

## The Oncogenic BRAF Kinase Inhibitor PLX4032/RG7204 Does Not Affect the Viability or Function of Human Lymphocytes across a Wide Range of Concentrations

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### Abstract

**Purpose:** PLX4032 (RG7204), an oncogenic BRAF kinase inhibitor undergoing clinical evaluation, has high response rates in early clinical trials in patients with advanced BRAF<sup>V600E</sup> mutant melanoma. Combining PLX4032 with immunotherapy may allow expanding the durability of responses. The effects of PLX4032 on immune cells were studied to explore the feasibility of future combinatorial approaches with immunotherapy for melanoma.

**Experimental Design:** Peripheral blood mononuclear cells (PBMC) and BRAF<sup>V600E</sup> mutant melanoma cells were exposed to increasing concentrations of PLX4032 and the cell viability, proliferation, cell cycle, apoptosis, and phosphorylation of signaling proteins were analyzed. Effects of PLX4032 on antigen-specific T-cell function were analyzed by specific cytokine release and cytotoxicity activity.

**Results:** The 50% inhibition concentration (IC<sub>50</sub>) of PLX4032 for resting human PBMC was between 50 and 150 μmol/L compared with an IC<sub>50</sub> below 1 μmol/L for sensitive BRAF<sup>V600E</sup> mutant melanoma cell lines. Activated lymphocytes were even more resistant with no growth inhibition up to concentrations of 250 μmol/L. PLX4032 had a marginal effect on cell-cycle arrest, apoptotic cell changes or alteration of phosphorylated signaling molecules in lymphocytes. Functional analysis of specific antigen recognition showed preserved T-cell function up to 10-μmol/L concentration of PLX4032, whereas the cytotoxic activity of PLX4032 was maintained up to high concentrations of 50 μmol/L.

**Conclusions:** The preserved viability and function of lymphocytes exposed to high concentrations of PLX4032 suggest that this agent could be a potential candidate for combining with immunotherapy strategies for the treatment of patients with BRAF<sup>V600E</sup> mutant melanoma.

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### Introduction

Two areas of clinical drug development have recently been providing significant excitement for the treatment of patients with advanced melanoma. One is the new generation of targeted agents that block driver oncogenic mutations showing unprecedented high response rates in early clinical trials. Specific oncogenic BRAF inhibitors, like PLX4032 and GSK2118436, are being developed for the

treatment of BRAF<sup>V600E</sup> mutated melanoma (1, 2), whereas c-kit inhibitors, like imatinib, have shown significant activity against melanomas with activating *c-kit* mutations (3). In both cases, the presence of a driver mutation in *BRAF* or *c-kit* is a prerequisite for response (3, 4). However, these responses frequently are of limited durability, which is a common feature of most oncogene-targeted therapies for cancer.

Novel immunotherapy avenues that build on the durable responses achieved with a variety of immune stimulating agents are also providing significant advances in the clinic. The most widely applicable are immune modulating antibodies that block negative immune signaling through CTLA4 or PD-1, or immune activating antibodies to CD40 or CD137. These agents provide evidence of reproducible immune cell-mediated tumor responses in a small subset of patients (5). However, the durability of these responses, similar to the durability of responses to high-dose interleukin-2 (IL-2), makes these agents a very attractive treatment option (6). It is conceivable to postulate that agents that specifically impact on the target tumor cells and prepare them for the immune effector cell attack induced by immunotherapy strategies may

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## Translational Relevance

Targeted therapies blocking oncogenic mutations, like c-kit and BRAF<sup>V600E</sup>, and novel immunotherapies with immune modulating antibodies or adoptive cell transfer therapy, are dramatically changing the treatment options of patients with advanced melanoma. The possibility of combining both approaches hinges upon showing lack of adverse effects of the oncogene-targeted inhibitors on lymphocytes. This work provides evidence that the oncogenic BRAF kinase inhibitor PLX4032 does not have major detrimental effects on lymphocytes. The results support the testing of combinatorial approaches of PLX4032 with immunotherapy in future clinical trials.

increase the effectiveness of tumor immunotherapy for melanoma.

Pharmacologic interventions with specific inhibitors of oncogenic events in cancer cells may be designed to sensitize cancer cells to immune attack, which has been termed "immunosensitization" (7, 8). An immune sensitizing agent should ideally block key oncogenic events in cancer cells, resulting in an increase in cell surface ligands for immune effector cells, and induce an intracellular proapoptotic cancer cell milieu, which would enhance the ability of immune effector cells like natural killer (NK) and cytotoxic T lymphocytes (CTL) to recognize and kill cancer cells. At the same time, immune sensitizing agents should not impair the viability or function of immune effector cells (8). Therefore, most chemotherapy agents administered concurrently with immunotherapy do not fit the definition of immune sensitizing agents, because they frequently kill activated and proliferating lymphocytes. However, some chemotherapy agents have target-sensitizing effects when they are carefully dosed and sequenced to foster cancer cell death without inducing adverse effects on immune effector cells (9). Examples of successfully tested immunosensitization approaches include proteasome inhibitors to improve NK cytotoxic activity (10), bcl-2 inhibitors to sensitize target cells to effector CTL responses (11) and epigenetic modification agents to sensitize target cells to NK and CTL responses (12–15).

PLX4032 is a class I RAF inhibitor that induces tumor regressions in a high proportion of patients with BRAF<sup>V600E</sup>-positive metastatic melanoma in early clinical trials (1). Blocking oncogenic BRAF<sup>V600E</sup> may synergize with tumor immunotherapy by the increased expression of melanosomal tumor-associated antigens upon mitogen-activated protein kinase (MAPK) pathway inhibition, as previously reported with a Mek inhibitor (16) and also with the related tool compound PLX4720 (17). In addition, tumor cell death with PLX4032 may induce the release of tumor antigens resulting in increased antigen cross-presentation to CTLs. Furthermore, this agent is likely to modulate the antiapoptotic environment in cancer cells resulting in increased sensitivity to proapoptotic signals from activated CTLs (18, 19). However, these potential

beneficial effects of PLX4032 blocking oncogenic BRAF<sup>V600E</sup> in melanoma cells have to be balanced with potential adverse effects of blocking wild-type BRAF and CRAF in activated lymphocytes (20). The MAPK pathway is critical for signaling from immune receptors, like the T-cell receptor (TCR) on T cells and the B-cell receptor (BCR) on B cells, and it is also critically involved in multiple intracellular pathways activated upon lymphocyte response to antigen encounter or cytokine stimulation (21). Therefore, as a first step to determine if PLX4032 could have a role as an immune sensitizing agent, we tested its effects on lymphocyte viability and function. Our studies show that lymphocytes are 1 to 2 logs less sensitive to the cytotoxic effects of PLX4032 than BRAF<sup>V600E</sup> mutant melanoma cell lines. At concentrations where PLX4032 has specific anti-tumor effects, it does not negatively impact the viability or function of T cells. These studies, together with the evidence that an analogue of PLX4032 favorably impacts on antigen presentation by melanoma cells and T-cell recognition (17), support the notion that PLX4032 could be combined with immunotherapeutic strategies in the clinic.

## Material and Methods

### Reagents and cells

PLX4032 (also known as RG7204 or RO5185426) was obtained under a materials transfer agreement (MTA) with Plexikon Inc. and provided by Dr. Gideon Bollag. PLX4032 was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific) and used as previously described (22). DMSO alone was used as vehicle control at the concentration of 0.0005% (unless noted, all percentages represent volume to volume), which is the highest concentration of DMSO in studies using PLX4032. Peripheral blood mononuclear cells (PBMC) were obtained by leukapheresis from a healthy subject (coded as H) under UCLA IRB approval #04-07-063, and from 3 patients with metastatic melanoma (coded as GA17, GA20, and GA21) while they were not in active therapy under UCLA IRB approval #06-06-093. PBMCs were cultured in RPMI 1640 with L-glutamine (Mediatech Inc.) containing 5% human AB serum (Omega Scientific), at a density of 1 million cells/mL. Proliferating PBMCs were cultured in 300 IU/mL of IL2 (Novartis) with 50 ng/mL of OKT3 (eBioscience) added for the first 48 hours of culture only. The human melanoma cell lines M229, M233, M238, and M249 were established from patient biopsies under UCLA IRB approval #02-08-067 and have been previously described (22). Melanoma cells were cultured in RPMI 1640 with L-glutamine containing 10% fetal bovine serum (FBS, Omega Scientific) and 1% penicillin, streptomycin and amphotericin (Omega Scientific). M249-A2.1 was generated by lentiviral transduction of M249 cells with LV-CMV-HLA-A2.1 as previously described (23). Stably expressing cells were obtained by limited dilution cloning. The HLA-A\*0201-transfected K562 (K562-A2.1) cell line was a kind gift of Dr. Cedric Britten (24). K562 cells were obtained from ATCC. Cell

lines were periodically shown to be mycoplasma-free by the Mycoalert assay (Lonza).

### Cell viability and proliferation assays

PBMC and melanoma cell lines were treated in triplicate with increasing concentrations of PLX4032 up to 250  $\mu\text{mol/L}$ , or DMSO as vehicle control, for up to 72 hours. Viability was analyzed using an ATP-based luminescent cell proliferation assay kit following the manufacturer's instructions (CellTiter-Glo Luminescent Cell Viability Assay; Promega). Flow cytometry-based intracellular staining was used to study proliferation of PBMC cultured in PLX4032 using Ki67-phycoerythrin (PE) antibody staining following the manufacturer's instructions (BD Biosciences) with a minimum of 30,000 lymphocyte events collected and with the inclusion of an isotype-matched antibody control.

### Cell-cycle analysis

PBMC and melanoma cells were incubated with 1 to 50  $\mu\text{mol/L}$  of PLX4032 or DMSO vehicle control for 72 hours, fixed (BD Cytotfix/Cytoperm; BD Biosciences), washed (BD Perm/Wash Buffer) and resuspended in 1 mL of 4',6-diamidino-2-phenylindole (DAPI) solution at a final concentration of 2  $\mu\text{g/mL}$ , 0.001% nonadherent-40, and 1% bovine serum albumin in PBS (Sigma-Aldrich). We acquired 12,000 cellular events in  $G_0$ - $G_1$  per sample for flow cytometric analysis.

### Assessment of apoptosis by flow cytometry

PBMC and melanoma cell lines were exposed to PLX4032 or DMSO as vehicle control for 72 hours. Evidence of apoptotic changes was compared with the effects of 1  $\mu\text{mol/L}$  of staurosporine as a positive control by Annexin V-FITC and propidium iodide (PI) staining (BD Biosciences), as previously described (22, 25). For the analysis of cleaved poly[ADP-ribose]polymerase (PARP), fixed and permeabilized cells were stained with anti-PARP-Alexafluor700 (clone F21-852; BD Biosciences). A minimum of 30,000 lymphocyte events per sample were collected by flow cytometry.

### Phosphoflow analysis

PBMC cultured in increasing concentrations of PLX4032 or vehicle control for 24 hours were analyzed for phosphorylated intracellular signaling molecules, as previously described (26). PBMC exposed to different concentrations of PLX4032 were fluorescently bar-coded with a combination of 0 or 6  $\mu\text{g/mL}$  of Ax350-NHS and 0 or 3  $\mu\text{g/mL}$  of Ax750-NHS to allow the simultaneous analysis of 4 different populations in 1 sample, as previously described (26, 27). Bar-coded samples were stained for 30 minutes using a combination of surface and intracellular staining antibodies detailed in Supplementary Table 2. All antibodies were directly conjugated and used at saturating concentrations. Between 100,000 to 300,000 lymphocyte events were collected by flow cytometry. All flow cytometric experiments were carried out after flow cytometric compensation using

anti-mouse Ig k/Negative Control FBS compensation particles (BD Biosciences). Flow cytometry was carried out in an LSRII (BD Biosciences), using biexponential axis and data were analyzed using FlowJo (Tree Star Inc.).

### Analysis of antigen-specific cytokine production

PBMC from a healthy donor were stimulated with anti-CD3 antibody (OKT3) and IL-2 for 48 hours and then transduced twice in retronectin-coated plates with a retrovirus expressing a high-affinity Melan-A/MART-1 TCR kindly provided by S.A. Rosenberg and R. Morgan (Surgery Branch, NCI), as previously described (28). The resulting HLA-A2.1-restricted MART-1 TCR-transduced T cells, as well as control untransduced T cells, were treated with 1 to 50  $\mu\text{mol/L}$  of PLX4032, or DMSO as the vehicle control. MART-1 TCR transgenic cells were then analyzed for specific cytokine release by coculture with stimulating MART-1<sup>+</sup> and HLA-matched (M202, M249-A2.1) or -mismatched (M249wt, M238) melanoma cells, as well as with MART-1<sub>26-35</sub> peptide pulsed or nonpulsed K562-A2.1 cells, at a 1:1 ratio for 24 hours. Supernatants were collected and analyzed for IFN- $\gamma$  release by ELISA following the manufacturer's instructions (eBioscience). In addition, quantification of released interleukin-4 (IL-4), IL-5, IL-10, and macrophage inflammatory protein-1  $\beta$  (MIP-1 $\beta$ ) was done following the manufacturer's instructions using the BioPlex kit (Bio-Rad).

### Cytotoxic T-cell assay

HLA-A2.1-restricted MART-1 TCR transduced T cells and control untransduced T cells cultured in PLX4032 for 24 hours at the above-mentioned conditions were used in a standard Cr<sup>51</sup> release assay using radioactively labeled melanoma cell lines M202 (HLA-A2.1, MART-1<sup>+</sup>), M249wt (non-HLA-A2.1, MART-1<sup>+</sup>), or M249-A2.1 (HLA-A2.1, MART-1<sup>+</sup>) at different ratios for 4 hours, as described previously (29).

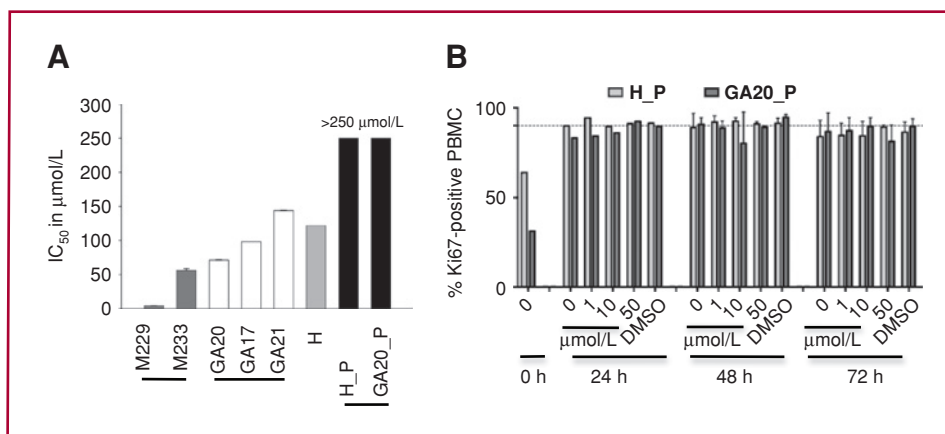
### Statistical analysis

GraphPad Prism (GraphPad Software) package program and Microsoft Excel were used for data presentation. Paired t-test was applied. At least 3 replicate and independent experiments were done with samples analyzed in duplicates or triplicates for each experiment.

## Results

### Effects of PLX4032 on PBMC proliferation and viability

The growth inhibitory effects of increasing concentrations of PLX4032 were tested against resting and anti-CD3 plus IL-2-activated PBMC compared with 2 melanoma cell lines used as positive and negative controls. M229 is a BRAF<sup>V600E</sup> mutant cell line that previously has been shown to be highly sensitive to PLX4032, with an IC<sub>50</sub> below 1  $\mu\text{mol/L}$ . In contrast, M233, also bearing the BRAF<sup>V600E</sup> mutation, was considered relatively resistant to PLX4032 in prior studies, with an IC<sub>50</sub> above 10  $\mu\text{mol/L}$

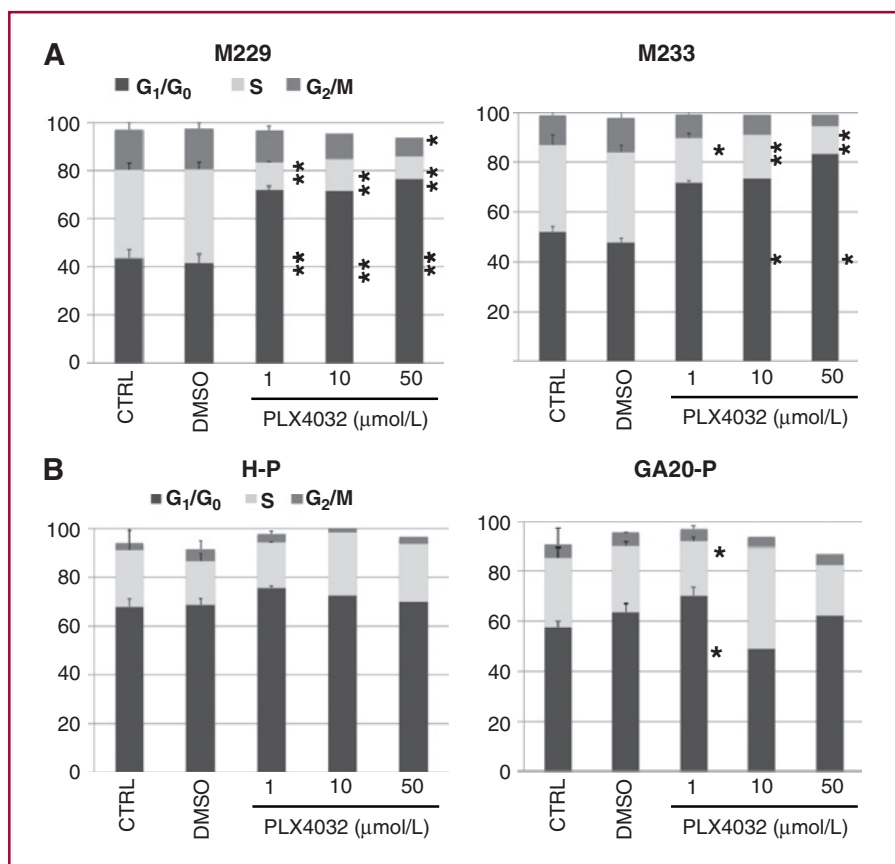


**Fig. 1.** Effects of PLX4032 on viability and proliferation of PBMC. A, IC<sub>50</sub> values of PLX4032 against BRAF<sup>V600E</sup>-mutated melanoma cell lines with prior demonstration of sensitivity (M229) and resistance (M233) to PLX4032 (dark gray columns) compared with resting PBMC from 3 patients with metastatic melanoma (GA17, GA20, and GA21; white columns), a healthy donor (H; gray column), and 2 samples of proliferating PBMC after activation with anti-CD3 and IL-2 (GA20-P and H-P; black columns). Data represent the mean of 3 experiments in triplicates. B, quantification of Ki67-Ax700 staining by flow cytometry of PBMCs cultured for 5 days in the presence of anti-CD3 and IL-2 and then exposed to PLX4032 or DMSO for 24 to 72 hours. Data represent the mean and SEM values from 3 independent replicate experiments.

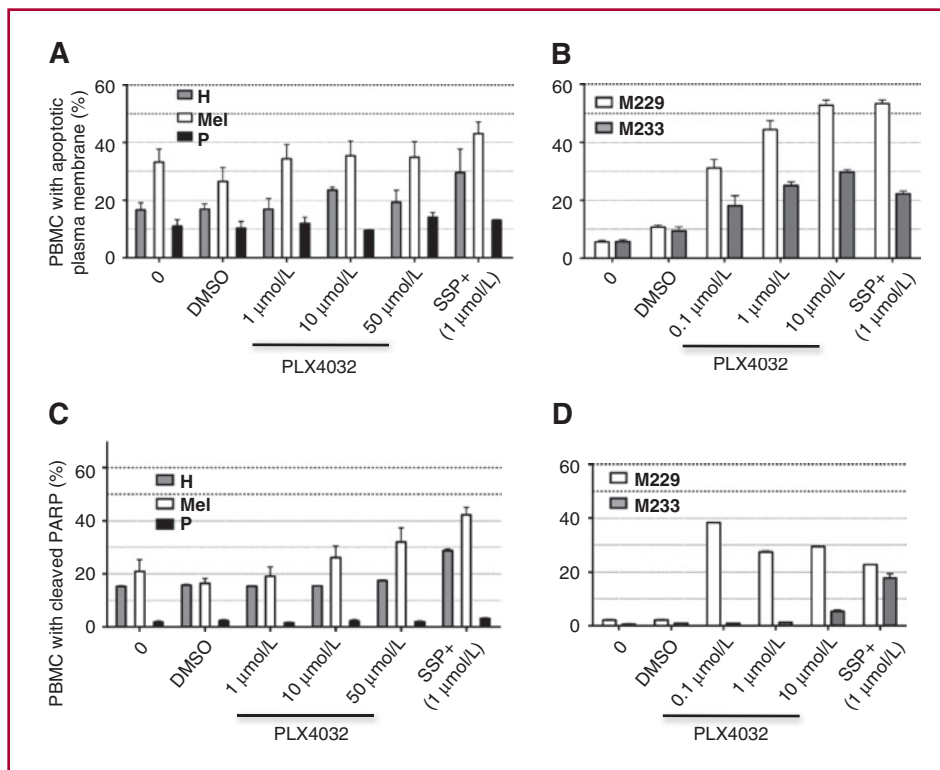
L (22). The IC<sub>50</sub> for resting PBMC obtained from a healthy donor and from 4 patients with metastatic melanoma (who were not in active treatment) was more than 50 μmol/L (Fig. 1A; Supplementary Fig. 1). Strikingly,

PBMCs activated with anti-CD3/IL-2 for 5 days were highly resistant to PLX4032, with no evidence of growth inhibition at the highest concentrations tested of 250 μmol/L of PLX4032.

**Fig. 2.** Analysis of cell-cycle progression of melanoma cell lines and activated lymphocytes cultured in PLX4032. A, 2 melanoma cell lines (M229, M233) and (B) 2 PBMC cultures stimulated in anti-CD3 and IL-2 for 5 days as described in Figure 1 (GA20-P, H-P) were analyzed for cell-cycle progression by DAPI staining using flow cytometry after exposure to PLX4032 for 72 hours. CTRL, control. Columns, means (n = 3); bars, SEM; \*, P < 0.05; \*\*, P < 0.01.







**Fig. 3.** Apoptosis in response to PLX4032 exposure. A and B, plasma membrane phosphatidylserine exposure through PI and Annexin V staining in PBMC (A) and melanoma (Mel; B) cell lines cultured in increasing concentrations of PLX4032 for 72 hours. C and D, cleavage of PARP (PARP-Ax700) into fragment p85 in PBMC (C) and melanoma cell lines (D) exposed to PLX4032 as described earlier. SSP at 1  $\mu\text{mol/L}$  was used as the positive control. Columns, means ( $n = 3$ ); bars, SEM.

### Cytostatic effects of PLX4032 on PBMCs

A pronounced cytostatic effect has been noted in BRAF<sup>V600E</sup> mutant melanoma cell lines exposed to PLX4032, even though some of the cell lines did not display cytotoxic sensitivity and had an IC<sub>50</sub> of more than 10  $\mu\text{mol/L}$  (22). Therefore, we evaluated whether different concentrations of PLX4032 interferes with the proliferation of anti-CD3/IL-2-stimulated lymphocytes. Five-day-activated PBMC from a healthy donor and a patient with melanoma were analyzed by intracellular flow cytometry for the proliferation-associated antigen Ki67. PBMCs were highly positive for Ki67 during the time course experiment, with no adverse effects induced by PLX4032 (Fig. 1B). We further studied the potential cytostatic effects of PLX4032 by flow cytometric analysis of cell-cycle progression using PI staining. Although PLX4032 had profound cytostatic effects against both the highly sensitive (M229) and resistant (M233) BRAF<sup>V600E</sup> mutant melanoma cell lines (Fig. 2A), effects on cell-cycle progression of activated lymphocytes exposed to concentrations of PLX4032 up to 50  $\mu\text{mol/L}$  were minimal (Fig. 2B).

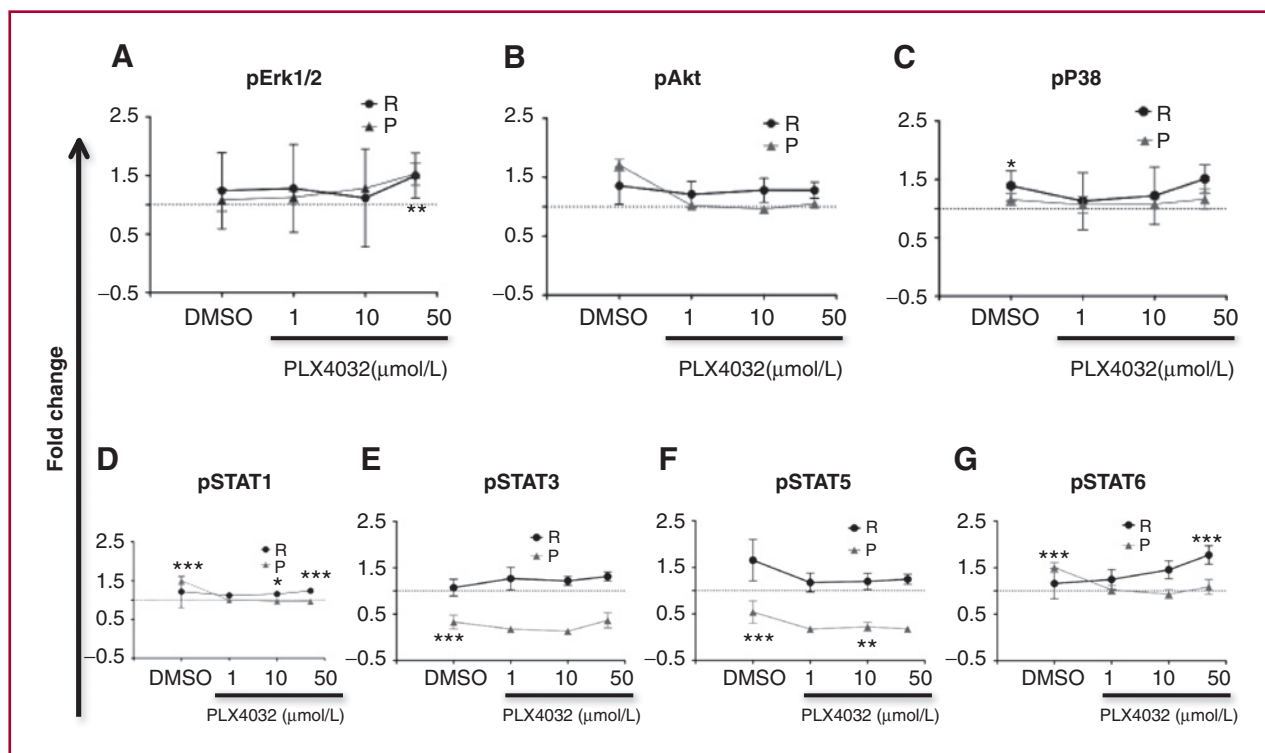
### Apoptotic changes in PBMCs cultured in the presence of PLX4032

Apoptotic cell changes detected by flow cytometric analysis using plasma membrane staining with Annexin V and PI, as well as intracellular staining for cleaved PARP, showed no major differences between PBMC samples exposed to vehicle control and samples exposed to increasing concentrations of PLX4032 (Fig. 3). This was evident

both in resting and proliferating PBMC, although proliferating PBMC had lower incidence of apoptosis compared with resting PBMC under both PLX4032 and DMSO control conditions. Consistent with our prior findings (22) there was significant apoptosis at all concentrations for the sensitive M229 cell line, whereas the equally BRAF<sup>V600E</sup> mutant melanoma cell line M233 was resistant to the apoptotic effects of PLX4032.

### Analysis of lymphocyte signaling networks after PLX4032 exposure

We analyzed the phosphorylation status of key signaling proteins downstream of immune cell receptors (TCR, BCR, cytokine receptors) on both resting and proliferating PBMC using phosphoflow. This analysis included signaling molecules downstream of BRAF and CRAF in the MAPK pathway. For these studies, we combined the data from the analysis of PBMC obtained from a healthy donor and 3 patients with melanoma as results were concordant. pERK1/2 and pp38 increased in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells exposed to 50  $\mu\text{mol/L}$  of PLX4032, whereas these changes were not evident in CD20<sup>+</sup> B cells (Fig. 4; Supplementary Figs. 3 and 4). Changes in pAkt showed a nonstatistically significant decrease in both T and B cells. The level of pSTAT1 and pSTAT6 decreased in proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T cells with increasing PLX4032 concentrations, whereas levels increased in resting CD8<sup>+</sup> T cells. pSTAT3 and pSTAT5 were significantly lower in proliferating CD8<sup>+</sup> T cells under all conditions compared with resting cells, and were maintained at similar levels in



**Fig. 4.** Phosphorylation of cell signaling proteins in CD8<sup>+</sup> T cells in the presence of increasing concentrations of PLX4032. Fold change compared with control in phosphoprotein levels for Erk1/2 (A), Akt (B), and p38 (C), as well as phosphorylated signal transducers and activators of transcription (STAT) proteins STAT1 (D), STAT3 (E), STAT5 (F), and STAT6 (G), in CD8<sup>+</sup> T cells. P, filled triangles, represent 5-day proliferating PBMCs. R, filled circles, represent resting PBMCs. DMSO (0.0005%). Y-axis, percentage of fold change compared with controls. Points, means ( $n = 3$ ); bars, SEM.

CD4<sup>+</sup> T cells. There were no changes other than a trend to decrease in pSTAT5 when exposed to increasing concentrations of PLX4032. Changes in phosphorylated STAT proteins in CD20<sup>+</sup> B cells were variable and showed no clear trend with increasing concentrations of PLX4032. Overall, these results show that exposure of PBMC to PLX4032 at a wide range of concentrations does not block signal transduction pathways important for T cell function, and may in fact increase MAPK signaling at higher concentrations.

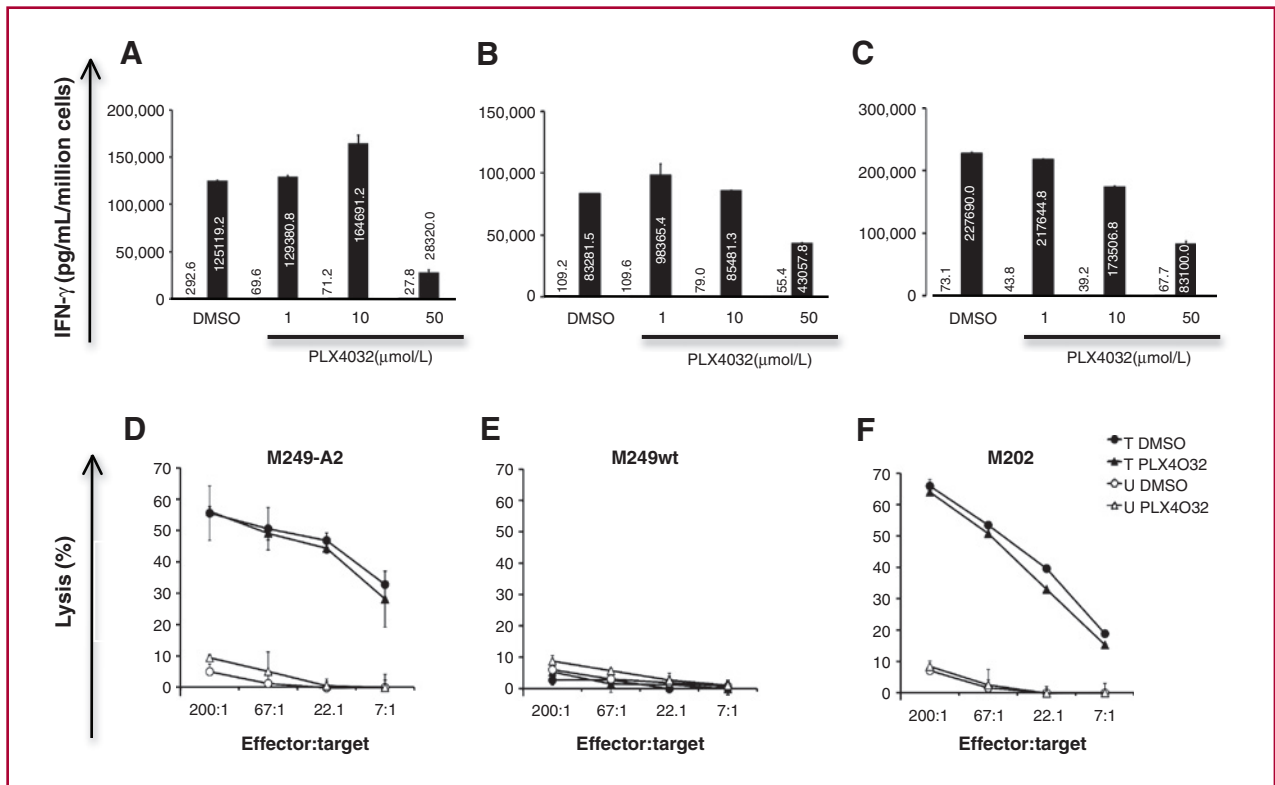
#### Effects of PLX4032 on antigen-specific cytokine release and cytotoxicity by T cells

To determine if PLX4032 alters the ability of T cells to respond to cognate antigen engagement by their TCR, activated PBMCs were retrovirally transduced with an HLA-A2.1-restricted TCR specific for MART-1 and were then exposed to PLX4032 or DMSO vehicle control. Their ability to respond with specific cytokine production to MART-1 was analyzed after coculture with HLA-matched or -mismatched melanoma cells or artificial antigen-presenting cells. Specific antigen recognition detected by IFN- $\gamma$  release was not affected at 1 and 10  $\mu\text{mol/L}$  of PLX4032, whereas there was a decrease at higher concentrations (Fig. 5A–C). A similar trend was evident for the Th2 cytokines IL-5 and IL-10, and for the chemokine MIP1- $\beta$  (Supplementary Fig. 5). Of note, one of the cell lines (M249) constitutively produces large amounts of IL-10,

which significantly decreased when cocultured with the TCR transgenic cells independent of exposure to PLX4032 (Supplementary Fig. 5D). To analyze the effects of PLX4032 on the cytotoxic activity of CTLs, HLA-A2.1-restricted MART-1 TCR transduced T cells were treated with PLX4032 or DMSO vehicle control and analyzed for cytotoxic activity against melanoma target cells. There was a specific lysis of HLA-A2.1<sup>+</sup> melanoma cells expressing MART-1 (M202 and M249-A2 cells), but no significant lysis of non-HLA-A2.1 targets (M249wt cells). The lysis of melanoma cells by MART-1-specific CTLs was neither enhanced nor suppressed with the exposure to PLX4032 up to concentrations of 50  $\mu\text{mol/L}$  (Fig. 5D–F).

#### Discussion

The studies presented herein show that the exposure of resting and proliferating PBMC to increasing concentrations of PLX4032 does not have a detrimental effect on their function at concentrations where this agent has marked antitumor activity against BRAF<sup>V600E</sup> melanoma cell lines. Most of our results are consistent with the notion that, at concentrations where PLX4032 has cytotoxic or cytostatic effects in melanoma cell lines, the viability and function of lymphocytes are maintained. These results provide preliminary information supporting the feasibility of combining oncogenic BRAF inhibitors with immu-



**Fig. 5.** Specific IFN- $\gamma$  secretion and lysis of HLA-A2.1 melanoma cells by the MART-1 TCR transgenic T cells. A–C, ELISA results showing IFN- $\gamma$  secretion by MART-1 TCR transgenic T cells exposed to increasing concentrations of PLX4032 for 24 hours. A, M202 (MART-1<sup>+</sup>, HLA-A\*0210<sup>+</sup>; black column), M238 (MART-1<sup>+</sup>, HLA-A\*0210<sup>-</sup>; white column). B, M249 (MART-1<sup>+</sup>, HLA-A\*0210<sup>-</sup>; white column), M249-A2.1 (MART-1<sup>+</sup>, HLA-A\*0210<sup>+</sup>; black column). C, K562-A2.1 (MART-1<sup>-</sup>, HLA-A\*0210<sup>+</sup>; white column), K562-A2.1 pulsed with MART-1<sub>26-35</sub> peptide (black column). Columns represent mean values from triplicate samples tested independently in 3 experiments; bars, SD. D–F, C<sub>r</sub><sup>51</sup> release assay of lytic activity against HLA-matched and -mismatched melanoma cells by MART-1 TCR transgenic cells exposed to 10  $\mu$ mol/L PLX4032 for 24 hours. U, untransduced; T, transduced with MART-1 TCR retrovirus. Columns represent mean values from triplicate samples tested independently in 4 experiments; bars, SD.

notherapy for the treatment of patients with advanced melanoma.

This conclusion was reached based on studies examining human PBMC exposed to PLX4032 *ex vivo*. It is acknowledged that these studies will require validation *in vivo*. It is possible that the *in vivo* effects exposure to PLX4032 may result in different effects on immune system function compared with our *ex vivo* studies. For example, as PLX4032 is more than 99% protein bound in serum, it is difficult to translate concentrations of PLX4032 used *in vitro* in 10% serum-containing culture media to concentrations achievable in patients with melanoma. Within the phase 1 testing of this agent, steady-state plasma concentrations were more than 80  $\mu$ mol/L (1, 4). However, the free concentration of PLX4032 should be less than 1% of this concentration, and the effective concentrations in tumors in humans have not been defined. Peak plasma concentrations of PLX4032 in mice dosed with 100 mg/kg daily were more than 100  $\mu$ mol/L. At this dose, the corresponding intratumor concentration of PLX4032 was 15  $\mu$ mol/L (30), suggesting that the range of concentrations studied by us may be relevant to the *in vivo* setting.

An indirect means to explore the effective concentrations of PLX4032 is the differential between highly sensitive and resistant melanoma cell lines tested *in vitro*. In our prior studies, BRAF<sup>V600E</sup> mutant melanoma cell lines were considered highly sensitive to PLX4032 when the IC<sub>50</sub> was below 1  $\mu$ mol/L, and relatively resistant when the IC<sub>50</sub> was above 10  $\mu$ mol/L. This is because the IC<sub>50</sub> of non-BRAF<sup>V600E</sup> mutant melanoma cell lines was generally between 10 and 50  $\mu$ mol/L; at that point, the concentrations of PLX4032 may have nonspecific effects that go beyond the targeted primary BRAF kinase inhibition (20). On the basis of these considerations, results showing that inhibitory growth and cytotoxic effects of PLX4032 require concentrations above 50  $\mu$ mol/L for resting lymphocytes, and the absence of proliferation inhibition with concentrations of more than 250  $\mu$ mol/L for activated lymphocytes, suggest that this agent has a wide margin between targeting BRAF<sup>V600E</sup> mutant melanoma and sparing lymphocytes from cytostatic or cytotoxic effects.

It is interesting to note that *in vitro* concentrations of 50  $\mu$ mol/L PLX4032 resulted in increased signaling through the MAPK pathway in T cells (but not B cells), detectable by the increase in phosphorylation of Erk1/2 and p38. As we

used the phosphoflow technique to analyze intracellular signaling, results are quantifiable and we can clearly define which cell subtype within PBMC mixtures is responsible for the phosphorylated signaling molecule changes (26). Increases in MAPK pathway output with PLX4032 and other class I RAF inhibitors in BRAF wild-type cells have been well documented recently (20, 31–33). This has been particularly evident in cells with oncogenic events upstream of wild type BRAF, like cells with activating RAS mutations or amplifications in receptor tyrosine kinases. Because the TCR functions as a surface receptor triggering signaling through the MAPK pathway upon antigen exposure or cross-linking CD3, it is not surprising that T cells should receive strong signaling through MAPK and have increased pathway output with exposure to PLX4032. Despite the increased MAPK signaling through Erk1/2 and p38 with high concentrations of PLX4032, the effector functions of lymphocytes decreased at concentrations beyond 50  $\mu\text{mol/L}$ . This may suggest that persistent signaling through the MAPK pathway enhanced by high concentrations of PLX4032 (likely supraphysiologic) would have a paradoxical effect of decreasing the ability of lymphocytes to respond to antigens encountered by releasing cytokines, as evidenced in our work.

Melanoma has long been regarded as an immune-responsive cancer. Recent results with the anti-CTLA4 antibody ipilimumab provided, for the first time, evidence that an immunotherapy strategy could change the natural course of metastatic melanoma and result in improvement in patient survival (34). The anti-CTLA4 antibody therapy adds to the body of evidence of immune responsiveness of melanoma to the prior FDA approved immunotherapeutic cytokines IFN- $\alpha$  and IL-2 (35), and the emerging evidence that the adoptive cell transfer (ACT) of large numbers of tumor antigen-specific T cells

results in high frequency of objective tumor responses in patients with advanced melanoma (36, 37). Therefore, it is a logical next step to combine melanoma immunotherapy with targeted therapy blocking activating oncogenes. Boni et al. reported that the PLX4032 analogue PLX4720 induces favorable changes in melanoma cells that predisposes them to become better targets of immunotherapy. In particular, their studies show an increase in melanosomal antigen expression by melanoma cell lines exposed to PLX4720, leading to increased ability to be recognized by melanoma-specific T cells. Complementary to these data, our results show that PLX4032 mostly spares T cells from cytostatic or cytotoxic effects, and maintains T-cell function at the concentrations of this agent that are clinically relevant. Together, these results support the consideration of combinations of class I BRAF inhibitors with immunotherapy for the treatment of patients with metastatic melanoma.

### Disclosure of Potential Conflicts of Interest

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