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TITLE: Evaluation of the immunologic impact of RAF Inhibitors to Guide Optimal Combination of RAF inhibitors and Immunotherapy for the treatment of Advanced Melanoma

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**ABSTRACT**

Our hypothesis is that combination therapy with MAPK inhibitors and immunotherapy will result in more rapid and durable control of melanoma than either modality alone. This hypothesis has gained support from several recent publications.1-4 Several proposed mechanisms for enhanced anti-tumor activity have been explored including changes in regulation of PD-L1 expression on tumor cells, changes in tumor antigen expression by tumor cells and changes in immune cell infiltration of the tumor microenvironment. So far, clear studies testing the role of enhanced tumor antigen release/antigen presentation or enhanced T cell activation have not been completed and this remains a fertile area to investigate the mechanistic basis for productive combination of targeted inhibitors and immunotherapies. In the present report, we review our findings to date and describe a pattern of paradoxical T cell activation by BRAF inhibitors that results in increased T cells upregulation of activation markers, cytokines and proliferation in vitro and in vivo. These findings represent one mechanism that may be exploited to maximize the clinical benefit of combination therapies. These finding are contrasted to our observations with MEK inhibitor treatment where T cell activation (including upregulation of PD-1, ICOS, CD25, CD69) are diminished in the presence of drug. This clearly sets the stage for the upcoming experiments in mouse models of melanoma testing the combination of checkpoint blockade and BRAF or MEK inhibitors in vivo.
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INTRODUCTION:

Our hypothesis is that combination therapy with MAPK inhibitors and immunotherapy will result in more rapid and durable control of melanoma than either modality alone. This hypothesis has gained support from several recent publications.¹⁴ Several proposed mechanisms for enhanced anti-tumor activity have been explored including changes in regulation of PD-L1 expression on tumor cells, changes in tumor antigen expression by tumor cells and changes in immune cell infiltration of the tumor microenvironment. So far, clear studies testing the role of enhanced tumor antigen release/antigen presentation or enhanced T cell activation have not been completed and this remains a fertile area to investigate the mechanistic basis for productive combination of targeted inhibitors and immunotherapies. In the present report, we review our findings to date and describe a pattern of paradoxical T cell activation by BRAF inhibitors that results in increased T cells upregulation of activation markers, cytokines and proliferation in vitro and in vivo. These findings represent one mechanism that may be exploited to maximize the clinical benefit of combination therapies. These finding are contrasted to our observations with MEK inhibitor treatment where T cell activation (including upregulation of PD-1, ICOS, CD25, CD69) are diminished in the presence of drug. This clearly sets the stage for the upcoming experiments in mouse models of melanoma testing the combination of checkpoint blockade and BRAF or MEK inhibitors in vivo.

BODY:
According to the Statement of Work we have focused on the following areas described below:

1) Test the impact of targeted inhibitors (PLX4720, AZD6244) on T cell activation in vitro and in vivo.

1a) Test the effect of targeted inhibitors on expression of clinically relevant markers (ICOS, CTLA-4, PD-1) in T cells activated in vitro.

1b) Evaluate the effect of targeted inhibitors on activation and expansion of tumor-antigen specific (pmel-1) transgenic T cells in vivo.

1c) Characterize the role of targeted inhibitors in releasing antigen by measuring expansion of tumor-antigen specific (pmel-1) tumors.

1d) Correlate pre-clinical findings by evaluating banked samples of T cells from patient previously treated with PLX4720 or AZD6244.

(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint blocking antibodies (CTLA-4, PD-1) in an immunocompetant mouse model of BRAF mutant melanoma.

2a) Combine targeted inhibitors with CTLA-4 blockade (or PD-1 blockade) and evaluate endpoints of therapeutic efficacy and immune markers in the tumor and periphery.

2b) Compare three schedules (concomitant/sequential/alternating) combining targeted inhibitors
with checkpoint blockade.

1a) Test the effect of targeted inhibitors on expression of clinically relevant markers (ICOS, CTLA-4, PD-1) in T cells activated in vitro.

This first aim has been explored and is described in the attached manuscript (Appendix 1). Specific observations that we report upon in this manuscript include a description of a pattern of paradoxical activation of T cells exposed to BRAF inhibitors that is reflected in the upregulation of activation markers and in T cell proliferation in vitro (measured by ki67 upregulation). This is seen in Jurkat cells (Appendix 1 Figure 1A) and in healthy donor CD4 and CD8 positive T cells (Appendix 1 Figure 1 C and D). This pattern of paradoxical activation is seen in T cells activated by anti-CD3 antibody and by T cells activated in an antigen specific fashion using peptide pulsed APCs (Appendix 1 Figure 2). The mechanism of paradoxical activation in T cells is best explained by increased signaling via phosphorylated ERK, as demonstrated in vitro (Appendix 1 Figure 3). The effect of BRAF inhibitor treatment is contrasted to the effect of the MEK inhibitor, which attenuates T cell activation (Appendix 1 Figure 3E). In fact, the paradoxical activation of T cells by the BRAF inhibitor may be reversed by the additional of a MEK inhibitor. Moreover, the addition of MEK inhibitor to T cell culture results in diminished upregulation of a host of activation markers including PD-1, CD25, ICOS, and CD69 (see Figure 1 below). Some markers appear to be more robustly inhibited (CD25 and PD-1) while other appear to be more modestly reduced (CD69, ICOS). Additional studies expanding the repertoire of activation markers evaluated and comparing BRAF and MEK inhibitors are ongoing.

**Figure 1.** General approach for in vivo stimulation of T cells in the presence of inhibitor
**Figure 2.** MEK inhibitors attenuate the upregulation of activation markers in T cells cultured in vitro. T cells cultured in the presence of vehicle control (veh) or MEK inhibitor PD325901 (PD901) were activated with anti-CD3 antibody. After twenty-four hours, expression of activation markers PD-1, CD69, CD25, and ICOS were measured by flow cytometry. Error bars represent SD for samples analyzed in triplicate.

**Figure 3:** AZD6244 effects Human CD4+ T cell activation in a concentration dependent manner. Purified human CD4+ T cells were activated with a combination of anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of AZD6244 ranging from .005µM to 20µM. Flow analysis for indicated (CTLA-4, ICOS, CD25, ICOS, PD-1, CD69) markers was performed on all CD4+ T cells.
1b) Evaluate the effect of targeted inhibitors on activation and expansion of tumor-antigen specific transgenic T cells in vivo.

This aim has been explored and is described in the attached manuscript (Appendix 1). Specific observations that we report upon in this manuscript expand upon the initial observations on T cells activated in vitro by testing the impact of BRAF inhibitors on T cell activation in vivo. Specifically, we report that T cell expansion after antigen-specific stimulation in increased in a dose-dependent fashion in the presence of a BRAF inhibitor. (Appendix 1 Figure 4 A) Furthermore, paradoxical ERK pathway activation is tested ex vivo in mice treated systemically with BRAF inhibitor and we find that BRAF inhibitor increases ERK signaling, as previously described in vitro. (Appendix 1 Figure 4 B and C)

Additional experiments comparing the effects of BRAF and MEK inhibitors on T cell expansion in vivo suggest that (as seen in vitro), these two inhibitors that can have similar effects on tumor cells, have very different effects on T cells. Specifically, T cells stimulated in an antigen-specific fashion in vivo have robust expansion in the presence of BRAF inhibitor, but greatly diminished in the presence of MEK inhibitor (Figure 2, below).
Figure 5. MEK inhibitors and BRAF inhibitors have opposing effects on T cells activated in vivo. Mice treated systemically with a vehicle control, the BRAF inhibitors PLX4720 or the MEK inhibitor PD325901 were immunized with peptide to expand antigen-specific TCR transgenic T cells. After 5 days, the expansion of antigen specific T cells was quantified by flow cytometry. Five mice were treated in each group and errors bars represent SD.

Figure 6. MEK inhibitors PD901 and AZD6244 have similar, inhibitor effects on antigen-specific T cells activated in vivo. Mice treated systemically with a vehicle control, the MEK inhibitor AZD6244 or the MEK inhibitor PD325901 were immunized with peptide to expand antigen-specific TCR transgenic T cells. After 5 days, the expansion of antigen specific T cells was quantified by flow cytometry. Five mice were treated in each group and errors bars represent SD.
Figure 7. Mice treated systemically with the MEKi AZD6244 or vehicle control were treated for 4-5 days. Spleens were harvested and immediately ex vivo, splenocytes were stimulated and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers.

Figure 8 (above). Mice treated systemically with the MEKi AZD6244 or vehicle control. Spleens were harvested, stimulated ex vivo and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers for CD4+ T eff cells (top left), CD8+ (top right panel) or Treg (bottom right panel).

Figure 9 (below). Mice treated systemically with the MEKi AZD6244 or vehicle control. Spleens were harvested, stimulated ex vivo and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers for CD4+ T eff cells (left), T reg (middle) or CD8+ (right) or Treg.
1c) Characterize the role of targeted inhibitors in releasing antigen by measuring expansion of tumor-antigen specific (pmel-1) tumors.

Technical challenges prevented us from generating meaningful data on this aim, despite several attempts.

1d) Correlate pre-clinical findings by evaluating banked samples of T cells from patient previously treated with PLX4720 or AZD6244.

During this period of funding, we obtained IRB approval for the analysis of human samples and developed a flow cytometry panel of this analysis, as demonstrated in Figure 3, below. However, the challenges related to the quality of banked PBMCs and small dataset precluded general conclusions related to this data.

![Figure 10. Detection of activation markers on human peripheral blood T cells in a newly developed multiparametric flow cytometry panel.](image)

(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint blocking antibodies (CTLA-4, PD-1) in an immunocompetent mouse model of BRAF mutant melanoma.

2a) Combine targeted inhibitors with CTLA-4 blockade (or PD-1 blockade) and evaluate endpoints of therapeutic efficacy and immune markers in the tumor and periphery.
Figure 11. Mice treated systemically with the BRAFi PLX4720 or vehicle control. Spleens, draining lymph nodes and tumors were harvested. Splenocytes, cells from draining lymph nodes, and tumor infiltrating immune cells were analyzed for % of indicated populations. Tumor cells were analyzed for surface expression of CD80, MHC, OX40L and PD-L1.
Figure 12. Mice treated systemically with the BRAFi, MEKi, combination or vehicle control according to a continuous, pulsatile early or pulsatile late schedule. Spleens, draining lymph nodes and tumors were harvested. Tumor infiltrating immune cells were analyzed for % of indicated populations.
Within each of these treatment groups, we evaluated the quantity and quality of immune infiltrate and made the following observations:

1. Continuous dosing of BRAF/MEKi results in reduced TIL, especially in MEKi and combination treated animals.
2. Pulsatile dosing appears to spare the depletion of TILs and in this case, combination dosing can increase the ratio CD8/Treg depending upon the timing of the pulsatile dose (see the far left panel in Figure 2b).

In addition, we compared the phenotypic markers (CTLA-4, PD-1, PD-L1, ki67, OX40, and GITR) expressed by CD8+, CD4+ FoxP3- and CD4+FoxP3+ (regulatory) T cells in mice treated with continuous versus pulsatile dosing of the BRAF inhibitor, the MEK inhibitor or the combination of both (Figure 3, 4, 5,6 ) and made the following observations:

1. Expression of T cell molecules can vary significantly depending upon treatment type and treatment regimen for example:
   a. CTLA-4 expression on CD8 T cells treated with vehicle vs PLX is much lower than in mice treated with AZD vs combo
   b. PD-1 expression is diminished in CD4+ T cells in mice treated with AZD or combo compared to those treated with vehicle or PLX
   c. OX40 expression is diminished in T regs treated with AZD or combo compared to Vehicle or PLX
   d. The effects on TILs may be quite different than the effect on immune cells in the LN

Figure 13. Evaluation of Exhaustion markers on TILs.
Figure 14. Evaluation of activation markers on TILs

A
Continuous

B
Pulsatile (early)

C
Pulsatile (late)

Figure 15. Evaluation of exhaustion markers on dLNs.
Figure 16. Evaluation of activation markers on dLN
b. Compare three schedules (concurrent/sequential/alternating) combining targeted inhibitors with checkpoint blockade.

Figure 17. Mice treated systemically with the BRAFi PLX4720 or vehicle control or combination BRAFi and anti-PD01 or anti-LAG-3 antibody on a concurrent schedule. Tumor growth measured in days post Tamoxifen induction.
The data represented in Figure 17 illustrates several points that have been instructive in developing this system further. First of all, response to the BRAFi across multiple spontaneous tumors is quite variable with some tumors showing relatively high sensitivity to BRAFi (top right panel, mouse #2), whereas other appear to have very low sensitivity. This represents a significant challenge since the diversity within this group would make it challenging to reliably detect differences between BRAFi alone and in combination with immunotherapy. To understand this observation better, we have grown out individual spontaneous tumors and tested their sensitivity to BRAFi and confirmed this diverse sensitivity. To meet this challenge, we selected a single BRAF mutant tumor cell line, WG492 that (1) transplantable and grows progressively in the syngeneic, immunocompetent B6 mouse and (2) is sensitive to BRAFi as demonstrated in Figures 18-19. This approach allows us to overcome the challenge of heterogeneous sensitivity to BRAFi (as well as more general heterogeneity of the spontaneous tumors).

Figure 18. Immunocompetant B6 mice transplanted with titrated numbers of syngeneic WG492 BRAF mutant tumor cell line. Tumor growth measured in days post implantation.
The second observation to emerge from the data in Figure 17 and similar experiments is that these spontaneous tumors appear to be relatively poorly immunogenic. It has been previously hypothesized that “spontaneous” tumor models may generate tumors that are less immunogenic since they have not acquired as many mutations (passive mutations that may be targets for an immune response) as would be acquired by tumors that develop over a longer period of time. The WG492 cell line offers us an opportunity to explore this possibility as well and we are presently working to introduce additional mutations in the WG492 cell line and test their immunogenicity/tumorogenicity.

Lastly, we were able to optimize the evaluation of the combined effect of BRAFi plus CTLA-4 blockade versus BRAFi plus PD-1 blockade vs triple therapy using this mouse model (WG492) (Figure 20). WG492 cells were implanted intradermally into C57BL/6 mice. Vemurafenib (PLX4032) treatment began two days post tumor implantation using 25mg/kg dose, administered p.o. BIDx5 for two weeks. The mice also received 100ug of aCTLA-4 and 250ug of aPD-1 treatments (via i.p. injections) three days post tumor implantation, every three days until the end of the experiment. The resulting tumor growth in two independent experiments is shown in Figure 21.

Figure 19. Immunocompetant B6 mice transplanted with syngeneic WG492 BRAF mutant tumor cell line. Tumor growth measured in days post implantation. Mice treated systemically with the BRAFi PLX4720 or vehicle control beginning on either 2 days or 4 days post implantation.
Figure 20. Optimized dosing of BRAFi and CTLA-4 blockade

Figure 21. Triple combination – CTLA-4 blockade, PD-1 blockade, and BRAFi.
KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by upregulation of T cell activation markers in vitro
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by upregulation of T cell activation markers in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by increased ERK signaling – supporting the mechanism of paradoxical activation
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by decreased ERK signaling.
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cell in vivo
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells in vivo.
- Development of a human T cell activation multiparametric flow cytometry panel
- Development and characterization of a cell line from the murine transgenic (BRAF/PTEN) model of spontaneous melanoma as a potential superior approach to testing combinations.
- Demonstration of modulation of the tumor microenvironment by BRAFi, MEKi and the combination in a mouse model of BRAF mutant tumor.

Alterations include:
- Diminished TIL in mice treated continuously with BRAFi, MEKi or combination
- Increased CD+ TIL in mice treated continuously with MEKi or combination
- Most favorable CD8/Treg ration in mice treated continuously with MEKi or combination

- Demonstration differential modulation of the tumor microenvironment depending upon continuous versus pulsatile dosing of BRAFi, MEKi and the combination in a mouse model of BRAF mutant tumor.
- Pulsatile dosing has a diminished effect in reducing TIL in tumors
- Pulsitile dosing (day 3) still demonstrated enhanced CD8/Treg ratio in MEKi treated mice

- Demonstration of altered T cell phenotype in TIL from mice treated with BRAFi, MEKi or combination
  - Increase CTLA-4 expression in CD8+ TIL in mice treated with MEKi or combination
  - Diminished PD-1 expression in CD4+ TIL in mice treated with MEKi or combination
  - Diminished OX40 expression in Treg TIL in mice treated with MEKi or combination
  - Differences between TIL and draining LN immune cells with regard to effect of BRAFi, MEKi, combination
• Testing of immunotherapy (CTLA-4 or PD-1 blockade) and targeted inhibitor (PLX4720) combinations in murine BRAF mutant melanoma model WG492.
  - Optimization of schedule for dosing agents established
  - Clear evidence for superior anti-tumor effect for BRAFi+CTLA-4 blockade or BRAFi+ PD-1 blockade versus monotherapy.

REPORTABLE OUTCOMES:
The following reportable outcomes have been accomplished during this funding period.

• Manuscripts published


• Abstracts and presentations

None during this reporting period

• Employment or research opportunities applied for and/or received based on experience/training supported by this award

Margaret Callahan, MD, PhD was appointed to a faculty position (Assistant Attending) at the Memorial Sloan-Kettering Cancer Center as a result of experience/training supported by this award.

Dr. Callahan and Dr. Merghoub have applied (as co-investigators) for a SU2C team science grant to study the effect of TKIs used in the treatment of NSCLC on immune cells, based upon experimental approaches and techniques developed based upon this award.

Dr. Callahan and Dr. Merghoub have applied for and received (as co-investigators) internal (MSKCC) funding to extend the work on MEKi as immune modulators based upon experimental approaches and techniques developed based upon this award.

CONCLUSION:

In these studies, we have characterized the effects of targeted inhibitors (BRAFi, MEKi, combination) on T cell activation in the presence of checkpoint blockade (CTLA-4 and PD-1) as well as the effects of targeted inhibitor/checkpoint blockade combinations on anti-tumor activity. These combinations show superior activity compared to targeted therapy or checkpoint blockade alone and shed a promising light on the further development of combination in the clinic. There are many additional questions to be pursued including:

1. How can the benefit of targeted inhibitor/checkpoint blockade combinations be maximized while limiting the potential toxicities?
2. How will the short-term benefits of combinations of targeted inhibitor/checkpoint blockade combinations compare to long-term effects? Is effective anti-tumor memory being generated and sustained?
3. How will the potential benefits of BRAFi and MEKi as partners for immunotherapies compare to a wider array of targeted inhibitors presently in use in the clinic?
REFERENCES: