Supplemental Data

**Eligibility**:

*Inclusion Criteria:*

Patients must meet all of the following inclusion criteria to participate in this study:

* Willing and able to provide written informed consent for the trial
* >18 years and <70 years of age on day of signing informed consent
* ECOG Performance Status 0-1
* Histologically or cytologically confirmed recurrent AML as defined by >5% myeloblasts in the bone marrow aspirate and/or biopsy
* Must have received at least 1 cycle of induction chemotherapy for front-line AML including:
  + Cytarabine continuous infusion + anthracycline +/- cladribine or etoposide for 1 or 2 cycles OR
  + Liposomal cytarabine and daunorubicin (CPX-351) OR
  + High dose cytarabine with or without fludarabine OR
  + Cladribine or clofarabine OR
  + >4 cycles of azacitidine/decitabine OR
  + The equivalent experimental therapy (as determined by PI)
* Cyoreduction allowed with hydroxyurea and/or leukapheresis for up to 14 days prior to treatment. Patients must be off hydroxyurea for >12 hours prior to day 1 of treatment.
* Serum creatinine <1.5 x upper limit or normal or creatinine clearance >60 mL/min in subjects with creatinine >1.5 x upper limit of normal
* Serum total bilirubin <1.5 x upper limit of normal unless due to Gilbert’s Disease, hemolysis, or leukemic infiltration
* AST and ALT <5x upper limit of normal
* INR/PT or aPTT <1.5 x upper limit of normal unless subject is receiving anti-coagulant therapy
* Female subject of childbearing potential should have a negative urine or serum pregnancy within 72 hours prior to receiving the first dose of HiDAC treatment and again prior to day 1 of pembrolizumab treatment.
* Female subjects of childbearing potential should be willing to use adequate methods of contraception.
* Male subjects must agree to adequate methods of contraception.
* Ability of the subject to understand and comply with study procedures.

*Exclusion Criteria:*

* Currently participating in or has participated in a study of an investigational agent or using an investigational device within 4 weeks of first dose of treatment
* Diagnosis of immunodeficiency or is receiving systemic steroid therapy or any other form of immunosuppressive therapy within 7 days of prior to first dose of HiDAC
* Known history of active TB
* Hypersensitivity to pembrolizumab or any of its excipients
* Prior monoclonal antibody within 4 weeks prior to HiDAC
* Prior chemotherapy, targeted small molecule therapy, or radiation therapy within 2 weeks prior to day 1 of treatment
* Grade >1 adverse events from prior therapies
* Known additional malignancy that is progressing or requires active treatment. Exceptions include basal cell carcinoma of skin, squamous cell carcinoma of skin that has undergone potentially curative therapy, or in situ cervical cancer that has undergone potentially curative therapy.
* Known active central nervous system (CNS) leukemia; subjects with previously treated CNS disease may participate provided they are stable (without evidence of active disease by imaging for at least 4 weeks prior to the first dose of treatment, and any neurologic symptoms have returned to baseline), have no evidence of new or enlarging brain metastases, and are not using steroids for at least 7 days prior to HiDAC
* Active autoimmune disease that has required systemic treatment in the past 2 years (i.e. with use of disease modifying agents, corticosteroids or immunosuppressive drugs). Replacement therapy (eg., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment
* Evidence of interstitial lung disease or a history of (non-infectious) pneumonitis that required steroids or current pneumonitis
* History or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the trial, interfere with subject’s participation for the duration of the study, or is not in the best interest of the subject to participate
* Known psychiatric or substance abuse disorders that would interfere with cooperation with the requirements of the trial
* Pregnant or breastfeeding, or expecting to conceive or father children within the projected duration of the trial
* Prior therapy with an anti-PD-1, anti-PD-L1, anti-PD-L2, anti-CD137, anti-CTLA-4 antibody
* Known history of HIV, active hepatitis B or hepatitis C
* Has received live vaccine within 30 days prior to HiDAC
* Uncontrolled intercurrent illness including but not limited to active and uncontrolled infection, symptomatic congestive heart failure, unstable angina, and uncontrolled symptomatic cardiac arrhythmia. Patients with infection under active treatment and controlled with antibiotics are eligible
* Acute promyelocytic leukemia (APL)
* Receipt of previous allogeneic stem cell transplant; receipt of previous autologous transplant for AML or non-AML condition is allowed

**Continuous Monitoring for Toxicity:**

Sequential boundaries were used to monitor unacceptable toxicity rate in the trial. An unacceptable toxicity was defined as any drug-related grade 3 non-hematologic toxicity (exceptions include infusion reactions, rash, fever, infection, nausea, fatigue and anorexia) persisting for >7 days despite supportive care), or any drug-related grade 4-5 non-hematologic toxicity (excluding infection). The accrual was planned to be halted if excessive numbers of unacceptable toxicities is equal to or exceeds b*n* out of *n* patients with full follow-up (**Supplementary Table 1**). This is a Pocock-type stopping boundary that yields the probability of crossing the boundary at most 0.05 when the rate of unacceptable toxicity is equal to the acceptable rate of 0.2.

The stopping boundary above guided enrollment as well as suspension of accrual (i.e., when to stop the trial if necessary). Initially 3 patients were enrolled. Enrollment was continued in the same manner so that at any point in the trial the number of patients with unacceptable toxicities and the number of patients in the follow-up does not exceed *bn* where *n* is the total number of patients accrued.

Dose modifications of pembrolizumab are outlined in **Supplementary Table 2.**

**Clinical Next Generation Sequencing (NGS) Assay:**

Pooled libraries were sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA), and variant review was performed using SOPHiA Genetics DDM™ software and Integrated Genome Viewer. All known or likely pathogenic variants were reported, if present at or above the assay’s limit of detection of 3-5% variant allele fraction (VAF). Paired sequence analysis of a normal germline sample was not performed with the clinical NGS platform. However, all variants were rigorously annotated utilizing internal variant databases, germline variant databases (e.g. gnomAD, ClinVar), and somatic variant databases (e.g. COSMIC). Variants of unknown significance and benign/likely benign variants were excluded. NGS panel with genes analyzed is shown in **Supplementary Table 3**. Two patients had local NGS assessment with similar methodology as above.

***Immune Biomarker Correlates:***

BM mononuclear cells were thawed and an aliquot was stained for AML blast markers (CD33 APC, CD133 VioGreen, CD117 PE, and CD34 VioBlue (Miltenyi) with F700 Live/Dead (BD Biosciences Cat. No 56499). Each sample was analyzed by flow cytometry to determine an AML marker that would positively select an enriched population of AML blasts. Cells were resuspended in MACS buffer with Bovine Serum Albumin and incubated with Microbeads (Miltenyi Biotech) that corresponded to the chosen marker for selection. Cells were applied to a MS cell magnetic cell separation column (Miltenyi Cat. No 130-042-201) and the flow-through contained blast reduced cells and the positive fraction eluted from the column contained the blast enriched cell population. DNA and RNA were extracted from selected cells (blast enriched) and RNA was extracted from unselected cells (blast reduced). Purity of the blast enriched populations ranged from 30% to 95%,

Nucleic Acid Extraction: DNA and RNA were extracted from AML blast selected cells with AllPrep DNA/RNA Mini kit (Qiagen Cat. No. 80204). RNA was extracted from the AML-reduced unselected cells with RNeasy Plus mini (Qiagen Cat. No 79134 if >500,000 cells, or RNeasy Plus micro if <500,000 cells (Qiagen Cat No. 74034). DNA and RNA quality were measured with TapeStation 2200 (Agilent) and concentrations were quantified using a Qubit 3.0 fluorometer (Life Technologies Q33216). The nucleic acid extractions were frozen until used in DNA and RNA sequencing library preparations.

*RNA Sequencing of AML-enriched blasts or AML-reduced from bone marrow:* Samples of total RNA were extracted from AML blasts (Qiagen RNeasy Plus mini Cat. No 79134 if >500,000 cells, or RNeasy Plus micro if <500,000 cells Cat No. 74034). Illumina TruSeq RNA Access (Cat. No. 20020189) sequencing libraries were used to convert total RNA into template molecules followed by sequence-specific capture of coding RNA. Sequencing was performed in the UNC-Chapel Hill High Throughput Sequencing Facility (HTSF) on an Illumina HiSeq 4000 platform using the Illumina HiSeq SBS 150 Cycles (PE-410-1001) with paired end 2 x 75 base read pairs.

*Enrichment of CD8 lymphocytes for Adapative Immune Receptor Repertoire Analysis and mRNA stranded sequencing libraries:* Cryopreserved PBMCs were thawed and recovered in complete AIM-V medium (Gibco) with 10% human AB serum (Gemini) at 370C, with 5% CO2 incubator for at least an hour. Cells were then washed with HBSS and resuspended in PBS with Bovine Serum Albumin, Miltenyi (Cat No. 130-091-376) and labeled with anti-CD8 Microbeads (Miltenyi Cat. No. 130-097-057 in the presence of anti-CD8 for CD8 purity before positive selection over an MS column (Miltenyi Cat, No. 130-042-201). Eluted CD8 lymphocytes were pelleted and lysed for RNA extraction (RNeasy® Plus Mini kit, Qiagen 74134 or RNeasyPlus Micro kit, Qiagen 74034). CD8+ enrichment and depletion from PBMC’s were determined by acquisition on a MACsQuant (Miltenyi) flow cytometer and analyzed with FlowJo software. Libraries were prepared for TCR profiling using the SMARTer Human TCR a/b Profiling Kit (Takara Bio USA, Inc., Cat. No 63516). Samples were pooled to a final concentration of 2 – 4 nM before dilution of the pool to 13.5 pM with 10% PhiX control v3 (Ilumina, Cat. No FC-110-3001). Sequencing was performed on an Illumina MiSeq® sequencer using the 600 cycle MiSeq Reagent Kit v3 (Illumina MS-102-3003) with paired-end 2 x 300 base pair reads.

*RNA Access Blast Analysis:* FastQC V0.11.4 was used to validate the quality of FASTQ files. Paired-end FASTQ files were aligned to an Ensembl transcriptome genome GRCh38 using Star V2.7.0a and gene counts were quantified using Salmon V0.8.2. Picard V1.86 was used to assess alignment quality of quantified BAMs. Differential gene expression was calculated from expression matrices output by Salmon and compared in R using the DESeq2 V1.22.2 Bioconductor package. Gene Set Enrichment Analysis (GSEA) was conducted using the GSVA V1.30.0 and limma V3.38.3 with the Hallmark gene sets from the Broad Institute (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp).(1)

*Adaptive Immune Receptor Repertoire Analysis:* MiXCR 2.1.9 was used to identify immune chains from paired-end FASTQ files using RNA seq parameters enumerated at https://mixcr.readthedocs.io/en/master/rnaseq.html. Reads were aligned with partial alignments allowed, followed by contig assembly and export. Diversity metrics were calculated using vdjtools V1.1.7.

*Flow cytometry:* The LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Life Technologies, Cat# L34968) was used for the T cell surface marker detection panel and Fixable Viability Stain 620 (BD Biosciences, Cat# 564996) was used for the T cell intracellular marker and transcription factor detection panel. BM and PB samples were pre-incubated with Human Trustain FC-Receptor Block (BioLegend, Cat# 422302) to prevent non-specific antibody binding prior to staining for flow cytometric analysis. Antibodies and manufacturers are listed in Supplementary Table 4. The intracellular staining for FoxP3 and TCF-1 was performed after cell permeabilization using Transcription Factor Buffer Set (BD Pharmingen, Cat#: 562574) as per manufacturer’s instructions. Flow cytometry data were biexponentially transformed, compensated using single stained controls and preprocessed (aggregates and dead cell removal) in FlowJo V10 (TreeStar). Pre-gated CD8+ T cells were then exported in R (version 4.0.2) for further analyses performed with a customized pipeline based on Nowicka M et al. (1) workflow. In particular, CD8+ T cells clusters were obtained using the FlowSOM algorithm and then visualized using the implementation of UMAP available in CATALYST R package. The different frequencies of the T cell subpopulations in CR and NR at the two timepoints (baseline and after treatment) were identified using the differential abundance analysis provided by the diffcyt R package.(2)

Supplemental Table 1: Reasons for Delay in Pembrolizumab Administration

|  |  |
| --- | --- |
| Reason for Delay | Day of Pembrolizumab administration |
| Uncontrolled infection- Enterobacter bacteremia | 19 |
| Grade 3 Hyperbilirubinemia | 19 |
| Grade 2 small intestinal mucositis | 15 |
| Logistical reasons (Day 14 was on a Sunday) | 15 |
| Typhlitis- Physician discretion | 15 |

Supplemental Table 2: Pembrolizumab-related Non-Hematologic Adverse Events

|  |  |  |  |
| --- | --- | --- | --- |
| **Toxicity** | **Grade 1-2** | **Grade >3** | **Total** |
| Cardiovascular  Heart Failure  Hypotension  Palpitations  QTc Interval Prolongation  Troponin I Increase | 1 (3%)  1 (3%)  1 (3%)  1 (3%)  1 (3%) | 0  0  0  0  0 | 1 (3%)  1 (3%)  1 (3%)  1 (3%)  1 (3%) |
| Electrolyte abnormalities  Hypocalcemia  Hypokalemia  Hypomagnesemia  Hyponatremia | 11 (30%)  8 (21%)  7 (18%)  5 (13%) | 0  1 (3%)  0  0 | 11 (30%)  9 (24%)  7 (18%)  5 (13%) |
| Endocrine  Hypothyroidism | 1 (3%) | 0 | 1 (3%) |
| Eye Disorders  Blurred Vision  Dry eye  Floaters | 1 (3%)  1 (3%)  1 (3%) | 0  0  0 | 1 (3%)  1 (3%)  1 (3%) |
| Gastrointestinal  Abdominal pain  Constipation  Diarrhea  Dry mouth  Mucositis/oral pain  Nausea | 1 (3%)  3 (8%)  1 (3%)  1 (3%)  4 (11%)  3 (8%) | 0  0  0  0  0  0 | 1 (3%)  3 (8%)  1 (3%)  1 (3%)  4 (11%)  3 (8%) |
| General Disorders  Anorexia  Bruising  Chills  Dehydration  Epistaxis  Fatigue | 4 (11%)  2 (5%)  2 (5%)  1 (3%)  1 (3%)  7 (18%) | 0  0  0  0  0  0 | 4 (11%)  2 (5%)  2 (5%)  1 (3%)  1 (3%)  7 (18%) |
| Hepatic  Alanine Aminotransferase Increase  Alkaline Phosphatase Increase  Aspartate Aminotransferase Increase  Hypoalbuminemia  Total Bilirubin increase | 13 (34%)  10 (26%)  9 (24%)  11 (30%)  11 (30%) | 2 (5%)  1 (3%)  2 (5%)  0  0 | 15 (41%)  11 (30%)  11 (30%)  11 (30%)  11 (30%) |
| Infections  Catheter-related infection  Clostridium difficile colitis  Febrile neutropenia  Hepatic infection  Lung infection  Mucosal infection  Typhlitis  Vaginal infection | 0  0  0  0  0  3 (8%)  0  1 (3%) | 3 (8%)  1 (3%)  23 (62%)  1 (3%)  10 (26%)  0  1 (3%)  0 | 3 (8%)  1 (3%)  23 (62%)  1 (3%)  10 (26%)  3 (8%)  1 (3%)  1 (3%) |
| Musculoskeletal  Arthralgia  Back pain  Bone pain  Myalgia | 2 (5%)  1 (3%)  2 (5%)  3 (8%) | 0  0  0  0 | 2 (5%)  1 (3%)  2 (5%)  3 (8%) |
| Nervous System  Dizziness  Dysguesia  Headache  Paresthesia | 1 (3%)  4 (11%)  2 (5%)  1 (3%) | 0  0  0  0 | 1 (3%)  4 (11%)  2 (5%)  1 (3%) |
| Pulmonary  Cough  Pulmonary edema | 2 (5%)  0 | 0  1 (3%) | 2 (5%)  1 (3%) |
| Renal  Creatinine increase  Hematuria  Proteinuria | 2 (5%)  4 (11%)  4 (11%) | 0  0  0 | 2 (5%)  4 (11%)  4 (11%) |
| Skin  Pruritus  Maculo-papular rash | 3 (8%)  4 (11%) | 0  2 (5%) | 3 (8%)  6 (16%) |

Supplemental Table 3: MRD Assessment in CR/CRi Patients

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Patient | Pre-treatment Disease Characteristics | Cytogenetics/FISH at CR | Flow Cytometry at CR | PCR at CR |
| 1 | Cytogenetics: t(9;11)  Mutations: None | Cytogenetics/FISH normal | N/A | N/A |
| 2 | Cytogenetics: Inv(3)  Mutations: DNMT3A | Cytogenetics: Persistence of inv(3) in 5/20 metphases  FISH: 2.0% positive for RPN1/MECOM | N/A | N/A |
| 3 | Cytogenetics: Normal  Mutations: NPM1, IDH2, PTPN11, DNMT3A | Cytogenetics: normal | N/A | NPM1; 2,554 transcripts/10,000 cell equivalents |
| 4 | Cytogenetics: Complex  Mutations: TP53 | Cytogenetics: Unable to be performed  FISH: Monosomy 7 present at 4.5% | N/A | N/A |
| 5 | Cytogenetics: t(9;11)  Mutations: FLT3-TKD (D835) | Cytogenetics: Normal  FISH: Normal | N/A | N/A |
| 6 | Cytogenetics: -7, Inv(3)  Mutations: CSF3R, ASXL1, ETV6, SETBP1 | Cytogenetics: Normal  FISH: 0.06% Inv(3)- RPN1/MECOM fusion detected | <0.1% detectable abnormal blasts | N/A |
| 7 | Cytogenetics: t(6;9)  Mutations: NRAS x 2 | Cytogenetics: Normal  FISH: Normal | N/A | N/A |
| 8 | Cytogenetics: Normal, FISH: 11q abnormality in 3.8%  Mutations: NPM1, FLT3-ITD | Cytogenetics: Normal  FISH: Normal | Undetectable (Limit 0.02%) | NPM1 PCR: Undetectable (Limit 0.01%) |
| 9 | Cytogenetics: t(9;11)  Mutations: None | Cytogenetics: None  FISH: Normal | Undetectable | N/A |
| 10 | Cytogenetics: Complex  Mutations: TP53 | Cytogenetics: Complex karyotype persistent  FISH: Del(5q) in 5.0% | 1.9% aberrant myeloblasts | N/A |
| 11 | Cytogenetics: Normal  Mutations: NPM1, CEBPA, IDH1 | Cytogenetics: Normal | Undetectable (Limit 0.02%) | NPM1: 520 transcripts/10,000 cell equivalents |
| 12 | Cytogenetics: t(9;11)  Mutations: KRAS | Cytogenetics: Normal  FISH: Normal | Undetectable (Limit 0.02%) | N/A |
| 13 | Cytogenetics: Normal  Mutations: RUNX1, KIT, BCOR | Cytogenetics: Unable to be assessed | Undetectable (Limit 0.02%) after 3 cycles of maintenance | N/A |
| 14 | Cytogenetics: Inv(16)  Mutations: None | Cytogenetics: Normal  FISH: Normal | Undetectable (Limit 0.02%) | CBFB-MYH11/ABL1 Ratio = 0.01192 |

Supplemental Table 4: Comparison of Outcomes Based on Prior HiDAC Therapy Versus Historical Controls

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | | **HiDAC + Pembrolizumab** | | | **Azacitidine + Nivolumab3** | | | **Monotherapy in R/R AML Historical Controls** | |
| **total** | **HiDAC-Refractory+** | | **Prior HiDAC++** | **HiDAC naïve** | **total** | **HMA-pretreated** | **HMA-naïve** | **HiDAC-HiDAC naïve** | **HMA- HMA naïve** |
| **number of patients** | 37 | 8 | | 12 | 17 | 70 | 45 | 25 | 314+++ | 6554 |
| **ORR** | 46% | 25% | | 42% | 59% | 33% | 22% | 52% | Unknown | 25% |
| **cCR** | 38% | 25% | | 33% | 47% | 22% |  | NR | 12-32% | 16% |
| **median OS** | 11.1 | 9.3 months | | 8.4 months | 13.6 months | 10.6 |  | NR | 5-8 months | 6-7 months |
| **irAE >=3** | 14% | 13% | | 8% | 18% | 11% |  | NR | N/A | N/A |

+ HiDAC Refractory defined as no response to HiDAC-based salvage chemotherapy or relapse <6 months after HiDAC

++ Prior HiDAC defined as HiDAC-based induction, salvage or consolidation chemotherapy >6 months prior to treatment

+++ Historical control references of HiDAC salvage chemotherapy for HiDAC-naïve AML patients from 3 randomized phase 3 studies:

1. VALOR Trial- Randomized Phase 3 Study of Vosaroxin + Cytarabine versus Cytarabine + Placebo in R/R AML (5)

* Eligibility: Primary refractory or first relapse; prior HiDAC chemotherapy included if >90 days from treatment
* Included Refractory subset (n=149) as HiDAC-naive
* CRc = 12%, median OS = 5 months

1. CLASSIC Trial- Randomized Phase 3 Study of Clofarabine + Cytarabine versus Cytarabine Alone in R/R AML (6)

* Eligibility: Primary refractory or first relapse; prior HiDAC chemotherapy included if >6 months from treatment
* Included Refractory AML subset (n=84) as HiDAC-naive
* CRc = 23%, median OS = 5.5 months

1. SWOG-8326- Randomized Phase 3 Study of HiDAC + Mitoxantrone versus HiDAC in R/R AML (7)

* Eligibility: Primary refractory or first relapse; all patients included on this study were HiDAC-naïve (n=81)
* CR rate = 32%, median OS = 8 months
* Some patients received re-induction of HiDAC based on residual leukemia on day 14 bone marrow biopsy (which was not done on our study)

NR= Not Reported

Supplemental Table 5: Treatment-Emergent Pembrolizumab-Related Grade >1 Adverse Events in Maintenance Phase

|  |  |
| --- | --- |
| Adverse Event | N=9 |
| Electrolyte abnormalities  Hyponatremia  Hypernatremia  Hypocalcemia | 1 (11%)  1 (11%)  1 (11%) |
| Endocrine  Hyperthyroidism  Hypothyroidism | 1 (11%)  1 (11%) |
| General  Anorexia  Arthralgia/Joint pain  Dysguesia  Edema  Fatigue  Fever  Nausea  Myalgia  Nail ridging  Vomiting | 1 (11%)  2 (22%)  1 (11%)  1 (11%)  2 (22%)  1 (11%)  1 (11%)  1 (11%)  1 (11%)  1 (11%) |
| GI  Diarrhea | 1 (11%) |
| Hematologic  Anemia  Leukopenia  Lymphopenia | 1 (11%)  2 (22%)  4 (44%) |
| Hepatic  Alkaline Phosphatase elevation  ALT increase  Hyperbilirubinemia | 2 (22%)  1 (11%)  1 (11%) |
| Neuro  Paresthesia | 1 (11%) |
| Pulmonary  Cough | 1 (11%) |
| Renal  Creatinine elevation | 1 (11%) |

Supplemental Table 6: Relapsed Patients Post-alloSCT

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Patient ID | Cytogenetics | Mutations | Overall Response | Relapse after alloSCT (days) |
| 1 | Inv(3) | DNMT3A | CR with MRD | 708 |
| 2 | Normal | DNMT3A, IDH2, NPM1 | CR with MRD | 416 |
| 3 | Inv(3), -7 | ASXL1, CSF3R, ETV6, SETBP1 | CR with MRD | 41 |
| 4 | t(9;11) | None | CR with no MRD | 132 |
| 5 | t(9;11) | KRAS | CR with no MRD | 184 |
| 6 | Inv(16) | None | CR with MRD (PCR) | 150 |

Supplemental Table 7: Patient Characteristics of MLL and Inv(3) Responders

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Genomic Subgroup | Age | Mutations | Prior Tx & Response | % Blasts Prior to Tx | MRD? | AlloSCT? | Pembro Maintenance? | Duration of CR |
| MLL Translocation- t(9;11) | 65 | None | 7+3-> CR  Lenalidomide consolidation (clinicaltrials.gov identifier NCT01578954)-> Relapse  CR1 duration = 9 months | 89 | FISH- normal  Flow cytometry not done | No | Yes, 7 cycles | 5.7 months |
| MLL Translocation- t(9;11) | 54 | FLT3-TKD (D835) | 7+3-> CR  4 cycles of HiDAC consolidation  Relapse (CR1 duration = 17 months) | 77 | FISH- normal  Flow cytometry not done | No | Yes, 3 cycles | 2.8 months |
| MLL Translocation- t(9;11) | 48 | None | CPX-351-> CR  CPX consolidation x 1 cycle  Azacitidine x 1 cycle-> relapse  HiDAC salvage-> no response | 13 | Flow cytometry- undetectable | Yes | No | 5.9 months |
| MLL translocation- t(9;11) | 54 | KRAS | Cladribine + 7+3-> no response | 6 | FISH- normal  Flow cytometry undetectable | Yes | Yes, 3 cycles | 8.7 months |
| Inv(3), -7 | 24 | ASXL1, CSF3R, ETV6, SETBP1 | AcIVP16 (Timed sequential therapy of cytarabine, idarubicin, etoposide)-> No response | 14 | FISH: 0.06% Inv(3)  Flow cytometry- undetectable | Yes | No | 2.6 months |
| Inv(3), der(3)t(3;18) | 39 | DNMT3A | AcDVP16 (Timed sequential therapy of cytarabine, daunorubicin, etoposide)-> No response | 21 | FISH positive at 2.0%  Flow cytometry not done | Yes | No | 25.3 months |

Supplemental Table 8: Continuous Monitoring for Toxicity Assessment

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Number of Patients, *n* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Boundary, *bn* | - | - | 3 | 4 | 4 | 5 | 5 | 5 | 6 | 6 | 6 | 7 | 7 | 7 | 8 | 8 | 8 | 8 | 9 | 9 |
| Number of Patients, *n* | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 |  |  |  |
| Boundary, *bn* | 9 | 10 | 10 | 10 | 11 | 11 | 11 | 11 | 12 | 12 | 12 | 13 | 13 | 13 | 13 | 14 | 14 |  |  |  |

Supplementary Table 9: Dose Modifications for Pembrolizumab

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **General instructions:**   1. Corticosteroid taper should be initiated upon AE improving to Grade 1 or less and continue to taper over at least 4 weeks. 2. For situations where pembrolizumab has been withheld, pembrolizumab can be resumed after AE has been reduced to Grade 1 or 0 and corticosteroid has been tapered. Pembrolizumab should be permanently discontinued if AE does not resolve within 12 weeks of last dose or corticosteroids cannot be reduced to ≤10 mg prednisone or equivalent per day within 12 weeks. 3. For severe and life-threatening irAEs, IV corticosteroid should be initiated first followed by oral steroid. Other immunosuppressive treatment should be initiated if irAEs cannot be controlled by corticosteroids. | | | | |
| **Immune-related AEs** | **Toxicity grade or conditions (CTCAEv4.0)** | **Action taken to pembrolizumab** | **irAE management with corticosteroid and/or other therapies** | **Monitor and follow-up** |
| Pneumonitis | Grade 2 | Withhold | * Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper | * Monitor participants for signs and symptoms of pneumonitis * Evaluate participants with suspected pneumonitis with radiographic imaging and initiate corticosteroid treatment * Add prophylactic antibiotics for opportunistic infections |
| Grade 3 or 4, or recurrent Grade 2 | Permanently discontinue |
| Diarrhea / Colitis | Grade 2 or 3 | Withhold | * Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper | * Monitor participants for signs and symptoms of enterocolitis (ie, diarrhea, abdominal pain, blood or mucus in stool with or without fever) and of bowel perforation (ie, peritoneal signs and ileus). * Participants with ≥ Grade 2 diarrhea suspecting colitis should consider GI consultation and performing endoscopy to rule out colitis. * Participants with diarrhea/colitis should be advised to drink liberal quantities of clear fluids. If sufficient oral fluid intake is not feasible, fluid and electrolytes should be substituted via IV infusion. |
| Grade 4 | Permanently discontinue |
| AST / ALT elevation or Increased bilirubin | Grade 2 | Withhold | * Administer corticosteroids (initial dose of 0.5- 1 mg/kg prednisone or equivalent) followed by taper | * Monitor with liver function tests (consider weekly or more frequently until liver enzyme value returned to baseline or is stable |
| Grade 3 or 4 | Permanently discontinue | * Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper |
| Type 1 diabetes mellitus (T1DM) or Hyperglycemia | Newly onset T1DM or  Grade 3 or 4 hyperglycemia associated with evidence of β-cell failure | Withhold | * Initiate insulin replacement therapy for participants with T1DM * Administer anti-hyperglycemic in participants with hyperglycemia | * Monitor participants for hyperglycemia or other signs and symptoms of diabetes. |
| Hypophysitis | Grade 2 | Withhold | * Administer corticosteroids and initiate hormonal replacements as clinically indicated. | * Monitor for signs and symptoms of hypophysitis (including hypopituitarism and adrenal insufficiency) |
| Grade 3 or 4 | Withhold or permanently discontinue1 |
| Hyperthyroidism | Grade 2 | Continue | * Treat with non-selective beta-blockers (eg, propranolol) or thionamides as appropriate | * Monitor for signs and symptoms of thyroid disorders. |
| Grade 3 or 4 | Withhold or  permanently discontinue1 |
| Hypothyroidism | Grade 2-4 | Continue | * Initiate thyroid replacement hormones (eg, levothyroxine or liothyroinine) per standard of care | * Monitor for signs and symptoms of thyroid disorders. |
| Nephritis and Renal dysfunction | Grade 2 | Withhold | * Administer corticosteroids (prednisone 1-2 mg/kg or equivalent) followed by taper. | * Monitor changes of renal function |
| Grade 3 or 4 | Permanently discontinue |
| Myocarditis | Grade 1 or 2 | Withhold | * Based on severity of AE administer corticosteroids | * Ensure adequate evaluation to confirm etiology and/or exclude other causes |
| Grade 3 or 4 | Permanently discontinue |
| All other immune-related AEs | Intolerable/ persistent Grade 2 | Withhold | * Based on type and severity of AE administer corticosteroids | * Ensure adequate evaluation to confirm etiology and/or exclude other causes |
| Grade 3 | Withhold or discontinue based on the type of event. Events that require discontinuation include and not limited to: Gullain-Barre Syndrome, encephalitis |
| Grade 4 or recurrent Grade 3 | Permanently discontinue |
| 1. Withhold or permanently discontinue pembrolizumab is at the discretion of the investigator or treating physician.   **NOTE:**  For participants with Grade 3 or 4 immune-related endocrinopathy where withhold of pembrolizumab is required, pembrolizumab may be resumed when AE resolves to ≤ Grade 2 and is controlled with hormonal replacement therapy or achieved metabolic control (in case of T1DM). | | | | |

Supplemental Table 10: Genes/exons Targeted by Clinical NGS Assay:

|  |  |  |
| --- | --- | --- |
| *ABL1* (exons 4-9) | *HRAS* (exons 2, 3) | *PTPN11* (exons 3, 7-13) |
| *ASXL1* (exons 9, 11, 12) | *IDH1* (exon 4) | *RUNX1* (all coding exons) |
| *BCOR* (all coding exons) | *IDH2* (exons 4, 5) | *SETBP1* (exon 4) |
| *BRAF* (exon 15) | *JAK2* (all coding exons) | *SF3B1* (exons 10-16) |
| *CALR* (exon 9) | *KIT* (exons 2, 8-13, 17-18) | *SRSF2* (exon 1) |
| *CBL* (exons 8, 9) | *KRAS* (exons 2,3) | *STAG2* (all coding exons) |
| *CEBPA* (all coding exons) | *MPL* (exon 10) | *TET2* (all coding exons) |
| *CSF3R* (all coding exons) | *MYD88* (all coding exons) | *TP53* (all coding exons) |
| *DNMT3A* (all coding exons) | *NOTCH1* (all coding exons) | *U2AF1* (exons 2, 6) |
| *ETV6* (all coding exons) | *NPM1* (exons 11, 12) | *WT1* (exons 6-10) |
| *EZH2* (all coding exons) | *NRAS* (exons 2,3) | *ZRSR2* (all coding exons) |
| *FLT3* (exons 13-15, 20) | *PPM1D* (all coding exons) |

Supplemental Table 11: Antibody Panels Used for Flow Cytometry

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Antibody | Fluorochrome | Clone | Company | Cat # | Isotype | Staining | Category |
| CD3 | PerCP-Cy5.5 | OKT3 | BioLegend | 317336 | Mouse IgG2a, κ | Surface | T cell |
| CD8 | BUV805 | SK3 | BD | 564910 | Mouse (BALB/c) IgG1, κ | Surface | T cell |
| CD56 | BV786 | 5.1H11 | BioLegend | 362550 | Mouse IgG1, κ | Surface | NK/NK-T |
| CD45RA | APC-Cy7 | HI100 | BioLegend | 304128 | Mouse IgG2b, κ | Surface | Memory |
| CCR7 | FITC | 150503 | BD | 561271 | Mouse IgG2a | Surface | Memory |
| CD27 | AF700 | O323 | BioLegend | 302814 | Mouse IgG1, κ | Surface | Stimulatory |
| CD28 | BV650 | CD28.2 | BioLegend | 302946 | Mouse IgG1, κ | Surface | Stimulatory |
| DNAM1 | BV711 | 11A8 | BioLegend | 338334 | Mouse IgG1, κ | Surface | Stimulatory |
| TIGIT | BV605 | A15153G | BioLegend | 372712 | Mouse IgG2a, κ | Surface | Inhibitory |
| CD57 | Pacific Blue | QA17A04 | BioLegend | 393326 | Mouse IgG1, κ | Surface | Senescence |
| KLRG1 | APC | 2F1/KLRG1 | BioLegend | 138412 | Syrian hamster IgG | Surface | Senescence |
| PD1 | PE | EH12.1 | BD | 560795 | Mouse IgG1, κ | Surface | Exhaustion |
| Tim 3 | PE-TR | [F38-2E2](https://www.biolegend.com/en-us/search-results?Clone=F38-2E2) | BioLegend | 345034 | Mouse IgG1, κ | Surface | Exhaustion |
| CD25 | PE-Cy7 | BC96 | e-Biosciences | 25-0259-42 | Mouse / IgG1, kappa | Surface | Activation |
| CD69 | BUV395 | 4B4-1 | BD | 745737 | Mouse BALB/c IgG1, κ | Surface | Activation |
| TCF1 | AF647 | [7F11A10](https://www.biolegend.com/nl-nl/search-results?Clone=7F11A10) | BioLegend | 655204 | Mouse IgG1, κ | Intracellular | Transcription Factor |
| FoxP3 | eFluor 450 | PCH101 | e-Biosciences | 48-4776-42 | Rat / IgG2a, kappa | Intracellular | Transcription Factor |

Supplemental Figure 1: Immune Biomarkers of CD8+ T cells in PB

A) Heatmap showing the 0-1 scaled MFI values of 12 markers in the CD8+ PB subsets from all samples (NR: n=13; CR: n=7). The median marker expression identifies the markers that characterize each cell subset. Each CD8+ subpopulation is colored according to the cluster identified using the FlowSOM algorithm.

CD8 activated effector: DNAM1+CD28+KLRG1+CD69+CD56+  
CD8 partially senescent: CD28+CD27+CD45RA+KRLG1+  
CD8 exhausted: CD28+CD27+PD1+KLRG1+  
CD8 DNAM1+CD28+: DNAM1+CD28+  
CD8 senescent: CD45RA+KLRG1+CD57+  
CD8 TEMRA CD57-: CD45RA+KLRG1+CD57-  
CD8 naive: CCR7+CD45RA+CD27+CD28+

B) UMAP plot split by timepoint and response. Note the different proportions of the PB CD8+ T cells subsets. UMAP plots were colored according to the seven CD8+ subpopulations identified and overlaid with contour plots (kernel density estimation). Designation: base- baseline/pre-treatment; post- post-treatment.   
  
C) Frequencies of CD8+ T cells subsets in PB. Boxplots showing the relative abundance of PB T-cell subpopulations at baseline CR (base\_CR) vs. NR (base\_NR) and post-treatment in CR (post\_CR) vs. NR (post\_NR). The boxes represent the interquartile range (IQR) and the horizontal line indicates the median. To perform differential test analysis and obtain adjusted p-values (padj) we used the diffcyt-DA-edgeR method from the diffcyt package. Asterisks indicate the significance level of differences in subsets frequencies between CR and NR at baseline and post-treatment (\*padj< 0.05,\*\*padj< 0.01,\*\*\*padj< 0.001).



Supplemental Figure 2: Gating strategy for CD8+ T-cells progenitor exhausted population (T-pex)

CD8+ T-cell subsets were gated based on the expression of CD45RA, CD27, PD1, CD28 and TCF1.

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Supplemental Figure 3: Frequency of Tregs at Baseline and After Treatment in CR vs. NR Patients

Chart, scatter chart

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BL = Baseline, Post = Post-HiDAC + Pembrolizumab at Recovery

Supplemental Figure 4: ssGSEA of RNA Seq of Pre-Treatment Leukemia Blasts

Volcano plot showing the differential expression of MHC gene sets determined by gene set enrichment analysis (GSEA). Analysis included upper quartile normalized gene expression data from all RNA access blast screen samples. Gene sets were selected from msigdb (Molecular Signatures Databse v7.4) by their inclusion of MHC genes and related genes.



Supplemental Figure 5: PD-L1 versus PD-L2 Expression on Pre-treatment Blasts and Non-Blasts in CR versus NR

Chart

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Supplemental Figure 6: Total Mutational Burden in CR versus NR patients

Chart, box and whisker chart

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