Phase I Study of Random Healthy Donor-Derived Allogeneic Natural Killer Cell Therapy in Patients with Malignant Lymphoma or Advanced Solid Tumors

Yaewon Yang, Okjae Lim, Tae Min Kim, Yong-Oon Ahn, Hana Choi, Hyejin Chung, Bokyung Min, Jung Hyun Her, Sung Yoo Cho, Bhumsuk Keam, Se-Hoon Lee, Dong-Wan Kim, Yu Kyeong Hwang, and Dae Seog Heo

Abstract

NATURAL IMMUNE SYSTEM CELLS, INCLUDING NATURAL KILLER (NK) CELLS, PLAY A CRUCIAL role in innate immune control of tumor development (1). The function of these cells is regulated by signals from activating and inhibitory receptors (2). Some MHC class I molecules, especially HLA-C, can be ligands for killer cell immunoglobulin-like receptors (KIR), which deliver inhibitory signals to NK cells (3). Among immune cells, natural killer (NK) cells, defined by CD56 or CD16 expression and the absence of CD3, play a critical role in innate immune control of tumor development (1). The function of these cells is regulated by signals from activating and inhibitory receptors (2). Some MHC class I molecules, especially HLA-C, can be ligands for killer cell immunoglobulin-like receptors (KIR), which deliver inhibitory signals to NK cells (3).

Interaction of the relevant self-MHC class I molecules with a given KIR results in inhibition of effector functions of autologous NK cells, even in the presence of additional activation signals (4). The limitation of self-MHC class I-mediated inhibition makes allogeneic NK cells potentially better effector cells for immunotherapy. Indeed, infusion of enriched alloreactive, haploidentical KIR ligand–mismatched NK cells has been shown to be safe, without graft-versus-host disease (GVHD), and to achieve significant clinical responses in cancer patients in human trials (4–8). Another benefit of allogeneic NK-cell treatment is that healthy donor–derived NK cells can be adoptively transferred with strong graft-versus-tumor (GVT) effect (9, 10).

In this study, we addressed the safety and clinical benefit of receiving allogeneic NK cells from random unrelated healthy donors, which may result in some cells having completely mismatched MHC class I allele expression between donor and recipient. This strategy not only allows for the extended possibility of donor-receptor KIR ligand–mismatch, but also overcomes limitations due to small potential donor pools. Even though the safety and efficacy of adoptive transfer of haploidentical NK cells in patients were confirmed (11), whether the expanded NK cells derived from random unrelated donors would be safe remains in question. Therefore, it must be ascertained if these cells do not cause any adverse effects by themselves in vitro without any beneficial combined therapy, including immunosuppressive drugs. To this end, we established an efficient method for the large-scale, ex vivo expansion of NK cells from peripheral blood mononuclear cells (PBMC) from random healthy donors under good manufacturing practice (GMP) conditions (12). These ex vivo expanded NK cells can be...
in vitro–expanded, random healthy donor–derived allogeneic NK cells, defined as MG4101, showed antitumor potency against various cancer cell lines in vitro and in SCID mice injected with human lymphoma cells (12). Based on these results, we have designed a phase 1 study of adoptive transfer of MG4101 into patients with malignant lymphoma or advanced, recurrent solid tumors.

**Patients and Methods**

**Patients**

Patients with malignant lymphoma or advanced, recurrent solid tumors who failed to benefit from standard treatment were enrolled in this study. All patients were at least 18 years old and had histologically or cytologically confirmed malignant lymphoma or solid tumors. Karnofsky Performance Scale >70, or Eastern Cooperative Oncology Group performance status of 0 to 2 (13, 14) with at least 3 months of expected survival. Exclusion criteria were immune deficiency, autoimmune diseases, other malignancies, severe allergic disorders, or exposure to cell-based therapy in the preceding 3 months. Subjects currently receiving or having received systemic therapy for any other malignancy in the preceding 4 weeks were also ineligible.

**Clinical trial design**

The primary objective of this single center, phase I noncomparative, dose-escalation study using MG4101 in patients with previously treated malignant lymphoma or advanced, recurrent solid tumors was to determine the safety, the MTD, and the maximum feasible dose (MFD) of MG4101 in humans. The secondary objectives were to evaluate the antitumor efficacy and persistence of MG4101. Tumor-related immune responses after MG4101 intravenous infusion were also evaluated. All the study samples were obtained following acquisition of the study participants’ written informed consent, in accordance with the Declaration of Helsinki. This trial was registered to ClinicalTrials.gov (NCT01212341) and was approved by the Institutional Review Board of Seoul National University Hospital (H-1004-027-315).

**NK-cell preparation and expansion**

PBMCs were isolated from random healthy donors, and NK cells were expanded as described previously under the conditions of GMP at Green Cross LabCell (12). Briefly, CD3+ T-cell–depleted PBMCs were expanded at a seeding concentration of 2 × 10^5 cells/ml in CellGro SCGM serum-free medium (CellGenix) with 1% auto-plasma, 1 × 10^6 cells/ml irradiated (2,000 rad) autologous PBMCs, 10 ng/ml of monoclonal antibody to CD3 (OKT3; Orthoclone), and 500 IU/ml of IL2 (Proleukin) in an A-350N culture bag (NIPRO). NK cells were fed fresh medium with 500 IU/ml of IL2 every 2 days until they were harvested on day 14.

After expansion, the cytotoxicity of MG4101 was evaluated by flow cytometric cytotoxicity assay against K562 as described (12). K562 was obtained from the ATCC and cultured in RPMI-1640 medium (GIBCO) supplemented with 10% FBS (GIBCO).

**Flow cytometric analysis of NK cells**

For the composition analysis of MG4101, NK cells were stained with the appropriate monoclonal antibodies to CD56 (B159), CD3 (UCHT1), CD16 (3G8), CD14 (M5E2), and CD19 (HB19; all from BD Biosciences). Samples were acquired on a BD LSR Fortessa, and data were analyzed using FlowJo software (TreeStar Inc.).

**Treatment protocol and evaluation of safety and efficacy**

MG4101 was administered intravenously one time (step 1) or repeatedly (step 2). The infusion protocol is described in Supplemental Fig. S1. This trial was designed using a traditional 3+3 method. Cohort 1 of step 1 was initiated with the infusion dose of 1 × 10^6 cells/kg of MG4101, and drug-related toxicities were assessed for 2 weeks. After safety assessment, cohort 3 (1 × 10^6 cells/kg, once weekly, triple infusion) and cohort 4 (3 × 10^6 cells/kg, once weekly, triple infusion) were sequentially proceeded. Next, the escalated dose of 1 × 10^7 cells/kg was adoptively transferred to cohort 2 of step 1. When the single dose of 1 × 10^7 cells/kg was defined as safe, cohort 5 (1 × 10^7 cells/kg, once weekly, triple infusion) and cohort 6 (3 × 10^7 cells/kg, once weekly, triple infusion) of step 2 proceeded sequentially. In the case of body temperature above 38°C or toxicities greater than grade 2 in absolute neutrophil count, platelet count, hemoglobin, serum creatinine, total bilirubin, or liver aminotransferase, the administration of MG4101 was withheld.

The safety profiles of step 1 and step 2 were assessed for 4 and 5 weeks after MG4101 administration, respectively. MTD was defined as one dose level below the dose at which dose-limiting toxicities (DLT) were observed in ≥33% of the participants. DLT was defined as any grade 4 toxicities, grade 3 toxicities lasting longer than 5 days, or GVHD of more than grade 2. If the maximum planned dose (3 × 10^7 cells/kg) of this study is evaluated to be tolerable, the MTD would not be determined and 3 × 10^7 cells/kg would be set as the MFD. Toxicities and adverse events were graded using the common toxicity criteria adverse events version 3.0 (15).

For the evaluation of the radiologic responses, chest CT scans before and 4 weeks after the initial infusion of NK cells were obtained and analyzed using RECIST criteria version 1.1 for solid tumors and Revised Response Criteria for Malignant Lymphoma (16, 17).

**Immune monitoring of recipients**

Flow cytometric analysis of the change in immune cell populations after MG4101 administration was performed on serially acquired PBMCs from recipients. Regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) were analyzed by lymphogating of CD4+CD25highFoxp3+CD127dim cells and Lin−CD14−HLA-DR−CD11b−CD15+ cells, respectively (18, 19). Various cytokines and chemokines in patient plasma were quantified with commercially available cytometric bead-based assays according to the manufacturers’ instructions (FlowCytomix; eBioscience).

**Persistence of administered NK cells**

Genomic DNA was extracted from serially acquired PBMCs of recipients. Nested PCR was performed to detect the presence of allo-HLA-DRB1 genes of donor NK-cell origin (20). HLA-DRB1 exon 2 or the DRw52-group–specific part of DRB1 exon2 was amplified in the first PCR, and either the HLA-DRB1 allele-specific or the group-specific amplification was performed in the second PCR. The sensitivity of nested PCR was analyzed by target gene amplification from samples containing serially decreased amounts of donor-derived DNA mixed with a fixed amount of recipient-derived DNA: 10%, 1%, 0.1%, 0.01%, and 0.001% (vol/vol ratio). As an internal positive amplification control, amplification of a fragment of the human growth hormone gene (hGH) was included (20).
Statistical analysis
Analyses for the demographic and clinical features were descriptive. The paired t test was used to compare the percentage and surface marker expression of immune cell subsets before and after therapy. The unpaired t test was used to compare the percentage of MDSCs between patients and healthy controls. A calculated P value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc.).

Results
Study population
Twenty eligible patients were enrolled from August 2010 to June 2012. Demographic characteristics of the enrolled patients are listed in Supplementary Table S1. The first lymphoma patient (C1-01) received a fifth line of prior chemotherapy and progressed. The other lymphoma patient (C4-01) received MG4101 as a third-line treatment. As for the patients with solid cancers, 17 patients (94.4%) had received prior chemotherapy and 10 patients (55.6%) had received prior radiotherapy.

Characterization of ex vivo-expanded NK cells
Because activated NK cells have been shown to contribute to stronger GVT effects than resting cells (9), we decided to use highly activated, ex vivo-expanded NK cells in this study. We have previously established a simplified and efficient method for GMP-compliant large-scale expansion of NK cells. MG4101 (12). In the present study, MG4101 products derived from random healthy donors were prepared for administration to cancer patients. The MG4101 was composed of enriched CD16+CD56+ (98.13 ± 1.98%) NK cells with minimal contamination of CD3+ T cells (0.41 ± 0.43%), CD14+ monocytes (0.40 ± 0.37%), and CD19+ B cells (0.15 ± 0.25%; Supplementary Fig. S2A). During the culture, NK cells were expanded 757.5 ± 232.2-fold (Supplementary Fig. S2C) with 92.9 ± 2.1% viability (Supplementary Fig. S2B). In a cytotoxicity assay, MG4101 showed potent cytolytic activity against K562 cells (Supplementary Fig. S2D). Similar to our previous results, we confirmed that MG4101 is composed of a highly pure population of CD3+CD16+CD56+ NK cells with potent antitumor activity (12).

Safety and toxicity profile
Toxicity profiles were evaluated in all 20 patients after MG4101 infusion and are summarized in Table 1. In step 1 (cohort 1 and cohort 2), none of the subjects showed DLTs and all the toxicities were grade 1 or 2. Furthermore, a serial dose increase of MG4101 in step 1 did not seem to cause a proportionate increase in toxicity. The only grade 2 toxicity in our study was chills, which occurred in 1 patient from cohort 2. In step 2, repeated injection of a higher dose of MG4101 correlated with increased incidence of adverse events, but all remained between grades 1 and 2. MG4101-related GVHD was not observed in any of the subjects. As the maximum planned dose of this study was found to be tolerable, the MTD was not determined and 3 × 107 cells/kg was set as the MFD. Further, toxicity-related suspension of MG4101 injection did not occur during our study.

Response to the MG4101
Responses to the MG4101 were evaluated in 17 patients, including 2 with lymphoma and 15 with advanced solid cancer. Three patients (C5-02, C6-01, and C2-03) were not evaluable due to incomplete treatment or follow-up loss. As for the lymphoma patients, C1-01 exhibited stable disease (SD) and C4-01 had progressive disease (PD). Of the solid cancer patients, 7 (47%) had SD and 8 (53.0%) had PD. Responses to the MG4101 therapy are summarized in Table 2. After MG4101 treatment, all of the lymphoma patients and 33% of solid cancer patients received further chemotherapies. The median progression-free survival (PFS) in patients with SD was 4 months (range, 2 to 18 months). To evaluate whether our observation could support the finding that KIR ligand–mismatched NK cells exhibit better GVT effects than KIR ligand–matched ones (9), we retrospectively analyzed the correlation between PFS and KIR expression pattern in cohorts 2, 5, and 6, in which each subject received more than 1 × 107 cells/kg of MG4101. We found that patients C5-01 and C6-03, who received higher numbers of incompatible KIR-expressing NK cells, had enhanced PFS compared with patients receiving lower amounts in each cohort, respectively (Table 3). We also evaluated whether the activating KIR B haplotype had any positive effects on outcome in our study (4). To this end, the KIR B haplotype was associated with a higher incidence of SD (Supplementary Fig. S3). Although our results should be interpreted with some caution due to the small size of the patient group, we believe that treatment with random healthy-donor allogeneic NK cells may provide better clinical benefit due to the increased pool of donors with higher incompatible KIR expression and B haplotype usage.

<table>
<thead>
<tr>
<th>Toxicities</th>
<th>Cohort 1 (percentage)</th>
<th>Cohort 2 (percentage)</th>
<th>Cohort 3 (percentage)</th>
<th>Cohort 4 (percentage)</th>
<th>Cohort 5 (percentage)</th>
<th>Cohort 6 (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General disorders and administration site conditions</td>
<td>1 (33.3%)</td>
<td>1 (33.3%)</td>
<td>–</td>
<td>–</td>
<td>1 (25%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Asthenia</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fatigue</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3 (75%)</td>
<td>–</td>
</tr>
<tr>
<td>Chills</td>
<td>–</td>
<td>1 (33.3%)</td>
<td>–</td>
<td>–</td>
<td>1 (25%)</td>
<td>–</td>
</tr>
<tr>
<td>Infections and infestations</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fatigue</td>
<td>3 (75%)</td>
<td>3 (75%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>sweats</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Headache</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nervous system disorders</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Headache</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Asthenia</td>
<td>3 (75%)</td>
<td>3 (75%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>Malaise</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rash</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
</tr>
</tbody>
</table>

*Only this patient was assessed as having grade 2 toxicity; the others had grade 1 toxicity.*
Persistence of adoptively transferred MG4101

Persistence of MG4101 was monitored by allo-HLA–specific nested PCR of sequentially obtained PBMCs from recipients after MG4101 administration. The persistence of MG4101 was assessed by visualization of donor-specific target gene amplification by agarose gel using hGH amplification as an internal control (20). Sensitivity was determined as described in Patients and Methods, and the detection limit of donor-specific gene amplification ranged between 0.01% and 0.001% (Supplementary Fig. S4). After administration of 1/C2 10^6 cells/kg, MG4101 was detected in the peripheral blood of recipients for the first 24 hours. In cohort 2, MG4101 was detected for up to 4 days, depending on the recipients. In the repeatedly infused cohorts, the persistence of MG4101 differed among recipients and gradually decreased following repeated administration (Supplementary Table S2 and Supplementary Fig. S4). The variation of persistence was independent of the number of cells administered. Compared with other NK cells in recently published clinical trials, the persistence of MG4101 was relatively short (6–9). It should be noted that in these other trials, allogeneic NK cells persisted for 1 to 2 weeks when administered along with immunosuppressive regimens in order to dampen the host T-cell response. However, in the present study, immunosuppressive regimens were not included. Therefore, the persistence of MG4101 for up to 4 days in recipients without any immunosuppressive drug cotreatment may in fact be comparable with the findings from these other clinical trials.

Immune monitoring after MG4101 infusion

The above findings of extended PFS in SD patients from cohorts 5 and 6 suggested that MG4101 could regulate the host immune response against tumors, including CD8^+ T-cell responses (9). There were no changes in the frequency of immune cell subsets, such as T cells, B cells, NK cells, and monocytes (data not shown). Based on previous observations, we decided to analyze surface expression of NKG2D on immune cells (Fig. 1A) to see whether MG4101 could affect host immune responses (21). Although no changes in the activation status of CD4^+ T cells (data not shown) and CD56^+ NK cells (Fig. 1B) were found after injection of MG4101, the expression of NKG2D-activating receptor significantly increased on CD8^+ T cells (P = 0.0073; Fig. 1C and Supplementary Fig. S5). It remains unclear if these NKG2D^+ CD8^+ T cells are alloreactive-specific or tumor antigen–specific T cells, but both cell types are widely accepted as key immune populations responsible for tumor clearance (22, 23).

### Table 2. Treatment schedule and response to NK-cell therapy

<table>
<thead>
<tr>
<th>Step</th>
<th>NK-cell infusion</th>
<th>Cohort (dose level)</th>
<th>NK-cell dose (x10^6/kg)</th>
<th>Case number</th>
<th>Best overall response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single</td>
<td>C1-01</td>
<td>1</td>
<td>C1-01</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1-02</td>
<td></td>
<td>C1-02</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1-03</td>
<td></td>
<td>C1-03</td>
<td>PD</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>C2-01</td>
<td>10</td>
<td>C2-01</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2-02</td>
<td></td>
<td>C2-02</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2-03</td>
<td></td>
<td>C2-03</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>C3-01</td>
<td>1</td>
<td>C3-01</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C3-02</td>
<td></td>
<td>C3-02</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C3-03</td>
<td></td>
<td>C3-03</td>
<td>SD</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>C4-01</td>
<td>3</td>
<td>C4-01</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4-02</td>
<td></td>
<td>C4-02</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4-03</td>
<td></td>
<td>C4-03</td>
<td>SD</td>
</tr>
<tr>
<td>5</td>
<td>Triple, once weekly</td>
<td>C5-01</td>
<td>10</td>
<td>C5-01</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5-02</td>
<td></td>
<td>C5-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5-03</td>
<td></td>
<td>C5-03</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5-04</td>
<td></td>
<td>C5-04</td>
<td>PD</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>C6-01</td>
<td>30</td>
<td>C6-01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C6-02</td>
<td></td>
<td>C6-02</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C6-03</td>
<td></td>
<td>C6-03</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C6-04</td>
<td></td>
<td>C6-04</td>
<td>PD</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not assessable.

### Table 3. Phenotypic analyses of mismatched KIR expression on donor NK cells

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Donor</th>
<th>2DL/3</th>
<th>2DL1</th>
<th>3DL1</th>
<th>Recipient genotype</th>
<th>Mismatched KIR (%)</th>
<th>Outcome</th>
<th>PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2-01</td>
<td>MG4101030</td>
<td>16.2</td>
<td>14.3</td>
<td>77.5</td>
<td>C1</td>
<td>14.1</td>
<td>SD</td>
<td>4</td>
</tr>
<tr>
<td>C2-02</td>
<td>MG4101035</td>
<td>72.5</td>
<td>24.9</td>
<td>5.6</td>
<td>C1</td>
<td>24.9</td>
<td>SD</td>
<td>3</td>
</tr>
<tr>
<td>C2-03</td>
<td>MG4101036</td>
<td>15.4</td>
<td>62.7</td>
<td>3.6</td>
<td>C1 C2</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>C5-01</td>
<td>MG4101040</td>
<td>18.3</td>
<td>45.9</td>
<td>14.5</td>
<td>C1</td>
<td>60.4</td>
<td>SD</td>
<td>16</td>
</tr>
<tr>
<td>C5-03</td>
<td>MG4101047</td>
<td>19.2</td>
<td>28.0</td>
<td>11.3</td>
<td>C1 C2</td>
<td>11.3</td>
<td>PD</td>
<td>0</td>
</tr>
<tr>
<td>C5-04</td>
<td>MG4101066</td>
<td>23.6</td>
<td>35.8</td>
<td>12.1</td>
<td>C1</td>
<td>35.8</td>
<td>PD</td>
<td>0</td>
</tr>
<tr>
<td>C6-02</td>
<td>MG4101044</td>
<td>8.4</td>
<td>10.3</td>
<td>8.5</td>
<td>C1</td>
<td>18.7</td>
<td>PD</td>
<td>0</td>
</tr>
<tr>
<td>C6-03</td>
<td>MG4101049</td>
<td>39.6</td>
<td>51.4</td>
<td>10.7</td>
<td>C1</td>
<td>51.4</td>
<td>SD</td>
<td>18</td>
</tr>
<tr>
<td>C6-04</td>
<td>MG4101053</td>
<td>46.0</td>
<td>38.7</td>
<td>16.9</td>
<td>C1 C2</td>
<td>16.9</td>
<td>PD</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Gray boxes indicate percentage of donor-versus-recipient mismatched KIRs from each of three KIR ligands.

Abbreviation: NA, not assessable.

*Mismatched KIR (%) indicates the total percentage of NK cells with mismatched KIRs.
Evidence of their important role in bridging innate and adaptive immunity, NK cells are potent sources of cytokines (24). To determine whether cytokine expression is altered in our trials, serum cytokine concentrations were analyzed. Whereas no marked differences were observed in production of cytokines important for Th1 or Th2 induction (data not shown), a significant reduction in TGF\(_{\beta}1\) was observed in cohort 6 (\(P = 0.0115\); Fig. 2C). Based on the negative effect of TGF\(_{\beta}1\) on the activation of effector cells (25), a reduction of TGF\(_{\beta}1\) might lead to NKG2D induction on CD8\(^+\) T cells. Thus, further analysis was performed to identify any changes in Treg and MDSC populations (Figs. 2 and 3), which are a main source of TGF\(_{\beta}1\) (25, 26). We found that Treg (\(P = 0.0653\); Fig. 2B) and MDSC populations (\(P = 0.00167\); Fig. 3C and D) decreased in cohort 6 after injection of MG4101, and the percentage of MDSCs in cancer patients before MG4101 treatment was also significantly higher than that of healthy controls (\(P = 0.0006\); Fig. 3B). Overall, our observations that injection of MG4101 was followed by reduced Treg and MDSC populations, a decrease in serum TGF\(_{\beta}1\), and induction of NKG2D on CD8\(^+\) T cells suggest that MG4101 treatment could be an effective therapy against cancer.

In addition to activation, the proper recruitment of effector cells to sites of inflammation is critical (27). For example, activated NK cells upregulate T-cell–recruiting chemokines (28). Thus, we assessed chemokine concentrations after MG4101 treatments specifically in cohort 6. Serially obtained sera were analyzed at all cycles of treatment for the presence of the chemokines MIG, MCP-1, MIP-1\(_a\), MIP-1\(_b\), RANTES, G-CSF, and IL8. G-CSF and IL8 concentrations did not significantly change (data not shown). In contrast, MIG, MCP-1, MIP-1\(_b\), and RANTES increased after MG4101 injections in all cohort 6 patients (Fig. 4). In the case of MIP-1\(_a\), increased cytokines coinciding with MG4101 injections were observed in only two cohort 6 patients (Fig. 4).

**Discussion**

This phase I trial using random healthy donor–derived allogeneic NK cells (MG4101) allows us to draw some clinical insights...
for patients with malignant lymphoma or advanced solid tumors. The underlying hypothesis for this investigation was that, given the previous successful treatment of leukemia by alloreactive haploidentical KIR ligand–mismatched NK cells, administration of MG4101 would be safe and exhibit enhanced clinical benefit over other therapies (5–8). This was because MG4101 had previously been shown to secrete effector cytokines and exhibit cytolytic activity against various cancer cell lines, but not against nontumor cells (12).

Because MG4101 consists of allogeneic NK cells derived from completely random donors, using it for treatment could result in GVHD; however, we found that engrafted MG4101 was tolerable at all doses in all cohorts. Our results clearly show that the repetitive administration of 3 × 10⁷ cells/kg/dose up to 6 × 10⁹ cells in total was safe without any sign of GVHD or serious adverse event. The lack of evidence of severe GVHD implied that MG4101 cells did not outgrow nor did they induce any nonspecific cytotoxicity against host tissues. Nested PCR analysis showed that MG4101 persisted for up to 4 days after a single injection and for several hours to 3 days after repeated injections. Several mechanisms can affect the persistency of administrated MG4101, including host allo-specific immune responses (29). Although we did not find a statistically significant correlation between antibody induction and the number of administrations, we observed more patient antibodies specific for donor NK cells after repeated injections than after a single injection (data not shown). For safety reasons, our protocols did not include immunosuppressive regimens that would have prevented outgrowth of NK cells derived from random unrelated donors. However, in vivo persistence of NK cells can be enhanced through several approaches. Miller and colleagues treated acute myelogenous leukemia patients with IL2-activated CD3-depleted haploidentical NK cells after cyclophosphamide plus fludarabine–induced immunosuppression and observed significant in vivo expansion and persistency of

Figure 2. Analysis of Tregs and TGFβ1 levels from patient peripheral blood. A, the percentage of Foxp3⁺CD127dim¹ Tregs was analyzed by flow cytometry after lymphogating on CD25⁺⁴CD4⁺CD3⁺ T cells. Gating strategy and representative FACS dot plots are presented. B, the percentage of Tregs from cohorts 5 and 6 was compared before and after MG4101 injections (n = 7). C, the blood concentrations of plasma TGFβ1 from cohort 6 were analyzed before (D0H0) and after (D35) MG4101 injections (n = 4). The concentration of soluble TGFβ1 was determined by cytometric bead-based assay.
considering immunosuppressive regimens to enhance clinical outcomes. For the next phase II study, we are currently evaluating a different immunosuppressive regimen, and the expansion and persistence of NK cells further increased (30). For the next phase II study, we are currently considering immunosuppressive regimens to enhance clinical benefit.

The other mechanism of action affecting the persistence of NK cells is massive infiltration into the tumor site. Administered donor-derived NK cells accumulate at the tumor site even though they are not detected in peripheral blood of recipients (31, 32). Several studies have shown a good inverse correlation between the number of tumor-infiltrating NK cells and progression of cancer (33). The infiltration of NK cells into tumors has been reported to be regulated by chemokine receptors, including CCR2, CCR5, CXCR3, and CX3CR1 (34–37). Although our small number of patients did not allow a statistically significant correlation, we found that CXCR3 expression on MG4101 tended to increase after large-scale expansion (12). CXCR3 binds chemokines CXCL9, CXCL10, and CXCL11, and expression of these chemokines is induced by IFNγ in a variety of cell types (38–40). We suggest that early IFNγ produced by MG4101 might enhance the expression of CXCL9–11 in the tumor mass, resulting in more active infiltration of MG4101 into the tumor. Thus, further studies to detect infused MG4101 not only in blood but also at the tumor site will help to define the kinetics of MG4101.

Although this phase I trial included a relatively small number of patients, our results suggest that MG4101-based immunotherapy is of potential benefit for cancer patients. Patients who received repeated injections of higher doses of MG4101 seemed to have better outcomes than patients injected one time with a lower dose, although this finding was not statistically significant. The mechanism explaining the clinical benefit seen in SD patients from cohorts 5 and 6 remains undefined; however, this may be due to either enhanced innate immunity or the enhancement of the T-cell–mediated adaptive immune response. After repeated injection of MG4101, we observed an increase in host CD8+ T cells expressing the NK2G2 activation marker. We have not characterized the nature of NK2G2+ CD8+ T cells, but we hypothesize that these cells may be alloreactive T cells, based on their rapid increase after MG4101 injection (data not shown).

Indeed, it has been shown previously that the antitumor immune response is induced during allogeneic transplantation, and alloreactive T cells were suggested to be a key contributor to the antitumor response (23). However, our results cannot rule out the possibility that induced NK2G2+ CD8+ T cells are tumor antigen–specific T cells, in which a mouse tumor model was triggered during NK-cell–mediated clearance of target cells (22). Further analysis using staining with tetramers of MHC class I loaded with tumor epitope peptides will be required to define the change in the NK2G2+ CD8+ T-cell response following MG4101 treatment.

Many studies have shown that increased frequencies of Tregs and MDSCs directly correlate with cancer progression (26, 41). Specifically, Tregs and MDSCs can inhibit NK and CD8+ T-cell activation through TGFβ, a negative immune regulator (41). In the current study, we found that treatment with MG4101 reduced Treg and MDSC populations as well as TGFβ1 secretion. The effects of NK cells against MDSCs have been studied in the context of regulation by chemokine receptors, including CCR2, CCR5, CXCR3, and CX3CR1 (34–37).

Figure 3. MDSC population in patient peripheral blood. A, the percentage of CD11b+ CD15– MDSCs was analyzed by flow cytometry with lymphogating on Lin− CD14+ HLA-DR+ cells. Gating strategy and representative FACS dot plots of patients and healthy controls are presented. B, the percentages of MDSCs were compared between healthy controls and patients before NK-cell therapy (n = 5). C, the percentages of MDSCs from cohort 6 (n = 5) were compared before (D0H0) and after (D35) MG4101 injections (n = 3). D, changes in MDSC populations were monitored for 5 weeks.

\[ D = \frac{\text{MDSC} \text{percentage before injection} - \text{MDSC} \text{percentage after injection}}{\text{MDSC} \text{percentage before injection}} \times 100 \]

Published OnlineFirst January 19, 2016; DOI: 10.1158/2326-6066.CIR-15-0118

www.aacrjournals.org Cancer Immunol Res; 4(3) March 2016 221
Figure 4. Analysis of plasma chemokines in patient peripheral blood. The blood concentrations of MCP-1, MIG, MIP-1α, MIP-1β, and RANTES from cohort 6 were monitored for 5 weeks after MG4101 injection (n = 4). The concentration of each chemokine was determined by cytometric bead-based assay.
of cancer by Sato and colleagues (42), who found that the frequency of MDSCs in non-Hodgkin lymphoma patients was increased and inversely correlated with that of NK cells, not that of T cells (42). Other evidence that activated NK cells can lyse the MDSCs has been published by Gleason and colleagues (43). Even though NK cells and their expression of FcγRII (CD16) are decreased in myelodysplastic syndromes (MDS) and inversely correlate with a substantial increase in MDSCs, the enhancement of CD16 signaling potently activates NK cells to lyse CD33− MDS and MDSC targets (43). Although we suggest a novel role for ex vivo–activated NK cells in overcoming the negative function of immune suppressor cells, identification of the underlying mechanism will require further study. To this end, additional analysis of interactions between NK and Treg or MDSC cells in a cancer model will not only provide valuable information, but may also improve efficacy in anticancre immunotherapy.

Several studies have examined the role of chemokines secreted from NK cells in experimental tumor models (24). In one report, significant induction of MIG, IP-10, RANTES, MCP-1, and IL8 from NK cells following IL2 administration suggested a role for NK cells in the initiation of the chemokine response (28). However, less is known about the chemokine response after the direct administration of NK cells. In our system, we found elevated MIG, MCP-1, MIP-1B, and RANTES concentrations, suggesting that activated NK cells secrete a broad array of T cell–attracting chemokines. These chemokines act together to recruit tumor-infiltrating T cells, resulting in a decreased incidence of recurrence and increased overall survival in cancer patients (28, 44–46). Moreover, our finding of secretion of T cell–attracting chemokines following administration of activated NK cells suggests an additional mechanism through which T cell infiltration to the tumor sites could be achieved.

In conclusion, the safety data for transplantation of MG4101 derived from unrelated random healthy donors will give increased opportunities to select donors that have either maximum KIR incompatibility against recipients or a potent KIR B haplotype. To enhance clinical benefit, we are currently considering a phase II study including immunosuppressive chemotherapy followed by MG4101 treatment, based on previous successful results involving lymphodepletive preparative regimens (9).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T.M. Kim, B. Keam, Y.K. Hwang, D.S. Heo
Development of methodology: O. Lim, T.M. Kim, H. Choi, H. Chung, B. Min, D.S. Heo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yang, O. Lim, T.M. Kim, Y.-O. Ahn, H. Chung, B. Min, J.H. Her, B. Keam, S.-H. Lee, D.-W. Kim, D.S. Heo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Yang, O. Lim, T.M. Kim, H. Choi, B. Keam, D.S. Heo
Writing, review, and/or revision of the manuscript: Y. Yang, O. Lim, T.M. Kim, H. Choi, S.Y. Cho, B. Keam, D.S. Heo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yang, H. Choi, S.Y. Cho, B. Keam
Study supervision: D.S. Heo

Grant Support
This study was supported by grants from Green Cross Corporation, MOGAM Biotechnology Institute, and the Innovative Research Institute for Cell Therapy, Republic of Korea (A062260). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 29, 2015; revised September 11, 2015; accepted December 2, 2015; published OnlineFirst January 19, 2016.

References

32. Bruno A, Felizario G, Albini A, Noonan DM. A think tank of TINK/TANKs:


26. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of

25. Wright GP, Ehrenstein MR, Stauss HJ. Regulatory T-cell adoptive immu-

24. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural

23. Barber LD, Madrigal JA. Exploiting bene


20. Bein G, Glaser R, Kirchner H. Rapid HLA-DRB1 genotyping by nested PCR


16. Gabrilovich DI, Nagata S. Myeloid-derived suppressor cells as regulators of


Human natural killer cells: a unique innate immunoregulatory role for the


Natural killer cells produce T cell-recruiting chemokines in response to


Infusion of haplo-identical killer immunoglobulin-like receptor ligand


of NK cells and their receptors affects clinical outcomes after hematopoietic


tumor-infiltrating/tumor-associated natural killer cells in tumor progres-


8. Morrison BE, Park SJ, Mooney JM, Mehdad B. Chemokine-mediated

recruitment of NK cells is a critical host defense mechanism in invasive


is essential for NK cell trafficking and host survival following Toxoplasma


neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that

modify experimental autoimmune encephalomyelitis within the central


ELR CXC chemokine with potent activity on activated T cells through


2. Luster AD, Ravetch JV. Biochemical characterization of a gamma interfer-


1. Pedroza-Pacheco I, Madrigal A, Saudemont A. Interaction between natural

killer cells and regulatory T cells: perspectives for immunotherapy. Cell Mol


Gleason MK, Ross JA, Warlick ED, Lund TG, Verneris MR, Wiemink A, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against


Fehniger TA, Herbein G, Yu H, Para MI, Bernstein ZP, O’Brien WA, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1


Arase N, Arase H, Hirano S, Yokosuka T, Sakurai D, Saito T. IgE-

mediated killing of target cells triggers robust antigen-specific T-cell-medi-


Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural


Wright GP, Ehrenstein MR, Stauss HJ. Regulatory T-cell adoptive immu-


Giudicelli J, Nagata S. Myeloid-derived suppressor cells as regulators of


Human natural killer cells: a unique innate immunoregulatory role for the


Natural killer cells produce T cell-recruiting chemokines in response to


Infusion of haplo-identical killer immunoglobulin-like receptor ligand


Foley B, Felices M, Cichocki F, Cooley S, Verneris MR, Miller JS. The biology

of NK cells and their receptors affects clinical outcomes after hematopoietic


Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U, et al. NK cells and the tumour microenvironment: implications for


Phase I Study of Random Healthy Donor–Derived Allogeneic Natural Killer Cell Therapy in Patients with Malignant Lymphoma or Advanced Solid Tumors

Yaewon Yang, Okjae Lim, Tae Min Kim, et al.


**Updated version**  
Access the most recent version of this article at:  
doi:10.1158/2326-6066.CIR-15-0118

**Supplementary Material**  
Access the most recent supplemental material at:  
http://cancerimmunolres.aacrjournals.org/content/suppl/2016/01/19/2326-6066.CIR-15-0118.DC1

**Cited articles**  
This article cites 46 articles, 20 of which you can access for free at:  
http://cancerimmunolres.aacrjournals.org/content/4/3/215.full#ref-list-1

**Citing articles**  
This article has been cited by 5 HighWire-hosted articles. Access the articles at:  
http://cancerimmunolres.aacrjournals.org/content/4/3/215.full#related-urls

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, use this link  
http://cancerimmunolres.aacrjournals.org/content/4/3/215.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.