK.

ERKi has been proposed to overcome MEKi resistance (35). Because MEKI or ERKi each can strongly suppress the MAPK pathway in normal cells, untoward toxicities are likely to arise with their combination. Type II RAFi as a single agent does not appear to be highly active in suppressing oncogenic MAPK signaling, which is consistent with low single-agent antitumor activity from early-phase clinical trials. Here, we showed that type II RAFi + MEKi is superior to type II RAFi + ERKi (SCH772984) in preventing and overcoming acquired MEKi resistance. This correlates with the superior ability of type II RAFi + MEKi to physically stabilize RAF/MEK and uncouple MEK/ERK interactions. The synergy derived from this allosteric mechanism may afford MEKi dose reduction and improve the therapeutic index, permitting triplet combination with immune-checkpoint blockade therapy.
We also presented evidence that type II RAFi + MEKi may have favorable T-cell impacts that directly rationalize combination with anti–PD-1/L1 therapy. Prior studies (14, 16) have analyzed the T-cell impacts of MEKi monotherapy. Here, we observed that type II RAFi + MEKi induce systemic levels of CD8+ TCM cells and reduce CD4+ TREG cells. Studies have implicated the importance of CD8+ TCM cells to adoptive immunotherapy or anti–PD-1 therapy (36, 37). Tumor antigen–specific CD8+ TCM cells (vs. TEM) are thought to exhibit more potent in vivo antitumor (i.e., eradication of large established tumors) recall responses (38, 39). Consistent with this, homing to secondary lymphoid tissues (e.g., spleen, lymph nodes) appeared to be required for optimal tumor eradication. That is, highly effective antitumor T cells were those that initially targeted secondary lymphoid tissues rather than peripheral/tumor sites (38). Intratumorally, we observed that type II RAFi + MEKi induced not only sustained immune infiltration but also the relative abundance of CD8+ TCM and TCM cells, their expression of PD-1/Ki-67 expression, and activation/exhaustion genes and signatures. Inside the tumor, CD8+ TCM cells acquire effector functions more rapidly than TEM cells. This superior tumor cytotoxicity in situ is thought to be a key mechanism through which preexisting CD8+ TCM cells mediate secondary or de novo priming of effector T cells in the tumor-draining lymph nodes. Intratumorally, type II RAFi + MEKi also promoted expansion and convergence of T-cell clonotypes, in contrast to MEKi monotherapy. Collectively, these findings rationalize triplet combination trials of type II RAFi + MEKi + anti–PD-1/ PD-L1 therapy in RAS/MAPK-hyperactivated cancers.

METHODS

Cell Lines

All cell lines and drug-resistant sublines were routinely tested for Mycoplasma and profiled and identified by RNA-seq and the GenePrint 10 system (Promega) at periodic intervals during the course of this study for banking and experimental studies. All cell lines were maintained in DMEM-high glucose with 10% heat-inactivated FBS (Omega Scientific) and 2 mmol/L glutamine in humidified, 5% CO2 incubator. To derive resistant sublines, parental human BRAFV600E, NRASQ61K, or NF1+/− melanoma cells seeded at low density were treated with BRAFi (PLX4032) + MEKi (AZD6244; for BRAFV600E/E mutant lines) or MEKi (trametinib; for NRASQ61K or NF1−/− lines) every 2 to 3 days for 12 to 15 weeks, and proliferative colonies were ring-isolated from NIL by exposure to 1 round of high-dose UVB radiation followed by ring-clonal selection and expansion.

Mice

C57BL/6 and NSG (NOD/SCID gamma) were obtained from the Radiation Oncology breeding colony at University of California, Los Angeles (UCLA). Male or female mice were used at 4 to 6 weeks of age. All animal experiments were conducted according to the guidelines approved by the UCLA Animal Research Committee.

 Constructs and Inhibitors

shNRAS, shCRAF, and shBRAF were subcloned into the lentiviral vector pLL3.7 as described (4, 7, 29, 40). NRAS overexpression and all knockdown constructs were packaged into lentiviral particles for infection. Experiments were carried out 3 days after transduction. Inhibitor(s) were obtained from the following sources: PLX4032 (Plexikon), trametinib in vitro and in vivo (LC Laboratories), BGB-283 and BGB-3245 (Beigene), RAF-709 (Cayman Chemical), LHX-254 (Selleckchem), cobimetinib (Selleckchem), binimetinib (LC Laboratories), and SCH772984 (Chemietek).

Cell Growth Assays

For clonogenic assays, cells were plated at single-cell density in six-well plates. Data presented are representative of at least two independent replicates. Inhibitor(s) and media were replenished every 2 days for the number of days noted. Colonies were fixed in 4% paraformaldehyde (PFA) and stained with 0.05% crystal violet. For temporal measurements of cell growth, cells were plated at single-cell density in 96-well plates and treated with indicated treatments every 2 to 3 days for 8 to 11 days. Cell viability in relative light units was measured using CellTiter-Glo every 2 to 3 days. Experiments were performed in triplicates. For cell counting, cells were plated at single-cell density in six-well plates, and experiments were performed in triplicates. Indicates treatment and media were replenished every 2 to 3 days and, after trypsinization, viable (trypan blue negative) cells were counted on the days noted. Additional plates for the last time point (11 days) were fixed in 4% PFA and stained with 0.05% crystal violet.

Protein Detection

Cells were lysed in IP lysis buffer (Thermo Fisher Scientific) with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) for IP and Western blotting. Dynabeads (Thermo Fisher Scientific) were used to immunoprecipitate proteins of interest based on the manufacturer’s protocol. For immunofluorescence (IF), tissues were fixed in 4% PFA and sucrose and cryoprotected in optimal cutting temperature (OCT) or in formalin followed by embedding in paraffin [formalin-fixed, paraffin-embedded (FFPE)]. For FFPE tissues, after deparaffinization and rehydration, tissue sections were subjected to heat for antigen retrieval. PFA/OCT sections were not subjected to antigen retrieval. Cell lines were fixed with 4% PFA. IF of both tissue and cell lines was performed with Alexa Fluor–conjugated secondary antibodies (Life Technologies). Nuclei were counterstained by DAPI. Duolink PLA (Sigma-Aldrich) was used to detect in situ protein/protein proximity interactions by following the manufacturer’s protocol. Fluorescence signals were captured with a Zeiss microscope (AXIO Imager A1) mounted with a charge-coupled device camera (Retiga EXi QImaging), and the images captured by Image-pro plus 6.0. PLA signals were quantified by counting signals per cell in each field (n = 5 fields per condition). IP, Western blots, IF, IHC, and PLA assays were performed using the following antibodies: CD4 (#183685 from Abcam), CD8 (14-0080-80 from Invitrogen/Thermo Fisher), BRAF (sc-5284 from Santa Cruz), CRAF (#53745, #12552 from Cell Signaling Technology), ERK (#9101, #4696 from Cell Signaling Technology), pERK1/2 (#5726, #4695 from Cell Signaling Technology), pMEK1/2 (#9154 from Cell Signaling Technology), MEK1/2 (#9126 from Cell Signaling Technology, sc-81504 from Santa Cruz), MEK1 (sc-6250 from Santa Cruz, #9146 from Cell Signaling Technology), p-90RSK (#11989 from Cell Signaling Technology), RSK1/2/3 (#9355 from Cell Signaling Technology), NRAS (sc-519, Santa Cruz), and TUBULIN (T9026 from Sigma-Aldrich).

PDX, Syngeneic Mouse Models, Treatments, and Tissue Collection

To develop PDX models, tumor fragments derived from NRASQ61K metastatic melanoma, PDAC, or NSCLC, with approval by the local Institutional Review Boards, were transplanted subcutaneously in sex-matched NSG mice (4–6 weeks old). One tumor fragment was implanted in each mouse. Tumors were measured with a caliper every 2 days, and tumor volumes were calculated using the formula

\[ \text{Volume} = \frac{1}{2} \times (\text{length} \times \text{width}^2) \]
(length × width)/2. Tumors with tumor volumes around 500 mm³ were randomly assigned into experimental groups. For experimental melanoma models, C57BL/6 mice were subcutaneously injected on both flanks with either one million NIL or NILER1-4 cells. Once tumors reached a size of 150 to 200 mm³, mice were assigned randomly into experimental groups. αCD8a and isotype control antibodies were administered intraperitoneally (200 μg/mouse) on day −1, day 0, and then twice a week. Special mice diets (for NSG and C57BL/6) were generated by incorporating trametinib at 1, 3, or 5 mg/kg to facilitate daily drug dosing and to reduce animal stress (Test Diet). BGG-283 (10 or 20 mg/kg/day) was administered to mice via oral gavage and SCH772984 (10 or 25 mg/kg/day) intraperitoneally. Tumors were excised from mice, minced, and digested to single-cell suspensions using the tumor dissociation kit and gentleMACS Octo Dissociator (Miltenyi Biotec). Spleens were manually homogenized, mashed through 45-μm filters into RPMI-1640 supplemented with 10% FBS. Red blood cells in single-cell suspensions were lysed using ACK lysis buffer (Lonza). Intracardiac blood samples were collected in the presence of heparin, and PBMCs were obtained through density centrifugation at 1,500 g for 30 minutes. Lymphocyte counts were performed from three high-power fields. TIL scores were defined as: 1 = 0–10% of total cells; 2 = 20–40% of total cells; 3 = 50–70% of total cells; 4 = 80–100% of total cells. These counts were confirmed using IHC for CD4 and CD8. The ratio of CD8+CD4+ cells was estimated in three distinct tumor regions with the highest TIL infiltration by counting CD4+ and CD8+ cells in the same area.

Histologic Evaluation
Following tissue fixation, paraffin embedding, and sectioning, histologic evaluation was performed by a dermatopathologist (P.O. Scumpia) blinded to the identity of the samples. TIL scoring was performed using a slight modification of standard methodology (41, 42). After identifying the tumor invasive margin and tumor core, lymphocyte counts were performed from three high-power fields. TIL scores were defined as: 1 = 0–10% of total cells; 2 = 20–40% of total cells; 3 = 50–70% of total cells; 4 = 80–100% of total cells. These counts were confirmed using IHC for CD4 and CD8. The ratio of CD8+CD4+ cells was estimated in three distinct tumor regions with the highest TIL infiltration by counting CD4+ and CD8+ cells in the same area.

Ex Vivo T-cell Activation
Splenocytes from NILER1-4 tumor-bearing mice with or without in vivo MAPKi treatment were collected and then seeded at 1 million cells/mL in 24-well plates, with or without anti-CD3 (145-2C11, 2 μg/mL) and anti-CD28 (37.51, 2 μg/mL). After 48 hours, media were refreshed using RPMI-1640 + 7.5% FBS + 0.1% β-mercaptoethanol + 30 U/mL IL-2, without antibodies. At 72 and 96 hours, cells were collected and analyzed by mass cytometry.

Mass Cytometry of Murine Tissues
Cells (2 × 10⁶ or fewer) were incubated with 2% of FBS in PBS with 25 μg/mL of 2,4-DG2 antibody at 4°C for 10 minutes prior to surface staining with an antibody cocktail at 4°C for 30 minutes in a 50-μL volume. Cells were incubated with 2.5 μmol/L 194Pp monoisotopic cisplatin (Fluidigm) at 4°C for 1 minute. Cells were then washed twice with FACS buffer and barcoded using palladium metal barcoding reagents according to the manufacturer’s protocol (Fluidigm). Subsequently, fixation and permeabilization were performed using the FOXP3 fix and permeabilization kit according to the manufacturer’s protocol (eBioscience). Cells were then stained with an intracellular stain antibody cocktail (FOXP3, Ki-67, granzyme B, T-bet, iNOS, and EOMES) for 30 minutes at room temperature. Cells were then washed twice with FOXP3 permeabilization buffer, twice with FACS buffer, and incubated overnight in 1.6% PFA PBS with 100 mmol/L iodide nucleic acid intercalator (Fluidigm). Cells were then washed twice with PBS with 0.5% BSA, filtered, and washed twice with water with 0.1% BSA prior to analysis. Samples were analyzed using a Helios mass cytometer based on the Helios 6.5.358 acquisition software (Fluidigm).

CyTOF Data Analysis
All the samples were preprocessed by CATALYST, including normalization, debarcoding, and compensation. The normalized fcs files were then uploaded into Cytobank (43), and data were gated to exclude beads and to include only live, single cells. The CD8+ and CD4+ T cells were gated from the CD45+CD3+ populations, and data were downloaded separately into individual files for each sample. We applied CyTCast (44) to perform the sSNE analysis separately on the manually gated CD4+ and CD8+ populations from PBMC, spleen, and lymph node samples. We selected 5,000 events/sample to ensure equal representation of cells across samples. For CD4+ T cells, 12 markers, including CD44, CD62L, CD25, CD69, CD366, FOXP3, PD-1, CTLA4, ICOS, EOMES, T-bet, and Ki-67, were used to cluster the cell populations. For CD8+ T cells, CD44, CD62L, CD25, CD69, CD366, granzyme B, PD-1, CTLA4, ICOS, EOMES, T-bet, and Ki-67 were used. We chose 3,000 iterations, perplexity of 30 and theta of 0.5, as the standard t-SNE parameters. Mean intensity values of markers in each cluster were calculated and visualized via heat maps. Cells were assigned to different populations on the basis of the local gradient expression of known markers, e.g., CD44, CD62L, Granzyme B, and FOXP3. Numbers of cells and percentages of different immune cell subsets were calculated for each sample. Ex vivo cultures stained with a reduced antibody panel (CD45, CD4, CD8, CD44, CD62L, FOXP3 ICOS, T-bet, PD-1, and Ki-67) were analyzed using the FlowJo software.

WES and RNA-seq Data and Analysis
Thirty-six PDx tumors and cell lines and matched normal (or surrogate normal) tissue specimens were subjected to WES and RNA-seq. Sequencing was performed using paired-end sequencing with read length of 2 × 150 bps based on either the Illumina HiSeq3000 or the NovaSeq V4 platform. We called single-nucleotide variants (SNV) and small indels as we reported previously (7, 28). Mutations were annotated for coding/noncoding alterations using the stand-alone version of Oncotator (45). Copy numbers were called using the intersection of copy-number calls derived from Sequenza (46) and VarScan2 (47). Recurrent gene alteration events were visualized using the OncoPrint tool, and the MEKi resistance-specific mutations on the MAP2K2 gene were visualized using MutationMapper (48, 49). GOF alterations include known oncogenic missense mutations (from COSMIC v88), copy-number amplification, and/or mRNA overexpression (22-fold up). LOF alterations include truncating mutations (nonsense, splice site, frameshift), copy-number loss, and/or mRNA down-expression (22-fold down). Missense/in-frame indels of unknown significance were counted as both GOF and LOF events. CNV-related differential gene-expression events were defined as concurrent copy-number gain and mRNA overexpression (at least 1.5-fold of genomic copy-number gain and 2-fold mRNA overexpression). We applied the same cutoffs for copy-number loss (at least 1.5-fold of copy-number loss and 2-fold mRNA down-expression). Paired-end, 2 × 150 bp RNA-seq reads were mapped to the Genome Reference Consortium Human Build 38 (GRCh38) reference genome using HISAT2 (50). Gene-level counts were generated by the htsseqcount (51) program, and we took log2 counts per million (CPM) as normalized gene-expression values. We added a pseudo CPM count of 0.1 to avoid taking the log of zero. The phylogenetic analysis was performed using the PHYLIP program as in our previous studies (7, 28).

For the syngeneic murine model NILER1-4, the paired-end reads were aligned to the mouse reference sequence (GRCh38) using the bowtie-mem algorithm. The aligned reads were then processed using Picard tools and GATK (version 4.0) for deduplication and base quality recalibration prior to mutation detection. SNVs and indels were identified with MuTect2 using WT C57BL/6J mouse as the germline control. Variants were filtered by FilterMutectCalls using GATK default thresholds. Accumulated mutations in NILER1-4, presumably caused by UV irradiation, were detected as novel variants compared with the parental line NIL.
Generation and Analysis of scRNA-seq Data

To sort TILs, single-cell suspensions of digested tumor were stained for 5 minutes at room temperature with Fc block (anti-CD16/32) and then stained with primary antibodies in staining buffer (PBS with 20% FCS) for 30 minutes. Sorting of live TILs (TER119+CD45−CD8+CD4− and TER119+CD45−CD4+CD8+) was performed on a BD FACSAria II (BD Biosciences). For each treatment condition, TILs from four different tumors were pooled together. Droplet-based 3′ end massively parallel scRNA-seq was performed by encapsulating sorted, live TILs into droplets, and libraries were prepared using Chromium Single Cell 3′ Reagent Kits v3 according to the manufacturer’s protocol (10x Genomics). The generated scRNA-seq libraries were sequenced using Illumina NovaSeq.

Alignment to GRCh38 reference genome, barcode, and unique molecular identifier (UMI) counting were performed by using Cell Ranger v2.1.0 (10x Genomics). Seurat package (52) was used for downstream analysis. In brief, cells with fewer than 500 genes detected or greater than 10% mitochondrial RNA content were excluded from analysis. Raw UMI counts were normalized to UMI count per million total and log-transformed. Variable genes were detected based on average expression and dispersion for each data set independently. We then use CellCycleScoring function to calculate scores of S and G2–M phase expression and dispersion for each data set independently. We then used the Scale-Batch function to integrate individual datasets previously reported (34).

Finally, scores of T-cell terminal exhaustion were assigned to each cell by using Seurat’s AddModuleScore function based on the gene sets previously reported (34).

Generation and Analysis of TCR-seq Data

Total RNAs were extracted from frozen tissues stored in RNA later using the QIAGEN All Prep DNA/RNA Mini Kit and the Ambion mirVana miRNA Isolation Kit. RNA (600 ng) was used as input to construct libraries with the QIAGEN QIAseq Immune Repertoire RNA Library Kit–T-cell Receptor Panel. Briefly, RNA was reverse-transcribed using a pool of TCR gene–specific primers against the constant region for the T-cell receptor alpha, beta, gamma, and delta genes. The resulting CDNA was then ligated to an oligo containing one side of sample index and UMI. After reaction cleanup, a single primer extension was used to capture the T-cell receptor using a pool of gene-specific primers. The resulting captured sequences were amplified and purified using QIAseq beads. Libraries were then sample indexed on the other side using a unique sample index primer and a universal primer to amplify the library and introduce platform-specific adapter sequences. The dual indexed sample PCR fragment was purified and then quantified for absolute quantification of amplifiable libraries (DNA with adaptors at both ends) in triplicate by real-time qPCR using the QIAGEN QIAseq Library Quant Array Kit. For sequencing, each library was diluted to 4 nmol/L, pooled, and denatured. Denatured library pool (1.2 pmol/L) was run with the QIAseq A Read 1 Primer on Illumina NextSeq 500 Mid Output Kit using v2.5 chemistry for 300 cycles with an asymmetrical paired-end 261/41 bp read for the CDR3 region.

Raw reads were analyzed from the QIAGEN GeneGlobe Data Analysis Center (https://www.qiagen.com/us/shop Genes-and-pathways/data-analysis-center-overview-page/), which estimates the abundance of reads of unique CDR3 sequence and generate TCR clonotype calls. Briefly, raw reads were trimmed and randomly down-sampled to control the oversequencing error in UMI and CDR3 sequences. Paired R1 and R2 were then merged into one read with trimmed V regions. Clonotypes were called by IMSEQ (53), which clustered highly similar CDR3 sequences. CDR3 calls that did not have at least one UMI supported by three reads were excluded from downstream analysis. R package tcr (54) was used to perform all the statistical analyses for TCR repertoires, including (i) size of the largest clone, top 3 clones, and large clones with frequency higher than 5%; (ii) diversity estimation using ecological diversity and Gini–Simpson index; (iii) similarities of TCR repertoires by calculating the Jaccard index and overlap coefficient between every pair of samples based on their unique alpha and beta chain CDR3 sequences. The Jaccard index was calculated using jaccard.index function in tcr package, and heat maps were generated using R heatmap package. The comparison of overlap coefficients intra- and across treatment groups was performed using the Wilcoxon rank-sum test.

Molecular Modeling

SASAs were calculated using the analytical module for surface calculation of the CHARMM molecular modeling package (55), with water molecules described as spheres with a radius of 1.4 Å. The value of SASA was buried upon the binding of MEK1 to BRAF was computed for the residues belonging to the BRAF P-loop (residues 465–469) and for MEK1 residues situated in the vicinity (i.e., residues 73–82 and 97–101) and by the difference in the SASA of these residues between the complex and the isolated MEK1 and BRAF proteins in the same conformation. The buried SASA was calculated for the apo and BGB-283–bound forms of BRAF to estimate its variation as a result of BGB-283 binding. The conformation of the MEK1/BRAF heterodimeric complex with the apo form of BRAF was taken from the experimental 3-D structure of the BRAF/MEK1 heterotrimer complex, involving the apo form of BRAF and the G-573/ACP-bound MEK1 (PDB ID 4MNE; ref. 56). Because the P-loop residues were not resolved in this structure, they were modeled using the MODELLER program (57). For this, 5,000 conformations of the P-loop were modeled with DOPE-based loop modeling classes of MODELLER. The 50 top-ranked conformations according to MODELLER were used to calculate the average SASA buried upon the binding of MEK1 to BRAF.

The conformation of the MEK1/BRAF heterodimeric complex with the apo and BGB-283–bound forms of BRAF was obtained by superimposing the experimental structure of the complex between BGB-283 and BRAF (PDB ID 4R5Y; ref. 20) on the experimental structure of the MEK1/BRAF heterodimeric complex (PDB ID 4MNE) using UCSF Chimera (58).

Statistical Analysis

No statistical methods were used to predetermine sample size. The paired test was performed to determine the statistical significance of differences between two variables. All statistical analyses were carried out using R and GraphPad Prism 7.

Data Availability

Raw sequencing files of RNA-seq, scRNA-seq, and TCR-seq data are available at the Gene Expression Omnibus (GEO accession number GSE18610). Mass cytometry data are deposited at FlowRepository (http://flowrepository.org/) using the experiment ID FR-FCM-Z34M. WES data have been made available through the Sequence Read Archive at the accession number PRJNA666070.

Authors’ Disclosures

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**Authors’ Contributions**

**A. Hong:** Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **M. Piva:** Conceptualization, resources, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **S. Liu:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **W. Hugo:** Formal analysis, investigation, visualization, and methodology. **S.H. Lomeli:** Resources, formal analysis, investigation, methodology, writing–review and editing. **W. Wang:** Resources, formal analysis, investigation, methodology, writing–review and editing. **V. Zoete:** Formal analysis, visualization, and methodology. **C.E. Randolph:** Data curation and methodology. **Z. Yang:** Resources, investigation, and methodology. **Y. Wang:** Resources, investigation, and methodology. **J.J. Lee:** Resources and investigation. **S.J. Lo:** Resources and investigation. **L. Sun:** Visualization. **A. Vega-Crespo:** Resources. **A.J. Garcia:** Methodology. **D.B. Shackelford:** Resources. **S.M. Dubinett:** Resources. **P.O. Scumpia:** Formal analysis, visualization, and methodology. **S.D. Byrum:** Methodology. **A.J. Tackett:** Methodology. **T.R. Donahue:** Resources. **O. Michelin:** Formal analysis, visualization, and methodology. **S.L. Holmen:** Resources, writing–review and editing. **A. Ribas:** Resources. **G. Moriceau:** Conceptualization, resources, data curation, formal analysis, supervision, validation, investigation, visualization, methodology, writing–review and editing. **R.S. Lo:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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CANCER DISCOVERY

Durable Suppression of Acquired MEK Inhibitor Resistance in Cancer by Sequestering MEK from ERK and Promoting Antitumor T-cell Immunity

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