**SF2523: Dual PI3K/BRD4 inhibitor blocks tumor immunosuppression and promotes adaptive immune responses in cancer**

**Supplementary methods:**

**Reagents**

MCSF was purchased from Peprotech. LPS from *E. coli* clone, 0111:B4, was purchased from Sigma- Aldrich. IL4 and IFNγ were purchased from Peprotech. JQ1 was a generous gift from James Bradner (Dana-Farber Cancer Institute, Boston, MA). SF2523 is obtained from Signal Rx Pharmaceuticals Inc. (San Diego, USA).

**Quantification of gene expression**

Total RNA was isolated from BMDMs and sorted tumor macrophages using the Qiagen RNAeasy kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. cDNA was prepared from 1 µg RNA sample using iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA (2 µL) was amplified by RT-PCR reactions with 1× SYBR green supermix (Bio-Rad, Hercules, CA) in 96-well plates on an CFX96 Real time system (Bio-Rad, Hercules, CA), using the program: 5 min at 95°C, and then 40 cycles of 20 s at 95°C, 1 min at 58°C and 30 sec at 72°C. The primer sets used for different sets of genes are previously described ([1](#_ENREF_1)). Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. Relative expression levels were normalized to Gapdh expression according to the formula, < 2^(Ct gene of interest-Ct Gapdh)> ([2](#_ENREF_2)).

**RNA sequencing**

Libraries for RNA seq were generated as described previously in ([3](#_ENREF_3)). Two independent preparations of resting and IL-4–treated macrophages were used to evaluate the transcriptional profile. Genes with an induction of ≥ 2-fold and *P* ≤ .05 were considered to be in M2 (as opposed to M0) and were considered M2 markers. For data analysis, FASTQ files were aligned to mouse genome build mm10 using STAR ([4](#_ENREF_4)). The aligned reads were then mapped to the mouse transcriptome with feature Counts ([5](#_ENREF_5), [6](#_ENREF_6)) using its default parameters. The read counts were then normalized in RPKM using the function from (47). Next, differential expression analysis was performed using the “make\_match\_panel” function from the CCAL python package, publicly available from: <https://github.com/UCSD-CCAL/ccal>. Genes significantly induced in M2 compared to M0 (empirical p-value≤ 0.05 and ≥ 2 fold) increase were considered as M2 markers. The same differential expression analysis was done for the transcriptional profiles in IL4-treated macrophages vs IL-4-treated macrophages in the presences of SF2523, resting vs LPS-treated macrophages, LPS-treated macrophages vs LPS-treated macrophages in the presence of JQ1, and LPS-treated macrophages vs LPS-treated macrophages in the presence of SF2523.

**Chromatin immunoprecipitation**

Twenty four hours after LPS/IL4 stimulation and/or JQ1 treatment, BMDMs were fixed in 1% formaldehyde at 37°C for 10 min, then subjected to chromatin immunoprecipitation (ChIP) as previously published ([11](#_ENREF_11)). In brief, cells were treated with JQ1 (1 μM), or vehicle control for 24 hours and then cross-linked with 1.1% formaldehyde, washed with PBS and frozen at -80°C. Antibody-conjugated beads were prepared by blocking 50 μL of protein A/G agarose beads with 0.5% BSA (w/v) followed by incubation with 6.25 μg of BRD4 antibody, 5 μg of normal rabbit IgG, or 5 μL of tri-methyl histone H3 (Lys4) antibody. Cross-linked cells were lysed with lysis buffer 1 (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-100), washed with lysis buffer 2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA pH 8.0 and 0.5 mM EGTA pH 8.0), and sonicated in lysis buffer 3 (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-lauroylsarcosine) for 10 cycles for 30 seconds each on ice (18 W) with 60 seconds on ice between cycles. Sonicated lysates were supplemented with Triton X-100 to 1% and cleared. Aliquots were reverse-crosslinked and digested with RNase A overnight and purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) for quantification of input chromatin. Sonicated, cleared chromatin (15 μg) was incubated overnight at 4°C with antibody-conjugated agarose beads. Beads were washed three times with wash buffer (50 mM Tris-HCl pH 8, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% Na-deoxycholate, and 0.1% SDS): one time with was buffer with 500 mM NaCl, one time with LiCl wash buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, and 0.5% Na-deoxycholate), and one time with TE. Chromatin was eluted in elution buffer (50 mM Tris-HCl pH 8, 10mM EDTA, and 1% SDS), reverse cross-linked, and digested with RNase A overnight and then purified as above. Then 2 ng of each sample was analyzed in duplicate or triplicate by qPCR. The fold difference was calculated as 2^[Ct(input) 2 Ct(ChIP)], and fold enrichment over an unrelated IgG Ab was assessed. Oligonucleotides were as described in Supplementary Table S1.

**Isolation of single cells from tumors and Flow cytometry**

Tumors were isolated, minced and then enzymatically dissociated in Hanks Balanced Salt Solution containing 0.5 mg/ml collagenase IV (Sigma), 0.1 mg/ml hyaluronidase V (Sigma), 0.6 U/ml Dispase II (Roche) and 0.005 MU/ml DNAse I (Sigma) at 37°C for 30-45 min as described before ([24](#_ENREF_24)). Cell suspensions were filtered through a 70 μm cell strainer and red blood cells were lysed with red cell lysis buffer (Pharm Lyse, BD Biosciences, San Jose, CA). Cells were then washed with PBS and were used for flow cytometry analysis or magnetic bead purification of CD11b, Gr1 or CD90.2 cells (Miltenyi Biotech MACS Microbeads) according to manufacturer’s protocol. For flow cytometry staining, single cells isolated from tumors were incubated with Aqua Live Dead fixable stain (Life Technologies, Carlsbad, CA) followed by FcR-blocking (BD Biosciences, San Jose, CA) and incubation with fluorescently labeled antibodies directed against CD45 (30-F11), CD11b (M1/70), Gr1 (RB6-8C5), F4/80 (BM8), MHC-II (AF6-120.1) from BD Pharmingen. For T cell analysis, cells were incubated with CD3 (145-2C11), CD4 (GK1.5), CD8 (53–6.7), followed by Near IR live dead stain (Life Technologies, Carlsbad, CA). For intracellular staining, cells were fixed, permeabilized using transcription staining buffer set (ebiosciences) and then incubated with fluorescently labelled antibodies to FoxP3 (clone MF23) from BD Biosciences. For FACS analysis of JQ1 or SF2523 treated BMDMs, cells were stained with CD11b, F4/80 antibodies and CD206 antibodies (BD Biosciences). Multicolor FACS analysis was performed on a BD Canto RUO 11 Color Analyzer. All data analysis was performed using the flow cytometry analysis program FloJo (Treestar).

***In vivo* tumor growth and metastasis experiments**

LLC cells or B16 (1 x 105) were injected subcutaneously into syngeneic 4-6 week old C57Bl/6 mice or 1 x 105 CT26 tumors were injected subcutaneously in Balb/c or nude mice and were treated with 40 mg/kg of JQ1 (dissolved in 30% (w/v) captisol) or 40mg/kg SF2523 (formulated in 15% DMA + 30% captisol) when tumors reached a tumor volume of 100 mm3. For adoptive transfer experiments, 5 X 105 LLC cells were mixed with IL4 stimulated macrophages (1:1 ratio) treated with or without JQ1. In some studies, CT26 injected tumors in Balb/c mice were treated with 200µg of anti-CD8 (clone YTS 169.4) or an isotype control (LTF-2) from Bio-X-cell administered ip on day -3, 0, 3, 6 and 10 day of tumor inoculation.

For experimental metastasis, B16 F10 luciferase melanoma cells (5 x 105) were injected intravenously as previously described [[19](#_ENREF_19)] and mice were treated with 40 mg/kg SF2523 (5 times a week) until lungs were harvested on day 15. The luciferase signal was monitored on IVIS by injecting luciferin on day 15 (n=5), before harvesting tumors. For spontaneous metastasis, 1 × 106 Panc02 were implanted orthotopically into the pancreas of syngeneic mice and were treated with 40 mg/kg SF2523 as described before [[24](#_ENREF_24)]. In order to determine the efficacy of SF2523 in reducing breast cancer metastasis, the PYMT murine breast cancer model was used [[26](#_ENREF_26)]. 9 week old PyMT+ female mice (with spontaneous breast tumors) were treated with 40 mg/kg SF2523 (thrice weekly) for 4 weeks (n=10). Total tumor burden was obtained from detecting the total mammary gland mass in PYMT+ mouse.

**Immunoblotting**

Whole cell extract of BMDMs and CD11b+ cells isolated from tumors were prepared and protein was quantitated with a Bio-Rad protein assay kit (Bio- Rad Laboratories, Hercules, CA) using BSA as a standard. Equal proteins from protein lysates were resolved by SDS-PAGE, followed by immunoblotting and probing with primary antibodies against anti-mannose receptor antibody (Abcam), anti-RELM alpha antibody (Abcam), arginase (BD Biosciences), and β-actin (Santa Cruz).

**Cell viability assays**

Cell viability assay was performed on JQ1 and SF2523 treated LLC cells using AlamarBlue® (Roche) reagent according to manufacturer’s protocol. Briefly, 1x 104 LLC cells were seeded in 96 well plate. After 24 hrs, different conc. Of inhibitors were added. After 48 hr, AlamarBlue® was added and plates were incubated at 37°C in 5% CO2 for 6 hours. Fluorescence signals were read as emission at 590 nm after excitation at 560 nm.

***In vitro* cytotoxicity assay**

CD90.2+ T cells were isolated from JQ1 treated and vehicle LLC tumors and then co-incubated with LLC cells (target cells) at 1.25:1, 2.5:1 and 10:1 ratios of T cells to tumor cells for 24 hrs followed by measurement of lactate dehydrogenase release (Cytotox non-radioactive cytotoxicity assay kit, Promega).

**References**

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