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## Autologous Stem Cell Transplant Recipients Tolerate Haploidentical Related-Donor Natural Killer Cell Enriched Infusions

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### Abstract

**BACKGROUND**—In the setting of allogeneic stem cell transplantation (alloSCT), infusing natural killer (NK) cells from a major histocompatibility complex (MHC) mismatched donor can mediate an anti-leukemic effect. Graft versus tumor (GvT) effect following autologous stem cell transplantation (ASCT) may result in less disease relapse.

**STUDY DESIGN AND METHODS**—We performed a phase I clinical trial to assess the safety and feasibility of infusing distantly processed donor NK enriched mononuclear cell (NK-MC) infusions from a MHC haplotype mismatched (haploidentical) donor to patients who recently underwent ASCT for a hematologic malignancy. On day 1, peripheral blood mononuclear cells (MC) were obtained by steady-state leukapheresis and sent from Boston to the Production Assistance for Cellular Therapies (PACT) facility at the University of Minnesota, where immunomagnetic depletion of CD3 cells was performed on day 2. NK-MC product were then returned to Boston on day 2 for infusion on day 3. Toxicity, cellular product characteristics and logistic events were monitored.

**RESULTS**—At a median of 90 days (range, 49–191) following ASCT, thirteen patients were treated with escalating doses of NK-MC per kg from  $10^5$  to  $2 \times 10^7$ . Adverse effects included grade 2 rigors and muscle aches, but no grade 3 or 4 events, and no GvHD or marrow suppression. One air courier delay occurred. NK-MC products were viable with cytotoxic activity after transport.

**CONCLUSION**—CD3-depleted, MHC mismatched allogeneic NK-MC infusions can be safely and feasibly administered to patients after ASCT following distant processing and transport, justifying further development of this approach.

### Keywords

Natural Killer Cell; Cellular Therapy; Transplantation- Stem Cell

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## INTRODUCTION

The goal of curing hematologic malignancies with autologous hematopoietic stem cell transplantation (ASCT) remains elusive for most patients. Relapse due to residual disease is the primary obstacle. The Center for International Bone & Marrow Transplant Research (CIBMTR) reported that from 2003–2008, primary disease accounted for 73% of deaths following ASCT.<sup>1</sup> Since high dose radiation and chemotherapy are inadequate in achieving cure for the majority of those patients, the development of innovative therapeutic modalities is needed.

Relapse of malignancy following ASCT is likely due to persistent clonogenic disease-specific stem cells that are resistant to high dose chemotherapy/radiation and escape autologous immune surveillance.<sup>2,3,4,5</sup> ASCT recipients also lack the graft-versus-tumor effect (GvT) of allogeneic hematopoietic stem cell (alloSCT) recipients. GvT is mediated by a complex milieu of cells and co-factors in the allograft, including donor T-cells, NK cells, cytokines and chemokines.<sup>6,7</sup>

In ASCT recipients, NK cells are generally inhibited by “self MHC” antigens through the activation of killer cell immunoglobulin like receptors (KIR) which recognize self MHC ligands, become activated and suppress NK cell cytotoxicity.<sup>8,9</sup> Although some tumors may have altered MHC expression,<sup>10,11</sup> this is not a universal finding in hematological malignancies.<sup>12,13</sup> This provides the rationale for testing the use of MHC mismatched allogeneic NK cells for cellular therapy to overcome such a potentially inhibitory mechanism. Initial studies from the Perugia group<sup>14,15,16</sup> suggested that allogeneic MHC and KIR mismatched NK cells may contribute to a graft versus leukemia effect with limited risk of graft versus host disease (GvHD), control advanced acute myeloid leukemia, and prevent recurrence after alloSCT. These observations were confirmed by other groups,<sup>17,18,19</sup> although some studies posit that a KIR/KIR ligand mismatch in itself is not sufficient for a GvT effect and that other NK cell receptors play an important part.<sup>20</sup>

The objective of this study was to use a Phase I design to determine whether distantly processed MHC mismatched natural killer cell enriched mononuclear cell (NK-MC) infusions from related donors can be safely administered in the months following an ASCT. Safety concerns focused on the risks of GvHD and marrow suppression. We sought to define the maximum dose of allogeneic NK-MC tolerated given the limits in production. The study was further designed to assess whether an apheresis MC product could be collected and shipped to a distant processing site where it would be manipulated and returned to the originating site as an NK-MC product with efficacy, viability, and sterility intact.

## MATERIAL AND METHODS

### Patient Eligibility

Between May 2007 and March 2010, a total of thirteen patients were enrolled (Table 1). All patients had received an ASCT for hematological malignancies: six patients had non Hodgkin’s lymphoma (NHL) [three mantle cell lymphoma, one diffuse large B cell lymphoma (DLBCL), one mixed follicular, and one anaplastic large cell (ALCL)]; five had multiple myeloma; and two patients had Hodgkin’s lymphoma. Eight patients had undergone ASCT in first remission after standard chemotherapy, while five patients were transplanted after salvage chemotherapy for recurrent or relapsed disease. Patient # 3 had received a previous ASCT ten years prior. We aimed to increase the probability of donor NK cell alloreactivity against host tumor cells by purposefully mismatching the donor-recipient human HLA antigens. Therefore, all donors shared only one MHC haplotype with their corresponding recipients. Patient-donor KIR mismatching was also recorded but not

used as a donor selection criterion. All patients and donors consented to participate in this study approved by the Committee on the Use of Human Subjects in Research at Tufts Medical Center. The study was conducted under IND # 12971.

## Study Design

All donors underwent leukapheresis at Tufts Medical Center with a COBE Spectra with granulocyte separation chamber and small volume collection chamber. Thirteen relatives each served as NK-MC donors: a parent (1), siblings (6) and children (6). The collected MC apheresis product was sent via overnight courier (AirNet Systems, Inc. Columbus, OH) to the PACT contracted cell processing facility at the University of Minnesota. Cell processing was initiated early on the morning of the day following the apheresis collection and arrival of the cells in Minnesota. The apheresis product was T cell - (CD3+) depleted using the Miltenyi Biotec CliniMACS Cell Selection System and CD3 MicroBeads reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Up to  $4 \times 10^{10}$  total cells ( $1.5 \times 10^{10}$  target cells) were labeled with CD3 MicroBeads and separated on an LS column which was placed in the magnetic field of the CliniMACS device. Cells were then re-suspended at  $2 \times 10^6$  cells/mL in X-VIVO 15, without gentamicin and phenol red (Lonza, Walkersville, Maryland), supplemented with 1000 U/mL IL-2 (Chiron Corporation, Emeryville, CA) 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). Prior to transportation from Minnesota back to Tufts Medical Center, each NK-MC product was required to meet established release criteria as follows: > 70% cell viability (7-AAD), <  $5 \times 10^5$ /kg CD3+ lymphocytes, > 20% CD3-/CD56+ NK-cells, negative gram stain and acceptable endotoxin level (<5 EU/kg).<sup>21</sup> Following product enrichment to >20% NK-cells, no further positive selection for CD56+ cells was performed.

Upon arrival in Boston, the cell preparation was washed with 5% human serum albumin and the MC concentration adjusted to the corresponding MC cell number appropriate for the dose escalation cohort. We also determined cytotoxicity of the allogeneic NK-MC by a standard flow cytometry assay using K562 as target cells at different ratios of effectors and targets.<sup>22</sup> Results were recorded but not used as a release criterion. To utilize a consistent endotoxin assay, a sample of the pre-infusion product was sent to Minnesota for testing.

The NK-MC infusion was administered in the outpatient setting as a one-time intravenous infusion over 5–15 minutes. Hydration with normal saline at 200cc/hr for two hours pre- and post-NK-MC infusion was given. Every patient received premedication with diphenhydramine. Corticosteroids or any other immunosuppressive medications were not allowed for 3 days prior to study entry. All patients were followed closely for adverse events and graded according to the Common Toxicity Criteria for Adverse Effects (CTCAE) v3.0.<sup>23</sup> The highest dose tolerated given the limited production capacity was also defined. However, if the dose of  $2 \times 10^7$  cells/kg was well tolerated, this was not considered as the true maximum tolerated dose (MTD) since the dose of  $2 \times 10^7$  MC/kg was the highest number of NK-MC obtainable by a single leukapheresis and single CliniMACS separation column. All patients were followed with regular clinical visits, laboratory exams and imaging studies, as clinically indicated. The initial clinical protocol called for a one time dose of Fludarabine ( $50 \text{ mg/m}^2$ ) the day before NK-MC infusion. After enrollment of the first patient, the protocol was amended and no immunosuppression was given prior to NK-MC infusion. To determine whether donor derived cells could be detected in the blood of recipients 24 hours after the infusions, chimerism analysis was performed on the DNA of peripheral blood samples of the three patients infused with the highest dose level of NK-MC ( $2 \times 10^7$  cells/kg). Chimerism was measured using a STR (short tandem repeat) based assay (Promega PowerPlex 16 STR-PCR system). STR analysis was performed using an ABI 3130xl Genetic Analyzer and the GeneMapper software (ABI).

## RESULTS

### Characteristics of the NK-MC Product

Before beginning the actual trial enrollment, three “dry runs” were performed to test the feasibility of shipping fresh and processed cell product across the country and to detect any problems that needed to be addressed before patient enrollment into the study. Those data are published in a separate paper <sup>24</sup>.

For the thirteen patients enrolled in the clinical trial, the transport of the donor cell collection from Boston to the University of Minnesota, the processing at that facility and the return shipment to Boston went without complications except in one case: the arrival at the Minnesota facility was delayed by eight hours due to a misplacement by the air courier of the box containing the leukapheresis product (donor # 3). This product had a lower CD56+ percentage (11%) after CD3 depletion. After consultation with the FDA it was decided to infuse the cells. No other transport related issues occurred. One additional product (donor #9) had a borderline low percentage of CD56+CD3- cells (19%) after processing in Minnesota. This product was released and shipped to Boston. All other NK-MC product release criteria from Minnesota were met. Patient # 8 received a lower than intended cell dose (dose given:  $0.64 \times 10^7$  MC/kg - intended dose:  $1 \times 10^7$  MC/kg) due to small clots present prior to incubation requiring the product to be filtered before infusion. All cell products prior to infusion in Boston had excellent viability of over 90% with one exception (85% in patient # 13). (Table 2). Gram stain and endotoxin tested negative and the microbiological cultures were negative in all cases. Cytotoxic activity of the pre-infusion cells against the K562 cell line at different effector:target (E:T) ratios showed killing of 11/13 patient samples of at least 20 % at E:T ratios of 25:1 and 50:1.

### Establishing a Safe Dose Range for NK- MC Product

All patients received one distantly processed, allogeneic NK-MC infusion from a MHC haplotype mismatched related donor. The NK-MC infusions were given 49–191 days (median 90 days) after the autologous stem cell transplant. NK-MC were infused at four escalating dose levels:  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$  and  $2 \times 10^7$  MC/kg recipient weight. There were three patients in each cohort except for dose level 2 ( $1 \times 10^6$  MC/kg), where an additional patient was enrolled as patient # 3 had received less CD56 + cells than intended (11% instead of > 20%) due to a delay of the cells in Minnesota.

The NK-MC infusions were well tolerated by all thirteen patients. Some grade 1 and 2 side effects occurred in patients receiving the higher dose levels (Table 3). All adverse events were transient, and events that prompted medications (meperidine 12.5mg IV, benadryl 25 mg IV) resolved quickly. None of the patients experienced any grade 3 or 4 side effects, or required hospital admission.

Although the NK-MC infusion products were T-cell depleted to the accepted level of CD3+ cells that would not cause GvHD ( $5 \times 10^5$ /kg), <sup>24</sup> there was still some concern that the remaining T-lymphocytes in the infused product would evoke GvHD in the immunocompromised ASCT recipient. However, none of the patients developed signs or symptoms of acute or chronic GvHD. Bone marrow suppression and graft rejection were other concerns, but regular follow- up with blood counts did not show any significant decline in blood parameters.

Chimerism analysis (STR-PCR) of blood MC performed on the three patients who had received the highest allogeneic NK-MC dose level did not detect donor derived NK-MC in the recipients. Although disease outcome was not a data point in this phase I study, survival is reported here for completeness (Table 1). Five patients developed disease recurrence

within 0.8–34.0 (median 3.4) months following NK-MC infusion [5.3–36.9 (median 6.8) months after ASCT]. Of the five relapsed patients, four belonged to cohorts 1 and 2, receiving one of the two lower NK-MC dose levels. At study conclusion, eight patients remained in clinical remission for 13.4–38.3 (median 23.9) months following NK-MC infusion 16.6 – 41.1 (median 26.8) months after ASCT.

## DISCUSSION

Recurrent disease remains the main cause of death after ASCT and is responsible for 73% of the mortality.<sup>1</sup> Variations or intensifications of the preparative regimen have only marginally improved the high recurrence rate. The rationale for NK cellular therapy is based on the hypothesis that this treatment modality kills malignant cells through the perforin/granzyme pathway and its way of inducing cell death is therefore different from chemotherapy and radiation.

Although alloSCT is the only curative treatment for some hematological malignancies, it carries a higher mortality risk and the quality of life of survivors is often compromised by chronic GvHD. Efforts are therefore directed towards achieving the benefits of the immunological graft versus tumor (GvT) effect in the autologous transplant setting without the risk of GvHD. Besides tumor specific T-cells, NK cells are good candidates as they do not cause GvHD.<sup>14,15,16,17</sup> Since recognition of target antigens by NK cells requires that the target does not express “self MHC”,<sup>8,9</sup> allogeneic NK cells are considered to be a superior source for anti-tumor cellular therapy than autologous NK-cells.

The potential GvT effect of allogeneic NK cells goes back to observations by Ruggeri et al.,<sup>14,15</sup> whose group has shown that in case of complete T lymphocyte depletion of the donor stem cell product, a clear GvT effect against AML persists when the donor NK cells express the KIR phenotype that is not activated by the corresponding recipient MHC. A large retrospective analysis of survival and relapse data from the National Marrow Donor Program (NMDP) database confirmed that relapse rates are lower in those donor/recipient combinations that are KIR/KIR ligand mismatched.<sup>19</sup> However, considering those transplants were performed in patient/donor combinations that were largely HLA-matched, the KIR phenotype and mismatching with the corresponding ligand were likely of relevance. Our study used one MHC haplotype mismatched donor MNC. KIR/KIR ligand mismatching was not considered for donor selection. The retrospective analysis of the KIR data confirmed that 11/13 patients were mismatched (Table 1).

Since no safety data are available for infusing MHC mismatched allogeneic NK cells early after ASCT, this study was designed as a phase I dose escalation safety and feasibility study. The two main concerns were whether the T-cell depletion of the donor CD3+ lymphocytes was sufficient to prevent GvHD and whether the infusion of MHC mismatched allogeneic NK would affect bone marrow function.

Patients tolerated the infusion of NK-MC up to the target dose of  $2 \times 10^7$ /kg without any serious side effects confirming earlier studies of allogeneic NK cell infusions.<sup>25–27</sup> At the higher dose levels, transient infusional symptoms (fever, rigors) were observed in some patients. This could be due to cytokine release from the infused MC or related to IL-2 that may have remained adherent to the cells despite washing. The T-cell depleted product had 1000 IU of recombinant IL-2 added to maintain the activity of the NK cells during the transit from Minneapolis to Boston. It is possible that patients would have tolerated even higher doses of NK-MC. The dose of  $2 \times 10^7$ /kg was chosen as an endpoint because this number could be obtained from the donor in a one day large volume leukapheresis.

Furthermore, processing this volume required only one immunomagnetic column for CD3 depletion, thereby keeping the costs at a reasonable level.

The release criteria from Minnesota called for a CD3+ content of less than  $5 \times 10^5$ /kg, which is higher than stated in the FDA guidelines for transfusion products. It has also been observed previously that transfusion-associated GvHD occurs more frequently when fresh product is transfused and when there is some MHC similarity/identity,<sup>25</sup> both conditions that were met in the patients treated here. It is unclear what may have prevented T-lymphocyte engraftment in patients and it remains speculative whether the allogeneic NK cells had a protective role against GvHD.<sup>25,28</sup>

NK enriched donor lymphocyte infusions have been given previously to patients after a T-depleted alloSCT.<sup>28</sup> The leukapheresis collection in that study was positively selected for CD56+ cells in a one step procedure resulting in a median CD56+ dose of  $10.6 \times 10^6$  cells/kg. It remains to be determined whether infusion of allogeneic NK-MC in the autologous transplant setting has any anti-tumor effect. Repeated infusions, the addition of IL-2 or any other cytokine may be necessary to see a benefit. We did not find any donor DNA in the recipient's blood 24 hours after infusion of the highest NK-MC dose level. Although this could have been due to the low sensitivity of the STR-PCR test, it is more plausible that the infused allogeneic cells were rejected by the recipient's T-cells. Considering that ASCT recipients are relatively immunocompetent, this is not an unlikely scenario.

The question is whether pre-infusion immunosuppression would allow longer survival of infused NK cells. Murine models show that depletion of immune cells before cell infusion enhances the antitumor effect of transferred donor cells.<sup>30</sup> Most adoptive immunotherapy studies now use the combination of fludarabine and cyclophosphamide for lymphodepletion.<sup>26,31</sup> In addition to eliminating recipient T-cells that could cause rejection, an immunosuppressive regimen would also eliminate immune cells competing with allogeneic NK for cytokines ("cytokine sinks") and limit the number of regulatory T lymphocytes (T-regs) that would suppress infused NK cells.<sup>32</sup>

Dudley et al.<sup>33</sup> evaluated the safety and efficacy of adding total body irradiation to a non-myeloablative chemotherapy preparative regimen prior to adoptive cell therapy in patients with metastatic melanoma. An objective response of 50–70% was reported. Additionally they noted increases in serum levels of IL-7 and IL-15 and hypothesized that these cytokines contributed to the persistence, proliferation, and activation of the adoptively transferred immune cells. IL-15 also remained increased in those patients with acute leukemia who had a tumor response to allogeneic NK cell infusions in the protocol by Miller et al.<sup>25</sup> Hence, there is sufficient evidence and rationale that supports incorporating immunosuppressive therapy prior to infusion and IL-15 after infusion in a phase II protocol in this patient population.

In addition to safety, our study also confirms that cell collection, cell processing and therapeutic application can be performed in distant locations without significant loss of cell number and viability, thus supporting the mandate of PACT. This organization was formed in 2003 by the National Heart, Lung, and Blood Institute (NHLBI) to provide centralized cell processing services.<sup>34</sup> Currently there are five cell processing facilities nationwide charged with implementing the transition of innovative cellular treatment concepts into clinical practice and supplying clinical grade cell products.

In summary, we confirm that infusion of CD3-depleted NK enriched MC infusions from a MHC-haplotype mismatched relative can be safely administered up to a tested dose of  $2 \times 10^7$  NK-MC cells/kg to recipients of a recent ASCT. NK-MC infusions were well tolerated with rigors and muscle aches being the only transient infusional side effects at the higher

doses. This study provides the platform for further studies with the objective to provide allogeneic cell based GvT effects for ASCT recipients.

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**Table 1**

Patient and Donor Characteristics\*

Patient	Age/Sex	Disease	Interval in days <sup>†</sup>	Donor Age/Relationship	Outcome <sup>‡</sup>	Days to outcome <sup>§</sup>
1	39 M	Multiple Myeloma	49	36 Brother	Relapse	144
2	77 F	NHL (Mantle)	77	55 Brother	Remission	1158
3	57 M	Multiple Myeloma	83	24 Son	Relapse	1036
4	59 M	Hodgkin's Disease	69	57 Sister	Remission	1059
5	47 F	NHL (DLBCL)	142	50 Brother	Relapse	163
6	27 F	Hodgkin's Disease	128	62 Mother	Relapse	489
7	63 M	NHL (Mantle)	133	39 Son	Remission	912
8	50 M	NHL (Follicular)	70	52 Sister	Remission	815
9	55 M	Multiple Myeloma	105	25 Son	Remission	710
10	62 M	Multiple Myeloma	104	29 Daughter	Relapse	380
11	56 F	NHL (ALCL)	121	29 Daughter	Relapse	188
12	56 M	NHL (Mantle)	191	43 Sister	Remission	698
13	62 F	Multiple Myeloma	90	33 Son	Remission	471

\* All patients were MHC haplotype mismatched. 11 patients were KIR-KIR ligand mismatched. Patients 8 and 9 were KIR-KIR ligand matched.

<sup>†</sup> Days from ASCT to NK MC infusion.

<sup>‡</sup> Outcome as of May 1, 2011.

<sup>§</sup> Days from ASCT.

NHL= Non Hodgkin's Lymphoma; DLBCL= Diffuse Large B-Cell Lymphoma; ALCL= Anaplastic Large Cell Lymphoma

**Table 2**

NK-MC Product Characteristics at Release (Minnesota) and Pre-infusion (Boston)

Patient	MC/kg Dose Level	CD56+/CD3- (%)	CD3+ (%)	Viability (%) Post wash
		Minnesota		Boston
1	10 <sup>5</sup>	26	0.6	98
2	10 <sup>5</sup>	28	0.8	93
3	10 <sup>5</sup>	11*	0.43	99
4	10 <sup>6</sup>	23	0.1	94
5	10 <sup>6</sup>	20	0.08	99
6	10 <sup>6</sup>	35	0.15	95
7	10 <sup>6</sup>	42	0.06	95
8	10 <sup>7</sup>	24	0.08	95
9	10 <sup>7</sup>	19	0.16	93
10	10 <sup>7</sup>	26	0.12	92
11	2 × 10 <sup>7</sup>	33	0.08	94
12	2 × 10 <sup>7</sup>	37	0.16	96
13	2 × 10 <sup>7</sup>	20	1.83	85
Median		26	0.15	95
Range		11–42	0.06–1.83	85–99

\* Product delayed 8 hours due to product misplacement by courier (AirNet).

**Table 3**

## Adverse Effects by Dose Cohort

Cohort/Dose (cells/kg)	ADVERSE EFFECTS			
	Grade 1	Grade 2	Grade 3	Grade 4
Cohort 1 $1 \times 10^5$	0	0	0	0
Cohort 2 $1 \times 10^6$	1- Pruritus 1- Flushing	1- Rigors	0	0
Cohort 3 $1 \times 10^7$	2- Muscle aches	1- Rigors	0	0
Cohort 4 $2 \times 10^7$	0	2- Rigors	0	0