SUPPLEMENTARY APPENDIX

Leukemia vaccine overcomes limitations of checkpoint blockade by evoking clonal T-cell responses in a murine acute myeloid leukemia model

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Supplemental Methods

Cell Lines: The murine AML cell line TIB-49 was purchased from ATCC. Cells were tested for Mycoplasma contamination (Myco-Alert Mycoplasma Detection Kit, LT07-318, Lonza). For all experiments, cell lines were transduced with luciferase/Mcherry using a lentiviral vector (pCDH-EF-eFFLy-T2A-mCherry) kindly provided by Prof. Irmela Jeremias from Helmholtz Zentrum München, Germany and then sorted to obtain a greater than 99% positive population. Cells were cultured at 37 °C in a humidified 5% CO2 incubator in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% fetal bovine serum (Atlanta Biologicals, Flower Branch, GA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Cellgro).

Murine LSK cells were obtained from transgenic C57BL/6J mice expressing mIDH2 (IDH2R140Q) and subsequently transduced with Hoxa9-GFP andMeis1a-YFP oncogenes as previously described[18].

Flow cytometry: Cells were analyzed for mCherry, CD62L, CD44, CD4, CD8, CD86 and CD25 expression by multichannel flow cytometry. Cells were incubated with FcR blocking reagent (Miltenyi, Bergisch Gladbach, Germany) for 10 min at room temperature followed by anti-CD62L APC (BD Pharmingen), anti-44-PE (BD Pharmingen), anti-CD4- BV (BioLegend, San Diego, CA), anti-CD8-APC-cy7 (BioLegend, San Diego, CA) or appropriate isotype control. Analysis was performed using FACS Aria (BD Biosciences, San Jose, CA) and Kaluza software (Beckman Coulter, Brea, CA).

For intracellular IFN-γ expression, T cells were pulsed with GolgiStop (1 μg/ml; BD Pharmingen) for 4–6 h at 37 °C then labeled with CD4-BV and CD8-APC-Cy7. Permeabilization with Cytofix/Cytoperm (BD Pharmingen) containing formaldehyde and saponin was performed for 30 min at 4 °C. Cells were washed twice in Perm/Wash solution and incubated with PE-conjugated IFN-γ (Invitrogen, Camarillo, CA) or a matched isotype control for 30 min. Cells were washed in 1 × Perm/Wash solution prior to analysis.

Murine survivin expression in TIB-49 cells was assessed using intracellular flow cytometric analysis using survivin (60.11) [Alexa Fluor® 647] mAbs (Novus, USA). Alexa Fluor® 647 Mouse IgG2a, κ was used as isotype control.

Vaccination with DC/AML fusions and/or treatment with checkpoint inhibitors in vivo: All animal studies were approved by BIDMC IACUC. Murine syngeneic DC/AML fusion cells were generated as previously described19. Briefly, DCs were generated from bone marrow mononuclear cells harvested from the femurs of C57BL/6J mice cultured in the presence of IL-4 and GM-CSF for 5-7 days. DCs were fused with TIB-49 mCherry AML cells or mutant IDH2/ Hoxa9-GFP/ Meis1a-YFP primary AML cells in the presence of PEG and exposed to 30Gy gamma irradiation. DC/AML fusion cells were quantified by determining the percentage of cells with co-expression of DC (anti-CD86-Alexa-647) and tumor (Mcherry) markers by flow cytometric analysis. C57BL/6J mice were inoculated retro-orbitally with 5×104 luciferase/mcherry TIB-49 murine leukemia cells (lmTIB) or 20×104 mutant IDH2/ Hoxa9-GFP/ Meis1a-YFP primary AML cells using tail vein injections. Cohorts of mice were assigned to treatment with 100x103 DC/AML fusion cells via subcutaneous injection 24 hours after AML challenge;

intraperitoneally with 200ug each of rat anti-mouse PD-1 (29F.1A10) rat IgG2a,k (Bio X Cell, NH USA); mouse anti-mouse TIM3 (T3A.1A10) mIgG1,k; rat anti-mouse RGMb (307.9D1) rat IgG2a,k or all three mAbs starting 4 days after AML challenge and continued every 3 days for 6 doses; or the combination of DC/AML fusion vaccine and mAbs treatment.

Bioluminescent imaging: BLI was performed using a Xenogen IVIS-50TM camera and analyzed with Living Image software (Caliper LifeSciences).

TCR diversity analysis: Targeted TCR diversity analysis was interrogated using SMARTer human α/β profiling kit (Takara, CA, USA). Initially total RNA was extracted and purified from mouse blood using RNeasy mini kit (Qiagen, Germantown, MD). See supplemental methods for details.

RNA quality was assessed using the RNA pico chip (Agilent 2100 bioanalyzer). Targeted TCR libraries were prepared from the high-quality RNA using the 5'-SMART (Switching Mechanism At the 5' end of RNA Template) approach. The TCR library quality was verified using HS DNA chips (Agilent 2100 Bioanalyzer). The high quality TCR libraries were sequenced on Illumina MiSeq Sequencer using the 600-cycle MiSeq Reagent kit v3 (Illumina, San Deigo, CA) with paired end (2X300 base pair reads).

The sequencing data was checked for quality control to remove low quality reads and aligned against TCR sequences from GenBank and IMGT database[20] using MiXCR software[21]. The aligned reads were assembled into TCR clones and their frequency or abundance was estimated. After normalization, the assembled clonotypes were analyzed to determine overall diversity of each sample using Inverse Simpson index

and rarefaction analysis. The diversity patterns of samples (i.e., Control, vaccine, and vaccine + checkpoint point inhibitors) were compared to gauge the overall variation in TCR repertoire that is associated with different groups. Comparative analysis between the treatment groups also involved identification of dominant clones and specific tracking analysis to identify TCR clones that are stimulated by vaccine treatment and further enhanced or maintained by combined vaccine and checkpoint-based therapy.

Single Cell RNA Sequencing: scRNA-Seq was performed on peripheral blood mononuclear cells isolated from control or cohorts treated with the DC/AML fusion vaccine, or vaccine + checkpoint point inhibitors. 10X Genomics chromium system was employed for capturing single cells in the context of uniquely barcoded primer beads together in tiny droplets enabling large-scale parallel single-cell transcriptome studies. The single cell suspensions were generated using a 10X Chromium Controller Instrument[21]. The libraries were prepared using the Chromium single cell 3' GEM, library and gel bead kit V3 (10X Genomics, Pleasanton, CA) and sequencing was performed using the massively parallel sequencing NextSeq 500 platform. Approximately 30,000-40,000 reads per cell were performed capturing the expression of approximately 500-1,000 transcripts.

Analysis for single cell RNA sequencing data: RNA sequencing data was analyzed using standard statistical algorithms after quality control filtering, alignment to the reference genome (mm10) to generate raw counts for transcripts from each cell type. The data was preprocessed by removing the outlier cells with very low or high number of features (i.e. <200 & >2500 transcripts) or high UMI mapping to mitochondrial genes (i.e. >15%). Genes that were detected in less than three cells were removed. The

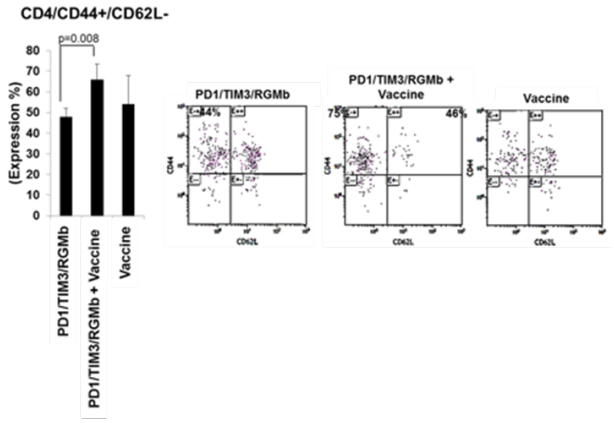
preprocessed raw count data was log normalized using Seurat R package (version 3.0) for unsupervised and supervised analysis[22]. The scRNA-Seq samples from control, vaccine and combo groups were merged using Find Integration Anchors and integration functions in Seurat to generate an integrated matrix of normalized data[23]. Normalized and preprocessed data was subjected to unsupervised analysis using PCA to identify the principal components with significant variation applied for uniform manifold approximation projection (UMAP) analysis to determine overall relationship among cells[23]. Transcriptome profiles were clustered and annotated to different cell types including T cells (CD3+), B cells (CD19+, CD79+,) and other immune cells based on expression of specific transcripts. Comparative analysis of the various cell types or subtypes in each cluster from control, vaccine and combination vaccine and checkpoint blockade cohorts. The distribution of various cell specific marker transcriptome profile was determined using feature plot function in the Seurat R package[24]. The significance of the differentially expressed transcripts was determined using t-stats ($P < 0.05$) and fold change (≥ 1.2).

Pathway, Functions and Systems Biology Analysis: Pathways, functions and systems biology analysis was performed using the Ingenuity Pathway Analysis software package (IPA 9.0) (Qiagen-detailed description available at http//www.ingenuity.com). The significance of effect on pathways and functional categories was determined using one-tailed Fisher's Exact test. The pathways, functions with a P value <.01 were considered statistically significant. The pathways, and functions with positive Z-score ≥ 2 and ≤ -2 were considered significantly activated and inhibited respectively.

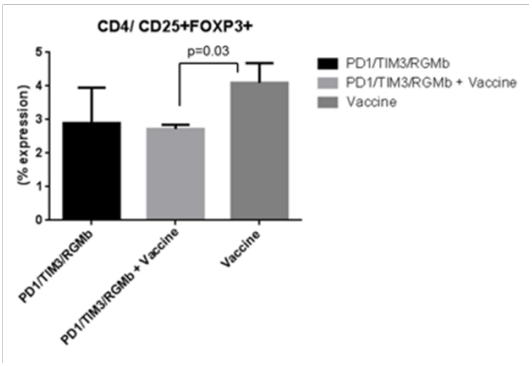
Supplemental Figures

Supplemental Figure 1:

A.



B.



Supplemental Figure 2: