The Infusion of *Ex-Vivo,* Interleukins-15 and -21-Activated Donor NK Cells after Haploidentical HCT in High-Risk AML and MDS Patients – A Randomized Trial

Supplementary Appendix

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Enrollment Criteria: Eligible acute myeloid leukemia (AML) patients had high-risk disease; *i.e.,* primary-refractory AML (refractoriness after at least two cycles of intensive induction chemotherapy or four cycles of decitabine chemotherapy), relapse-refractory AML (refractoriness to salvage chemotherapy after recurrence), AML in early-relapse (complete remission (CR) duration <6 months), AML in the first CR with high-risk chromosomal abnormality at diagnosis (–5, del(5q), t(6;9), –7, del(7q), or ≥3 abnormalities), AML in the first CR with measurable residual disease, and AML in the second CR. Eligible myelodysplastic syndrome (MDS) patients had intermediate-2 or high risk disease according to the International Prognostic Scoring System.[1] Other eligibility criteria included; age over 18 years, Karnofsky performance status ≥70, and adequate organ function (serum total bilirubin <5 mg/dL, aspartate aminotransferase <5 times the upper normal limit, serum creatinine <3 mg/dL, and no clinically evident cardiac or pulmonary dysfunction).

Haploidentical HCT Procedure: The haploidentical hematopoietic cell transplantation (HCT) methodology used was similar to that reported previously.[2-5] Patients received busulfan (Busulfex; Otsuka Pharmaceuticals, Rockville, MD) 3.2 mg/kg daily intravenously (i.v.) on days –7 and –6 (day 0 being the first day of donor cell infusion), fludarabine (Genzyme Corporation, Cambridge, MA) 30 mg/m^2 daily i.v. on days –7 to –2, and anti-thymocyte globulin (ATG; Thymoglobulin; Genzyme Corporation) 2.5 mg/kg/day on days –3 to –1. In addition, methylprednisolone 2 mg/kg was administered i.v. daily on days –4 to –1. For graft-*vs*-host disease (GVHD) prophylaxis, patients received cyclosporine 1.5 mg/kg i.v. every 12 hours starting on day –1, which was switched to oral dosing when oral feeding became feasible. The blood cyclosporine concentrations were monitored at least weekly and were referenced to a 100 ng/mL to 300 ng/mL range. Starting 30 to 60 days after HCT, the cyclosporine dose was decreased by 10% every 2 to 4 weeks in patients without evidence of GVHD. Post-HCT i.v. methotrexate was not administered.

Supportive Care for HCT: The supportive care for HCT included intravenous (i.v.) diphenylhydantoin treatment during busulfan administration, oral ciprofloxacin, and i.v. micafungin

from the start of conditioning therapy. granulocyte colony-stimulating factor (G-CSF) 450 μg was infused intravenously daily starting on day 5 until absolute neutrophil count (ANC) recovered to 3,000/μL. Gamma-globulin (0.5 g/kg) was administered i.v. starting day 7 of HCT, biweekly until day 91, and monthly until day 180. For the purpose of leptomeningeal leukemia prophylaxis, all patients received methotrexate 12 mg intrathecally prior to the conditioning therapy. After HCT, AML patients received 3 additional doses of intrathecal methotrexate after recovery of their platelet count >50,000/μL.

Donor NK Cell Generation: Cells collected from the donors in the donor natural killer cell infusion (DNKI) group of patients were transported to the Korean Ministry of Food and Drug Safety (KMFDS)-inspected laboratory located in the hospital for natural killer (NK) cell production. The methodology for NK cell production and quality control has been described previously.[3,4,6] Briefly, $CD3^+$ cells were depleted from the leukapheresis product by the RosetteSep system (StemCell Technologies Inc., Vancouver, BC, Canada). CD3-depleted cells were then cultured in α-MEM media (Welgene, Daegu, Korea) supplemented with human interleukin (IL)-15 (10 ng/mL, PeproTech, Rocky Hill, NJ, USA), human IL-21 (10 ng/mL, PeproTech), and hydrocortisone (10^{-6} M, Stemcell Technologies Inc.) in a humidified 5% CO_2 atmosphere at 37°C. One half of the media was replaced with fresh complete media twice weekly. After 2 to 3 weeks of cultivation, cells were harvested, suspended in human albumin solution (5%), and infused into patients after the following quality assurance procedures. NK cell products were immunophenotyped by flow cytometry after fluorescence labeling. Cell viability was determined by labeling differentiated cells with propidium iodide and analyzing them by flow cytometry. The ability of donor NK cells to kill target cells (K562 and Raj) was measured using calcein-release assay (Abcam, Cambridge, UK). Donor NK cell interferon-γ secretion was determined in the culture supernatant by enzyme-linked immunosorbent assay. In addition, culture supernatants were tested for bacterial, viral, and mycoplasma contamination using appropriate methods.

Patient Monitoring and Evaluation: The criteria for neutrophil and platelet engraftment[2] and acute

and chronic GVHD[7,8] have been described. Bone marrow was biopsied within 2 weeks prior to conditioning therapy and again 4 to 5 weeks after HCT. In refractory AML and MDS, CR was defined as the recovery of a peripheral ANC to >1,000/μL and evidence of donor cell regeneration in a bone marrow aspirate with ≤5% blasts. In patients with persistent leukemia after HCT, the day of progression was defined as the day on which leukemia blasts reappeared in the peripheral blood. In patients with documented CR, leukemia progression was defined as documentation of leukemic blasts in the peripheral blood, >5% leukemic blasts in a bone marrow sample, extramedullary leukemia, or at least two consecutive detections of marker gene transcripts by reverse transcriptase-PCR.[9] Until day 100 of HCT, patients were monitored for cytomegalovirus (weekly using pp65 antigenemia assay or quantitative PCR for viral DNA) and Epstein-Barr virus (biweekly using quantitative PCR) reactivation. Serum ATG levels were measured on the days of DNKI.[10] Post-transplantation hematopoietic chimerism was assessed in the unfractionated peripheral blood mononuclear cells (PBMCs) at 1, 3, and 6 months after HCT by a PCR-based procedure using short tandem DNA repeats.

Post-HCT Immune Parameter Monitoring: The immunologic status of the patients was monitored to investigate the effect of DNKI on immune reconstitution. Peripheral blood (15 mL) was obtained from the patients before the initiation of conditioning therapy and at 1, 3, 6, and 12 months after HCT. Four patients in the study who had been randomized to the DNKI group but did not receive this treatment were excluded. Patients who have experienced disease progression at the time of analysis were also excluded. The numbers of patients in the DNKI and no-DNKI groups, respectively, were 36 and 36 pre-HCT, 32 and 24 at 1 month after HCT, 23 and 18 at 3 months, 16 and 14 at 6 months, and 13 and 8 at 12 months. Mononuclear cells were isolated by density gradient centrifugation using Ficoll (GE Healthcare, WI). The following lymphocyte subsets were measured by flow cytometry (CyFlow Cube 6; Sysmex Corporation, Kobe, Japan): $CD3^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD3^+/CD4^+/CD25^+$, $CD3^+/CD45RA^+$, $CD3^+/CD45RO^+$, $CD19^+$, $CD3^-/CD56^+$, $CD3^-/CD56^+/CD16^+$, $CD3^-/CD56^+/NKG2D^+$, $CD3^-/CD56^+/CD69^+$, $CD3^-/CD56^+/NKp30^+$, $CD3^-/CD56^+/NKp44^+$, $CD3^-$

/CD56^{+}/NKp46^{+}, CD3^{-}/CD56^{+}/KIR3DL1^{+}, CD3^{-}/CD56^{+}/CD158a^{+}, CD3^{-}/CD56^{+}/CD158b^{+}, and CD3^{-}/CD56^{+}/CD94^{+}/NKG2A^{+}. Serum cytokines were measured by enzyme-linked immunosorbent assay. In addition, cell cytotoxicity against K562 and Raj cells was assessed using a calcein-release assay (Abcam, Cambridge, UK) with an effector-target ratio of 10:1.

scRNA-Seq: The single-cell RNA sequencing (scRNA-seq) analysis was approved by the Institutional Review Boards of the participating institutions. We performed scRNA-seq using 10X chromium 3' v3 chemistry. The raw scRNA-seq reads were aligned to the Homo sapiens reference genome, GRCh38, using cellranger v6.0.0 pipeline from 10X genomics. The detailed statistics for each scRNA-seq experiment are provided in Supplementary Tables S5 and S6. A total of 87,584 individual PBMCs were profiled in 21 scRNA-seq experiments by cellranger. After filtering, 79,068 cells were used for further analysis. For each cell, log-normalization was performed by scaling to a constant total read count per cell (100,000) and log-transformation. We employed canonical correlation analysis to integrate the data and reduce batch effects. For visualization, we performed principal component analysis[11] with 2,000 highly variable genes for initial dimensionality reduction and employed UMAP to reduce Principal Component Analysis-dimensions into a 2D space. All of these aforementioned analyses were performed using Seurat v4.0.3 package[11] in R (v 4.1.0).

Single cell annotation using label transfer: We projected the seven study patients' PBMC data onto an annotated PBMC CITE-Seq reference dataset using Seurat.[11] The annotated PBMC CITE-Seq reference dataset is based on a CITE-Seq dataset of 211,000 human PBMCs with large cell-surface protein marker panels extending up to 228 antibodies.[11] We mapped this dataset onto our reference using only the transcriptome data and transferred our level 2 annotations, revealing the presence of multiple high-resolution lymphoid subsets. Using a label transfer algorithm, we classified our patients' PBMCs into 8 major and 31 sub-cluster cell types.

NK cell subclustering: NK cells were subclustered using a graph-based clustering approach in Seurat.[11] We compared gene expression among the NK cell clusters to characterize NK cell subclusters. Using various NK cell markers, we identified NK cell subclusters and then validated

them by calculating known gene signatures scores using the AddModuleScore function in Seurat R package.

Differentiation trajectory analysis of CD56^{bright} NK cells to memory-like NK cells: Using UMAP, the dimension reduction of NK cells was performed. Monocle3[12] was then employed to identify the cell state transition. Monocle3 uses approximate graph abstraction to identify single-cell trajectories based on pseudotime ordering by learn_graph function.

T cell clonality identification using TRUST4: To identify T cell clonality from the PBMC scRNA-seq data, we used TRUST4 (version 1.0.2)[13] to infer the CDR3 sequences of T cell receptors from scRNA-seq bam files. From the results, we determined that T cells with more than two clones are highly expanded clones. Next, to validate T cell clonality, we compared inferred highly-clonally-expanded T cells with not-expanded T cells using gene set enrichment analysis.

ARACNe-VIPER pipeline for the construction of a gene regulatory network and the identification of master TFs: The ARACNe algorithm was used to reconstruct a gene regulatory network. The R package VIPER was then applied to calculate the activities of TFs using this network. TFs with a significantly different viper score between NK cells from DNKI *vs* no-DNKI patients were considered master TF candidates. In addition, TFs with an activity score with high correlation with the NK cell differentiation pseudotime trajectory were identified.

Data availability: Both the raw and processed scRNA-seq data used in this study have been deposited in the NCBI Gene Expression Omnibus database with an accession code of GSE154392.

Statistical Analysis: Achievement of complete remission after HCT was recorded in patients with refractory AML and MDS and compared using chi-square test. Times to disease progression, engraftment, GVHD, graft failure, non-relapse mortality (NRM), and viral reactivation were measured from HCT and estimated using the cumulative incidence function in the competing risks framework. Graft failure, disease progression, and NRM were considered mutually competing risks. Graft failure, disease progression, and NRM were considered competing risks for the analyses of engraftment,

GVHD, and viral reactivation. Theses time-event variables were compared using Gray test.[14] Progression-free survival (PFS) and overall survival (OS) were measured from HCT and estimated using the Kaplan-Meier method. For PFS, disease progression and NRM were recorded, whereas for OS, death from any cause was recorded. PFS and OS were compared using the log-rank test. In the subgroup analyses, subdistribution hazard ratios were estimated using Fine-Gray proportional hazard regression model.[15] Cumulative incidence and survival analyses were performed using EZ-R software version 1.51. For other analysis, IBM SPSS software version 21 was used. In addition, continuous variables were compared using non-parametric Mann-Whitney test.

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