Inhibition of p-STAT3 Enhances IFN-α Efficacy against Metastatic Melanoma in a Murine Model

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Abstract

Purpose: Melanoma is a common and deadly tumor that upon metastasis to the central nervous system has a median survival duration of <6 months. Activation of the signal transducer and activator of transcription 3 (STAT3) has been identified as a key mediator that drives the fundamental components of melanoma malignancy, including immune suppression in melanoma patients. We hypothesized that WP1193, a novel inhibitor of STAT3 signaling, would enhance the antitumor activity of IFN-α against metastatic melanoma.

Experimental Design: Combinational therapy of STAT3 blockade agents with IFN-α was investigated in a metastatic and an established syngeneic intracerebral murine tumor model of melanoma. The immunologic in vivo mechanisms of efficacy were investigated by T-cell and natural killer (NK) cell cytotoxic assays.

Results: IFN-α immunotherapy was synergistic with WP1193 showing marked in vivo efficacy against metastatic and established intracerebral melanoma. At autopsy, it was noted that there was a decreased trend in mice with melanoma developing leptomeningeal disease treated with combinational therapy. The combinational approach enhanced both NK-mediated and T-cell-mediated antitumor cytotoxicity.

Conclusions: The immune modulatory effects of STAT3 blockade can enhance the therapeutic efficacy of IFN-α immunotherapy by enhancing both innate and adaptive cytotoxic T-cell activities. This combinational therapy has the potential in the treatment of metastatic melanoma that is typically refractory to this type of immune therapeutic approach. Clin Cancer Res; 16(9); 2550–61. ©2010 AACR.

Patients with stage IV melanoma, especially upon metastasis to the brain, have a median survival duration of <6 months (1) despite multimodality therapy, and the prognosis is even more dire in patients who develop leptomeningeal disease (LMD), who have a median survival of <2 months. Several large, cooperative-group adjuvant trials have documented a 24% to 38% reduction in the relative relapse risk with the use of high-dose IFN-α for stages II and III melanoma with overall survival prolongation (2). In contrast, no large cooperative-group trials have shown significant prolongation of survival with IFN-α for inoperable stage IV melanoma and especially for those with central nervous system (CNS) melanoma and metastasis. IFN-α is known to have powerful effects on immune cells, including enhancing natural killer (NK) cell tumor cytotoxicity (3), dendritic cell maturation, Th1 skewing, enhancement of T-cell survival, inducing immunologic memory (4), and inhibiting the invasive ability of cancer cells (5). Therapeutic strategies using IFN-α in melanoma patients with LMD have included the direct intraventricular administration of IFN-α, which resulted in the clearance of malignant cells in the cerebrospinal fluid (6) but was confounded by significant and sustained neurotoxicity (7). Thus, there is a clear clinical need to identify treatment modalities that exert therapeutic effects in melanoma patients with CNS disease.

A key transcription factor that drives the fundamental components of melanoma tumorigenesis and metastasis has been identified—the signal transducer and activator of transcription 3 (STAT3; ref. 8). Growth factors and cytokines, including interleukin-6 (IL-6), activate Janus-activated kinase 2, which then activates STAT3 by phosphorylation of the tyrosine residue (Tyr705) in the STAT3 transactivation domain (p-STAT3) resulting in translocation into the nucleus and the expression of a variety of target genes. STAT3 is frequently overactivated in most cancers, including melanoma, and propagates...
p-STAT3 has divergent functions within the various immune cell populations. Tregs, and STAT3 binds to the first intron of the FoxP3 gene (23). We have previously shown that the number and functional activity of these FoxP3+ Tregs can be inhibited with WP1066, the small molecule inhibitor of the p-STAT3 pathway (24). Furthermore, the Th17 immune subset, which is induced by the IL-6/STAT3 pathway, has been shown to promote melanoma growth (25). Thus, STAT3 seems to be a key molecular hub for inhibiting immune surveillance and clearance of malignancy. Our hypothesis was that the addition of a STAT3 inhibitor would enhance the therapeutic efficacy of IFN-α for advanced melanoma, including within the typically treatment refractory CNS, and this would be secondary to enhanced NK and cytotoxic T cell–mediated tumor cytotoxicity.

Materials and Methods

Tumor cell lines and murine models. The B16/F10 murine melanoma cell line was derived from a spontaneous melanoma in the C57BL/6J mouse of the H-2B background and was provided by Dr. Isaiah Fidler (University of Texas M.D. Anderson Cancer Center). The B16 model system is known for its propensity to develop LMD (26). The B16 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 and 95% air. All cell lines were grown in antibiotic-free medium and were free of Mycoplasma contamination (27).

For the in vivo experiments, we used 4- to 6-week-old female C57BL/6J mice maintained in the University of Texas M.D. Anderson Cancer Center Isolation Facility in accordance with Laboratory Animal Resources Commission standards and conducted according to an Institutional Animal Care and Use Committee–approved protocol (08-06-11831). A mouse was euthanized when it became unable to reach food or water. To induce intracerebral tumors in C57BL/6J mice, B16 cells were collected in logarithmic growth phase, washed twice with PBS, mixed with an equal volume of 10% methyl cellulose and RPMI 1640, and loaded into a 250-μL syringe (Hamilton) with an attached 25-gauge needle. The needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments). The intracerebral tumorigenic dose for the B16 cells was 5 × 10^5 in a total volume of 5 μL. To induce metastatic disease, 1 × 10^5 cells in a total volume of 100 μL were injected into the tail vein.
Cell proliferation/survival assay. For cell proliferation assays, B16 cells were seeded at a density of 1,000 per well in 96-well culture plates and were treated with IFN-α (0-1,250 units/mL; PBL Interferon Source) with or without WP1193 (0.5 or 1.0 μmol/L). After 72 hours of treatment, 25 mL of 5 mg/mL MIT (Sigma-Aldrich) solution were added to each well, and the cells were cultured for 3 hours at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The cells were lysed with 100 μL/well of lysing buffer (50% dimethylformamide, 20% SDS [pH 5.6]) and incubated at room temperature overnight. Cell proliferation and viability were evaluated by reading the absorbance at 570 nm.

Immunoblotting analysis. Murine melanoma B16 cells and splenocytes were used for protein isolation and immunoblotting analysis as described below. B16 cells were seeded at a density of 2 × 10⁶ per well in six-well culture plates and incubated at 37°C in an atmosphere containing 5% CO2 with RPMI 1640 overnight. Afterward, B16 cells were cultured in the absence or presence of WP1193 (5 and 10 μmol/L). After 3.5 hours, the B16 cells were further cultured in the absence or presence of 2,000 units/mL of IFN-α for 30 minutes. For the splenocyte preparations, spleens from two 4- to 6-week-old female mice were harvested and disassociated into a single-cell suspension. After erythrocytes were lysed with 1× RBC lysis buffer (eBioscience), splenocytes were washed once with RPMI 1640 and were ready as effector cells for the standard cytotoxicity assay. The ratios of splenocyte effector cells to B16 target cells were 30:1 and 100:1. After 48 hours of incubation, the CFSE-labeled B16 melanoma cells were removed from the plates with trypsin-EDTA (0.05%) and analyzed by FACS. The B16 cells that were stained with propidium iodide (PI; BD Biosciences) to distinguish viable cells from nonviable cells. B16 cells that were stained with CFSE and PI were considered nonviable. Flow cytometric acquisition of the B16 target cells was done with a FACSCalibur flow cytometer (BD Biosciences), and data analysis was done using FlowJo software (TreeStar).

NK cell and T-cell cytotoxicity assay against melanoma cells. Splenocytes were prepared as described above. NK1.1+CD3− NK effector cells or CD3+CD8+ T effector cells were sorted from splenocytes on a FACS Aria Cell Sorter (BD Biosciences) with FITC-conjugated antimonu NK1.1 (eBioscience), PE-conjugated anti-CD3, and allophycocyanin (APC)-conjugated anti-CD8 antibodies (Miltenyi Biotec). B16 target cells were prepared as described above. The ratio of NK1.1+CD3− NK effector cells or CD8+ T effector cells to B16 target cells was 10:1 and 5:1, respectively. We were limited to minimizing the titration ratios of effector to targets secondary to limitations of purification and the logistics of the assay set up. In the presence of NK1.1+CD3− NK cells or CD8+ T cells, treatment groups consisted of B16 target cells alone, B16 cells with 2 μmol/L of WP1193, B16 cells with 2,000 units/mL of IFN-α, and B16 cells with 2 μmol/L of WP1193 and 2,000 units/mL of IFN-α. After 48 hours of incubation, the CFSE-labeled B16 melanoma cells were removed from treatment with WP1193 for 16 days. Single-cell suspensions were prepared from bone marrows, lymph nodes, spleens, thymuses, and peripheral blood of mice. Single cells were surface-stained by FITC-conjugated anti-CD4 (L3T4), PerCP-conjugated anti-CD8 (53-6.7), and allophycocyanin-conjugated anti-CD25 (PC61), and the cells were further subjected to intracellular staining with phycoerythrin (PE)–conjugated monoclonal antibodies (mAb) to mouse FoxP3 (clone FJK-16s; eBioscience) using staining buffers and conditions specified by the manufacturer.

Ex vivo splenocytes cytotoxicity assays. Spleens from 4- to 6-week-old female mice in the above treatment scheme were harvested and disassociated into a single-cell suspension. After erythrocytes were lysed with 1× RBC lysis buffer (eBioscience), splenocytes were washed once with RPMI 1640 and were ready as effector cells for the standard cytotoxicity assay. For the splenocyte preparations, spleens from two 4- to 6-week-old female mice were harvested and disassociated into a single-cell suspension. After erythrocytes were lysed with 1× RBC lysis buffer (eBioscience), splenocytes were washed once with RPMI 1640 and were ready as effector cells for the standard cytotoxicity assay. The mixtures were further cultured in the absence or presence of 2,000 units/mL of IFN-α for 30 minutes. After 1.5 hours, the splenocytes were further cultured in the absence or presence of 2,000 units/mL of IFN-α for 30 minutes. Afterward, all the above B16 cells and splenocytes were pelleted and rinsed with ice-cold PBS at 1,500 rpm for 5 minutes. The cells were lysed for 30 minutes in ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA] containing 1% Triton X-100 and phosphatase and protease inhibitors (Sigma-Aldrich). The lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatants were collected and quantified for protein content. Equal amounts of proteins (65 μg) were electrophoretically fractionated in 8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with specific antibodies against p-STAT3 (Tyr705), STAT3 (Cell Signaling Technology, Inc.), and β-actin (Sigma-Aldrich). Autoradiography of the membranes was done using Amersham enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences). The densities of the protein bands compared with the β-actin protein control were measured with the Image J program provided by NIH (http://rsb.info.nih.gov/ij/index.html).

Determination of inhibition of Tregs in vivo. To ascertain the inhibition of Tregs within the bone marrow, lymph node, spleen, thymus, and peripheral blood compartment, non–tumor-bearing mice were treated with IFN-α or “empty” plasmid control, WP1193, or IFN-α in combination with WP1193 for 16 days. Single-cell suspensions were prepared from bone marrows, lymph nodes, spleens, thymuses, and peripheral blood of mice. Single cells were surface-stained by FITC-conjugated anti-CD4 (L3T4), PerCP-conjugated anti-CD8 (53-6.7), and allophycocyanin-conjugated anti-CD25 (PC61), and the cells were further subjected to intracellular staining with phycoerythrin (PE)–conjugated monoclonal antibodies (mAb) to mouse FoxP3 (clone FJK-16s; eBioscience) using staining buffers and conditions specified by the manufacturer.
the plates with trypsin and analyzed by FACS. Then, B16 cells were stained with PI (BD Biosciences) to distinguish viable cells from nonviable cells. B16 cells that were stained with CFSE and PI were considered nonviable. Flow cytometric acquisition of the B16 target cells was done with a FACSCalibur flow cytometer (BD Biosciences), and data analysis was done using FlowJo software (TreeStar).

Detection of NK cell receptors. Spleens from 4- to 6-week-old female mice were harvested and dissociated into a single-cell suspension as described above. Splenocytes were seeded at a density of 4 × 10⁶ cells per well in 24-well culture plates and cultured with RPMI 1640 in absence or presence of WP1193 (2 μmol/L) and/or INF-α (2,000 units/mL) for 24 hours. Afterwards, the cells were harvested and washed twice in PBS with 5% FCS, resuspended in staining buffer, and labeled with FITC-conjugated or PE-conjugated anti-mouse NK1.1 (eBioscience) to identify the NK population. To stain in duplicate, 10⁶ cells were transferred to 96-well plates and cultured with RPMI 1640 in absence or presence of WP1193 (2 μmol/L) and/or INF-α (2,000 units/mL) for 24 hours. Afterwards, the cells were harvested and washed twice in PBS with 5% FCS, resuspended in staining buffer, and labeled with FITC-conjugated or PE-conjugated anti-mouse NK1.1 (eBioscience) to identify the NK population.

To ascertain the MHC I, MHC II, and NK cell ligands on B16 melanoma cells. To ascertain the MHC I, MHC II, and NK cell ligands on melanoma cells, B16 cells were seeded at a density of 2 × 10⁶ per well in 24-well culture plates and cultured with RPMI 1640 in the absence or presence of WP1193 (2 μmol/L) and/or INF-α (2,000 units/mL) for 24 hours. Afterwards, the cells were harvested and washed twice, and 10⁶ cells in duplicate were Fc blocked with purified rat anti-mouse CD16/CD32 serum (BD Biosciences) for 15 minutes at room temperature. Secondary staining was done with FITC-conjugated rat anti-mouse CD94 (KLRD1) mAb (LifeSpan Biosciences), biotin-conjugated rat anti-mouse NKG2C mAb (AbD Serotec), PE-conjugated rat anti-mouse NKG2D (CD314) mAb (Biolegend), FITC-conjugated rat anti-mouse Nkp46 (NCR1) mAb (R&D Systems), or Alexa Fluor 647-conjugated rat anti-mouse CD226 (DNAM-1) mAb (Biolegend). Negative control wells were stained with the corresponding isotypes. After incubation, the cells were washed twice with FACS buffer and then analyzed with a BD FACSCalibur with gates set for viable splenocytes.

Detection of MHC I, MHC II, and NK cell ligands on B16 melanoma cells. To ascertain the MHC I, MHC II, and NK cell ligands on melanoma cells, B16 cells were seeded at a density of 2 × 10⁶ per well in 24-well culture plates and cultured with RPMI 1640 in the absence or presence of WP1193 (2 μmol/L) and/or INF-α (2,000 units/mL) for 24 hours. Afterwards, the cells were harvested and washed twice, and 10⁶ cells in duplicate were Fc blocked with purified rat anti-mouse CD16/CD32 (BD Biosciences) for 15 minutes at room temperature. Secondary staining was done with FITC-conjugated rat anti-mouse CD94 (KLRD1) mAb (LifeSpan Biosciences), biotin-conjugated rat anti-mouse NKG2C mAb (AbD Serotec), PE-conjugated rat anti-mouse NKG2D (CD314) mAb (Biolegend), FITC-conjugated rat anti-mouse Nkp46 (NCR1) mAb (R&D Systems), or Alexa Fluor 647-conjugated rat anti-mouse CD226 (DNAM-1) mAb (Biolegend). Negative control wells were stained with the corresponding isotypes. After incubation, the cells were washed twice with FACS buffer and then analyzed with a BD FACSCalibur with gates set for viable splenocytes.

Immune therapeutics. pORF:INF-α (IFN-α) plasmid was obtained from InvivoGen. Hydrodynamic gene transfer consisted of a single i.v. injection of 3 μg endotoxin-free pORF plasmid encoding murine IFN-α or pORF control plasmid DNA (InvivoGen) in 2 mL of saline, as previously described (28). This results in the sustained in vivo expression of IFN-α at serum levels of 900 pg/mL for >30 days after administration (29). The STAT3 inhibitor WP1193 was synthesized and supplied by Dr. Priebe (University of Texas M.D. Anderson Cancer Center). WP1193, a third-generation analogue inhibitor of the p-STAT3 pathway, was dissolved in a mixture of 20 parts DMSO to 80 parts polyethylene glycol 300 (Sigma-Aldrich) at titered concentrations and delivered in a final volume of 100 μL. Before use, WP1193 was stored as a lyophilized powder at 4°C.

Treatment for the established tumors was with WP1193 (starting on day 3) and/or IFN-α (starting on day 5) after B16 tumor cell challenge. Mice were injected i.v. with 2 mL (3 μg) of IFN-α plasmid in saline once. Mice were treated with a subtherapeutic dose of 30 mg/kg of WP1193 by oral gavage (e.g.) in a vehicle of DMSO/polyethylene glycol 300 (20 parts/80 parts) on Monday, Wednesdays, and Fridays for five treatments (the metastatic model) and nine treatments (the intracerebral model). For the metastatic model, the mice were euthanized on day 14 after the B16 cell i.v. injection. Lungs were dissected, and B16 nodules in the lungs were counted. For the intracerebral model, survival was observed. When mice were treated in the therapeutic range of 40 mg/kg, >80% of animals survived long term (>70 days) and synergy with IFN-α could not be assessed (24). Ten mice per experimental group were used, including treatment with the DMSO/polyethylene glycol 300 vehicle alone in the control group.

Statistics. Kaplan-Meier product-limit survival probability estimates of overall survival were calculated (30), and log-rank tests (31) were done to compare overall survival between treatment groups and the control arm. Ex vivo or in vitro data are presented as means ± SEM of three repeated experiments. Student's t test was done. A P value of <0.05 was considered statistically significant.

Results

WP1193, an inhibitor of p-STAT3. Using molecular modeling and medicinal chemistry approaches, we designed and developed a panel of unique small molecule inhibitors (32) that block STAT3 phosphorylation (33, 34), in vitro and in vivo, based on the caffeic acid benzyl ester/AG490 scaffold. WP1193 is a third-generation analogue that has an additional aromatic ring on the benzyl amine moiety (Fig. 1A) and was selected for these studies based on its potential to be a potent immune modulator. Both IFN-α and WP1193 have direct cytotoxic effects on B16, with the combination therapy exerting an additive effect (Fig. 1B). WP1193 can inhibit p-STAT3 in B16 cells and in immune cells (Fig. 1C and D, respectively).

In vivo treatment of mice with IFN-α and WP1193 inhibits bone marrow–derived Tregs but combinational therapy is not synergistic. To ascertain the in vivo effects of IFN-α and
WP1193 and in combination on Tregs, non–tumor-bearing mice were treated for 16 days. Both WP1193 and IFN-α significantly inhibited the number of Tregs (CD4+Foxp3+) in the bone marrow by 31% and 78%, respectively, compared with the control (\(P < 0.05\) and \(P < 0.01\); Fig. 2). WP1193 and IFN-α also significantly inhibited the number of Tregs (CD4+Foxp3+) in the peripheral blood by 20% and 46%, respectively, compared with the control (\(P < 0.05\); data not shown). However, the combination of IFN-α and WP1193 was not additive or synergistic for inhibiting the number of Tregs in either the bone marrow or the blood. Furthermore, WP1193 or IFN-α alone or in combination did not inhibit the number of Foxp3+ Tregs in the thymus, lymph nodes, or spleen (data not shown). This suggested to us that an additive inhibition of Tregs would not be a mechanism of efficacy with combinational therapy.

In vivo treatment of mice with IFN-α or WP1193 enhances melanoma immune-mediated cytotoxicity. To ascertain the immunologic mechanisms of combinational therapy, we examined splenocytes cytotoxic responses directed against B16 melanoma cells by NK cells and CD8+ cells. The NK1.1+CD3− (NK) cells and CD3+CD8+ T cells from...
spleens of 4- to 6-week-old mice were isolated, cocultured with CFSE-labeled B16 target cells, and treated with RPMI 1640 (control), WP1193 (2 μmol/L), IFN-α (2,000 units/mL), or IFN-α (2,000 units/mL) + WP1193 (2 μmol/L) for 48 hours to assess NK or T-cell cytotoxicity against B16 cells. Both the NK and CD8+ T-cell populations from the WP1193 (P = 0.01 and P = 0.02, respectively) or IFN-α–treated group (P = 0.0002 and P = 0.0006, respectively) increased cytotoxic clearance of the B16 target cells compared with the control group (Fig. 3B). Furthermore, there was enhanced cytotoxic clearance of the B16 target cells in the NK cells or CD8+ T cells treated with both IFN-α and WP1193 compared with the NK cells or CD8+ T cells treated with either WP1193 or IFN-α alone (P < 0.01; Fig. 3B).

**MHC I and NK activating ligands are expressed on melanoma cells but are not further enhanced by combination therapy.** Because we observed NK and CD8+ T cell–mediated antitumor cytotoxicity was enhanced with combinational therapy, to ascertain if either IFN-α, WP1193, or both were augmenting the expression of MHC or NK-activating receptors or their ligands, splenocytes and B16 cells were treated with RPMI 1640 (control), WP1193 (2 μmol/L), IFN-α (2,000 units/mL), or IFN-α (2,000 units/mL) + WP1193 (2 μmol/L) for 24 hours. The NK-activating ligands (Rae-1, H60, and CD155), MHC (I and II) on B16 cells, and the NK-activating receptors (NKG2D, KLRD1, NKp46, and DNAM-1) on NK1.1+ NK cells were analyzed by flow cytometric analysis. MHC I but not MHC II, was expressed on B16. IFN-α enhanced MHC I expression, but this was not further enhanced with WP1193 (Fig. 4A). Additionally, B16 expressed H60, Rae-1, and CD155; however, neither IFN-α nor the WP1193 treatment altered the mean fluorescent intensity on the surface, indicating that these treatments do not alter the receptor density of the NK ligands (Fig. 4B). Furthermore, NKG2D, KLRD1, NKp46, and DNAM-1 were expressed on the NK cells but also did not seem to be upregulated by either WP1193 or IFN-α, indicating that these treatments do not alter the receptor density of the NK receptors (Fig. 4C).

**STAT3 blockade enhances the efficacy IFN-α against metastatic melanoma.** To determine whether the IFN-α and STAT3 blockade combination therapy yielded a synergistic efficacy against the process of metastasis, IFN-α (i.v.) or WP1193 (o.g.) was given alone or in conjunction with each other in C57BL/6j mice with systemic, metastatic melanoma. To observe the synergistic effect of STAT3 blockade and IFN-α, the subtherapeutic dose of WP1193 (30 mg/kg) was used in this study. The number of pulmonary metastasis was quantified 14 days after tumor inoculation. The number of metastasis for tumor-bearing mice without further intervention was 28 ± 12. Neither subtherapeutic WP1193 (34 ± 17, P = 0.31 compared with control) nor IFN-α alone (28 ± 11, P = 0.48 compared with control) inhibited B16 pulmonary metastasis (Fig. 5A). However, the number of pulmonary metastasis was significantly reduced for WP1193 + IFN-α combinatorial therapy (9 ± 5) compared with control (P < 0.05), WP1193 alone (P < 0.05), or IFN-α alone (P < 0.05; Fig. 5A).

**Treatment of established intracerebral melanoma with both IFN-α and STAT3 blockade is efficacious.** To determine whether the IFN-α and STAT3 blockade combination therapy yielded a synergistic efficacy against established CNS tumors, IFN-α (i.v.) or WP1193 (o.g.) was given alone or in conjunction with each other to C57BL/6j mice in which intracerebral melanoma had been established via intracranial injection of log-phase B16 tumor cells. Kaplan-Meier survival curves were plotted for those mice. Upon death, the etiology was confirmed to be tumor progression. Median overall survival for tumor-bearing mice without further intervention was 17 days [95% confidence interval (95% CI), 16, not estimable; n = 11] and was significantly enhanced by all therapies, including with either subtherapeutic WP1193 (18.5 days; 95% CI, 17, NA; P < 0.04

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**Fig. 3.** Cytotoxicity of B16 in vitro produced by splenocytes from mice treated with WP1193, IFN-α, or both. A, the splenocyte effector cells from mice that were treated with either WP1193 or IFN-α induced modest tumor lysis. However, splenocyte effector cells from mice that were treated with the combination of WP1193 and IFN-α potently enhanced B16-specific lysis (P < 0.01). B, cytotoxicity of B16 in vitro produced by NK and CD8+ T cells treated with WP1193, IFN-α, or both. The NK and CD8+ immune effector cell populations were sorted as described and were treated with either WP1193, IFN-α, or both. Similar to the data in A, either treatment induced modest tumor lysis. However, both effector immune cell populations treated with the combination of WP1193 and IFN-α potently enhanced B16-specific lysis (P < 0.01). This experiment was reproduced twice in its entirety with identical findings.
Regulation of MHC and NK-activating receptors and their respective ligands by WP1193 and IFN-α. Splenocytes or B16 cells were treated with WP1193, IFN-α, or both, and MHC and the NK-activating receptors and ligands were subsequently analyzed by flow cytometric analysis. The isotype control is shown by the dashed black line and the respective target antigen by a solid black line.

A, B16 cells stained for surface expression of MHC I and MHC II after exposure to WP1193, IFN-α, or the combination of WP1193 and IFN-α.

B, B16 cells stained for surface expression of the NK-activating receptor ligands H60, Rae-1, and CD155 after exposure to WP1193, IFN-α, or the combination of WP1193 and IFN-α.
compared with control; \( n = 12 \) or IFN-\( \alpha \) alone (27.5 days; 95% CI, 21, NA; \( P < 0.01 \) compared with control; \( n = 8 \); Fig. 5B). Moreover, median overall survival (40 days; 95% CI, 31, NA; \( P < 0.001 \); \( n = 11 \)) was significantly longer for WP1193 + IFN-\( \alpha \) combinatorial therapy than for IFN-\( \alpha \) alone (\( P < 0.02 \)). For the mice treated with the combinatorial therapy of WP1193 and IFN-\( \alpha \), 27% survived long term (>84 days; \( P < 0.001 \) compared with the control group), and there was at least a 135% increase in median survival time when the experiment was terminated to perform the tumor rechallenge experiments.

To determine whether mice with intracerebral tumors treated with both WP1193 and IFN-\( \alpha \) were able to generate long-lasting protective immune memory, mice that survived for 84 days after the initial tumor cell implantation were reinoculated with B16 cells in the contralateral hemisphere. Upon rechallenge, in the animal group that had received WP1193 and IFN-\( \alpha \), the median survival time was 16 days, which did not differ significantly from the median survival time (17 days) of naive, control mice. There were no long-term survivors in the rechallenged group (data not shown), indicating that long-lasting immunity was present in the rechallenged group.

\[\text{Fig. 4. Continued.}\] C, NK cells labeled with anti-NK1.1+ antibody from murine splenocytes stained for surface expression of the NK-activating receptors NKG2D, KLRD1, NKp46, and DNAM-1 after exposure to WP1193, IFN-\( \alpha \), or the combination of WP1193 and IFN-\( \alpha \).
immune memory was not induced by the combination therapy.

**IFN-α inhibits in vivo death secondary to LMD.** Because IFN-α has previously been shown to inhibit invasion and metastasis, we hypothesized that the combination of IFN-α and WP1193 might exert efficacy against metastasis. To test this hypothesis, we performed an intracerebral injection of B16 melanoma cells into the brains of C57BL/6J mice. The number of pulmonary metastasis was quantified 14 days after tumor inoculation. The number of metastasis for tumor-bearing mice without further intervention was 28 ± 12. Neither subtherapeutic WP1193 (34 ± 17, P = 0.31 compared with control) nor IFN-α alone (28 ± 11, P = 0.48 compared with control) inhibited B16 pulmonary metastasis (A). However, the number of pulmonary metastasis was significantly reduced for WP1193 + IFN-α combinatorial therapy (9 ± 5) compared with control (P < 0.05). WP1193 alone (P < 0.05), or IFN-α alone (P < 0.05). B, survival data from C57BL/6J mice treated with WP1193, IFN-α, or both after B16 cells were established in the brain. Median overall survival for mice with intracerebral tumors without further intervention (n = 11) was 17 days. C57BL/6J mice with established intracerebral B16 cells treated with a subtherapeutic dose of WP1193 (o.g., n = 12) showed a 9% increase in their median survival time to 18.5 days (P < 0.04 compared with control); in mice with established tumor treated with IFN-α alone (n = 8), there was a 62% increase in median survival to 27.5 days (P < 0.01 compared with control). In those mice with established tumors treated with the combination of IFN-α and WP1193 (n = 11), there was a 135% increase in median survival to 40 days that was significantly longer compared with IFN-α alone (P < 0.02). In mice that survived long-term subsequent rechallenge by injection of B16 cells into the contralateral hemisphere, there was insufficient power to draw statistically meaningful conclusions; however, when we assessed all mice who died of LMD, the median survival was 16.7 ± 0.7 days in those that died of LMD and 18.5 ± 1.5 days in those that died of tumor. Furthermore, in the subtherapeutic WP1193 group, median survival was 17.7 ± 0.3 days in mice that developed LMD and 20 ± 2.1 days in those that died of tumor. In contrast, within the IFN-α treatment group, the median survival of mice with progressive tumor was 25.8 ± 2.5 days, which was further increased to 29.7 ± 5.5 days in the combination treatment group. Within individual treatment groups, there was insufficient power to draw statistically meaningful conclusions; however, when we assessed all mice who died of LMD, the median survival was 16.9 ± 0.4 days compared with 24.7 ± 2 days for mice dying of tumor progression (P = 0.0006), indicating this model system conforms to the negative prognostic influence of LMD observed in human patients.

**Discussion**

In this report, we showed that the combination of IFN-α and p-STAT3 blockade can exert efficacy against metastatic melanoma, including intracerebral established CNS melanoma. Patients with CNS melanoma, especially those with LMD, are typically refractory to currently available standard therapies, and our preclinical data would suggest that this combination might have clinical utility. This is notable considering that immunotherapeutic approaches for melanoma have been disappointing (35). The cytokine IFN-α is currently approved by Food and Drug Administration for patients with surgically resected stage III melanoma without evidence of radiographic measurable disease (36) and is not particularly efficacious against CNS disease. In our experimental model systems of metastatic and CNS melanoma, IFN-α induced a modest therapeutic response. However, when IFN-α was used in combination with WP1193, marked therapeutic efficacy was seen, and there seemed to be a trend to a diminished propensity for the development of LMD. This warrants further investigation in murine models of established LMD with larger cohorts of animals. Previous immune therapeutic approaches to treat melanoma LMD have included the intrathecal administration of IL-2 (37) and IFN-α (6). IL-2 enhances NK cell activity, activates cytotoxic T cells, stimulates IFN-γ release, and activates macrophages, and IFN-α has direct antiproliferative effects on tumor cells.
activates NK cells and cytotoxic T cells, and enhances antigen presentation and MHC expression. Clinical trials of IL-2 in melanoma patients with LMD showed a high rate of tumor clearance from the cerebrospinal fluid (38–40); however, treatment resulted in meningeal irritation, fever, brain edema, seizures, stupor, and one death (38–40). Similarly, IFN-α in clinical trials also showed clearance of malignancy within the cerebrospinal fluid (6); however, treatment was confounded by profound neurotoxicity (7). Our data would suggest that systemic IFN-α in combination with WP1193 might be a novel approach to inhibit the development of LMD.

The combination of IFN-α and WP1193 enhanced tumor cytotoxicity mediated by both the NK and CD8+ T-cell populations. It was not surprising to observe an enhancement of CD8+ T-cell tumor cytotoxic activity with the combination of STAT3 blockade and IFN-α, because this was consistent with our previously published data of p-STAT3 inhibitors (21) and other investigators work that showed that IFN-α enhances CD8+ cytotoxic responses (41). Because melanomas have been shown to evade immune detection by downmodulating MHC (42–44), immunologic responses that are antigen-MHC independent, such as NK-mediated cytotoxicity, are appealing for overcoming at least one mechanism of immune therapeutic resistance. To determine whether the enhancement of NK-mediated cytotoxic function was related to upregulation of NK-activating receptors or ligands by treatment with the combination of IFN-α and WP1193, we assessed the expression levels on NK cells and B16, respectively. The B16 cells expressed the MHC I, Rae1, H60, and CD155, indicating that they would be capable of triggering NK cytotoxic responses, resulting in tumor clearance similar to findings in a previous report (45), but treatment did not alter the expression of the ligands. Furthermore, we did not find changes in the NK-activating receptor expression levels of NKG2D, KLRD1, NKp46, or DNAM-1. It is possible that there was a transient increase in the NK-activating receptors that was not identified under the current experimental conditions.

We have shown that the p-STAT3 inhibitors inhibit Tregs in murine models of melanoma and from melanoma patients (24, 46). IFN-α has been shown to augment IL-10 production, and IFN-α–treated dendritic cells induce IL-10–producing Tregs (47). Additionally, in studies of human melanoma patients treated with high-dose IFN-α, Wang et al. showed an enhancement of Tregs in the lymph nodes, but the Treg population was not analyzed in the bone marrow and blood (48). Furthermore, we found that IFN-α induces the immune suppressive p-STAT3, and others have shown that p-STAT3 is a promoter of FoxP3 expression in Tregs. Thus, we hypothesized that the p-STAT3 inhibitors would enhance the therapeutic efficacy of IFN-α by inhibiting the induced Tregs. Although there is inhibition of the number of Tregs in both the bone marrow and blood with IFN-α and WP1193, there was no additive effect on inhibiting the number of Tregs. Interestingly, IFN-α showed inhibition
of the number of Tregs in bone marrow and the peripheral blood and slight enhancement of the numbers of Tregs in the lymph nodes, and so a paradox arises as to the mechanism of IFN-α in inhibiting Tregs that we observed in vivo. Within a few days after hydrodynamic gene transfer of IFN-γ, the total bone marrow cellularity drops with the CD4 T-cell population being the most affected, which is consistent with other reports of IFN-α (49). Within the CD4 fraction, the FoxP3+ Treg numbers are even more suppressed compared with non-Treg CD4+ T cells, thus the reason why, in the IFN-γ treatment group, the Treg numbers were most dramatically inhibited within the bone marrow. Using sorted Tregs from a FoxP3-GFP reporter mouse from IFN-α–treated or control mice, we did not see a decrease in the suppressive activity (data not shown). Thus, IFN-α inhibits the relative number of Tregs but not their suppressive activity, whereas WP1193 only modestly inhibits the number of Tregs in the bone marrow compared with IFN-α but does suppress their functional activity, as we have previously shown (24, 46). Alternatively, we may not have appreciated an additive effect on Treg inhibition, because these studies were conducted in non–tumor-bearing animals, in which Treg induction may have been minimal.

In conclusion, the combination approach of WP1193 and IFN-α enhances both NK and CD8+ cytotoxicity and seems to be a promising potential treatment modality for melanoma patients with CNS disease, who currently have very few therapeutic options available and who are typically excluded from clinical trials.

Disclosure of Potential Conflicts of Interest

W. Priebe and A.B. Heimberger hold patents on WP1193.

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