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SHORT COMMUNICATION

CpG-based immunotherapy impairs antitumor activity of BRAF inhibitors in a B-cell-dependent manner

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Combining immunotherapy with targeted therapy has increasingly become an appealing therapeutic paradigm for cancer treatment due to its great potential for generating durable and synergistic antitumor response. In this study, however, we unexpectedly found that two types of CpG-based tumor peptide vaccine treatments consistently negated the antitumor activity of a selective BRAF inhibitor in tumors with *BRAF* mutation rather than showing a synergistic antitumor effect. Our further studies demonstrated that CpG alone was sufficient to dampen BRAF inhibitor-induced antitumor responses, suggesting that the impaired antitumor activity of the BRAF inhibitor observed in mice receiving CpG-based peptide vaccine is mainly dependent upon the use of CpG. Mechanistically, CpG increased the number of circulating B cells, which produced elevated amounts of tumor necrosis factor-α (TNFα) that contributed to the increased tumor resistance to BRAF inhibitors. More importantly, B-cell depletion or TNFα neutralization can restore the antitumor effect of BRAF inhibition in mice receiving CpG treatment, indicating that TNFα-secreting B cells play an indispensable role in BRAF inhibitor resistance induced by CpG. Taken together, our results strongly suggest that precautions must be implemented when designing combinatorial approaches for cancer treatment, because distinct regimens, despite their respective therapeutic benefit as monotherapy, may together provide antagonistic clinical outcomes.

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INTRODUCTION

Combining targeted therapy with immunotherapy has increasingly become an appealing therapeutic strategy for cancer treatment due to its great potential for improved overall efficacy and durable antitumor response.[1,2] Indeed, using animal models and patient samples, we and others have demonstrated a synergistic outcome of combining targeted therapy with immunotherapy.[3–6] For example, co-administration of a selective BRAF inhibitor with adoptively transferred T lymphocytes results in significantly enhanced tumor control in *BRAF*-mutant melanoma.[6] Additionally, immune checkpoint blockade using anti-PD1 antibodies synergizes with BRAF inhibition, providing increased therapeutic benefit when compared with the targeted therapy alone.[3] It is not known whether targeted inhibition of BRAF synergizes with other immunotherapeutic strategies, such as cancer vaccines, which would generate *de novo* anti-cancer immune responses.[7,8]

Among various regimens of cancer vaccines that have been designed and shown to benefit cancer patients, administration of peptides harboring tumor-specific T-cell epitopes may represent a convenient vaccination strategy due to the ease of peptide synthesis and purification. Adjuvants are often used together with these peptides to stimulate the immune response to the antigen. CpG oligodeoxynucleotides (CpG-ODN), short single-stranded synthetic DNA molecules containing unmethylated cytosine-guanine motifs, represent one type of the widely employed vaccine adjuvants due to its potency in promoting antigen-specific immune responses.[9,10] CpG motifs are abundant in microbial genomes but rare in vertebrate genomes, and thus are considered as pathogen-associated molecular patterns.[11] They can be recognized by the pattern recognition receptor, Toll-like receptor 9, which is constitutively expressed on B cells and plasmacytoid dendritic cells in humans and rodents.[12] Therefore, CpG can activate Toll-like receptor 9 on B cells and plasmacytoid dendritic cells and thereby regulate subsequent immune response to vaccines. Notably, in multiple murine tumor models, CpG adjuvants are essential for inducing activation and accumulation of cytotoxic T lymphocytes that are specific to tumor antigens.[13,14]

There are five classes of CpG ODNs (Class A, Class B, Class C, Class P and Class S) based on their sequences and secondary structures.[15] Among these, Class A and Class B CpG ODNs are the most frequently used adjuvants to treat patients with melanoma, lung, ovarian, breast and colon cancers,[16] and display ability to enhance T-cell-mediated antitumor response. In a clinical study, melanoma patients treated with CpG-based peptide vaccines demonstrated increased amounts of circulating Melan-A-specific $CD8^+$ T cells when compared with treatment without CpG adjuvants.[17] Based upon these previous observations, we hypothesize that combination of CpG-based peptide vaccines and BRAF inhibitors can generate synergistic antitumor effects. Unexpectedly, however, our results showed that using CpG as the vaccine adjuvant impaired the antitumor activity of BRAF inhibitors in mouse models of *BRAF*-mutant melanoma, and this depends on increased levels of tumor necrosis factor-α (TNFα) produced by B cells. These results strongly suggest that precautions must be taken when combining targeted therapy

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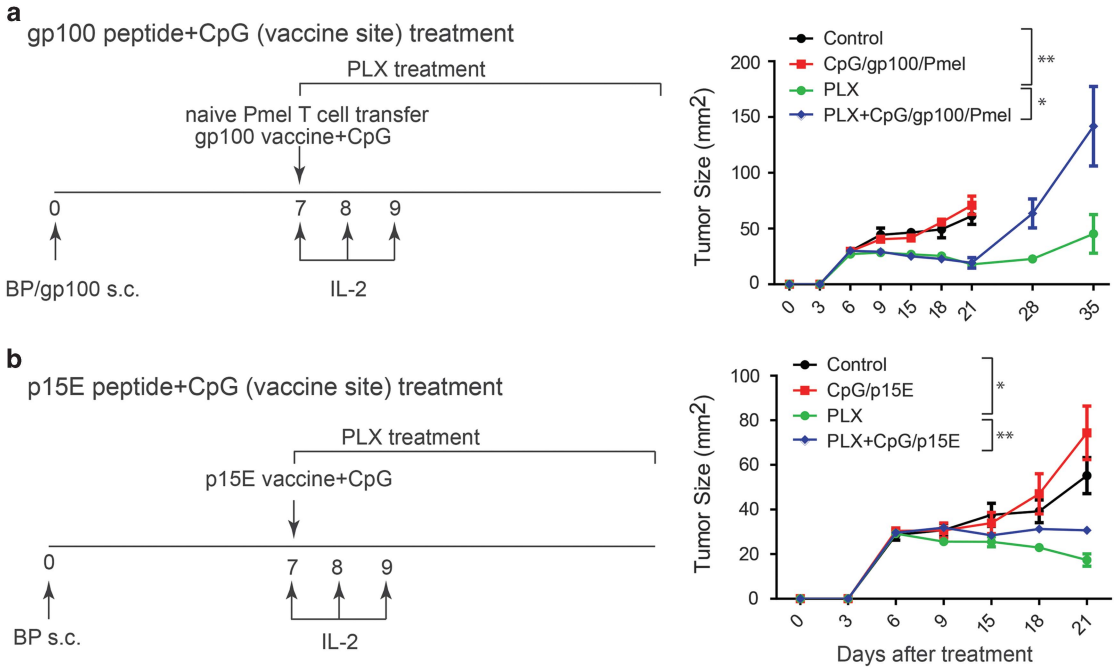

Figure 1. BRAF inhibitor-induced antitumor responses are impaired when combined with CpG-based peptide vaccines. (**a**) A previously established murine melanoma cell line bearing *BRAF* and PTEN mutation (BP) was provided by Dr Wargo (MD Anderson Cancer Center)[3] and maintained in RPMI 1640 with 10% FCS and 100 μg/ml Normocin (Invivogen, San Diego, CA, USA). To generate gp100-expressing murine cell line (BP/gp100), BP cells were transduced with the lentiviral vector encoding full-length human *gp100* as previously described.[34] Tumor growth was induced by subcutaneous injection of 5×10^5 BP/gp100 cells in 6- to 8-week-old female C57BL/6 mice (from Charles River NCI, Frederick, MD, USA). Seven days after tumor challenge, tumor-bearing mice were treated with a selective BRAF inhibitor PLX4720 (Plexxikon, Berkeley, CA, USA; 100 mg/kg in 3% DMSO and 1% methylcellulose by oral gavage daily[35]), tumor vaccine with 50 μg CpG (CpG-ODN-2216 synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA) and injected intratumorally once per week for 3 weeks) and 100 μg synthetic, high-affinity H-2D^b-restricted hgp100$_{25–33}$ peptide (KVPRNQDWL, purchased from Peptides International (Louisville, KY, USA) at a purity >95% and injected subcutaneously), or both. For groups containing the tumor vaccine, 1×10^3 Pmel-1T cells (isolated from Pmel-1 TCR/Thy1.1 mice from in-house breeding colonies) were intravenously administered and 100 μg anti-CD40 (Bioxcell, West Lebanon, NH, USA) was intraperitoneally injected on day 7, and 1×10^5 IU rhIL-2 protein (Prometheus Laboratories Inc, San Diego, CA, USA) were intraperitoneally injected on days 7, 8 and 9. (**b**) Tumor growth was induced by subcutaneous injection of 5×10^5 BP cells in 6- to 8-week-old female C57BL/6 mice. Seven days after tumor challenge, tumor-bearing mice were treated with PLX4720, tumor vaccine with 50 μg CpG and 100 μg H-2K^b-restricted mouse P15E$_{134–141}$ peptide (KSPWFTTL, purchased from Peptides International at a purity >95% and injected subcutaneously), or both. For groups containing the tumor vaccine, 100 μg anti-CD40 was intraperitoneally injected on day 7, and 1×10^5 IU rhIL-2 protein were intraperitoneally injected on days 7, 8 and 9. Tumor-bearing mice treated with vehicle (3% DMSO and 1% methylcellulose) were used as control. Tumor growth was monitored every 3 days by measuring the perpendicular diameters of tumors. $N=5$ mice per group. Data expressed as mean ± s.e.m. *$P<0.05$, **$P<0.01$, two-way ANOVA plus *post hoc* Turkey test.

and immunotherapy because these two distinct regimens, despite their respective therapeutic benefits, may together provide antagonistic, rather than synergistic, clinical outcomes.

RESULTS AND DISCUSSION

CpG-based tumor vaccines inhibit *in vivo* antitumor activity of BRAF inhibitors

FDA-approved BRAF inhibitors have become the frontline treatment option for melanoma patients harboring *BRAF* mutations. To develop and evaluate novel combinatorial therapies, we set out to determine the *in vivo* therapeutic effect of combining cancer vaccines with a selective BRAF inhibitor, PLX4720 (PLX). We employed two well-established models of tumor vaccines: (1) gp100 peptide vaccination plus adoptive transfer of gp100-specific T cells from *Pmel-1* mice[4] and (2) p15E peptide vaccination.[18] These different vaccination regimens focused on distinct antigenic peptides and induced exogenous and endogenous antitumor responses, respectively. CpG-ODN-2216, which displays a great potency to induce T-cell mediated antitumor immune response in murine vaccination models,[18,19] was included in both vaccination approaches as the adjuvant. A spontaneous melanoma cell line established from *Tyr:CreER;BRAF$^{V600E/+}$; PTEN$^{lox/lox}$* mice (designated as BP hereafter) mice was utilized for tumor growth studies in a syngeneic transplantable mouse model.[3] Specifically, BP tumor cells stably expressing the melanoma antigen gp100 (BP/gp100) were generated and subcutaneously implanted into mice and allowed to grow for 7 days before treatment with PLX, tumor vaccines or both. Our results demonstrated that BRAF inhibition alone resulted in significantly impaired tumor growth when compared with vehicle control (Figure 1a). However, when combined with the vaccination regimen comprising gp100 peptide, CpG adjuvant, adoptively transferred Pmel-1 T cells, anti-CD40 and IL-2, the BRAF inhibitor-induced antitumor responses appeared to be significantly weakened (Figure 1a). Similarly, reduced antitumor activity from combined CpG-based vaccines and BRAF inhibitors was also observed when the second vaccination regimen targeting the endogenously expressed antigen P15E was used (Figure 1b). Together, these results indicated that, regardless of the type of antigen or the involvement of T-cell transfer, peptide-based vaccines with CpG adjuvant consistently impaired the tumor-regressing capacity of BRAF inhibitors.

The antagonism between BRAF-targeted therapy and CpG-based tumor vaccines was an unexpected and intriguing finding. When administered as a single agent, BRAF inhibitors[20] or CpG vaccination with melanoma antigens[17,21] can induce tumor regression in animal studies and patient cohorts. However, our results demonstrated that when implemented as a combination

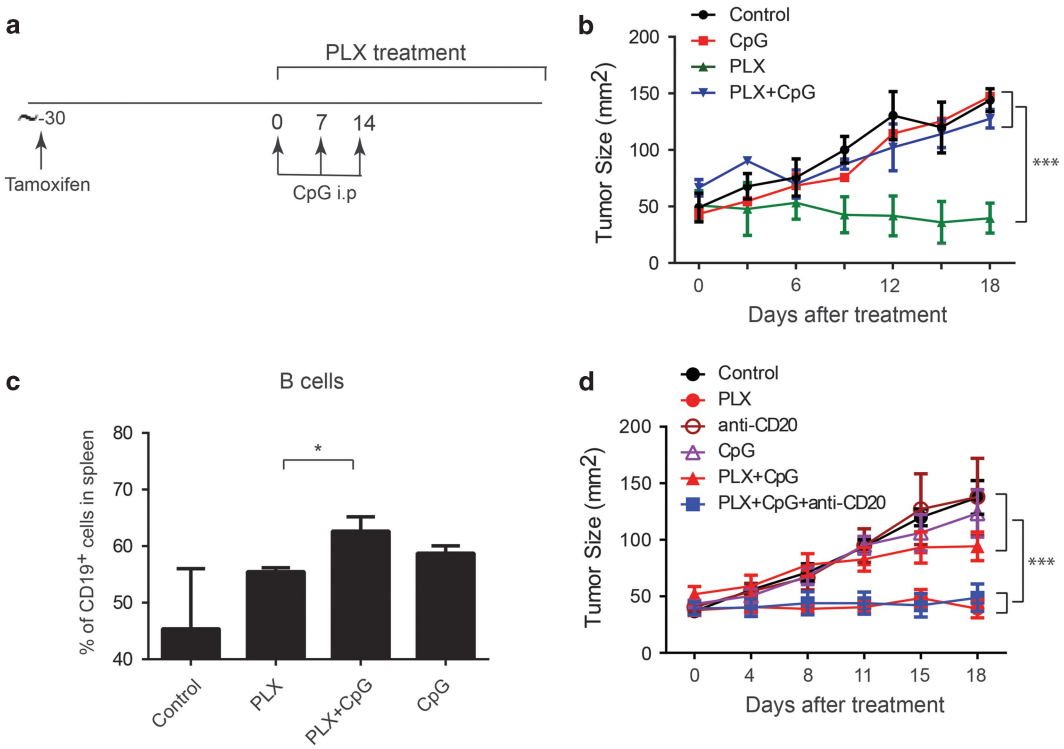

Figure 2. CpG negates the antitumor activity of BRAF inhibitors in a B-cell-dependent manner. To induce spontaneous melanoma in genetically modified mice, Tyr:CreER; $PTEN^{lox/lox}$; $BRAF^{V600E/+}$ (BP) mice on a C57BL/6 background (6–8 weeks of age) were treated with 50 mg ml^{-1} 4-hydroxytamoxifen (Sigma, St Louis, MO, USA) to induce the expression of Cre as previously described.[23] Mice with measurable tumors received indicated antitumor treatments. (**a**, **b**) Tumor-bearing BP mice were treated with daily oral gavage of 100 mg/kg PLX4720 (PLX), intraperitoneal injection of 50 μg CpG once per week for 3 weeks, or both. Tumor growth was monitored every 3 days by measuring the perpendicular diameters of tumors. $N=5$ mice per group. (**c**) Single-cell suspensions were prepared from the spleens of tumor-bearing BP mice treated with PLX, CpG or both, and stained with anti-CD4, CD8 and CD19 antibodies (BD Biosciences, San Jose, CA, USA). The percentages of various immune cell populations (T cells and B cells) in stained samples were analyzed by flow cytometry using a FACSCANTO II cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA). $CD19^+$ B cells in spleen in each group were shown. $N=5$ per group. (**d**) Tumor-bearing BP mice were simultaneously treated with PLX, CpG or both, as described in (**a**, **b**). Also, 50 μg of anti-mouse CD20 Ab IgG2a kindly provided by Biogen Idec (San Diego, CA, USA) were injected intravenously in tumor-bearing BP mice once per week for 2 weeks to deplete B cells either alone or together with PLX and CpG. Isotype control antibody purchased from Sigma were similarly injected as a control. Tumor sizes were monitored every 3 days (Control group: $N=7$; PLX group: $N=6$; anti-CD20 group: $N=5$; CpG group: $N=4$; PLX+CpG group: $N=6$; PLX+CpG+anti-CD20 group: $N=6$). Data expressed as mean ± s.e.m. $^*P<0.05$, $^{***}P<0.001$, two-way ANOVA (**b**, **d**) or one-way ANOVA (**c**) plus *post hoc* Turkey test.

therapy, CpG-based vaccines resulted in diminished BRAF inhibitor-mediated antitumor activity. Although BRAF inhibition can synergistically improve therapeutic capacity of several types of immunotherapy, such as adoptive T-cell transfer[5,6] and immune checkpoint blockade,[3,22] our seemingly counterintuitive results suggest that combining targeted therapy with immunotherapy may not always generate synergistic outcomes.

CpG negates the antitumor activity of BRAF inhibitors in a B-cell-dependent manner

The various components in the tumor vaccine may be responsible for the deficient antitumor activity when combined with BRAF inhibition. However, we reasoned that CpG adjuvant was the most likely candidate, because the adverse impact of the vaccine appeared to be antigen-independent and regimen-irrelevant based upon results from the two distinct vaccination models. To test our hypothesis, we administered CpG intraperitoneally in combination with PLX (Figure 2a) and assessed antitumor responses in BP transgenic mice, a tamoxifen-inducible spontaneous mouse model that physiologically recapitulates the development of human melanoma.[23] Our results confirmed that systemic administration of CpG alone was sufficient to dampen BRAF inhibitor-induced antitumor responses (Figure 2b), suggesting that CpG is largely responsible for the differential antitumor activity observed between the BRAF inhibitor alone and the combination therapy.

We next sought to determine the mechanism that underlies the adverse effect of CpG on the combination therapy. CpG may have an effect on different types of cells, including tumor cells, dendritic cells and T cells. Thus, it is possible that CpG directly changes tumor cell biology and function, which in turn may impair the antitumor effect induced by the BRAF inhibitor. To test this possibility, we treated BP tumor cells *in vitro* with or without CpG for 24 h and measured cell growth by CellTiter Blue assay. As shown in Supplementary Figure S1, both high and low concentrations of CpG failed to alter the growth of tumor cells, suggesting that the antagonistic effect of CpG in combination with the BRAF inhibitor could not be explained by the direct effect of CpG on tumor cells.

Given its potent immunomodulatory function,[24] CpG likely alters immune factors that influence the therapeutic response to BRAF inhibitors. Indeed, flow cytometry analysis revealed that the percentage of splenic B cells was significantly increased in mice treated with the combination therapy when compared with the cohorts treated with the BRAF inhibitor alone (Figure 2c). To characterize the functional relevance of the increased number of B cells induced by CpG, we administered an anti-CD20 antibody intraperitoneally to deplete B cells *in vivo* and assessed how this impacted the role of CpG in the combination therapy. Upon

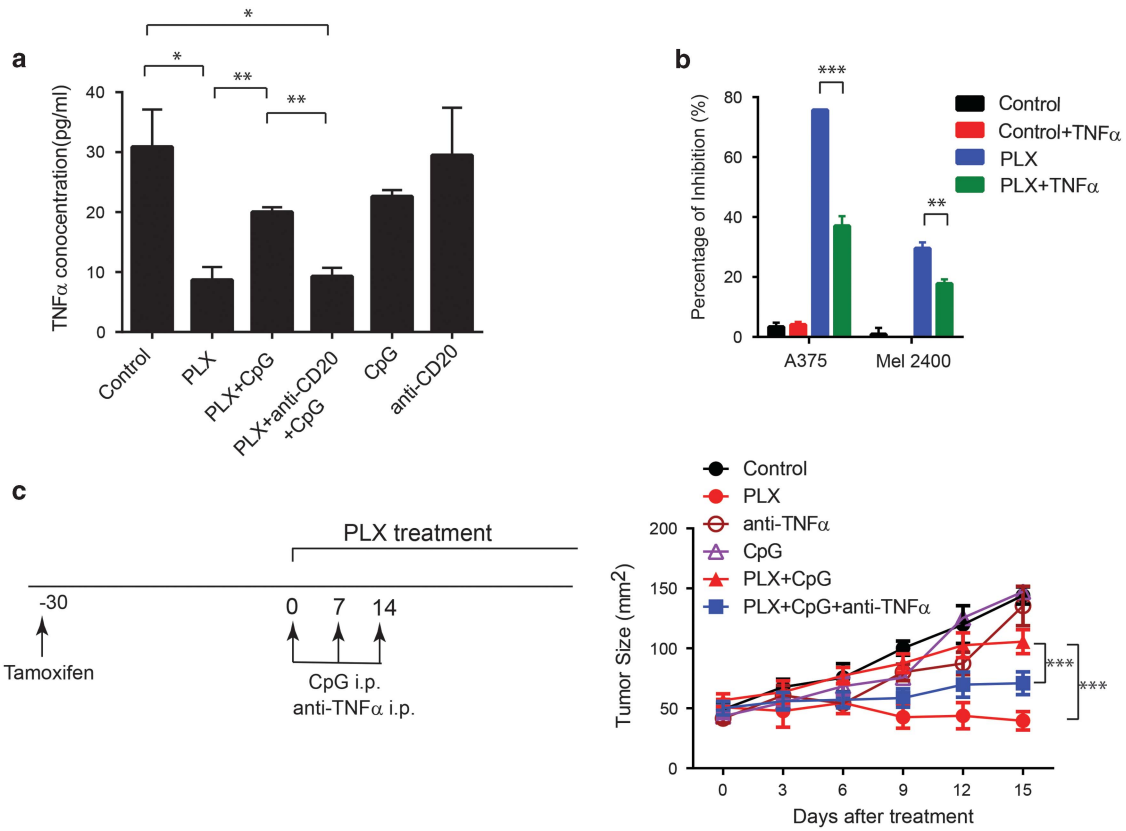

Figure 3. CpG treatment dampens BRAF inhibitor-induced tumor regression through B-cell-derived TNFα. (**a**) Sera samples from tumor-bearing BP mice treated as in Figure 2d were collected and measured by MILLIPLEX mouse cytokine/chemokine panels I (premixed 32-plex) and II (premixed 8-plex) according to the manufacturer's protocol (EMD Millipore, Billerica, MA, USA). The concentration of each cytokine and chemokine in serum was determined using a Luminex 200 system (Luminex Corporation, Austin, TX, USA). Serum expression of TNFα from different treatment groups are shown. $N=3$ per group. (**b**) Melanoma tumor cells A375 and 2400 were seeded at 1000 cells per well and stimulated with 1 μM PLX, 20 ng/ml TNFα (Bioxcell) or both. Three days later, cell viability was determined using CellTiter Blue assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Data expressed as mean ± s.e.m. of triplicates from two independent experiments. (**c**) Tumor-bearing BP mice were treated with vehicle, PLX, CpG, or combination of PLX and CpG as described in Figure 2. To neutralize TNFα, mice were injected with 100 μg of rat anti-mouse TNFα IgG1 (Bioxcell) intraperitoneally once per week for 2 weeks, either alone or together with PLX and CpG. Tumor sizes were monitored every 3 days. (Control group: $N=5$; PLX group: $N=4$; anti-TNFα group: $N=5$; CpG group: $N=4$; PLX+CpG group: $N=6$; PLX+CpG+anti-TNFα group: $N=6$). Data expressed as mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, one-way ANOVA (**a**, **b**) or two-way ANOVA (**c**) plus *post hoc* Turkey test.

anti-CD20 administration, the percentage of $CD19^+$ B cells was significantly reduced in blood, spleen and tumor, whereas that of $CD4^+$ and $CD8^+$ T cells, Gr-1^+ granulocytes and $CD11b^+$ monocytes were unaltered (Supplementary Figures S2a and b). As illustrated in Figure 2d, tumor growth in BP mice was effectively suppressed by BRAF inhibitor treatment, but not in the presence of systemic CpG administration. However, the antitumor response was restored upon B-cell depletion by anti-CD20 antibody. These data indicate that B cells play an indispensable role in the resistance to BRAF inhibitors in the presence of systemic CpG treatment.

B-cell-derived TNFα contributes to CpG-induced resistance to BRAF inhibition.

To mechanistically identify how CpG-induced B cells augment resistance to BRAF inhibitors, we further characterized the profile of serum cytokines in treated mice through a Luminex multiplex assay. Among the assessed cytokines, the expression pattern of TNFα was identified to correlate with the magnitude of antitumor responses upon different types of treatment. Specifically, treatment with the BRAF inhibitor PLX resulted in significantly decreased levels of serum TNFα, which was upregulated when combined with CpG treatment (Figure 3a). Importantly, addition of a B-cell-depleting anti-CD20 antibody impeded the increase in TNFα observed with CpG treatment (Figure 3a), implicating B cells as a major source of TNFα. We further confirmed that treatment of purified B cells with CpG *in vitro* induced the production of TNFα (Supplementary Figure S3a). In addition, mice injected with CpG had higher TNFα in splenic $CD19^+$ B cells than in those without CpG treatment (Supplementary Figure S3b). Furthermore, in a transplantable model of BP tumors, treatment of tumor-bearing mice with CpG-based vaccine dramatically increased the percentage of $TNFα^+$ cells in the $CD19^+$ B-cell population in the spleen (Supplementary Figure S4), indicating that CpG-based vaccine can also increase TNFα-producing B cells in the spleen. These results are consistent with previous reports showing CpG-induced TNFα production from B cells.[25,26] In line with previous studies demonstrating an essential role for TNFα in the resistance to mitogen-activated protein kinase signaling inhibition,[27–29] our *in vitro* assays in two patient-derived melanoma cell lines showed that the inhibition of tumor cell growth by the BRAF inhibitor was significantly mitigated when combined with TNFα (Figure 3b). More importantly, upon administration of a neutralizing anti-TNFα antibody in tumor-bearing BP mice, inhibition of *in vivo* tumor growth was largely restored as compared with the combined PLX and CpG treatment (Figure 3c). Taken together, these results

suggest that B-cell-secreted TNFα is an essential mediator for the CpG-induced resistance to BRAF inhibitor therapy.

Previous studies have shown that B cells, but not T cells, express Toll-like receptor 9 and can thus be specifically activated by CpG,[24,30] which is consistent with the increased production of B-cell-derived TNFα upon systemic CpG treatment observed in our study. Notably, multiple lines of evidence have implicated an important cancer-promoting role for B lymphocytes through various immunoregulatory mechanisms, potentially involving downregulated NK cell activity, less intratumoral $CD8^+$ T cells and increased function of regulatory T cells.[31–33] Our results added B-cell-derived TNFα as another possible layer of immunomodulatory mechanisms, because co-treatment with anti-TNFα antibodies partially rescued the impaired antitumor activity in response to the combinatorial therapy. TNFα is known to suppress apoptosis and promote growth in melanoma, thereby inducing resistance to mitogen-activated protein kinases pathway inhibitors, including those targeting BRAF.[27–29] These observations suggest that better antitumor therapeutic outcomes may be achieved by simultaneously disrupting TNFα function or its downstream nuclear factor-κB pathways with targeted therapy or combinatorial immunotherapy. However, it needs to be noted that, in addition to TNFα, other B-cell-derived factors may also be accountable, because tumor resistance to the combined CpG and BRAF inhibitor treatment was not completely alleviated upon TNFα neutralization.

Among many currently available treatment approaches, targeted therapy and immunotherapy have demonstrated impressive clinical benefit for cancer patients. To seek even better antitumor responses, recent studies have exploited the possibility of combining targeted therapy and immunotherapy, which have shown improved therapeutic outcomes in animal models and clinical trials.[3,5,6,22] However, it remains unclear how and why the involvement of one therapeutic approach could affect the effect of another. Our study provides evidence that CpG-based immunotherapy can upregulate the production of B-cell-derived TNFα and thereby negatively influence antitumor responses to BRAF inhibitors. Given the established role of TNFα in suppressing antitumor activity of mitogen-activated protein kinase inhibitors,[27–29] it is highly likely that CpG treatment can also result in enhanced resistance to mitogen-activated protein kinase inhibitors. This implicates the adverse impact that CpG-based immune treatment may have when combined with mitogen-activated protein kinases pathway inhibition in general.

Taken together, our study demonstrated that CpG treatment can impair the antitumor activity of BRAF inhibitors by enhancing the production of TNFα by B cells. More importantly, our results strongly suggest that precautions must be implemented when designing combinatorial approaches for cancer treatment, because distinct regimens, despite their respective therapeutic benefit, may together provide antagonistic, rather than synergistic, clinical outcomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

PH and WP conceived and supervised the research study. LH, ZW, CL, CX, RM, JM, HK and WP conducted experiments, acquired and analyzed data. LH, ZW and WP wrote the manuscript.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)