Supplemental material

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SUPPLEMENTAL MATERIALS AND METHODS

Patient Eligibility

Eligible patients ($\geq$ 18 years) had histologically confirmed, measurable disease by RECIST v1.1, with at least 2 tumor masses accessible for biopsy purposes, ECOG performance status < 2, and a life expectancy of at least 12 weeks. Prior treatment with chemotherapy, immunotherapy or targeted therapy was allowed, if these treatments did not include dasatinib. Patients were excluded if they: i.) had received chemotherapy or radiotherapy within 4 weeks prior to entering the study, ii.) had not yet recovered from adverse events due to agents administered more than 4 weeks earlier, iii.) had documented c-KIT mutations, iv.) were receiving other investigational agents, v.) had active brain metastases, vi.) had a history of allergic reactions attributed to compounds of similar chemical or biologic composition to dasatinib or any of the components of the vaccine being administered, vii.) had a history of bleeding abnormalities or ventricular arrhythmias or viii.) had any active infection. The protocol was conducted per Declaration of Helsinki principles.

Patient Randomization and Assignment to Treatment Arm

Patients were randomized in a 1:1 ratio to the two study arms, stratified by BRAF mutation status. Patient randomization to treatment arm was conducted using the University of Pittsburgh Cancer Institute (UPCI) randomizer which was maintained by the Biostatistics Facility of the UPCI (https://randomize.upci.pitt.edu/randomizer/home.seam). The randomizer was accessed each time a new patient was enrolled, at which time only the assignment for that current patient was viewable. In all cases, the protocol coordinator (AR) assigned patients to interventions according to the randomizer's output.

Autologous αDC1/TBVA Peptide Vaccine Generation

Patients underwent a 3h apheresis procedure at baseline, for collection of a total volume of white blood cells between 125-250 ml. Vaccines were prepared according to cGMP guidance in the Immunologic Monitoring and Cellular Products Laboratory (IMCPL) at the Hillman Cancer Center. Elutriated leukapheresis product fractions enriched in monocytes were plated in DC medium (Cell Genix) with 1000 U/mL rhGM-CSF (Genzyme and Sanofi) and 1000 U/mL rhIL-4 (Cell Genix), with fresh cytokines added on day 3-4 of culture. On culture day 5, immature DC (iDC) were matured overnight by addition of clinical grade rhIL-1β (10 ng/mL), rhTNF-α (10 ng/mL), rhIFN-α (3,000 U/mL), rhIFN-γ (1,000 U/ml), and poly-I:C (20 μg/ml) at 37°C in 5% CO_2, as previously described[S1]. Harvested mature αDC1 cells were then loaded with a cocktail of the certified

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synthetic $DLK1_{310-318}$ (ILGVLTSLV), $EphA2_{883-891}$ (TLADFDPRV), HBB_{31-39} (RLLVVYPWT), $NRP1_{433-441}$ (GMLGMVSGL), $RGS5_{5-13}$ (LAALPHSCL), $TEM1_{691-700}$ (LLVPTCVFLV) peptides (at a final concentration of 1 μg/mL of each peptide in CellGenix media) for 3-4 hours at 37°C, 5% CO_2. Criteria for release of patient αDC1/peptide vaccines included: sterility by Gram stain and bacteriologic culture; negative *Mycoplasma*; endotoxin lower than 5.0 EU/kg of body weight; greater than 70% expression of both CD86 and HLA-DR on αDC1 cells as determined by flow cytometry. The first vaccine was administered fresh, with subsequent vaccines cryopreserved in 10% DMSO/40% AB serum/50% CellGenix medium until time of use when the cells were thawed prior to injection. Fresh and thawed vaccines were shown to be viable and phenotypically stable for at least 4h at 4°C. Freshly prepared patient αDC1 vaccine cells were also analyzed for secretion of IL-12p70 and IL-10 into culture supernatant after *in vitro* stimulation as previously decsribed[1] using cytokine-specific ELISAs (BD Biosciences).

IFNγ ELISPOT Assays

Patient peripheral blood T cell responses against peptides in the vaccine formulation were examined using standardized IFNγ ELISPOT assays using HLA-A2$^+$ T2 cells as an antigen-presenting cell. T2 cells were left untreated or pulsed with individual vaccine-associated TBVA peptides or HLA-A2-presented peptides derived from alternate TBVA-associated proteins (i.e. $NRP1_{331-339}$ [GLLRFVTAV], $PDGFR\beta_{890-898}$ [ILLWEIFTL], $VEGFR1_{770-778}$ [TLFWLLLTL], $VEGFR2_{773-781}$ [VIAMFFWLL])[S2,S3] or melanoma-associated proteins (i.e. $MART1_{26-35}$ [EAAGIGILTV], $gp100_{209-217}$ [ITDQVPFSV], $TYR_{368-376}$ [YMNGTMSQV])[S4] overnight at 37°C, prior to washing in PBS and addition to IFNγ ELISPOT wells. Triplicate determinations were performed. Plates were analyzed on a CTL Technologies ImmunoSpot reader (Cleveland, OH).

Flow Cytometry

Baseline and on-treatment patient peripheral blood mononuclear cells were analyzed for frequencies of CD4$^+$Foxp3$^+$ Treg, HLA-DR^{neg}CD3^{neg}CD11b$^+$CD14$^+$CD15^{neg}CD19^{neg}CD33$^+$ monocytic MDSC (M-MDSC) and HLA-DR^{neg}CD3^{neg}CD11b$^+$CD14^{neg}CD15$^+$CD19^{neg}CD33$^+$ polymorphonuclear MDSC (PMN-MDSC) using the following directly-conjugated antibody probes: α-FoxP3-PE antibody (eBioscience), α-HLA-DR-FITC (BD Pharmingen), α-CD3-PE-Cy7 (BD Pharmingen), α-CD4-FITC (BD Pharmingen), α-CD19-PE-Cy7 (BD Biosciences), α-CD33-APC (BD Pharmingen), α-CD11b-Pacblue (BD Pharmingen), α-CD14-PerCP (BD Biosciences), and α-CD15-PE (BD Pharmingen). Data analysis was performed using FACSDiva™ software (BD Biosciences). For Treg, cells were first stained using α-CD4 mAb, then washed and

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fixed/permeabilized with FoxP3/Transcription Factor staining buffer set (eBioscience) per the manufacturer's instruction. Permeabilized cells were then intracellularly stained with α-FoxP3 antibody. Fluorescent positivity was determined by utilizing appropriate antibody isotype controls.

Vaccine DC were also phenotyped for cell surface molecules not involved in cell product release by flow cytometry using the following fluorescently labeled Abs: α-CD266-APC (BioLegend), α-CTLA4-BV786 (BD Biosciences), α-Gal-9-PE (BD Biosciences), α-ICOSL-BV421 (BD Biosciences), α-OX40L-PE (BioLegend), α-PD-L1-BV421 (BioLegend), α-PD-L2-BV711 (BD Biosciences) and appropriate isotype-matched control Abs (BioLegend). Live cell gating was established using Zombie Aqua Viability Dye (BioLegend).

Serum Testing

Baseline and on-treatment (week 5) patient serum specimens were analyzed using a commercial ELISA specific for human CXCL10/IP-10 (R&D Systems) by the IMCPL. The IMCPL also analyzed patient sera for levels of soluble checkpoint molecules using the multiplex Luminex Immuno-Oncology Checkpoint Marker Panel detecting soluble forms of BTLA, CD27, CD28, CD80, CTLA-4, GITR, HVEM, IDO, LAG-3, PD-1, PD-L1, PD-L2, TIM-3 and 4-1BB. In these assays, sera from 19 healthy male and female donors (age range 23-65) were included as normal controls.

RNA Isolation and Gene Expression Analyses

Total RNA was isolated from vaccine αDC1, tumor biopsies and peripheral blood mononuclear cells and subjected to qRT-PCR and GeneChip™ Human Genome U133A 2.0 Arrays (Thermo Fisher Scientific) profiling performed by the University of Pittsburgh Genomics Research Core, to the Oncomine TCRB-LR Assay and to the Oncomine™ Immune Response Research Assay (OIRRA; Thermo Fisher Scientific, Carlsbad, CA) under an institutional material transfer agreement (MTA). TCRβ chain repertoire libraries were constructed by multiplex PCR utilizing FR1 and constant gene targeting primers via the Oncomine TCRB-LR assay, then sequenced using the Ion Torrent S5 to a target depth of 1.5M raw reads per library. To evaluate T cell receptor convergence, we searched for instances where TCRβ chains were identical in amino acid space but had distinct nucleotide sequences owing to N-addition and exonucleotide chewback within the V-D and D-J junctions of the complementary determining region 3 (CDR3). Targeted gene expression profiling of pre- and post-treatment tumor biopsies was performed via the Oncomine Immune Response Research Assay (OIRRA) using total RNA input.

Biostatistics and Bioinformatics Analyses

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All correlative data analyses were conducted using R (version 3.4.3) and Bioconductor. Efficacy and safety were assessed in all patients who received one or more cycles of vaccine +/- dasatinib. The association between variables and response was examined by comparison between responders and non-responders with the two-sided two-sample *t* test, Wilcoxon signed rank or Fisher's exact test, when appropriate. Survival data were analyzed with the Kaplan-Meier method and compared with log-rank tests. For exploratory endpoint survival analyses, the R packages survminer (v 0.2.4) and survival (v 2.11-4) were used. Association of variable with survival endpoints was examined with the Cox proportional hazards model. Comparisons between datasets were performed using non-parametric tests, as detailed in the figure legends. A p-value of 0.05 or less was considered statistically significant. No adjustments for multiple comparisons were performed. Kaplan-Meier analysis was used to estimate the median OS and PFS. Log rank tests were used to compare the OS and PFS between different study arms. Serum cytokines with detection rate < 50% were dichotomized, and those with $\geq$ 50% detection rate were treated as continuous endpoints. Associations of DC cell surface markers and secretion levels of IL-12p70 and IL-10 with vaccine antigen response was determined by performing a student's t-test of the expression level for patients who were vaccine antigen responders against those who were non-responders. Flow cytometry analyses of vaccine αDC1 and patient peripheral blood MDSC and Treg cells were processed to compare absolute and percentage cell counts at baseline. Change between time points was determined by calculating both the difference and fold change between the events, with statistical differences assessed using a student's t-test. Serum levels were determined by ELISA or Luminex for CXCL10 and soluble checkpoint proteins, respectively, at baseline vs. 5 weeks on-treatment. Change between time points was determined by calculating both the difference and fold change between the time points. Statistical testing was performed using student's t-test. Spearman's correlation coefficients were calculated to determine associations present between differentially expressed genes (DEG) in patient mDCs and tumor biopsy tissues and patient immune/clinical responses to treatment. Gene set enrichment was performed on transcriptional data sets from patient DC and tumor (baseline and on-treatment) samples using the GSEA software (V.4.1.0), a joint project of the University of California-San Diego and the Broad Institute[S5,S6].

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