Generation of IDH1m HLA.A2/DR1 (dTG)-syngeneic tumor cell line

Primary astrocytes isolation[1]: cortical tissue from the brain of 4 neonatal dTG mice was dissected and incubated with 0.25% Trypsin to generate a single-cell suspension. The cells were washed, resuspended in astrocyte culture medium (DMEM + 10% FBS + 1% P/S), and plated in a flask coated with poly-L-lysine. Media was changed 2 days later and again every 3 days for a total of 11 days when the adherent cells reached confluency. The resulting astrocyte culture was depleted of microglia using the CD11b microbeads magnetic cell sorting kit (Miltenyi Biotec, #130-049-601) following the manufacturer's protocol.

Astrocytes transformation: $3x10^5$ cells were plated per well in a 6-well plate pre-coated with poly-L-lysine. The following day, the cells were transfected using FuGENE HD (Promega, #E2311) with 0.5µg/well pT2/C-Luc/PGK-SB1.3 (Addgene plasmid #20207), 0.5µg/well pT/Caggs-NrasV12 (Addgene #20205), 0.5µg/well pT2/shp53/GFP4/mPDGFβ (a gift from Dr.John Ohlfest), and 1.5µg/well pT3.5/Caggs-HsIDH1R132H (a gift from Dr. David Largaespada) following Promega's protocol. After several passages, transformed colonies were observed. To isolate a uniform population, the resulting pool of transformed cells underwent single-cell cloning. Multiple clones were screened for the expression of IDH1m and GFP *in vitro* and their ability to reproducibly form tumors *in vivo*. Cells isolated *ex-vivo* were expanded, maintained in complete DMEM (includes 10% FBS, 1% P/S, 1mM Na-Pyr, 1X NEAA, 2mM GlutaMAX, and 10mM HEPES) and were used in the remainder of the studies (referred to as dTG-$IDH1^{R132H}$). For experiments evaluating responses to Class I TAAs, the dTG-$IDH1^{R132H}$ cells were transduced with a MMLV retroviral construct expressing the $hgp100_{(209–217)}$, $hTYR_{(368–376)}$, and h/$mTRP2_{(180–188)}$ peptides (Fig. 5E), and a blasticidin resistance gene. The plasmid was synthesized by VectorBuilder. The resulting dTG-$IDH1^{R132H}$-TAA^{+} cells (Fig. 5D) were selected using 10µg/ml blasticidin (maintenance 5µg/ml).

Pharmacodynamics and pharmacokinetic analyses

In vitro (Cell Pellet) LC-MS/MS Analysis

Cell pellets were lysed with 170-uL of methanol:water (80:20 [v/v]).

- AG-881:

Calibration standards and quality control (QC) samples were prepared in methanol:water (80:20 [v/v]). A 10 uL aliquot of calibration standards and QCs was mixed with 120 uL of methanol:water (80:20 [v/v]) in a 96-well plate. Cell lysate (130 uL) was aliquoted into the plate. All samples were mixed with 250 uL of acetonitrile containing internal standard AGI-0028187 (1 ng/mL) for protein precipitation. The mixture was vortexed at 800 rpm for 4 min and centrifuged at 4000 rpm, at 4°C for 10 min. A 300 µL aliquot of supernatant was transferred to a clean plate and dried down. The dried extract was reconstituted in 100 µL acetonitrile:water:formic acid (50:50:0.1 [v/v/v]), and 1 µL of the final sample was injected onto the LC-MS/MS system for analysis which was carried out on a SCIEX Triple Quad™ 6500+ with Exion LC™ AD system. A reversed-phase gradient method using a Waters ACQUITY UPLC HSS T3 Column (100Ã, 1.8µm, 2.1 mm X 50 mm) maintained at 50 °C, provided chromatographic separation. Mobile phases A and B used were water with 0.1% formic acid and acetonitrile with 0.1% formic acid respectively, at a total flow rate of 600 µL/min. AG-881 and it's internal standard were ionized under a positive ion electrospray mode and monitored using the multiple-reaction monitoring (MRM) transitions of m/z 459.1→260.2 and m/z 421.0→266.1. The peak area ratios of analyte relative to internal standard were used for AG-881 quantitation. Linearity was achieved in the AG-881 concentration range from 1 ng/mL to 30,000 ng/mL. A concentration factor of 13X was factored into final calculated concentrations.

- 2-HG:

Calibration standards and quality control (QC) samples were prepared in methanol:water (80:20 [v/v]). A 30 uL aliquot of cell lysate, calibration standards and QCs was mixed with 30 uL of internal standard ([$^{13}C_5$]2-HG, 200 ng/mL), followed by protein precipitation with 200uL of

acetonitrile. The mixture was vortexed and centrifuged and an aliquot of 180 µL of supernatant was transferred to a clean plate and dried down. The dried extract was reconstituted in 200 µL acetonitrile:water:formic acid (25:75:0.1 [v/v/v]), and 10 µL of the final sample was injected onto a a SCIEX 4000QTRAP™ with Waters ACQUITY UPLC® LC-MS/MS system for analysis. Chromatographic separation was performed using a gradient elution on a Thermo Scientific™ Hypersil GOLD™ aQ C18 Polar Endcapped Column (175Å, 3 µm, 3 × 100mm) maintained at 40 °C. Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A and B respectively at a total flow rate of 700 µL/min. MS detection was done on electrospray ionization, negative ion mode and the MRM transitions of m/z 147.0/129.0 and m/z 152.0/134.0 were monitored for 2-HG and [$^{13}C_5$]2-HG respectively. The peak area ratios of the analyte relative to the internal standard were used for 2-HG quantitation. Linearity was achieved in the 2-HG concentration range from 1 ng/mL to 30,000 ng/mL in methanol:water (80:20 [v/v])

In vivo (Plasma/tissue) LC-MS/MS Analysis (AG-881)

A non- validated protein precipitation method was used for analysis of AG-881 in plasma samples. Calibration standards and quality control (QC) samples were prepared in blank mouse plasma. Brain/tumor samples were homogenized using a FastPrep homogenizer, with 10 volumes (volume-by-weight [v/w]) of homogenizing solution (80:20 methanol in water [v/v]) to get a homogenate with a dilution factor of 11. A 10-µL aliquot of calibration standards, QCs, unknown plasma and tissue homogenate were mixed with 200 µL of acetonitrile containing the stable labeled internal standard (IS) AGI-0028187 at 25 ng/mL, for protein precipitation. The mixture was vortexed and centrifuged. A 100 µL aliquot of supernatant was mixed with 100 µL of water with 0.1% formic acid and vortexed to mix. A 5 µL injection volume was analyzed on a SCIEX Triple Quad™ 6500+ with Exion LC™ AD system. A reversed-phase gradient method using a Waters ACQUITY UPLC HSS T3 Column (100Ã, 1.8µm, 2.1 mm X 50 mm) maintained at 50 °C, provided chromatographic separation. Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A and B respectively, at a total flow rate of

600 µL/min. AG-881 and it's internal standard were ionized under a positive ion electrospray mode and monitored using the multiple-reaction monitoring (MRM) transitions of m/z 459.1→260.2 and m/z 421.0→266.1. The peak area ratios of analyte relative to internal standard were used for AG-881 quantitation. Linearity was achieved in the AG-881 concentration range from 1 ng/mL to 30,000 ng/mL.

In vivo (Tissue) LC-MS/MS Analysis (2-HG)

The concentrations of 2-HG in brain/tumor were determined using a qualified LC-MS/MS method. Tissue samples were homogenized using a FastPrep homogenizer with 10 volumes (volume-by-weight [v/w]) of methanol:water (80:20 [v/v]) to get a homogenate with a dilution factor of 11. Calibration standards and quality control samples for 2HG were prepared in surrogate matrix methanol:water (80:20 [v/v]). In a 96-well plate, 30 µL aliquot of calibration standards, quality control samples, tissue homogenates were mixed with 30 µL internal standard ([$^{13}C_5$]2-HG at 200 ng/mL) followed by addition of 200 µL of acetonitrile to each sample for protein precipitation. The mixture was vortexed and centrifuged, and 200 µL of supernatant of was transferred to a clean plate and dried down under vacuum followed by reconstitution in 150 µL acetonitrile:water:formic acid (25:70:0.1 [v/v/v]). A 5 µL aliquot of the final samples were injected for analysis on a SCIEX 4000QTRAP™ with Waters ACQUITY UPLC®. Chromatographic separation was performed using a gradient elution on a Thermo Scientific™ Hypersil GOLD™ aQ C18 Polar Endcapped Column (175Å, 3 µm, 3 × 100mm). The column temperature was kept at 40 °C. Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A and B respectively at a total flow rate of 700 µL/min. Electrospray ionization method for MS detection was used on negative ion mode and the MRM transitions monitored for 2-HG and [$^{13}C_5$]2-HG were m/z 147.0/129.0 and m/z 152.0/134.0 respectively. The peak area ratios of the analyte relative to the internal standard were used for 2-HG quantitation. Linearity was achieved in the 2-HG concentration range from 1 ng/mL to 30,000 ng/mL in methanol:water (80:20 [v/v]).

In vivo (Plasma) LC-MS/MS Analysis (2-HG)

A qualified LC-MS/MS method with surrogate analyte approach was used to quantitate 2-HG in mouse plasma samples. Surrogate analyte ([$^{13}C_5$]2-HG was used to prepare calibration standards and quality control samples in blank mouse plasma matrix. A 30-µL aliquot of calibration standards, quality control samples and unknown plasma samples was mixed with 30 µL internal standard ([$^{13}C_5D_5$]2-HG at 1000 ng/mL) and further precipitated with 200 µL of acetonitrile. The mixture was vortexed and centrifuged and a 200-µL aliquot of supernatant was transferred to a clean plate and dried down. The dried extract was reconstituted in 150 µL acetonitrile-water-formic acid (25:75:0.1 [v/v/v]), and a 10- µL aliquot was injected onto an LC-MS/MS system, SCIEX 4000QTRAP™ with Waters ACQUITY UPLC®, for analysis. A Thermo Scientific™ Hypersil GOLD™ aQ C18 Polar Endcapped Column (175Å, 3 µm, 3 × 100mm), maintained at 40 °C, was used for chromatographic separation of analytes. Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phase A and B respectively for gradient elusion, at a total flow rate of 700 µL/min. MS detection was done on electrospray ionization, negative ion mode and the MRM transitions monitored for 2-HG, [$^{13}C_5$]2-HG and [$^{13}C_5D_5$]2-HG were m/z 147.0/129.0, m/z 152.0/134.0 and m/z 157.00/139.10 respectively. The peak area ratios of the surrogate analyte relative to the internal standard were used for 2-HG quantitation. Linearity for [$^{13}C_5$]2-HG was achieved in the concentration range from 10 to 30,000 ng/mL in mouse plasma.

Nanostring data normalization and analysis

Background correction was performed with the Background Thresholding option in nSolver software (v4.0), whereby a threshold count value was set to 11, and all counts which fell below that value were adjusted to match it. All the subsequent computational analyses were performed using R (v4.0.3). For the subsequent differential expression (DE) analysis, the factors of

unwanted variation were estimated based on the housekeeping genes and implemented into the design matrix, according to the RUVSeq workflow.[2] DE analysis was performed with the aforementioned design matrix using the DESeq2 R package (v1.30.0).[3] For multiple testing, p-values were adjusted with the Benjamini-Hochberg method.

Flow cytometry

Single cell suspensions (0.5-1x10^6 cells) of spleens, lymph nodes, BILs, or TILs were pre-incubated with blocking solution (BioLegend, 156604). After 10 min, a cocktail of fluorophore-conjugated antibodies resuspended in 50 µl FC buffer (1X PBS, 0.5% FBS, 2 mM EDTA) was added directly to each tube, and samples were incubated at 4°C for 20 min in the dark. Samples were washed with excess buffer and resuspended for analysis. Samples were analyzed using Invitrogen Attune NxT (Thermo Fisher Scientific).

ELISpot assay

Following completion of the study, available samples from each treatment group were thawed, and TILs were isolated by subsequently enriching the cell suspensions for leukocytes through a 70%/30% Percoll gradient separation, followed by depletion of $CD11b^+$ leukocytes using the CD11b microbeads magnetic cell sorting kit (Miltenyi Biotec). The resulting $CD11b^-$ TILs were added to peptide-loaded APCs and co-cultured at 37°C for 7 days. APCs were prepared from the splenocytes of naïve 6-10-week-old dTG mice by depletion of $CD3^+$ lymphocytes using the Mouse CD3 T-cell Positive Selection Kit (BioLegend, #480031) followed by 18hrs treatment with 50ng/ml IFNɣ. Prior to co-culture with TILs, class I (TAAs) or class II (R132H) peptides were added to the APC suspension at 10µg/ml. After 7 days, viable lymphocytes were collected and counted from the *ex vivo* restimulation cultures. IFNɣ ELISpot assay (Mouse IFNɣ ELISpot Kit, R&D Systems, EL485) was performed by co-culturing 2x10^4 restimulated TILs with freshly prepared peptide-pulsed APCs at a 1:5 (TIL:APC) ratio for 36hrs at 37°C. Media only and each cell type cultured alone were used as negative controls and TILs + 1X cell activation cocktail (BioLegend, 423302) was used as a positive control. IFNɣ spots were developed according to

the kit's manufacturer protocol. Spots were imaged and quantified using the CTL S6 Universal-V Analyzer ELISpot Reader (ImmunoSpot).

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