

# A Novel Small Molecule Inhibitor of Signal Transducers and Activators of Transcription 3 Reverses Immune Tolerance in Malignant Glioma Patients

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## Abstract

**Overcoming the profound immunosuppression in patients with solid cancers has impeded efficacious immunotherapy. Signal transducers and activators of transcription 3 (STAT3) has recently emerged as a potential target for effective immunotherapy, and in this study, we describe a novel small molecule inhibitor of STAT3 that can penetrate the central nervous system (CNS) in mice and in physiologically relevant doses *in vitro* and reverse tolerance in immune cells isolated from glioblastoma multiforme (GBM) patients. Specifically, it induces the expression of costimulatory molecules on peripheral macrophages and tumor-infiltrating microglia, stimulates the production of the immune-stimulatory cytokines interleukin 2 (IL-2), IL-4, IL-12, and IL-15, and induces proliferation of effector T cells from GBM patients that are refractory to CD3 stimulation. We show that the functional enhancement of immune responses after STAT3 inhibition is accompanied by up-regulation of several key intracellular signaling molecules that critically regulate T-cell and monocyte activation. Specifically, the phosphorylation of Syk (Tyr<sup>352</sup>) in monocytes and ZAP-70 (Tyr<sup>319</sup>) in T cells are enhanced by the STAT3 inhibitor in marked contrast to toll-like receptor and T-cell receptor agonists, respectively. This novel small molecule STAT3 inhibitor has tremendous potential for clinical applications with its penetration into the CNS, easy parental administration, direct tumor cytotoxicity, and potent immune adjuvant responses in immunosuppressed cancer patients.** [Cancer Res 2007;67(20):9630–6]

## Introduction

In patients with solid tumors, systemic immune responses are unable to overcome the immunosuppressive tumor microenvironment (1). Patients with malignant gliomas are profoundly immunosuppressed with impaired T-cell responses secondary to impaired T-cell receptor signaling, immunosuppressive cytokines, regulatory T cells, and dysfunctional antigen-presenting cells (APC; ref. 2). We have found that CD11b/c<sup>+</sup>CD45<sup>+</sup> glioma infiltrating

microglia/macrophages from postoperative tissue specimens of glioma patients had surface MHC class II expression but lacked expression of the costimulatory molecules CD86, CD80, and CD40 critical for T-cell activation and thus were unable to stimulate naive T cells. Also, within malignant gliomas, there is a lack of effector/activated CD8 T cells. To overcome these profound immunologic impairments in responsiveness, compounds that are capable of modulating the local immune responses may provide synergistic effects.

Signal transducers and activators of transcription (STAT), in particular, STAT3, has recently emerged as a potential target for effective immunotherapy (3). The role of STAT proteins in the development of tumors is varied but STAT3 is constitutively activated in many common tumors, including high-grade human gliomas (4). Inhibition of STAT3 signaling leads to an inhibition of cancer cell growth and to the induction of apoptosis. In addition, STAT3 has been shown to be a potent regulator of inflammatory responses. STAT3 suppresses macrophage activation *in vitro* (5–7) and limits the host's inflammatory response (8). When STAT3 has been blocked, either with a dominant negative variant or an antisense oligonucleotide, macrophages have been shown to increase the production of interleukin-12 (IL-12) and RANTES and reverse systemic tolerance (9). WP1066, a novel small molecule STAT3 inhibitor, has proapoptotic and antiproliferative activity in a variety of tumor cell types, including human malignant glioma cell lines U87-MG and U373-MG, indicating that WP1066 has potent intrinsic antiglioma activity *in vivo* (10).

In this study, we show that STAT3 blockade by a novel small molecule can reverse immune tolerance in glioblastoma multiforme (GBM) patients. We determined that this STAT3 inhibitor could act as a potent immune adjuvant by up-regulating costimulatory molecules CD80 and CD86 in not only peripheral blood monocytes but also glioma-infiltrating microglia/macrophages and induce effector T cell-stimulating cytokines. Furthermore, this STAT3 inhibitor is able to activate and induce proliferation of effector T cells from GBM patients that are refractory to CD3 stimulation. In addition, it is speculated that STAT3 signaling can regulate downstream activation of nuclear factor- $\kappa$ B (3). Tyrosine phosphorylation of Syk initiates downstream signaling events during human monocyte activation, whereas the phosphorylation of Tyr<sup>319</sup> in Zap-70 plays a critical role in mediating T-cell activation via signaling through the T-cell receptor. We show augmentation of phosphorylation of key intracellular signaling molecule Syk (Tyr<sup>352</sup>) in monocytes and ZAP-70 (Tyr<sup>319</sup>) in T cells, thereby providing a critical mechanism by which this molecule can act upon immune cells and overcome immunosuppression in the tumor microenvironment.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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## Materials and Methods

### Human Subjects

Patients' tumors ( $n = 11$ ) were graded pathologically as GBM by a neuropathologist according to the WHO classification. Peripheral blood was drawn from the patients intraoperatively. This study was conducted under protocol LAB03-0687, which was approved by the institutional review board of The University of Texas M. D. Anderson Cancer Center, and informed consent was obtained. Peripheral blood from normal donors was obtained from Gulf Coast Regional Blood Center, (Houston, TX).

**Antibodies and reagents.** WP1066 was synthesized and supplied by Dr. Priebe. The cell surface was stained with PE, FITC or allophycocyanin-labeled antibodies against the following proteins: CD11b, CD14, CD80, and CD86 (PharMingen). For intracellular cytokine staining, PE-labeled or FITC-labeled antibodies against IL-1 $\beta$ , IL-2, IL-4, IL-15, and IL-12 (R&D Systems) were used. Intracellular STAT3 was assessed using phosphorylated Stat3 Alexa Fluor 488 conjugate (Cell Signaling Technology). Appropriate isotype controls were used for each antibody. CFSE was from Invitrogen. Purified lipopolysaccharide (LPS) was from *Escherichia coli* 0111:B4 (Sigma-Aldrich Corp.).

**Animal experiments.** WP1066 was injected i.p. at doses of 100 mg/kg every other day for 2 weeks in nude mice. Plasma, central nervous system (CNS) tissue, and U87-MG flank tumors were harvested and, after extraction, were analyzed for WP1066 content using tandem liquid chromatography/mass spectrometry.

### Isolation of Microglia/Macrophages from Human Brain Tumor Tissue

To purify microglia/macrophages from human brain tissue, we used a modified Percoll gradient isolation technique as previously described (11). This technique minimized artificial activation of the microglia/macrophages, and these cells were isolated within 3 h of surgical resection. Briefly, after resection, freshly isolated tumor was mechanically dissociated and layered onto successive density gradients. Microglia/macrophages were collected from the interface between the 1.065 and 1.03 g/mL layers and washed, and their viability determined by the trypan blue dye-exclusion method.

**Isolation of monocytes from human peripheral blood.** Due to the technical difficulties in obtaining large quantities of microglia/macrophages from human glioma specimens, the precursors, monocytes (12), were used in several experiments. Peripheral blood mononuclear cells (PBMC; from GBM patients or normal donors) were removed from the buffy coat layer and washed. Untouched monocytes were isolated via negative selection using a monocyte bead isolation kit and Midi-MACS magnetic separation column (Miltenyi Biotec). The isolated monocytes were 97% positive for CD11b+ cells.

**Stimulation of microglia/peripheral blood monocytes with WP1066.** Cells (microglia or monocytes) were added to 96-well plates at  $1 \times 10^6$  cells per well. LPS (5  $\mu$ g/mL) or WP1066 (0.1, 1, or 10  $\mu$ mol/L) was added to each well at the indicated dose and incubated for various time points at 37°C, 5% CO<sub>2</sub> along with unstimulated controls. In addition to the stimulation, monocytes were also incubated with supernatant from U-87 cell culture, previously shown to contain immunosuppressive cytokines [IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ); ref. 13]. Stimulated cells were then washed in media and used either for direct fluorescence-activated cell sorting (FACS) analysis or in functional assays.

### Surface Marker and Intracellular Cytokine Staining

Cells were blocked for nonspecific binding using purified anti-CD16 antibody (PharMingen) for 20 min, and after washing, the cells were incubated with the fluorescence-labeled primary antibody or isotype control for 30 min at 4°C. For the intracellular cytokine or STAT3 analysis, microglia/macrophages or monocytes were fixed with Cytofix/Cytoperm (BD Biosciences), washed in PermWash (BD Biosciences), and then stained with fluorescence-labeled monoclonal antibodies or isotype controls for 30 min at 4°C. Cells were analyzed with a FACSCalibur flow cytometer using Cellquest software (BD Immunocytometry Systems).

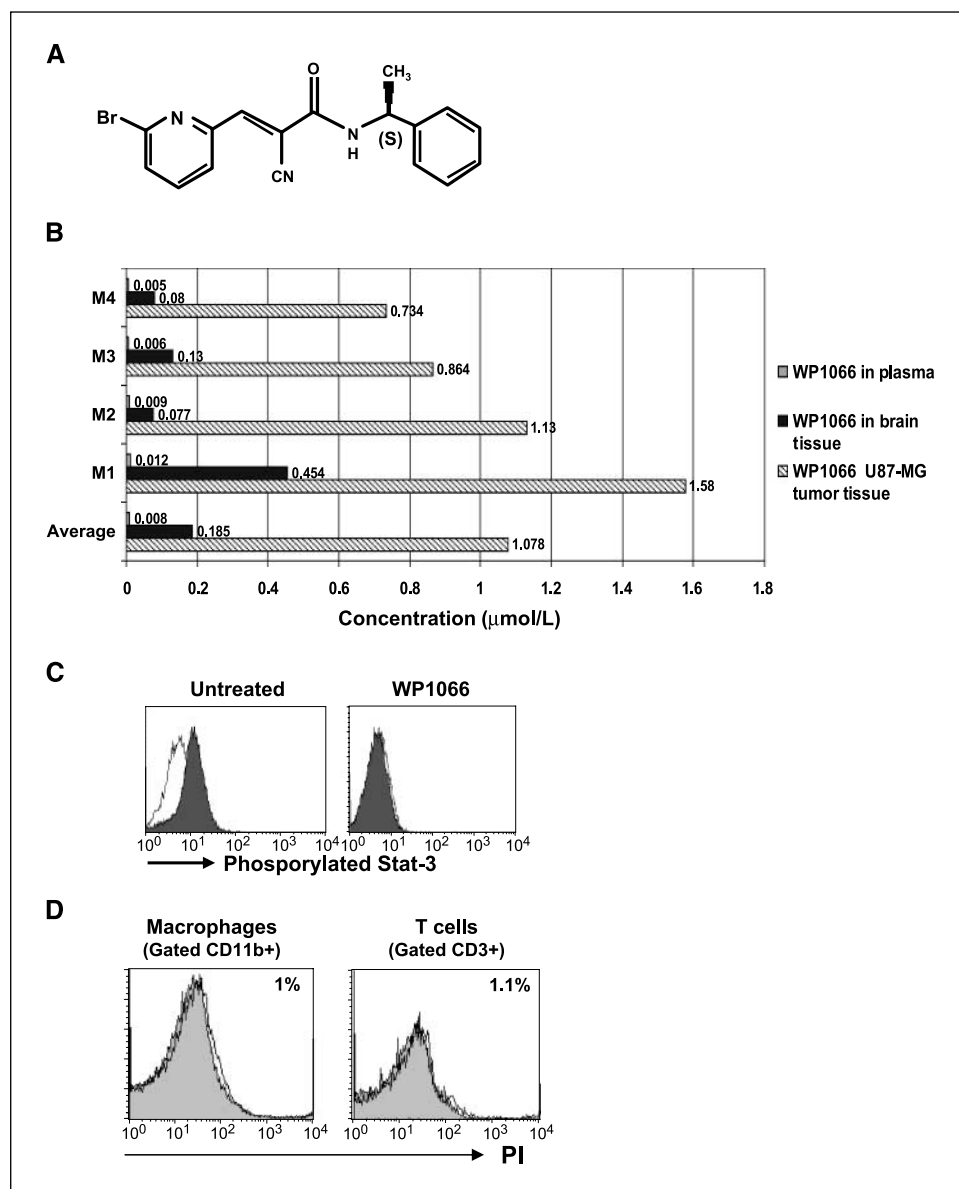
**T-cell proliferation assay.** Stimulator cells (antigen-presenting cells) were monocytes isolated from the peripheral blood of the respective glioma patients or monocytes from normal donors. Monocytes were isolated and purified as described above. To isolate autologous responder T cells, PBMCs from the same monocyte source were labeled with CD8 microbeads (Miltenyi Biotec) and passed through a magnetic separation column (LS MACS Column, Miltenyi Biotec) placed in the magnetic field of a MACS separator (Miltenyi Biotec). CD8<sup>+</sup> cells were retained on the column, whereas the unlabeled cell fraction ran through. The cells were washed and determined to be 97% CD8<sup>+</sup> T cells by flow cytometry. Responder CD8<sup>+</sup> T cells were then labeled with CFSE (11), whereas the antigen-presenting monocytes were prestimulated with either LPS or WP1066 as described above. For the proliferation assay, in a 96-well plate, CFSE labeled responder CD8<sup>+</sup> T cells ( $0.75 \times 10^5$  cells per 50  $\mu$ L per well) were added to the autologous stimulator monocyte population at a range of 0.75 to  $2.25 \times 10^5$  cells per 50  $\mu$ L per well (for final responder/stimulator ratios of 1:1, 1:2, and 1:3) and incubated for 4 days at 37°C, 5% CO<sub>2</sub> with either purified. Controls included T cells incubated in media alone, each stimulator in media alone, and T cells stimulated with purified anti-CD3 antibody or anti-CD3 + anti-CD28 antibodies. After incubation, cells were labeled with anti-CD11b and anti-CD8 antibodies to identify the CFSE stimulator cells and CFSE+ responder T cells, respectively, for analysis by FACS.

**Western blot analysis of cell signaling molecules.** Monocytes from GBM patients were purified as described above. T cells from the same patients were isolated with human CD3 microbeads and a magnetic separation column (LS MACS column, Miltenyi Biotec) with the exception of one patient who lacked sufficient PBMCs for both monocyte and T-cell isolations. Monocytes or T cells were seeded at a density of  $1 \times 10^6$  cells per well in six-well culture plates and were incubated at 37°C, 5% CO<sub>2</sub>, with either the medium or medium supplemented with 5  $\mu$ mol/L WP1066. After 2 h, monocytes and T cells were stimulated for 5 min with 2  $\mu$ g/mL LPS and 5  $\mu$ g/mL anti-CD3 antibody, respectively, in the presence or absence of WP1066. Subsequently, cells were lysed in buffer containing 1% Triton-X and protease inhibitors (Sigma-Aldrich). Protein aliquots (20  $\mu$ g) from each monocyte and T-cell lysate were electrophoretically fractionated in 8% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and immunoblotted with antiphosphotyrosine monoclonal antibody 4G10 (Cell Signaling Technology, Inc.). Autoradiography of the membranes was done using Amersham enhanced chemiluminescence Western blotting detection reagents (GE Healthcare). After stripping the membrane, it was reblotted with antibody to phosphorylated p72Syk (Tyr<sup>352</sup>; Cell Signaling Technology, Inc.). With subsequent restripping, the membrane was reblotted with antibodies to Syk, phosphorylated Hck, Hck, and Lyn (Cell Signaling Technology, Inc.). For the T-cell membranes, after stripping, the membrane was reblotted with antibody to phosphorylated ZAP-70 (Tyr<sup>319</sup>; Cell Signaling Technology, Inc.). With subsequent restripping, the membrane was reblotted with antibodies to ZAP-70, p56-Lck (Cell Signaling Technology, Inc.), and  $\beta$ -actin (Sigma-Aldrich), respectively. Semiquantification of protein on the Western blots was done using Scion Image software (Scion Co.).

**Statistics.** Statistical analyses were done using the Student's *t* test, and all values with  $P < 0.05$  was considered significant.

## Results and Discussion

Current immune stimulators/adjuvants are insufficient to overcome the immunosuppressive influences of the tumor microenvironment in patients with malignant human gliomas and solid cancer (1). Therefore, we investigated whether a pharmacologic derivative of the natural compound, caffeic acid (WP1066; Fig. 1A), a small molecule inhibitor of STAT3 activity could overcome these immunosuppressive factors. In the murine model, WP1066 has a plasma half-life of >4 h, and studies in the animals bearing flank tumors showed selective uptake of the compound by tumors, which showed a higher drug content than any other tissue. WP1066 delivered i.p. at doses of 100 mg/kg every other day for up to 2 weeks or i.v. at doses of only



**Figure 1.** A novel small molecule, WP1066, inhibits STAT3 activity and has significant CNS and glioma tumor penetration *in vitro* and *in vivo*. **A**, chemical structure of WP1066. **B**, administration of WP1066 resulted in significant CNS penetration and accumulation within malignant gliomas. WP1066 was injected i.p. at doses of 100 mg/kg every other day for 2 wk in nude mice. Plasma, CNS tissue, and U87-MG flank tumors were analyzed for WP1066 content using tandem liquid chromatography/mass spectrometry. **C**, untreated and WP1066-treated normal donor PBMCs were assessed for phosphorylated STAT3 activity (*shaded histogram*) compared with appropriate isotype controls (*clear histogram*) by intracellular staining with a phosphorylated Stat3 Alexa Fluor 488 conjugate and analysis by flow cytometry. **D**,  $1 \times 10^6$  normal donor PBMCs incubated at 37°C, 5% CO<sub>2</sub> with different doses of WP1066 (0.1–5 µmol/L) for 2 and 4 h. Cells were stained with fluorescently labeled anti-CD3 and anti-CD11b antibodies and propidium iodide (PI) and analyzed by flow cytometry. Macrophages and T cells were gated on CD11b<sup>+</sup> and CD3<sup>+</sup> populations, respectively, and cell death (%) was quantified as CD11b<sup>+</sup>PI<sup>+</sup> or CD3<sup>+</sup>PI<sup>+</sup> populations in the respective plots. Plots represent the 5 µmol/L maximum dose (*clear histogram*) compared with control untreated cells (*shaded histogram*) incubated under similar conditions.

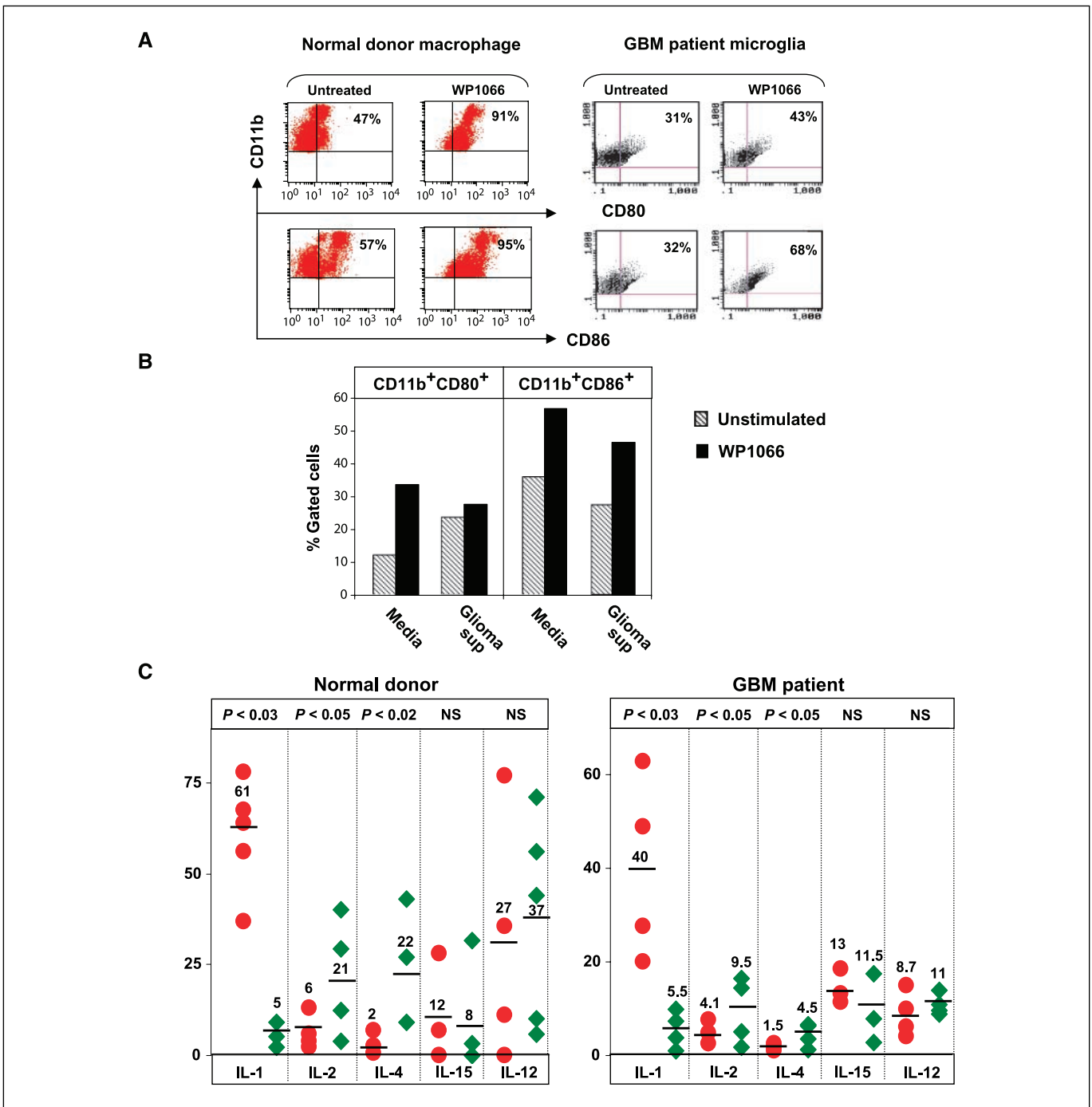
10 mg/kg (data not shown) achieved plasma concentrations in excess of 1 µmol/L, CNS concentrations in excess of 62 µg/g (0.1854 µmol/L) of tissue and in U87-MG malignant glioma-bearing animals, and concentrations of 362 µg/g (1.07 µmol/L) of tumor. These data show that WP1066 can effectively cross the blood-brain barrier and achieve CNS concentrations, a critical factor when determining therapies for glioma patients (Fig. 1B).

STAT3 is constitutively activated in many tumors (4), and WP1066 has been shown to inhibit STAT3 and have proapoptotic activity in a variety of tumor cell types, including malignant gliomas (10). The proapoptotic activity WP1066 possesses may contribute to an induced neutropenia and lymphopenia, but at doses achievable *in vivo* (1 nmol/L to 5 µmol/L) that inhibit STAT3 within immune cells (Fig. 1C), cell viability is not compromised (Fig. 1D), suggesting that WP1066 could be used as an immune adjuvant.

We recently reported that microglia/macrophages isolated from human gliomas lacked expression of the costimulatory molecules CD86 and CD80 and were unable to activate naive T cells (11). In

normal PBMCs, when STAT3 was blocked, macrophages increased production of IL-12, induced Th1 responses, and reversed systemic tolerance (9). WP1066 is capable of up-regulating CD80 and CD86 on both normal donor PBMCs and also on tumor-infiltrating microglia/macrophages freshly isolated from GBM patients (Fig. 2A). This up-regulation of costimulation could even be achieved when human monocytes were incubated with glioma supernatant known to contain immunosuppressive cytokines (IL-10 and TGF-β; Fig. 2B). Furthermore, known immune activators, such as LPS, were insufficient in up-regulating these costimulators on microglia isolated directly from GBM patients (11), indicating that WP1066 is a potent agent for inducing costimulation.

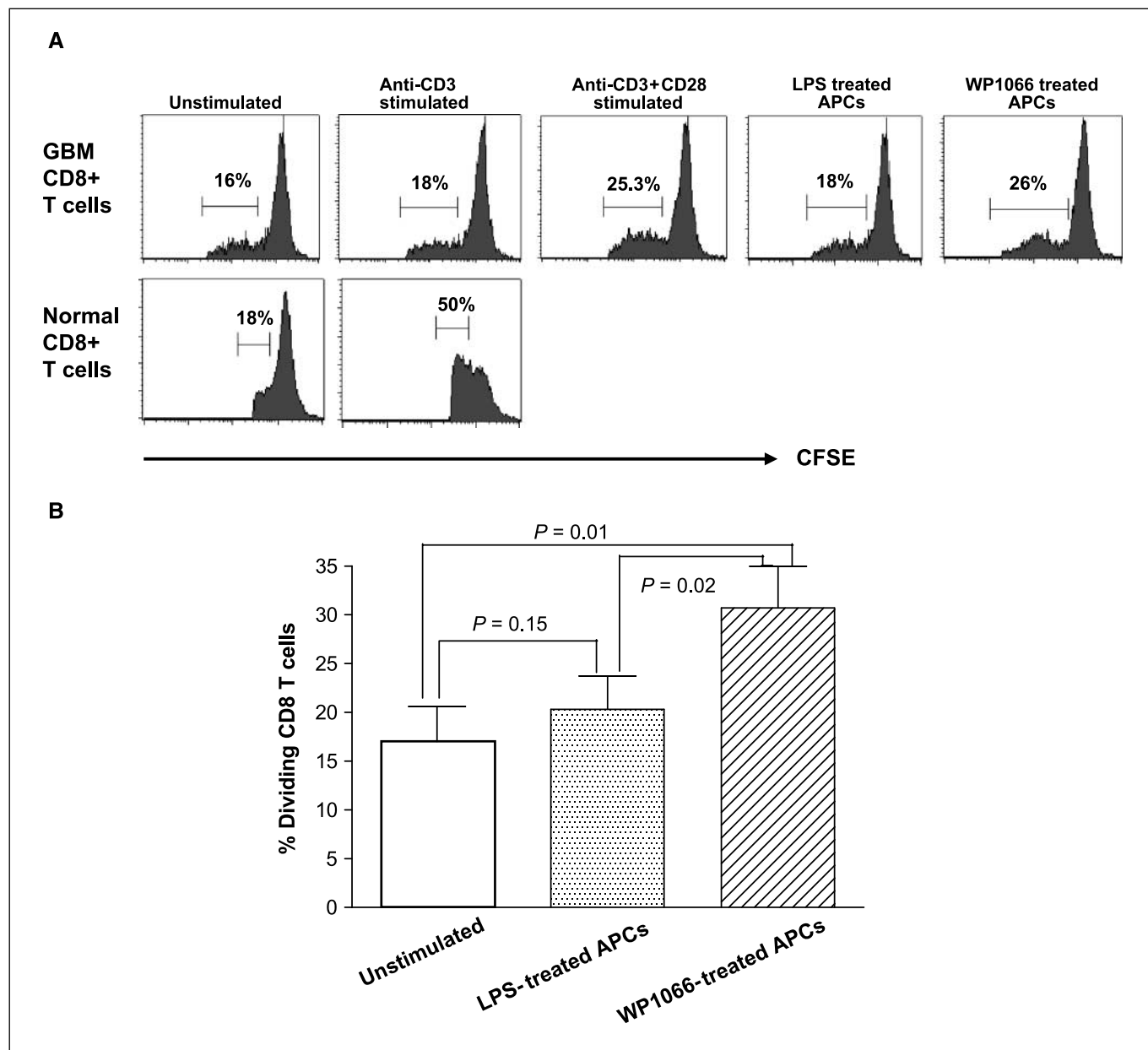
Human glioma patients have also been shown to be deficient in immunostimulatory cytokines (13). WP1066 can induce the crucial cytokines that stimulate T-cell effector function, such as IL-2, IL-4, IL-12, and IL-15 (Fig. 2C) from macrophages isolated from normal donors and from GBM patients. IL-2 and IL-15 have been used successfully in cancer immunotherapy to induce proliferation of



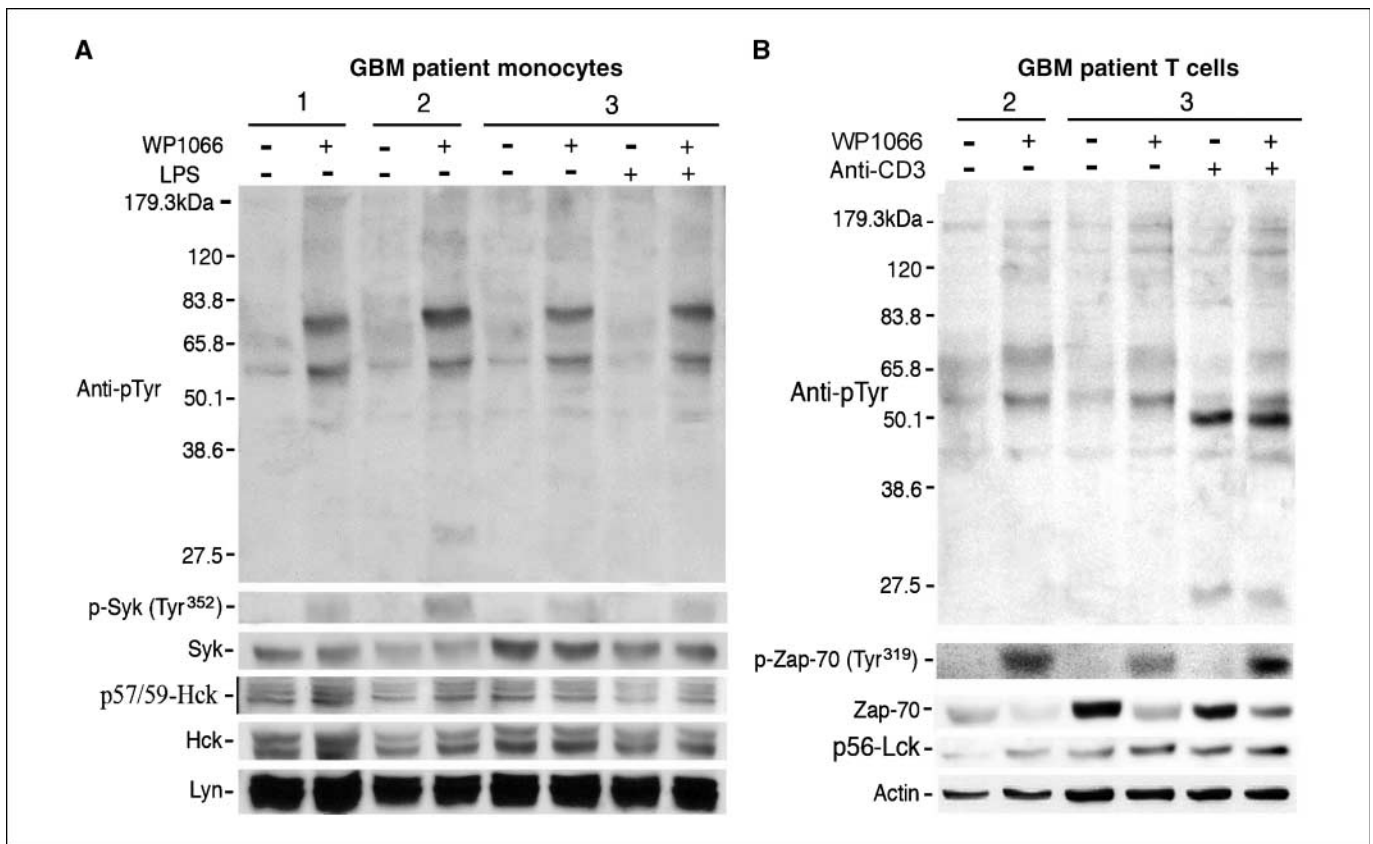
**Figure 2.** WP1066 is a powerful immune modulator in glioma patients. *A*, peripheral blood macrophages and glioma-infiltrating microglia/macrophages up-regulate costimulatory molecules when incubated with STAT3 inhibitor WP1066. PBMCs from normal human donors ( $n = 5$ ) and glioma-infiltrating microglia were purified from freshly resected GBM patient tumor tissue ( $n = 3$ ) and were incubated for 24 h at 37°C, 5% CO<sub>2</sub>, in either medium [RPMI 1640 + 10% fetal bovine serum (FBS)] or medium supplemented with 1 μmol/L WP1066. Cells were then stained with fluorescent antibodies to CD11b<sup>+</sup>/CD45<sup>+</sup> and either CD80 or CD86 and analyzed by flow cytometry. Numbers in the top right quadrant of each graph denote the percentage of CD11b<sup>+</sup>-gated cells that express either CD80 or CD86. Despite the isolation of purified microglia/macrophages, the size of these cells, and other factors contribute to a significant double-positive, autofluorescent population upon flow cytometry analysis. We have previously described our analysis strategy to exclude these cells (11). Briefly, the double-positive population within the isotype control is gated, and the percentage of cells present in this autofluorescent population is determined (Supplementary Fig. S1). To exclude all autofluorescence, this number is then subtracted from any double-positive subset analyzed within the same gate in the test samples. *B*, PBMCs from normal donors were incubated in either control media (RPMI 1640 + 10% FBS) or U-87 glioma supernatant and stimulated with 1 μmol/L WP1066 as indicated. Cells were then stained with fluorescent antibodies to CD11b<sup>+</sup> and either CD80 or CD86 and analyzed by flow cytometry, as described in (*A*), to determine the percentage of CD11b<sup>+</sup>CD80<sup>+</sup> and CD11b<sup>+</sup>CD86<sup>+</sup> cells. *C*, PBMCs from normal human donors ( $n = 7$ ) were incubated for 4 h at 37°C, 5% CO<sub>2</sub>, in either medium or medium supplemented with WP1066 (1 μmol/L) or LPS (1 μg/mL). Cells were then labeled with CD11b/CD14 macrophage markers, stained, and analyzed for intracellular cytokine production. Percentages of macrophages that were CD11b<sup>+</sup>CD14<sup>+</sup> and also positive for the respective cytokine were calculated by flow cytometry analysis. A scatter plot representative of all samples analyzed for macrophage intracellular cytokine production was determined with LPS-treated cells (circles) and WP1066-treated cells (diamonds). Black bars, mean values of each subgroup. *P* values determining statistical significance between LPS-stimulated groups and WP1066-stimulated groups were determined using Student's *t* test. NS, not statistically significant.

tumor-infiltrating lymphocytes (14, 15). IL-4 induces antitumor factors (16) and can cause APC differentiation characterized by efficient antigen uptake and processing (17). We also show lower levels of IL-1 $\beta$  are induced by WP1066 when compared with LPS stimulation. Recent data have shown that IL-1 $\beta$  is abundant at tumor sites and play an important role in invasiveness and metastasis of tumor cells (18, 19). Our data indicate that WP1066 can prime APCs for efficient antigen presentation and, in addition, induces antitumor immunostimulatory cytokines.

T cells isolated from peripheral blood of GBM patients and incubated with allo-CD11b<sup>+</sup> macrophages are impaired in their proliferative response to anti-CD3 stimulation. When additional costimulation is provided with anti-CD28 antibody, T-cell proliferation is augmented in these patients (Fig. 3A). Furthermore, LPS-treated macrophages induced only minimal T-cell proliferation ( $20.3 \pm 5.9\%$ ) from newly diagnosed GBM patients ( $n = 3$ ) compared with unstimulated controls ( $17.0 \pm 3.6\%$ ;  $P = 0.15$ ; Fig. 3B). In contrast, physiologic doses of WP1066-treated macrophages



**Figure 3.** A, WP1066-treated APCs can stimulate strong proliferative responses in normally refractive T cells from the peripheral blood of GBM patients. CFSE-labeled CD8<sup>+</sup> T cells isolated from newly diagnosed GBM patients were incubated with untreated autologous APCs (CD11b<sup>+</sup>/CD14<sup>+</sup>) and anti-CD3 antibody, with anti-CD3 antibody + anti-CD28 antibody and with LPS-treated (5  $\mu$ g/mL) or WP1066-treated (1  $\mu$ mol/L) autologous APCs. In all cases, cells were incubated for 4 d at 37°C, 5% CO<sub>2</sub>, followed by surface staining for T-cell markers (CD3, CD8) and analysis by flow cytometry. The number on each plot indicates the percentage of CD8<sup>+</sup>-gated cells that have undergone cell division via CFSE dilution. These data are representative of three separate experiments. All APCs were treated with the respective stimulants (control medium alone, LPS, or WP1066) for 24 h and then washed before incubation with T cells in the proliferation assay. B, proliferative response of CD8<sup>+</sup> T cells from GBM patients stimulated with LPS-treated or WP1066-treated APCs. The percentage of proliferating CD8<sup>+</sup> T cells was calculated based on the histogram in (A). LPS-treated macrophages induced only minimal T-cell proliferation from newly diagnosed GBM patients ( $n = 3$ ) compared with unstimulated controls ( $P = 0.15$ ). In contrast, physiologic doses of WP1066-treated macrophages potentially induced T-cell proliferation compared with unstimulated macrophages ( $P = 0.01$ ) and LPS-stimulated macrophages ( $P = 0.02$ ). The  $P$  value was determined using Student's  $t$  test.



**Figure 4.** WP1066 enhances phosphorylation of intracellular signaling molecules. Monocytes and T cells isolated from the PBMCs of GBM patients were incubated with either the medium or medium supplemented with 5  $\mu\text{mol/L}$  WP1066. After 2 h, monocytes and T cells were stimulated for 5 min with 2  $\mu\text{g/mL}$  LPS and 5  $\mu\text{g/mL}$  anti-CD3 antibody, respectively. Subsequently, cells were lysed, electrophoretically fractionated in 8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10. *A*, for monocyte membranes, subsequently the same membrane was stripped and reblotted with antibodies to phosphorylated p72Syk (Tyr<sup>352</sup>), Syk, phosphorylated Hck, Hck, and Lyn. *B*, for the T-cell membranes, after stripping, the membrane was reblotted with antibodies to phosphorylated ZAP-70 (Tyr<sup>319</sup>), ZAP-70, p56-Lck, and  $\beta$ -actin.

potently induced T-cell proliferation ( $30.7 \pm 7.4\%$ ; compared with unstimulated macrophages,  $P = 0.01$  and LPS-stimulated macrophages,  $P = 0.02$ ; Fig. 3*B*) even without additional costimulation, as shown in an example (Fig. 3*A*). Together with APCs, WP1066 could stimulate T-cell proliferation that is profoundly impaired in GBM patients compared with CD3-stimulated controls.

To determine how WP1066 induces these potent immune responses, T cells and monocytes isolated from GBM patients were treated with WP1066 and subsequently lysed and analyzed by immunoblotting for critical signaling proteins. In contrast to LPS or anti-CD3 antibody, WP1066 induced or enhanced tyrosine phosphorylation in both monocytes and T cells, producing the most striking increase in levels of proteins of 70 to 75 kDa and 50 to 60 kDa (Fig. 4*A* and *B*). Further analysis revealed that WP1066 markedly induced phosphorylated p72Syk (Tyr<sup>352</sup>) by 184% and phosphorylated ZAP-70 (Tyr<sup>319</sup>) by 179% in monocytes and T cells, respectively, compared with untreated cells. Less effect was observed on levels of the Src family protein tyrosine kinase p57/p59-Hck in monocytes, which was increased by 17%, and p56-Lck in T cells, which was increased by 77% (Fig. 4). The tyrosine phosphorylation of Syk initiates downstream signaling events during human monocyte activation (20), whereas the phosphorylation of Tyr<sup>319</sup> in Zap-70 plays a critical role in mediating T-cell activation via signaling through the T-cell receptor (21). Both Syk and ZAP-70 can be phosphorylated through autophosphorylation and transphosphor-

ylation. Although WP1066 did not affect the total protein level of Syk (Fig. 4*A*), it markedly decreased the total protein level of ZAP-70 (Fig. 4*B*), suggesting that WP1066 may change localization and/or stability of ZAP-70. The tyrosine phosphorylation of critical signaling proteins induced/enhanced by WP1066 are likely pivotal to the WP1066-induced restoration of immune function in GBM patients and explains the mechanisms of potent immune activation.

Several immunotherapeutic clinical trials in glioma patients have shown promise, but in patients with advanced cancers and grossly evident disease, the objective response rates have remained low (1). Potent immune activators are necessary to counteract the immunosuppressive factors that can overwhelm an induced immune response. Small molecule inhibitors with well-characterized mechanisms of immune modulation, such as WP1066, can be effectively and efficiently used in the setting of immunotherapy and/or vaccine administration.

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## A Novel Small Molecule Inhibitor of Signal Transducers and Activators of Transcription 3 Reverses Immune Tolerance in Malignant Glioma Patients

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