Adoptive immunotherapy with haploidentical natural killer cells and Anti-GD2 monoclonal antibody m3F8 for resistant neuroblastoma: Results of a phase I study

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ABSTRACT
Natural killer (NK) cell-mediated antibody-dependent toxicity is a potent mechanism of action of the anti-GD2 murine monoclonal antibody 3F8 (m3F8). Killer immunoglobulin-like receptor (KIR) and HLA genotypes modulate NK activity and are key prognostic markers in m3F8-treated patients with neuroblastoma. Endogenous NK-cells are suppressed in the setting of high tumor burden and chemotherapy. Allogeneic NK-cells however, demonstrate potent anti-neuroblastoma activity. We report on the results of a phase I clinical trial of haploidentical NK-cells plus m3F8 administered to patients with high-risk neuroblastoma after conditioning chemotherapy. The primary objective was to determine the maximum tolerated NK-cell dose (MTD). Secondary objectives included assessing anti-neuroblastoma activity and its relationship to donor-recipient KIR/HLA genotypes, NK function, and donor NK chimerism. Patients received a lymphodepleting regimen prior to infusion of haploidentical CD3-CD56+ NK-cells, followed by m3F8. Overall and progression free survival (PFS) were assessed from the time of first NK-cell dose. Univariate Cox regression assessed relationship between dose and outcomes. Thirty-five patients received NK-cells at one of five dose levels ranging from <1 × 10^6 to 50 × 10^6 CD3-CD56+ cells/kg. One patient experienced grade 3 hypertension and grade 4 pneumonitis. MTD was not reached. Ten patients (29%) had complete or partial response; 17 (47%) had no response; and eight (23%) had progressive disease. No relationship was found between response and KIR/HLA genotype or between response and FcyRIII receptor polymorphisms. Patients receiving >10 × 10^6 CD56+ cells/kg had improved PFS (HR: 0.36, 95%CI: 0.15–0.87, p = 0.022). Patient NK-cells displayed high NKG2A expression, leading to inhibition by HLA-E-expressing neuroblastoma cells. Adoptive NK-cell therapy in combination with m3F8 is safe and has anti-neuroblastoma activity at higher cell doses.

Introduction

Neuroblastoma (NB) comprises >8% of malignancies in children, but disproportionately accounts for 15% of all pediatric oncology deaths.\(^1\) Despite aggressive multimodality therapy, long-term progression-free survival (PFS) for high-risk (HR) NB is <50%.\(^2\) The prognosis for relapsed NB is much worse: a recent review reported 5-year post-relapse overall survival (OS) for stage 4 NB of 8%.\(^3\) Thus, newer therapies are urgently needed for metastatic and relapsed disease. NB is the first pediatric solid tumor for which monoclonal antibody (MoAb)-based immunotherapy has been proven to be effective.\(^4\) The murine anti-GD2 MoAb 3F8 (m3F8) induces complete remission (CR) in chemoresistant osteomedullary NB,\(^5\) and improves survival in stage 4 NB patients in first\(^6\) and subsequent remissions.\(^7\) However, m3F8 is relatively ineffective against high disease burden: both osteomedullary and soft tissue.\(^8\) Furthermore, dose-intensive chemotherapy for HR-NB depletes endogenous leucocyte stores and compromises antibody-dependent cell-mediated cytotoxicity (ADCC), a major anti-NB mechanism of m3F8.

Natural killer cells (NK-cells) can target NB via multiple pathways. They express the low-affinity FcyRIII receptor required for binding m3F8 and triggering NK-mediated ADCC; multiple other receptors bind to activating ligands expressed on NB.\(^9\)–\(^11\) NK-cells are tolerized to cells expressing self-MHC class I molecules and are cytotoxic to cells lacking self-MHC class I molecules. Recognition of MHC class I ligands requires T-cell engagement through the CD8 receptor and is thereby achieved by killing tumor cells lacking self-MHC class I ligands ("missing self"). The same KIR receptors signal inhibition by cells expressing cognate self-HLA class I ligands, thereby achieving tolerance to self.\(^14\)–\(^15\) Resting NB tumors are generally deficient in MHC class I expression,\(^16\) ideal for NK
recognition and killing. Under mild inflammatory conditions, however, MHC class I expression can be induced on NB cell surface, thus inhibiting licensed NK-cells expressing KIR for self-MHC.

NK response, both effector and inhibitory, therefore can be predicted based on genotyping of KIR and MHC class I. We previously reported that m3F8-treated NB patients who lacked HLA ligands for their inhibitory KIR (“missing KIR ligand”) had significantly improved PFS and OS compared to those in whom all KIR ligands were present. A similar observation was also made with the anti-GD2 immunocytokine hu14.18-IL218 though not with the anti-GD2 antibody dinutuximab suggesting that NK cells might vary in their utilization of monoclonal antibodies for ADCC. Our preclinical findings with m3F8 indicated that endogenous unlicensed NK-cells, capable of recognizing targets with “missing ligand,” are the primary mediators of NK-mediated ADCC in patients. While endogenous NK-cells are important in controlling NB progression, chemotherapy-treated patients and those with high tumor burden often have poorly functioning NK-cells, hampering robust ADCC. We hypothesized that adoptively transferred allogeneic NK-cells would have potent ADCC and anti-NB cytotoxicity, especially if donor-recipient KIR/HLA genotype combinations predictive of donor NK activation due to “missing self” could be selected. This was tested in a phase I clinical trial in which adoptively transferred haploidentical NK-cells were combined with m3F8 in patients with chemoresistant or relapsed HR-NB (ClinicalTrials.gov NCT00877110). The primary objective was to determine the toxicity and maximum tolerated dose (MTD) of haploidentical NK-cells when combined with m3F8. Haploidentical NK-cells have been shown to be safe in adults and are readily available from parents of young children with NB. Pilot studies have tested haploidentical NK-cells in children after autologous stem cell transplant and with chemotherapy plus MoAb. To create a lymphodepleted host environment for enhanced donor NK-cell survival, we treated patients with a high-dose cyclophosphamide-based chemotherapy regimen with known anti-NB activity prior to NK-cell infusion and m3F8 therapy (Fig. 1). We now report the results of this first phase I trial of the combination of haploidentical NK-cells and anti-tumor MoAb in children.

Results

Patient and donor characteristics

Thirty-five patients (11 female; 24 male) with a median age of 5.6 (range 2–14.7) years received a total of 43 treatments. Six patients were re-treated: five and one with 1 and 2 additional treatments respectively. Median time from diagnosis to therapy was 15 (range 6–69) months. All patients were heavily prior treated with high-dose chemotherapy. Patients were classified into 3 groups bases on their pre-therapy disease status (a) Primary refractory (n = 13): patients who had incomplete response to induction therapy but had never had disease progression. (b) Secondary refractory (n = 13): patients who had relapse or disease progression, then received interim therapy with stable disease prior to enrolling on protocol. (c) Progressive disease (PD) (n = 9): patients received protocol therapy at time of relapse or disease progression. Mothers were NK-cell donors for 21 patients and fathers for 14. Leukapheresis was performed using peripheral venous access in all donors except one, who required insertion of a femoral central line. Clinical details and HLA, KIR and FCGR3A genotyping are presented in Table 1.

NK-cells

Since a variable number of NK-cells were isolated, allowance was made for infusion of any number of NK-cells isolated, as long as the dose conformed to the desired or lower cell dose. This led to the final number of patients treated at each dose level to differ from the characteristic phase I 3+3 dose-escalation schema. Planned and actual dose levels and NK-cell numbers are shown in Table 2. An adequate number of NK-cells were isolated in 100% (6/6) patients at dose level 1. At dose levels 2, 3 and 4, planned numbers of cells were isolated for 75%

Table 1. Clinical features and results of genotyping on patients and donors.

| MYCN status (n = 34) | N (%)| Amplified | 9 (26)| Non-amplified | 25 (74) |
|---------------------|------|-----------|-------|---------------|--------|
| Prior ASCT | Yes | 9 (26) |
| No | 25 (74) |
| Prior m3F8 therapy | Yes | 13 (37) |
| No | 22 (63) |
| Disease status prior to study entry | Primary refractory | 13 (37) |
| Secondary refractory | 13 (37) |
| Progressive disease | 9 (26) |
| "Missing KIR Ligand" | Yes | 21 (60) |
| No | 14 (40) |
| "Missing Self" | Yes | 12 (34.3) |
| No | 23 (65.7) |
| Donor FCGR3A polymorphisms | F/F | 12 (34.3) |
| F/V | 21 (60) |
| V/V | 1 (2.9) |
| Unknown | 1 (2.9) |
| Host FCGR3A polymorphisms | F/F | 21 (60) |
| F/V | 9 (25.7) |
| V/V | 4 (11.4) |
| Unknown | 1 (2.9) |

Abbreviations: ASCT: autologous stem cell transplant; KIR: Killer immunoglobulin-like receptor.

1"Missing KIR ligand" denotes those patients who lack any HLA ligand for their donor’s inhibitory KIR, regardless of HLA ligands in the donor.

2"Missing Self" denotes those patients who lack HLA ligands present in the donor.
Three infusions in two patients were considered to be unsuccessful (i.e. <1×10^6 cells/kg were isolated, comprising dose level 0). Release criteria were met for all cell products except one, in which NK-cell viability was 61% (<70%). Mean NK-cell purity was 96.3 ± 5.1%; residual CD3+ cells 0.2 ± 0.3%; and viability 92.5 ± 7%.

**Toxicities**

Almost all toxicities were expected and related to m3F8, prior therapy, disease activity, or conditioning chemotherapy. These included grade 4 myelosuppression and lymphopenia, grade 3 febrile neutropenia, grade 2 pain and urticaria and grade 2 bronchospasm (entire list shown in Table 3). Two patients, however, experienced unusual toxicities. One patient treated at dose level I developed dose-limiting toxicity (DLT); grade 3 hypertension, leading to an additional 3 patients being treated at that dose level. The same patient also had grade 4 pneumonitis in the setting of febrile neutropenia after receiving chemotherapy, NK-cells and 4 doses of m3F8. A second patient at dose level I, although asymptomatic, developed grade 2 left ventricular dysfunction (on echocardiogram). Both patients recovered completely from the toxicities. No patient experienced graft-versus-host disease (GvHD). Thirty-two (90%) patients experienced febrile neutropenia, which was uncomplicated except for the first patient described above. No other DLTs were encountered and maximal tolerated dose (MTD) for NK-cells was not reached. Nevertheless, because of the low success rate in isolating >30×10^6 cells/kg, further accrual was discontinued. Human anti-mouse antibody (HAMA) was noted in 3/33 (9%) of patients tested, all previously treated with anti-GD2 MoAbs. Donors did not experience adverse events.

**Responses**

Overall response rate (complete remission [CR] + partial remission [PR]) was 29%: 5 CR, 5 PR, 17 no response (NR), and 8 PD. Responses were observed in osteomedul- lary as well as soft tissue disease, although all responses in the latter were modest (<PR) (Table 4). Site specific responses were as follows: improvement in MIBG scores in 20/35 (57%), complete responses on bone marrow (BM) testing in 9/16 (56%) and objective responses in soft tissue NB (< PR) in 4/14 (29%). Responses were noted at all NK-cell dose levels, and no relationship was found between responses (assessed both by modified International Neuroblastoma Response Criteria [INRC] and by site-specific response) and dose level, MYCN-status, HLA/KIR interaction (“missing KIR ligand” or “missing self”), or FCGR3A polymorphism in host or donor (p > 0.2 for each) (Table 5). No correlation was found between NK-dose considered as a continuous variable, and percentage change in MIBG score (rho = −0.11, 95%CI: −0.43–0.23, p = 0.51). However, all 4 patients with major reductions in MIBG scores (reduction of >10) (Fig. 2; response shown in a representative patient) received NK-cells at levels 2–4. Of the 6 patients who received >1 NK infusions, incremental reductions in MIBG scores were noted in 3. Patients with PD at enrollment had the worst outcomes: 0/9 CR/PR versus 10/24 for all others (p = 0.05) and lowest reduction in MIBG score (p = 0.01).

**Survival**

Because most patients went on to receive other anti-NB therapies, survival rates could not solely be attributed to

### Table 2. Planned and actual dosage of haploidentical NK cells administered.

| Planned dose level (No. NK cells) | Planned No. of patients | Planned No. of patients treated | Planned No. of infusions | Actual No. of infusions | Mean±SD NK cells/infusion (million/kg) |
|-----------------------------------|-------------------------|--------------------------------|--------------------------|------------------------|--------------------------------------|
| 0 (<1×10^6/kg)                    | 0                       | 3 (9%)                         | 0                        | 3                      | 0.4 ± 0.27                           |
| 1 (1–4.99×10^6/kg)                | 6                       | 11 (41%)                       | 6                        | 11                     | 3.54 ± 1.45                          |
| 2 (5–9.99×10^6/kg)                | 6                       | 6 (17%)                        | 6                        | 11                     | 8.53 ± 1.52                          |
| 3 (10–30×10^6/kg)                 | 6                       | 14 (40%)                       | 13                       | 16                     | 16.80 ± 5.38                         |
| 4 (30–50×10^6/kg)                 | 6                       | 1 (3%)                         | 9                        | 1                      | 32.6                                 |
| Total                             | 21                      | 35                              | 34                       | 42                     |                                      |

Abbreviations: NK: natural killer; No.: number; SD: standard deviation. *raw value as mean could not be calculated.

### Table 3. Numbers of patients experiencing toxicities.

| Toxicity Grade: numbers of patients | 1   | 2   | 3   | 4   |
|-------------------------------------|-----|-----|-----|-----|
| Anemia                              | 3 (9%) | 27 (77%) | 5 (14%) | 3 (9%) |
| Lymphopenia                         | 35 (100%) | 35 (100%) | 35 (100%) | 35 (100%) |
| Neutropenia                         | 6 (17%) | 29 (83%) | 6 (17%) | 35 (100%) |
| Hypotension                         | 4 (11%) | 4 (11%) | 5 (14%) | 3 (9%) |
| Non neutropenic fever               | 16 (46%) | 3 (9%) | 1 (3%) | 2 (6%) |
| Urticaria                           | 3 (9%) | 22 (63%) | 4 (11%) | 3 (9%) |
| Anorexia                            | 8 (23%) | 3 (9%) | 3 (9%) | 2 (6%) |
| Diarrhea                            | 7 (20%) | 20 (57%) | 5 (14%) | 3 (9%) |
| Vomiting                            | 7 (20%) | 20 (57%) | 5 (14%) | 3 (9%) |
| Febrile neutropenia                 | 2 (6%) | 2 (6%) | 5 (14%) | 3 (9%) |
| Elevated alanine aminotransferase    | 2 (6%) | 2 (6%) | 5 (14%) | 3 (9%) |
| Elevated aspartate aminotransferase | 4 (11%) | 4 (11%) | 5 (14%) | 3 (9%) |
| Hypomagnesemia                      | 9 (26%) | 22 (63%) | 4 (11%) | 3 (9%) |
| Hypokalemia                         | 12 (46%) | 1 (3%) | 3 (9%) | 2 (6%) |
| Hyponatremia                        | 19 (54%) | 35 (100%) | 35 (100%) | 35 (100%) |
| Pain                                | 11 (31%) | 1 (3%) | 3 (9%) | 2 (6%) |
| Hypertension                        | 1 (3%) | 7 (20%) | 1 (3%) | 3 (9%) |
| Flushing                            | 1 (3%) | 8 (23%) | 1 (3%) | 3 (9%) |
| Left ventricular dysfunction        | 1 (3%) | 3 (9%) | 1 (3%) | 3 (9%) |
| Hyperbilirubinemia                  | 3 (9%) | 2 (6%) | 3 (9%) | 2 (6%) |
| Hypokalemia                         | 19 (54%) | 35 (100%) | 35 (100%) | 35 (100%) |
| Fatigue                             | 11 (31%) | 3 (9%) | 3 (9%) | 2 (6%) |
| Bronchospasm                        | 3 (9%) | 3 (9%) | 3 (9%) | 2 (6%) |

*pRelated to chemotherapy; **Related to m3F8.*
protocol therapy. Median PFS and overall survival (OS) for the entire group were 7.4 (95%CI: 4.6–16.3) and 30.7 (95%CI: 17.1–49.5) months respectively. In univariate analysis, patients treated at dose levels 2–4 had superior PFS compared to those treated at dose levels 0–1 (p = 0.018). Patients with PD immediately prior to treatment had significantly inferior PFS and OS. No other factors impacted survival. In a bivariate mode incorporating dose level and pre-treatment status, PFS remained inferior in PD patients (HR: –0.007) and marginally superior for dose-levels >1 (HR: 0.42, 95%CI: 0.17–2.24%).

**NK-cell chimerism, phenotype and function**

Circulating donor NK-cells could be detected in 2/15 (13%) and 0/11 patients tested 7 and 14 days post-infusion respectively. The two patients with circulating NK cells at day 7 were treated at dose level 1 and 2 respectively. Intriguingly, both patients with detectable NK-cells had durable CR. Donor NK phenotype and functional studies were performed for the first 13 products. These demonstrated normal response capacity, and confirmed that NK populations responded to m3F8-mediated ADCC of NB target cells according to NK education predicted by HLA/KIR genotyping (Table 6). Although previous reports have indicated that post-treatment inflammatory environment can augment NK function, this was not evident in the 19 patients tested (Fig. 3). Nearly all patients had a sustained high frequency of cells expressing the inhibitory receptor NKG2A (median±SD 88.8±22.4%), consistent with a less mature NK repertoire. In comparison, healthy adult individuals exhibited fewer NKG2A-expressing NK cells (54.6±15.8%; p<0.001) (Fig. 4). Given that NB cell lines express HLA-E, the inhibitory ligand for NKG2A, the high frequency of NKG2A+ cells in NB patients could undermine an optimal anti-tumor NK response. Indeed, IFN-γ-induced upregulation of HLA on the neuroblastoma target cell line BE(2)N resulted in inhibition of NKG2A-expressing NK-cells, even in the setting of m3F8 activation. Specific to NKG2A-expressing NK-cells, this inhibition could be reversed by the anti-NKG2A blocking antibody (Fig. 5).

**Discussion**

Our results indicate that haploidentical NK-cell therapy in combination with CTV-chemotherapy and m3F8 is safe in children. The safety of adoptive NK-cell therapy has been previously reported in children with leukemia. Two prior studies report on haploidentical NK cell infusions administered with chemotherapy and anti-GD2 antibodies in 15 patients with neuroblastoma, though neither investigated the combination in a phase I design. In one study, haploidentical NK cells were infused 2–5 days after autologous stem cell transplant along with the anti-GD2 antibody hu14.18K322A. Encountered toxicities were primarily related to the myeloablative pre-plant regimen and responses were not evaluable. In another pilot study, 13 patients with resistant neuroblastoma received varying doses of haploidentical NK cells plus hu14.18K322A

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**Table 4.** Responses.

| Response | CR | PR | NR | PD |
|----------|----|----|----|----|
| Best response by INRC (n = 35) | 5 (14%) | 5 (14%) | 17 (49%) | 8 (23%) |
| Best site-specific responses | | | | |
| Bone marrow disease (involved by histology pre-treatment in 16 patients) | 9 (56%) | N/A | 7 (44%) | N/A |
| MIBG scan (n = 35) | 5 (14%) | 5 (14%) | 11 (31%) | 5 (14%) |
| Soft tissue disease (detected pre-treatment in 14 patients) | 0 | 0 | 9 (64%) | 5 (36%) |

*Objective responses not amounting to PR were noted in 1/11 and 4/9 patients deemed to have NR on MIBG-avid skeletal and soft tissue disease respectively. Abbreviations: CR: complete remission; INRC: International Neuroblastoma Response Criteria; MIBG: metaiodobenzylguanidine; N/A: not applicable; NR: no response; PD: progressive disease; PR: partial response.*

**Table 5.** Relationships between response and dose and other factors.

| Factors tested | Modified INRC | Bone Marrow Histology | MIBG scan (Reduction in MIBG score) |
|----------------|--------------|-----------------------|-----------------------------------|
|                | CR/PR n (%)  | NR/PD n (%)           | p-value                           | Median (Range) reduction in MIBG score | p-value |
| NK cell Dose Level | 0–1 | 3 (21.3) | 11 (78.6) | 0.70 | 4 (28.6) | 10 (71.4) | >0.95 | –1 (–5 to +2) | 0.71 |
|                  | 1–2 | 7 (33.3) | 14 (66.7) | 0.71 | 5 (26.3) | 14 (73.7) | 0.65 | –1 (–18 to +5) | 0.45 |
| MYCN Negative | 8 (80) | 19 (79.2) | >0.95 | 8 (88.9) | 18 (78.3) | 0.65 | –1 (–18 to +5) | 0.56 |
|                  | Positive | 2 (20) | 5 (20.8) | 1 (11.1) | 5 (21.7) | 0.65 | –1 (–18 to +5) | 0.23 |
| Missing KIR Ligand | No | 4 (40) | 10 (40) | >0.95 | 3 (33.3) | 9 (37.5) | >0.95 | –1 (–18 to +5) | 0.23 |
|                  | Yes | 6 (60) | 15 (60) | 0.65 | 6 (66.7) | 15 (62.5) | 0.65 | 0 (–2 to +2) | 0.50 |
| Missing Self | No | 7 (70) | 16 (64) | >0.95 | 5 (55.6) | 16 (66.7) | 0.69 | –1 (–18 to +5) | 0.56 |
|                  | Yes | 3 (30) | 9 (36) | 0.69 | 4 (44.4) | 8 (33.3) | 0.71 | –1 (–10 to +2) | 0.23 |
| Donor Polymorphism | F/F | 5 (50) | 7 (29.2) | 0.27 | 4 (44.4) | 8 (34.8) | 0.70 | –1 (–18 to +5) | 0.85 |
|                  | F/V or V/V | 5 (50) | 17 (70.8) | 0.27 | 5 (55.6) | 15 (65.2) | 0.69 | –2 (–2 to +2) | 0.14 |
| Host Polymorphism | F/F | 8 (80) | 13 (54.2) | 0.25 | 4 (44.4) | 16 (69.6) | 0.24 | –2 (0 to –10) | 0.14 |
|                  | F/V or V/V | 2 (20) | 11 (45.8) | 0.24 | 5 (55.6) | 7 (30.4) | 0.24 | 0 (–18 to +5) | 0.14 |

*Abbreviations: CR: complete remission; INRC: International Neuroblastoma Response Criteria; MIBG: metaiodobenzylguanidine; N/A: not applicable; NR: no response; PD: progressive disease; PR: partial response.*
given after various combinations of chemotherapy. The contribution of NK cells in observed toxicities and responses could not be ascertained.\textsuperscript{24} In both studies, infused NK cell numbers ranged from $4 \times 10^6$ to $60 \times 10^6$ NK cells/kg.

We could harvest up to $30 \times 10^6$ pure NK-cell populations from a majority of donors. Lower dose levels were achievable in 100% of donors, but isolation of NK-cell numbers $> 5 \times 10^6$/kg was feasible only in 67%. The primary reason for the inability to isolate higher NK-cell numbers was loss of cells after overnight incubation in the presence of IL-2, possibly due to apoptosis from cytokine over-activation. This loss was especially marked when incubation media contained 1000 IU/ml of IL-2 (first 10 products) and reduced when the IL-2 concentration was decreased to 500 IU/ml. Future adoptive NK-cell therapy protocols may benefit from removal of ex vivo IL-2 stimulation altogether. Alternatively cytokines, such as IL-15, IL-12, and IL-18 may be more beneficial for NK survival and activation.\textsuperscript{30}

NK-cell infusion was not toxic; in particular, no patient developed GvHD or delayed blood count recovery. As expected with CTV-chemotherapy, almost all patients developed febrile neutropenia, requiring treatment with IV antibiotics. We had chosen this high-dose regimen not only for its anti-NB effect and to prevent the possibility of GvHD in our significantly immunosuppressed patients, but with the expectation that via its lymphodepleting effects, we would improve donor NK-cell survival. Despite severe lymphodepletion, we did not observe significant NK-cell persistence, possibly due to induced apoptosis from ex vivo IL-2 stimulation. To preserve lymphodepleting effects while minimizing myelosuppression, future adoptive NK-cell therapies could use single-agent cyclophosphamide conditioning.

This phase I study was not designed to evaluate the antitumor effect of NK-cells, since the regimen also included chemotherapy and anti-GD2 MoAb; and responses in

**Table 6.** Correlation of NK cytotoxicity and donor-recipient HLA/KIR phenotyping.

| Pt# | Donor inhibitory KIR | Donor KIR ligands | Recipient KIR ligands | NKGA2 % NK cells | Global expression on total NK (%) | NK cytotoxic response to 3F8-mediated ADCC (%) |
|-----|----------------------|-------------------|-----------------------|------------------|----------------------------------|-----------------------------------------------|
| 1   | 2DL2, 3DL1           | C1, Bw4           | C1, C2, Bw4           | 51               | 0                                | 33.6                                          |
| 2   | 2DL1, 2DL3, 3DL1     | C1, C2, Bw4       | C1                    | 76.2             | 20.3                             | 29.3                                          |
| 3   | 2DL1, 2DL3, 3DL1     | C1, Bw4           | C1, Bw4               | 63.7             | 10                               | 18.9                                          |
| 4   | 2DL1, 2DL3, 3DL1     | C1, C2, Bw4       | C1, Bw4               | 58.7             | 17.5                             | 18.5                                          |
| 5   | 2DL1, 2DL2, 3DL3     | C1, Bw4           | C1                    | 45.6             | 15.5                             | 13.5                                          |
| 6   | 2DL1, 2DL2, 3DL3     | C1, C2, Bw4       | C1                    | 63.5             | 3.51                             | 41.6                                          |
| 7   | 2DL1, 2DL3, 3DL1     | C1, C2            | C1                    | 54.5             | 29.4                             | 28.7                                          |
| 8   | 2DL1, 2DL2, 3DL3     | C1, C2, Bw4       | C1                    | 59.8             | 19.4                             | 27.4                                          |
| 9   | 2DL1, 2DL3, 3DL1     | C1                | C1, C2, Bw4           | 55.4             | 15.3                             | 27.4                                          |
| 10  | 2DL1, 2DL2, 3DL3     | C1                | C1                    | 46               | 10                               | 29.5                                          |
| 11  | 2DL1, 2DL2, 3DL1     | C1, C2            | C1, C2, Bw4           | 48.6             | 18.8                             | 22                                             |
| 12  | 2DL1, 2DL2, 3DL1     | C1, Bw4           | C1, C2, Bw4           | 48.9             | 6.74                             | 23.9                                          |

Abbreviations: ADCC: Antibody-dependent cell mediated cytotoxicity; NP: non performed; Pt: Patient.

Donor inhibitory KIR shown in bold face indicate self-HLA-specific inhibitory KIR predictive of licensed population; Global expression % depicted in bold face indicates missing self-HLA KIR+ NK cells; Italicized expression % indicates missing ligand KIR+ NK cells.
patients with resistant NB have been reported with a che-
mimunotherapy regimen consisting of irinotecan, temo-
zolomide, dinutuximab and GM-CSF. However, in our
study, a dose-response effect for NK-cells appeared to be
present with robust MIBG responses and improved PFS in
patients receiving \(10^6\) NK-cells/kg and durable CRs in
patients with NK-cell persistence implying that adoptively
transferred NK-cells contributed to the anti-NB effect of the
therapeutic combination. Although direct correlations are
difficult to make, the 29% CR+PR rate observed in our
study was higher than the 15% response rate we had previ-
ously observed for the CTV (in which the dose of topotecan
was 8 mg/m\(^2\)/cycle compared to 7.2 mg/m\(^2\)/cycle in our
study) combination administered without any concurrent
m3F8 or NK-cells in a similar patient population. These
observations lend credence to our hypothesis that allogeneic
NK-cells can mediate ADCC and tumor control and that
higher NK-cell numbers might be more beneficial. We
safely re-treated patients with additional cycles of protocol
therapy and observed incremental responses in three of six
patients. Alternative approaches to increase NK-cell num-
bers include ex vivo expansion with IL-21, or in vivo
expansion with exogenous IL-15 or IL-15 agonist
complex. The timing of therapeutic agents was primarily
dictated by logistical issues (most patients received all ther-
apy including NK-cell infusions and m3F8 on an out-
patient basis). However, given our observation that circulat-
ing NK-cells could not be detected >14 days after infusion,
concurrent administration of NK-cells and anti-GD2 ther-
apy could possibly be more effective. We did not discern a
clear benefit of KIR/HLA mismatch, either via the mecha-
nisms of “missing self-HLA” or “missing KIR ligand”,
although the comparison groups in this phase I study were
too small and heterogeneous to yield adequate power for
such an analysis. Therefore, the utility in selecting a donor
based on KIR/HLA genetics for this treatment approach
remains unknown.

Donor NK phenotyping and functional studies revealed NK
responsiveness predicted by KIR and HLA genetics and consis-
tent with previous observations in normal individuals. Our
patients had a high frequency of NKG2A+ NK-cells which
may reflect a repertoire that is immunologically naive in this
pediatric population or a derangement in NK recovery
following chemotherapy. The high percentage of NKG2A+ NK-cells could dampen NK-ADCC, but this inhibition could be relieved by anti-NKG2A blocking antibody which might have a potential clinical role to enhance NK-cell responses in patients. For this trial, donors were not selected on the basis of presence of an adaptive NKG2C FcR- population, an NK population found in CMV-seropositive individuals and potent mediators of ADCC. Future trials using this selection criterion may result in higher response to m3F8, but will continue to face challenges achieving higher cell doses and longer in vivo survival.

Results from this phase I trial haploidentical NK-cells in combination with MoAb indicate that adoptively transferred NK-cells, when activated by ADCC-eliciting MoAbs, can exhibit anti-tumor activity. Based on the lessons learned in this study with regard to NK-cell isolation and regimen toxicity, our current follow-up study (NCT02650648) combines cyclophosphamide conditioning, haploidentical NK-cells, a MoAb with superior ADCC properties (hu3F840) and in vivo administration of IL-2.

**Donor selection**

Prior to initiating therapy, prospective donors and patients underwent HLA and KIR genotyping by high-resolution HLA-A, B and C genotyping (Histogenetics) and PCR-SSP respectively. The following KIRs were genotyped: KIR2DL1, KIR2DS1, KIR2DL2/S2, KIR2DL3, KIR3DL1, KIR3DS1. Related donors confirmed to be HLA-haploidentical to the patient and with negative serological testing for HIV, HTLV I and II and West Nile virus, underwent leukapheresis for a total of 10–15 liters. Donors whose class I KIR ligands were not present in the patient and who possessed the corresponding cognate inhibitory KIR receptors were prioritized. For those donor-recipient pairs where no KIR ligand incompatibility was found, donors with activating KIR were prioritized over donors who lacked all

**Methods**

**Patient selection**

Patients with HR-NB (stage 4 disease diagnosed at > 18 months of age or MYCN-amplified ≥ stage 3 tumor at any age) and a history of chemoresistance to high-dose induction chemotherapy), or NB patients relapsing with metastatic disease were eligible. The presence of evaluable (microscopic BM metastases, abnormal scintigraphic studies) or measurable (CT or MRI) NB ≥ 1 month after completion of systemic therapy was required for protocol eligibility. Patients with BM positivity for NB as their only evaluable disease were excluded. Prior m3F8 therapy was permitted. Patients with life-threatening infections or > grade 2 toxicity according to the National Cancer Institute’s Common Toxicity Criteria version 3.0 (CTC v3.0) were excluded.
activating KIR. In this latter group, we first prioritized KIR2DS1, followed by centromeric activating KIR. \(^{42}\)

### NK-cell isolation

NK-cells were isolated from a donor leukapheresis product in a two-step process using the CliniMACS System (Miltenyi Biotec). Briefly, the cell product was first depleted of T-lymphocytes by anti-CD3 antibody-coated paramagnetic particles. The CD3\(^-\) effluent fraction was then enriched for NK cells with the CliniMACS CD56 reagent. Cells were incubated overnight with 500–1000 U/mL IL-2 (Chiron) at a concentration of 2\(\times\)10\(^6\) cells/ml, then washed before infusion into the patient the following day. Release criteria for the final product prior to patient infusion included: <2\(\times\)10\(^7\)/kg CD3\(^+\) cells, \(\geq\)90% CD3\(^-\)CD56\(^+\) purity by flow cytometry, and \(\geq\)70% viability.

### Study design

The protocol was approved by the institutional review board (IRB) of Memorial Sloan Kettering Cancer Center (MSKCC). Written informed consent was obtained from patients or their guardians. Patients received cyclophosphamide 70 mg/kg intravenously (IV) on days 1 and 2, topotecan 2.4 mg/m\(^2\)/day IV on days 1–3 and vincristine 0.067 mg/kg IV on day 1. Donor leukapheresis followed by NK-cell isolation was performed on day 4, and NK-cells were infused on day 5. m3F8 (20 mg/m\(^2\)/day IV) was infused over 0.5–1.5 hours on days 8–12. (Fig. 1) The study was planned with a standard 3+3 design, patients received NK cells at one of 4 escalating dosage levels: 1\(\times\)10\(^6\), 5\(\times\)10\(^6\), or 10\(\times\)10\(^6\) cells/kg and 30\(\times\)10\(^6\) cells/kg, and 30\(\times\)10\(^6\) cells/kg. If DLT was encountered in 1/3 patients at a dose level, 3 additional patients were planned to be treated at that level. If the targeted NK-cell dose was not achieved but otherwise met release criteria, all isolated NK-cells were infused, and the patient counted at the actual dose level. If <1\(\times\)10\(^6\)/kg were isolated, all cells were infused (termed dose level 0). As a result, >3–6 patients were treated at each dose level. After enrollment was completed on dose level 1, the protocol was amended to allow up to 2 additional NK-cell infusions if patients met all the following criteria: (a) no DLT, GvHD or HAMA response, \(^{43}\) (b) no PD, (c) had an objective response and (d) continued to meet all eligibility criteria. In an effort to reduce severe myelosuppression, chemotherapy for subsequent treatments was restricted to cyclophosphamide 50 mg/kg/day IV on days 1 and 2. NK-cell and m3F8 doses remained unchanged for subsequent cycles.

### Toxicity monitoring

Toxicities and acute GvHD were assessed by CTCv3.0 and previously described criteria. \(^{44}\) Toxicties clearly related to chemotherapy, >grade 2 toxicities clearly related to co-interventions, prior therapy or disease activity, and grade 3 fever, rash or hypotension related to m3F8 were not DLT. All other >grade 3 toxicities were considered DLT. Patients were monitored for toxicity at least once weekly with physical examination, complete blood counts, liver function tests, BUN and serum creatinine.

### Response assessment

Disease status was assessed after each treatment and then at least every three months with CT or MRI, MIBG scan, and BM aspirates and biopsies. Disease status was defined International Neuroblastoma Response Criteria \(^{45}\): CR: no evidence of disease; very good partial remission: >90% decrease in volume of primary tumor and no other evidence of active disease; PR: 50–90% decrease in volume of measurable disease, \(\geq\)50% reduction in modified Curie score on MIBG scan \(^{46}\) but <CR; no new lesions, \(\leq\)1 positive BM site allowed, no response (NR): <25% increase in volume of any existing soft-tissue lesion, <50% reduction in MIBG score; PD: any new lesion, increase of any measurable lesion by >25%. In addition, for comparison between groups, objective response was defined as any improvement in modified Curie score. For purposes of statistical analyses, responses were grouped into CR/PR versus SD/PD.

### Correlative studies

In addition to HLA and KIR genotyping, donor and patient NK phenotype and function, FCGR3A polymorphisms, chimerism, and HAMA were assessed. NK-cell chimerism was evaluated by quantitative PCR for DNA polymorphisms. NK phenotype was evaluated by multi-parameter flow cytometry for cell-surface expression of CD94/NKG2A and inhibitory and activating KIR, as previously described. \(^{17}\) Functional response of NK populations was measured flow cytometrically by CD107a mobilization to the NK-sensitive line K562 and to the NB cell lines LAN-1, BE(1)N and SKNLH in the presence of m3F8. \(^{15}\) In some studies, activation of NK cells by target cells was performed in the presence of the anti-NKG2A blocking antibody (clone Z199, Beckman Coulter). HAMA was detected using ELISA \(^{47}\) with a titer of >1000 U/ml being considered positive. FCGR3A polymorphisms were evaluated as previously described. \(^{48}\) Allelic discrimination of FCGR3A was identified as [F/F], [V/V] or [F/V].

### Statistical methods

Relationships between response and dose level (dose levels were grouped as levels 0–1 and 2–4) and other factors were assessed with Fisher’s Exact Test and the Wilcoxon Rank Sum test where appropriate. The relationship between continuous dose and MIBG score percent change was assessed with Spearman’s Rank Correlation. Wilcoxon Signed Rank Test was used to assess if the change in MIBG score from pre to post-treatment was significant. Kaplan-Meier estimates and plots were generated for OS and PFS in the full sample, and also stratified by dose level. The log-rank test was used to assess the relationship between dose with OS and PFS. Univariate Cox proportional hazards regression was used to assess the relationship between potential predictors and OS/PFS. Factors significant at p = 0.05 were considered for multivariate analysis with dose. Due to the
hypothesis generating nature of the study, no adjustments were made for multiple hypothesis testing. All analyses were performed using SAS 9.4 (The SAS Institute, Cary, NC). OS and PFS was calculated from the time of first dose until death or PD respectively.

Disclosure of potential conflicts of interest

The authors declare the following conflicts of interest: anti-GD2 antibodies have been licensed by Memorial Sloan Kettering Cancer Center (MSK) to Ymabs Therapeutics Inc., both MSK and NK Cheung have financial interest in this company; Shakeel Modak and Kim Kramer are consultants to Ymabs Therapeutics Inc.

Acknowledgments

We thank Alison Slocum, Chandresh Undhad, and other members of the Center for Immune Cell Therapies at MSK for their work on NK cell isolation. We thank Joe Olechnowicz for editorial assistance.

Funding

This work was supported by the NIH National Cancer Institute (P30 CA008748), NIH R01 CA164356, FDA R01 FD-R-004128, Alex’s Lemonade Stand Foundation, Society of Memorial Sloan Kettering Cancer Center, and the Cancer Center Core grant (NIH P30 CA008748, NIH O01AI069197).

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