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Decitabine, Vorinostat, and Haploidentical Natural Killer Cell Infusions with IL-2 Stimulation in High-Risk Myelodysplastic Syndrome

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Abstract

Myelodysplastic Syndromes (MDS) describe a heterogeneous group of hematologic malignancies with variable prognoses and allogeneic hematopoietic stem cell transplantation the only known potential cure. Standard therapy with Hypomethylating Agents (HMA) change the natural history of the disease; however, response duration is limited. Additional treatments are needed. Between 2013 and 2015, we enrolled high-risk MDS patients on a clinical trial utilizing a combination of decitabine, vorinostat, and haploidentical related Natural Killer (NK) cells followed by IL-2 stimulation. Patients received 2 cycles utilizing a fresh NK cell product with cycle 1 and a cryopreserved NK cell product with cycle 2. Nine patients were treated on this study conducted at the University of Minnesota and the Mayo Clinic in Rochester.

We observed an overall response rate of 56% (5 of 9) including Marrow Complete Remission (mCR) (n=3), partial cytogenetic response (n=2), stable disease (SD) (n=3), and progressive disease (PD) (n=1). Toxicity was minimal. The majority of grade 3 events were infectious (36%) or gastrointestinal (25%) and grade 4 events were limited to cytopenias. Donor NK cell expansion was not observed but endogenous NK cell killing was increased and was present after both cycle 1 and cycle 2. Our older high-risk MDS population tolerated sequential decitabine, vorinostat, and haploidentical NK cell infusions with IL-2 administration and experienced good response rates. Unlike previous studies using more potent lymphodepletion, we did not observe donor NK cell expansion with this outpatient regimen and thus further study with a more intensified immunosuppressive platform is warranted.

Keywords: Decitabine; MDS; Natural Killer Cells; Vorinostat

Key Points

- Sequential decitabine, vorinostat, haploidentical natural killer cells and IL-2 was well tolerated in an older and high-risk MDS population.
- Response rates were faster than expected with chemotherapy alone and extended to the highest risk population, therapy-related MDS.
- Host immunity remains one of the main barriers to allogeneic NK cell adoptive transfer, and more immunosuppressive conditioning is required for transient persistence and *in vivo* expansion of donor NK cells.

Introduction

Myelodysplastic Syndromes (MDS) are a complex and heterogeneous group of clonal stem cell disorders manifested by diverse clinical and biologic paths with varying needs for transfusions, risk of infection, and risk of progression to acute myeloid leukemia. The FDA approval of three new therapeutic agents for MDS between 2002-2006 (azacitidine, lenalidomide, and decitabine) changed the treatment paradigm for this spectrum of diseases. However, despite hundreds of active clinical trials, no further FDA approvals for MDS treatment have occurred since 2006 and stem cell transplantation remains the only curative therapy. New therapies that change the natural history of MDS are needed.

The pathophysiology of MDS is complex, diverse, and still not completely understood. Structural alterations in DNA play a role in the pathogenesis of the disease due to loss or alteration of genetic material involved in proliferation, differentiation, or apoptosis. Epigenetic changes can also alter gene expression and impact disease biology [1]. More recent data regarding molecular mutations have highlighted further understanding of MDS biology and prognosis [2,3]. Immune dysfunction likely also plays a role in MDS development or propagation as evident by the clinical responses to immunosuppressive therapies [4,5] and the data highlighting natural killer cell dysfunction [6,7] and alterations in regulatory T cells (Tregs) [8,9]. Epigenetic modulation has been extensively studied in MDS. Azacitidine and decitabine, the two FDA approved Hypomethylating Agents (HMAs) produce responses of 40-60% with a median time to response of 3 months [10-13]. Histone deacetylase inhibitors (HDACi) show generally low single agent response rates around 10-20% [14-15]. Combination studies with HMAs and HDACi in MDS have produced a wide range of responses [16,17]. Interestingly, HDACi, vorinostat, has also been cited as a possible immune modulating drug based on an acute GVHD mouse model that showed diminished inflammatory cytokines and the development of acute graft versus host disease while not impairing T cell responses to host antigens after transplant [18].

The University of Minnesota has pioneered Natural Killer (NK) cell therapy in patients with acute myeloid leukemia over the last 10+ years using a platform of immunosuppressive and lymphodepleting chemotherapy with cyclophosphamide and fludarabine with subsequent infusion of enriched haploidentical natural killer cells followed by low dose subcutaneous IL-2 administration. This platform yields remission rates of approximately 30% in highly refractory/relapsed AML patients and response is associated with the persistence and *in vivo* expansion of functional donor derived NK cells [19,20]. Morbidity associated with this regimen includes a prolonged hospital stay, high fevers, and capillary leak, some inherent to the treatment of patients with refractory leukemia and some at least possibly related to therapy. While the outcomes have been favorable for patients with refractory leukemia, the lack of sustainable remission at 1 year in the absence of definitive therapy such as stem cell transplant indicated that this adoptive cell therapy in its current form is not curative, and would likely be too high risk for an older population of MDS patients. However, these results suggest potential efficacy of immunotherapy with NK cells in MDS if combined with other active agents in MDS.

Based on solid single agent efficacy data with decitabine, studies documenting a synergistic anti-tumor effect in MDS with vorinostat, and the potential lymphodepleting effect of vorinostat, we developed the following outpatient clinical trial: sequential decitabine and vorinostat to allow for transient host acceptance of haploidentical donor NK cells followed by IL-2 for added anti-tumor effect. Study registered on clinicaltrials.gov (NCT01593670).

Methods

Patients

Eligible patients had a diagnosis of high risk MDS defined as one or more of the following:

- International Prognostic Scoring System (IPSS) Category: INT-2 or High Risk
- International Prognostic Scoring System-Revised (IPSS-R) Category: High or Very High (Score of 5 or above)
- WHO Classification: RAEB-1 or RAEB-2 or CMML (not highly proliferative)
- High risk cytogenetic abnormalities as defined by presence of monosomy 7, complex karyotype, or monosomal karyotype
- Therapy related MDS (t-MDS); or MDS with severe cytopenias including neutropenia (ANC $\leq$ 0.8), platelet or PRBC transfusion dependence.

Patients could be treatment naïve (untreated/minimally treated with a maximum of 2 cycles of hypomethylating agents (azacitidine or decitabine) without evidence of treatment failure) or enrolled after treatment failure (defined as those with no response or disease progression after hypomethylating agents, 7+3 type induction chemotherapy, or immunosuppressive therapy such as ATG+CSA). Patients were age $\geq$ 18 years, had an ECOG PS of 0-2, had an available related HLA-haploidentical NK cell donor by at least Class I serologic typing at the A&B locus, and acceptable organ function (creatinine < 2, ALT/AST < 5 X ULN, oxygen saturation > 90% on room air, cardiac ejection fraction > 40% and QTc < 500 msec). Patients with active infection, on corticosteroids, with a prior history of use of HDAC inhibitors, active HIV, other potentially life-threatening malignancies, and

those who were pregnant were ineligible. Related donors (sibling, parent, offspring, parent or offspring of an HLA identical sibling) who were 18-75 years of age, at least 40 kg, in good health, and with a HLA-haploidentical donor/recipient match by at least Class I serologic typing at the A&B locus were used.

Treatment

The study was IRB approved and conducted at two institutions, The Mayo Clinic and the University of Minnesota, in compliance with Good Clinical Practice and the code of Helsinki. All patients on this Phase II therapeutic trial were treated with decitabine 10 mg/m2/day, days 1-5, with oral vorinostat 200 mg twice daily days 6-15, [21-23] on an outpatient basis followed by an infusion of CD3-/CD19- enriched donor NK cells on day 17 and a short course of Interleukin-2 (IL-2) to facilitate NK cell survival and expansion on Days 17,19, and 22 (corresponding to the day of the NK cell infusion, and 2 and 5 days after infusion). This schedule was designed to give a brief period of *in vivo* stimulation to the donor cells without stimulating recipient Treg as we have seen previously with 2 weeks and 6 doses of IL-2 [20].

Two courses of treatment were separated by 6 - 8 weeks. Clinical response was formally assessed 4 - 6 weeks after the 2nd course or at time of study removal in the setting of early study termination, based on International Working Group (IWG) criteria. Patients all received standard supportive care including antibacterial prophylaxis and antifungal therapy while neutropenic (until ANC >1000 x 2 days).

Donor Apheresis occurred on day 15 of course 1 only with subsequent cell product manufacturing. Peripheral blood cells were collected by a single apheresis from the haploidentical related donor performed on the Amicus Apheresis system (Fenwal Inc., Lake Zurich IL, USA) over a fixed time of 5 hours with a flow rate of 65 ml/min, and cycle volume of 1400 ml. Cell product manufacturing was performed at the University of Minnesota Molecular and Cellular Therapeutics (MCT) cGMP Facility.

Mayo Clinic participated as a second site in this study and utilized overnight courier services [24]. The apheresis product and a tube of donor blood (2ml EDTA) was sent from Mayo Clinic by overnight courier the day of collection (day 15). At the MCT Facility, the apheresis product was held overnight and then enriched for NK cells with the large-scale CliniMacs device (Miltenyi) by depletion of CD3+ cells to remove T-lymphocytes and depletion of CD19+ cells to remove B-lymphocytes. The NK cell product was then divided in two with one part cryopreserved (controlled rate) with CryoStor10 (BioLife Solutions, Bothell, WA) at 1:1 ratio cell volume to CryoStor10 and placed in vapor phase of liquid nitrogen in a continuously monitored storage tank until day 17 of course #2. The remaining NK cell enriched product was activated by incubation with 1000 U/ml IL-2 in X-VIVO 15, without gentamicin and phenol red (Lonza, Walkersville, Maryland) and 10% human AB serum, heat-inactivated (Valley Biomedical Products and Services, Inc., Winchester, VA) in VueLife™ Teflon® (FEP) bags (American Fluoroseal Corporation, Gaithersburg, MD).

For U of MN patient products, the cells were processed with lot release testing per the CMC and infused on day 17. For Mayo patient products, the cells with IL-2 were shipped to Mayo's Human Cellular Therapy lab on day 16 by overnight courier for arrival the morning of day 17 (the day of the NK cell infusion). Partial lot release testing on the pre-IL-2 product including sterility testing (Gram stain, endotoxin), cell viability, flow cytometry analysis was performed prior to shipping the cells to Mayo. Results were confirmed with Mayo prior to the NK cell product infusion. At Mayo, the cells were washed and final lot release performed.

Patients were premedicated with acetaminophen 650 mg PO and diphenhydramine 25 mg PO/IV within 1 hour before and 4 hours after the NK cell infusion. Demerol 25-50 mg IV was given for chills/rigors during the NK cell infusion.

The NK cells were infused without a filter or pump, slowly by gravity over at least 15 minutes but no longer than 1 hour. The targeted infused cell dose of $CD3^-$ $CD19^-$ selected NK cell product was within the range of 2-3 x 10^7 cells/kg. However, cell infusion proceeded regardless of cell dose if prior defined product collection parameter of > 3-fold NK cell enrichment between the apheresis and final products was met. Tubing was flushed on completion of the NK cells with normal saline to ensure all of the cells are infused. Vital signs were completed before the NK cell infusion, every 15 minutes during the infusion, and then every 30 minutes x 2, then per routine. Monitoring time totaled at minimum 2 hours after NK cell infusion. Patients were monitored for adverse effects of the NK cell infusion such as rash, acute allergic reaction, bronchospasm, respiratory distress, and acute vascular leak syndrome. Corticosteroids were avoided from time of NK infusion to Day+25. Subcutaneous IL-2 was given at 6 million units on days 17,19 and 22 (the day of the NK cell infusion and 2 and 5 days after infusion) with acetaminophen 650 mg PO and diphenhydramine 25 mg PO/IV given before and 4 hours after each dose of IL-2.

The second treatment course began 6 to 8 weeks after the start of course 1, identical to the first course but substitution of the frozen NK cell product for the fresh product, unless medically unsafe. On the day prior to infusion of the second dose, NK cells were removed from storage, thawed, washed and placed in culture as above. Cells were incubated overnight for 16-24 hours (37°C/5% CO_2) and washed twice as above to prepare for infusion. Cells intended for patients at Mayo were shipped as above and washed by the Mayo lab before infusion. Target dose for infusion was > 1 x 10^7 NC/kg for the frozen/thawed/re-cultured product. Patients

with disease refractory to prior treatment prior to study enrollment underwent a bone marrow aspirate prior to the start of the 2nd treatment course to monitor for rapidly progressive disease.

Patients received two courses of study treatment unless the following occurred: patient withdrawal of consent or non-compliance, lack of recovery to pre-study counts, development of grade 4 non-hematologic toxicity, evidence of rapid disease progression defined as > 20% peripheral blood blasts suggesting progression to AML, or concurrent medical illness that in the treating physician's opinion made ongoing treatment unsafe.

Correlative Studies Methods

Cytotoxicity assay: NK product cells, both before and after activation by overnight incubation with IL-2, and peripheral blood mononuclear cells (PBMC) isolated from patient blood on day 14 after first and second NK cells infusions were assayed for the ability to lyse highly NK-sensitive K562 target cells. Graded numbers of NK product cells or PBMC were added to Cr51-labeled K562 cells and incubated at 37C. After 4 hours the amount of Cr51 released from the target cells into the supernatant was measured and the % specific lysis was calculated as follows:

% specific lysis = (Cr51 released in presence of effector cells-Cr51 released in medium alone) / (Cr51 released by detergent lysis of target cells – Cr51 released in medium alone) x 100.

The presence of donor NK cells in the blood was determined using either molecular or flow cytometry based chimerism analysis. Molecular analysis used short tandem repeat genotyping, the standard technique used to monitor engraftment following allogeneic hematopoietic stem cell transplant. Flow cytometry analysis used Phycoerythrin (PE) labeled antibodies to HLA Class I molecules that were present only on either the donor or recipient cells. Antibodies used were HLA-A3 PE (Invitrogen) and HLA-B7 PE (Thermo Fisher).

Statistical Analysis

The primary endpoint of this pilot study was clinical response at the end of two cycles of therapy. Secondary endpoints include evaluation of safety, tolerability, the proportion of patients who become transfusion independent, the association of the *in vivo* NK cell expansion with clinical response, and 1-year overall survival.

Results

The study opened in June 2013 and accrued patients through May of 2015. The goal was to come up with a safe outpatient preparative regimen allowing transient persistence of donor derived NK cells similar to what we have seen with high intensity lymphodepleting chemotherapy. Nine patients were treated: six patients at the U of Minnesota and 3 at the Mayo Clinic. Five females and 4 males were treated at a median age 63 (range 38-79). The majority of patients were treatment naïve (n=8) or had received only 1 cycle of prior HMA without documented treatment resistance. One patient had prolonged prior azacitidine exposure with initial response but then eventual disease progression. (Table 1) includes disease characteristics and all patients' responses to therapy and long-term outcomes.

Patient Number	Prior MDS Therapy	Pre-Study Bone Marrow Biopsy	Therapy Completed	Post-Study Bone Marrow Biopsy	Overall Response	Survival Status
001 Age: 50	None	T-MDS/ RAEB-1: Blasts: 6% Cytogenetics: -5, +8, dic(5;8),dic (7;17) [8]	2 cycles	No morphologic dysplasia and no increase in blasts Cytogenetics: FISH: 0% (+8, -5), 12% (TP53)	CR	Deceased Died 24 months post HCT with heart failure in CR
002 Age: 67	None	T-MDS/ RCMD: Blasts: 2-3% Cytogenetics: -7[11] and del7q [9]	2 cycles	Residual dysmegakaryopoiesis + 15% remaining CLL Blasts: 1% Cytogenetics: -7 [0] and del 7q [2]	HI–neutrophils PR (Cytogenetic)	Deceased Died <2 months post HCT with residual CLL and DAH

003 Age: 54	None	RAEB-1 Blasts: 2% PB, 3% BM Cytogenetics -7 [19]	1 Cycle [A]	40% cellular, ongoing erythroid dysplasia. Blasts: no increase Cytogenetics: FISH for -7 (11%)	PR (Cytogenetic)	Deceased Died post HCT with non-engraftment
004 Age: 66	1 Cycle of Decitabine with no documented resistance	RAEB-2 Blasts: 9% BM and 2% PB Cytogenetics: del 5q (FISH 60%)	2 Cycles	RAEB-2 Blasts: 10% Cytogenetics: Del 5q (FISH 46%)	SD	Deceased Died of progressive disease Off treatment
005 Age: 79	None	RAEB-1 Blasts: 5% Cytogenetics: Complex with del 5q, del 7q, del 3q, +8, -1 -16, -17, -18, -20 [20]	1 Cycle (missed final dose of IL-2)[B]	RAEB-1 Blasts: 4-5% Cytogenetics: Complex [20]	SD	Deceased Died with active MDS and infections
006 Age: 64	Progressive Disease on Azacitidine	RAEB-2 Blasts: BM 11-15% by flow Cytogenetics: del 7q [12]	2 Cycles	Leukemic Transformation	PD	Deceased Died with AML
007 Age: 63	None	CMML-2 Blasts: 10% PB and 9% BM Cytogenetics: 50 XY, +8, +10, +21, +22 [20]	2 cycles	Initial Post-Study Marrow: < 5% blasts Cytogenetics still +8, +10, +21, +22 [20] Repeat Marrow 1 month later prior to HCT admission: CMML-2 with 15% blasts	Transient CR Then Back to CMML-2/SD	Deceased: Died post HCT

008 Age: 38	1 cycle of Decitabine with no documented resistance	Therapy related MDS/ RAEB-1 Blasts: 5-8% Cytogenetics: t (3;21) [19]	Completed all of Cycle 1 + Cycle 2 Decitabine + Vorinostat of cycle 2[C]	No morphologic evidence of residual MDS No increase in blasts	CR	Alive: 22 months post study completion 18 months post HCT but with disease relapse 10 months post HCT
009 Age: 58	None	RCMD Blasts < 5% Cytogenetics: -7 [9], del 20 [5], 46 XY [6]	2 cycles	Persistent RCMD Blasts < 5% Cytogenetics: -7 [7], del 20q [2], 46 XY [11]	SD	Alive: Active MDS and intermittent treatment with Decitabine 20 months post study treatment

CR= complete morphologic remission; PR = partial response; HI = hematologic improvement; SD = stable disease; PD = progressive disease; MDS = Myelodysplastic syndrome; T-MDS = Therapy related MDS; RAEB = Refractory Anemia with Excess Blasts; RCMD = Refractory Cytopenias with Multilineage Dysplasia; HCT = hematopoietic stem cell transplant; BM = bone marrow; PB = peripheral blood
A = Removed from study prior to cycle 2 due to neutropenic fevers/infection and eligibility to move directly to transplant
B= Removed from study due to coexisting pulmonary process making ongoing therapy risky
C= NK cells held due to grade 3 cholestatic transaminitis

Table 1: Disease Characteristics and Treatment Response.

Given our previous experience that responses from a single NK cell infusion effect did not last beyond a year in refractory AML patients, we designed the study to divide a single apheresis into two cell infusions, one given fresh and one given cryopreserved. Pre-clinical studies previously showed that immediately cryopreserved NK cells did not recover function when tested *in vitro* and in a xenogeneic model *in vivo* [25]. The starting apheresis product contained a total of $2.2 \pm 0.16 \times 10^{10}$ mononuclear cells comprised of 10.6±1.2% NK cells and 59.6±4.0% T cells. After T and B cell depletion under GMP conditions, [26,27] the final NK cell product contained $8.0 \pm 7.3 \times 10^4$ T cells and NK cells were enriched 4.6±0.7 fold. The final NK cell dose between the fresh and frozen product, despite the overnight recovery in IL-2, differed significantly between the fresh and frozen product (Table 2).

Product Characteristics	Fresh Cell Infusion	Frozen Cell Infusion	P-Values
NC/kg Lot release	1.43E+07	1.06E+07	0.64
NC/kg	2.04E+07	1.50E+07	0.51
NK%	45.91%	19.91%	0.009
NK/kg	8.43E+06	2.59E+06	0.0008
CD3%	0.32%	0.20%	0.72
CD3/kg	6.30E+04	2.00E+04	0.34

Table 2: Product data: CD3/CD19 depleted IL-2 Product Characteristics.

The difference in final NK cell dose may overestimate the difference between the fresh and thawed products given the CD56 down-regulation on the thawed product, since incubation of the thawed product overnight with IL-2 rescued cytotoxic function, and both fresh and thawed products exhibited potent cytotoxicity against K562 targets (Figure 1).

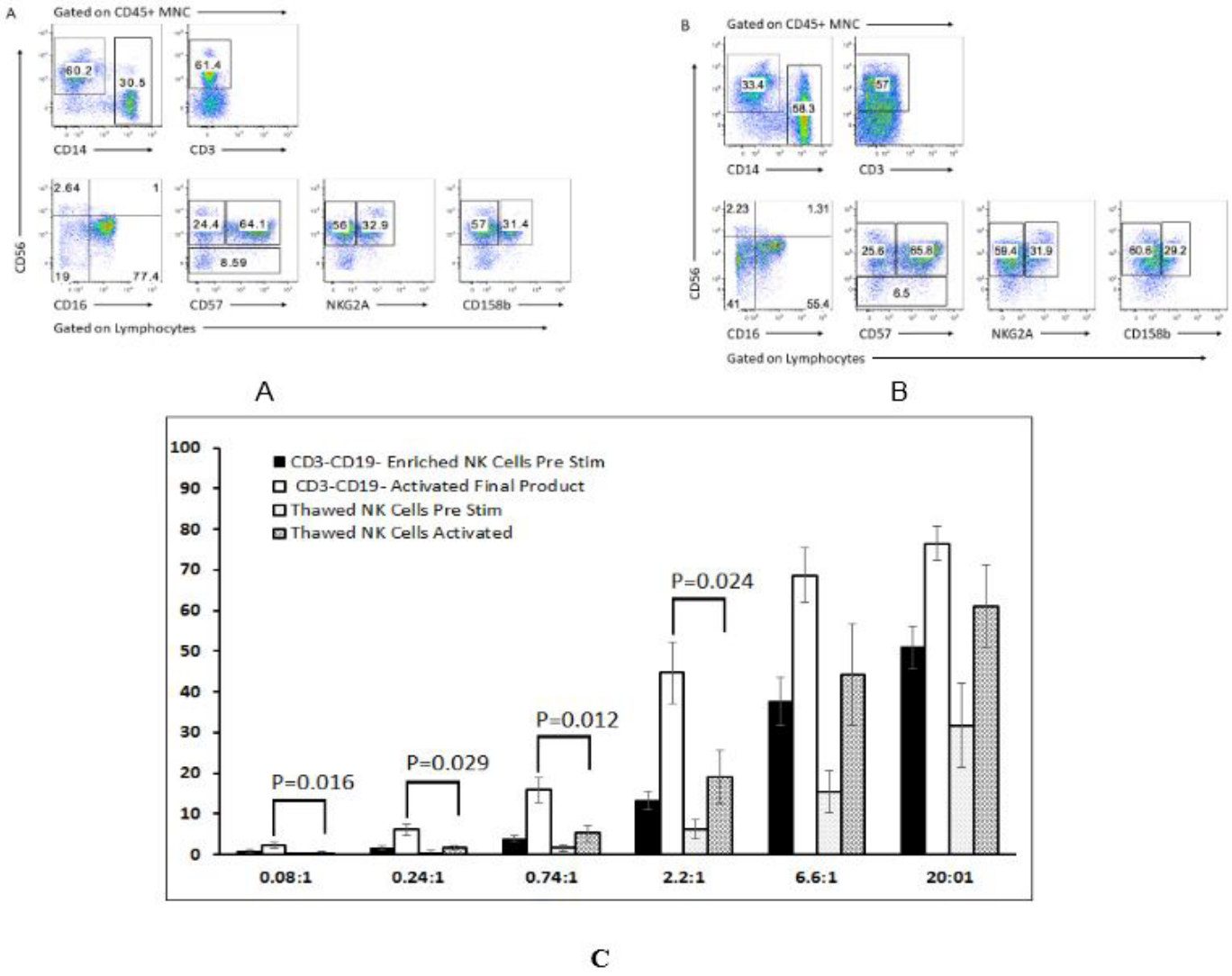

Figure 1: Fresh and Thawed Activated NK Cell Products Maintain NK Cell Phenotype and Exhibit Potent Cytotoxic Activity against K562 Targets.

A. Phenotype of a representative fresh, overnight activated product. Plots in the top row were gated on CD45 bright+ cells that fall in the Mononuclear cell (MNC) light scatter gate. Plots on the bottom were further gated on Lymphocytes using a light scatter gate.

B. Phenotype of the same product shown in panel A when thawed and overnight activation with IL-2. Plots were gated as described for panel A.

C. CD3 and CD19-depleted, enriched NK cells prepared under GMP conditions were tested both fresh and after thawing and both before and after overnight activation with 1000 U/mL IL-2 for cytotoxic activity against K562 target cells (fresh cells: n=9, thawed cells: n=3). Cytotoxic activity by cells tested immediately post thaw was low but was rescued by overnight activation with IL-2. Both fresh and thawed activated final product cells exhibited potent cytotoxic activity, however cytotoxic activity exhibited by the thawed product was roughly 3-fold lower than cytotoxic activity by fresh activated cells.

Six patients received the two cycles of planned therapy. Two patients came off study after cycle 1. A final patient received cycle 1 and the chemotherapy part of cycle 2 and missed the NK cells + IL-2 of cycle 2. Of the nine patients treated on the study, two achieved a durable marrow CR prior to moving to stem cell transplant, one achieved a transient marrow CR (< 2 months) prior to reverting back to CMML-2 status/stable disease. Two patients had partial cytogenetic improvement, three had stable disease, and the one patient with progressive disease was the only patient enrolled on the protocol with prior azacitidine refractory disease. Thus, overall the response rate (marrow CR+partial cytogenetic improvement) was 56% (5 of 9 with response.) Completion of protocol defined 2 cycles did not appear to be required for response as one of the patients who achieved a CR and went on to transplant did not receive the second round of NK cell infusion + IL-2 injections.

Overall survival at 1 year was a secondary endpoint of the study. Seven of the nine treated patients died with a median time to death from start of study treatment of 174 days (range 87-693). Only 2 patients remain alive at the time of analysis: 1 patient alive approximately 22 months post study treatment and 18 months post HCT but with disease relapse 10 months post HCT; the second survivor alive 20 months post study treatment but on MDS directed therapy.

Five patients went on to allogeneic stem cell transplant. Four patients died of transplant complications (non-engraftment in 1 who received a Double Umbilical Cord Transplant (DUCBT), 1 who had coexistent CLL and MDS with persistent CLL post-HCT, one with late complication of heart failure 2 years post-HCT, and one multi-organ system failure day +35 post DUCBT with no count recovery). Of the remaining 3 deaths, 2 patients died from progressive disease and 1 died from infections and ongoing MDS without further therapy.

All patients were eligible for toxicity assessment. Adverse events and serious adverse events noted in (Table 3) illustrating counts of AEs by category. The majority of adverse events were grade 3 or less. The majority of grade 4 events (96%) were either thrombocytopenia/neutropenia/ leukopenia, inherent to MDS disease biology and felt to be unrelated to the study immunotherapy. The majority of grade 3 events were attributable to infections (36%) or gastrointestinal symptoms (25%).

Category	Adverse Event Detail	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
Arm: Decitabine, Vorinostat, NK, IL-2							
Infections	Febrile neutropenia	0	0	3	0	0	3
	Infections	0	0	7	0	0	7
	Urinary tract infection	0	1	0	0	0	1
Ear and labyrinth disorders	Ear pain	0	1	0	0	0	1
Gastrointestinal disorders	Diarrhea	0	1	1	0	0	2
	Nausea/Anorexia	0	2	3	0	0	5
	Cholecystitis	0	0	1	0	0	1
	ALT/AST/Bilirubin increased	1	3	2	0	0	6
Constitutional	Chills	0	2	2	0	0	4
	Fatigue	0	1	1	0	0	2
	Pain	0	1	0	0	0	1
	Arthralgia	0	2	0	0	0	2
Abnormal Blood Tests	Cholesterol high	1	0	0	0	0	1
	WBC/ANC decreased	1	0	3	10	0	14
	Platelets decreased	0	0	1	15	0	16
	Hypocalcemia	0	0	0	1	0	1
Renal Disorders	Renal calculi	0	0	2	0	0	2

Respiratory Disorders	Dyspnea/Hypoxia	0	2	0	0	0	2
Skin Disorders	Skin and subcutaneous tissue disorders	1	0	0	0	0	1
Vascular disorders	Hypertension	0	0	1	0	0	1
	Hypotension	0	1	1	0	0	2
Total		4	17	28	26	0	75

Table 3: Adverse Events.

Correlative Studies

In 8 of 9 evaluable patients, there was no evidence of donor chimerism (n=6 by molecular analysis and n=2 by flow cytometry analysis) at 7 and 14 days after either of the NK cell infusions. NK cell cytotoxicity assays revealed increased NK cell activity in the blood 14 days after the second adoptive transfer despite the absence of any donor chimerism, suggesting that the enhanced NK cell function was recipient in nature (Figure 2). This suggests that while the donor NK cells did not persist or expand, the complete therapy package resulted in increased NK cell function in the blood that improved between 14 days after cycle one to 14 days after cycle 2.

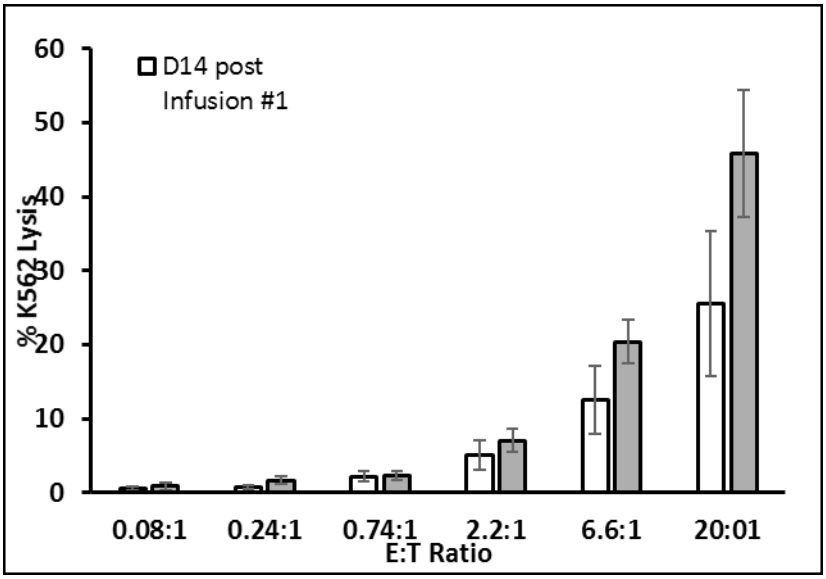

Figure 2: Effect of activated NK cell product infusions on endogenous NK cell cytotoxic activity. Although donor chimerism was not detected on day 14 after either the first or second infusions of activated NK cells, recipients who received the second infusion had increased NK cytotoxic activity in the blood on day 14 (n=3) when compared with NK activity in the blood 14 days after the first infusion of NK cells (n=7), suggesting an effect of the two NK cell infusions on endogenous NK function. Lysis was determined using a 4-hour 51Cr release assay with K562 target cells.

Discussion

High risk MDS remain a challenging group of diseases to treat. Few FDA approved therapies exist and those that are available often take months to produce a response [10-13]. Months of therapy equate to potentially months of neutropenia with increased infection risk and months of anemia and thrombocytopenia with increased transfusion needs. Therapies with lower toxicity (unlike AML-type induction chemotherapy) and faster response rates could shorten the duration of neutropenic risk, shorten the duration of transfusion needs and diminish risk of iron overload with resulting improvement in quality of life and cost of care.

Our clinical trial aimed to develop a platform of outpatient chemotherapy plus immunotherapy to improve clinical responses. While our trial enrollment met our safety goals, the trial was stopped early because of the lack of donor NK cell persistence and expansion using a low-intensity outpatient preparative regimen. Despite the small trial size, we observed interesting clinical response trends:

- Faster than expected responses as compared to historical responses to HMA +/- HDACi studies,
- High responses in higher risk t-MDS patients.
- Our good outcomes may be associated with *in vivo* NK cell function seen 14 days after NK cell infusions, albeit from host origin.

Toxicity was relatively low in this study of older, high-risk MDS patients. The majority of all treatment occurred in the outpatient setting with admissions primarily only for neutropenic fevers/infections. Grade 4 toxicities were isolated to cytopenias. The remaining adverse events were primarily infectious (febrile neutropenia/pneumonia/UTI) and gastrointestinal (mild nausea/anorexia/diarrhea). The rates of adverse effects were similar to other studies with hypomethylating agents [10-13]. Thus, the addition of NK cell infusions and IL-2 was well tolerated and did not alter the toxicity compared to the standard of care.

Standard HMA responses occur within 4 to 6 months, with CR rates ranging from 20-40% [10-13]. Our 56% response rate occurred within 2 rounds of therapy, with less chemotherapy exposure in comparison to standard therapy, raising the possibility that the NK cell immunotherapy may be adding to the benefit to standard therapy. Additionally, two patients with therapy-MDS in our study achieved a marrow complete remission (2 out of 3 patients, 67%), a rate higher than expected in comparison to the published ORR of 30-40% suggesting a possible additional benefit of immune therapy in this population of patients with MDS that may have more intrinsic chemotherapy resistance [28,29].

Long-term survival in our cohort of patients was low which is not surprising given the high-risk patient population. Deaths were primarily due to transplant related mortality and progressive disease. Only 2 long term survivors remained, one of which had a complete remission and went on to hematopoietic stem cell transplant only to relapse later and the other with ongoing MDS on alternative therapy. Additional work is needed to improve survival in this high-risk group of patients.

Our study utilized and compared fresh and frozen donor NK cells split from a single donor collection. We observed similar NK cell cytotoxicity after cytokine activation of the frozen/thawed products. However, in contrast to AML patients receiving high dose cyclophosphamide and fludarabine prior to NK cell infusion, our correlative NK cell assays showed no NK cell persistence from either donor NK cell product 7 and 14 days after adoptive transfer. This result is important and highlights the fact that host immunity remains one of the main barriers to allogeneic NK cell adoptive transfer, and more immunosuppressive conditioning is required for transient persistence of donor NK cells. In summary, our older high-risk MDS population tolerated sequential decitabine, vorinostat, and haploidentical NK cell infusion with subsequent IL-2 favorably in the outpatient setting. While we observed some clinical responses in high-risk MDS patients, the study numbers were too low to draw definitive conclusions, and the immunosuppressive platform did not achieve the goal of donor NK cell persistence and *in vivo* expansion. To overcome the lack of *in vivo* NK cell persistence in future trials, we will explore additional strategies using expanded "off the shelf" NK cells with augmented immunosuppressive conditioning to enhance efficacy.

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Authorship Contributions

EW constructed the original manuscript (introduction, clinical methods, results, discussion, and tables), JM contributed substantial input into the manuscript results and discussion, JC and PR contributed the NK correlative study methods, correlative study results and figures, DM contributed the cell processing methods. All authors reviewed and contributed feedback for the final edits.

Conflicts of Interest: None to disclose

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