

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

Yaewon Yang^{1,2}, Okjae Lim³, Tae Min Kim^{1,2}, Yong-Oon Ahn², Hana Choi³, Hyejin Chung³, Bokyung Min³, Jung Hyun Her³, Sung Yoo Cho³, Bhumsuk Keam^{1,2}, Se-Hoon Lee^{1,2}, Dong-Wan Kim^{1,2}, Yu Kyeong Hwang³, and Dae Seog Heo^{1,2}

Abstract

Natural killer (NK) cells with mismatched killer cell immunoglobulin-like receptor–ligand pairs have shown efficacy and been proven safe in treatment of cancer patients. *Ex vivo*-expanded and highly activated NK cells (MG4101) had been generated under good manufacturing practice conditions, which demonstrated potent anticancer activity *in vitro* and *in vivo* in preclinical studies. The current phase I clinical trial was designed to evaluate safety and possible clinical efficacy of repetitive administrations of MG4101 derived from random unrelated healthy donors into patients with malignant lymphoma or advanced, recurrent solid tumors. The maximum dose (3×10^7 cells/kg, triple infusion) was tolerable without

significant adverse events. Of 17 evaluable patients, 8 patients (47.1%) showed stable disease and 9 (52.9%) showed progressive disease. We also evaluated the capacity of MG4101 to influence host immune responses. Administration of MG4101 augmented NKG2D expression on CD8⁺ T cells and upregulated chemokines that recruit T cells. In contrast, administration of MG4101 reduced regulatory T cells and myeloid-derived suppressor cells and suppressed TGFβ production. In conclusion, administration of a large number of MG4101 cells was not only safe and feasible, but also exhibited efficacy in maintaining the effector arm of the host immune response. *Cancer Immunol Res*; 4(3); 215–24. ©2016 AACR.

Introduction

One of the innovative therapies developed against cancers refractory to current therapies is treatment involving immune cells. 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–recipient 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 vivo* 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*

¹Department of Internal Medicine, Seoul National University Hospital, Seoul, Korea. ²Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea. ³Cell Therapy Team, MOGAM Biotechnology Institute, Yongin, Gyeonggi-do, Korea.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

Y. Yang and O. Lim contributed equally to this article.

Current address for H. Choi and Yu Kyeong Hwang: Cell Therapy Research Center, Green Cross LabCell, Yongin, Gyeonggi-do, Korea.

Corresponding Authors: Tae Min Kim, Seoul National University Hospital, 28 Yeongeong-Dong, Jongno-Gu, Seoul 110-744, Korea. Phone: 82-2-2072-3559; Fax: 82-2-764-2199; E-mail: gabriel9@snu.ac.kr; and Yu Kyeong Hwang, Cell Therapy Team, MOGAM Biotechnology Institute, 341 Bojeong-dong, Giheung-gu, Yongin, Gyeonggi-do, 446-799, Korea. Phone: 82-31-260-9853; Fax: 82-31-260-9808; E-mail: ykhwang@mogam.re.kr

doi: 10.1158/2326-6066.CIR-15-0118

©2016 American Association for Cancer Research.

vivo-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 I 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; Orthocon), 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 (HIB19; 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 Supplementary 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⁺CD25^{bright}Foxp3⁺CD127^{dim} 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).

Table 1. Toxicity profiles

Toxicities	Number of patients (percentage) \leq grade 2					
	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5	Cohort 6
General disorders and administration site conditions	1 (33.3%)	1 (33.3%)	-	-	1 (25%)	3 (75%)
Asthenia	-	-	-	-	-	3 (75%)
Fatigue	1 (33.3%)	-	-	-	1 (25%)	1 (25%)
Chills	-	1 (33.3%) ^a	-	-	-	1 (25%)
Infections and infestations	-	-	-	-	-	3 (75%)
Sweating/fever	-	-	-	-	-	2 (50%)
Febrile infection	-	-	-	-	-	1 (25%)
Nervous system disorders	-	-	-	-	-	2 (50%)
Headache	-	-	-	-	-	2 (50%)
Musculoskeletal and connective tissue disorder	-	-	-	-	-	2 (50%)
Myalgia	-	-	-	-	-	1 (25%)

^aOnly this patient was assessed as having grade 2 toxicity; the others had grade 1 toxicity.

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 \pm 1.98%) NK cells with minimal contamination of CD3⁺ T cells (0.41 \pm 0.43%), CD14⁺ monocytes (0.40 \pm 0.37%), and CD19⁺ B cells (0.15 \pm 0.25%; Supplementary Fig. S2A). During the culture, NK cells were expanded 757.5 \pm 232.2-fold (Supplementary Fig. S2C) with 92.9 \pm 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 3×10^7 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 1×10^7 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 2. Treatment schedule and response to NK-cell therapy

Step	NK-cell infusion	Cohort (dose level)		NK-cell dose ($\times 10^6$ /kg)	Case number	Best overall response
		1	2			
Step 1	Single	1		1	C1-01	SD
					C1-02	PD
					C1-03	PD
		2		10	C2-01	SD
					C2-02	SD
					C2-03	NA
		3		1	C3-01	SD
					C3-02	PD
					C3-03	SD
4		3	C4-01	PD		
			C4-02	PD		
			C4-03	SD		
Step 2	Triple, once weekly	5		10	C5-01	SD
					C5-02	NA
					C5-03	PD
		6		30	C5-04	PD
					C6-01	NA
					C6-02	PD
		C6-03	SD			
		C6-04	PD			

Abbreviation: NA, not assessable.

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 1×10^6 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 3. Phenotypic analyses of mismatched KIR expression on donor NK cells

Cohort	Donor	Phenotype (%)			Recipient genotype	Mismatched KIR (%) ^a	Outcome	PFS	
		2DL2/3	2DL1	3DL1					
C2-01	MG41011030	16.2	14.1	77.5	C1	Bw4	14.1	SD	4
C2-02	MG41011035	72.5	24.9	5.6	C1	Bw4	24.9	SD	3
C2-03	MG41011036	15.4	62.7	3.6	C1	C2	Bw4	NA	-
C5-01	MG41011040	18.3	45.9	14.5	C1		60.4	SD	16
C5-03	MG41011047	19.2	28.0	11.3	C1	C2	11.3	PD	0
C5-04	MG41011066	23.6	35.8	12.1	C1	Bw4	35.8	PD	0
C6-02	MG41011044	8.4	10.3	8.5	C1		18.7	PD	0
C6-03	MG41011049	39.6	51.4	10.7	C1	Bw4	51.4	SD	18
C6-04	MG41011053	46.0	38.7	16.9	C1	C2	16.9	PD	0

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

Abbreviation: NA, not assessable.

^aMismatched KIR (%) indicates the total percentage of NK cells with mismatched KIRs.

Downloaded from <http://aacrjournals.org/cancerimmunolres/article-pdf/4/3/215/2350089/215.pdf> by guest on 07 October 2024

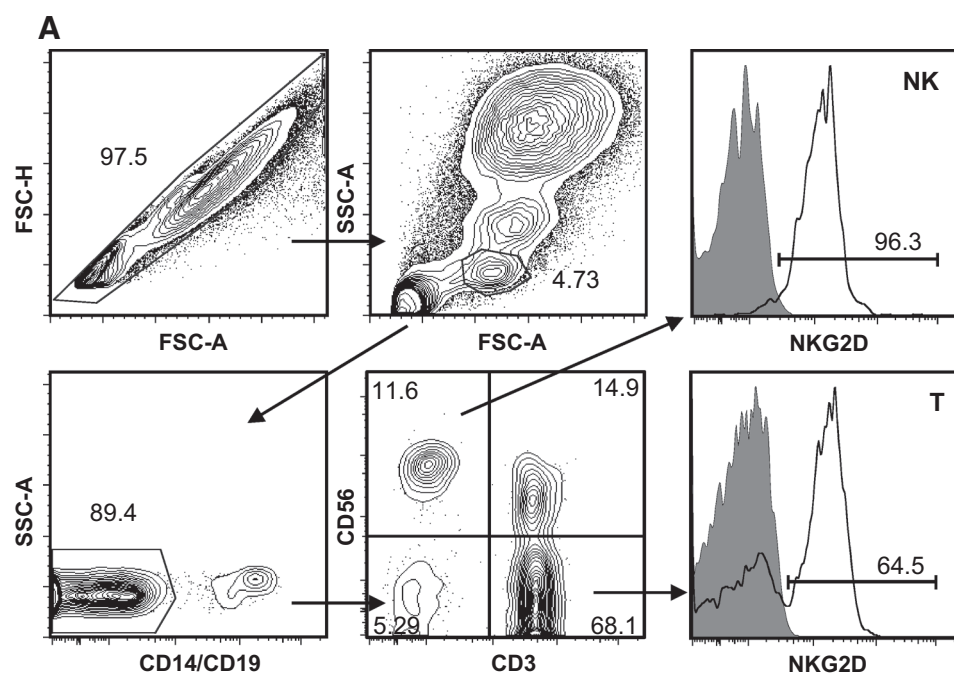
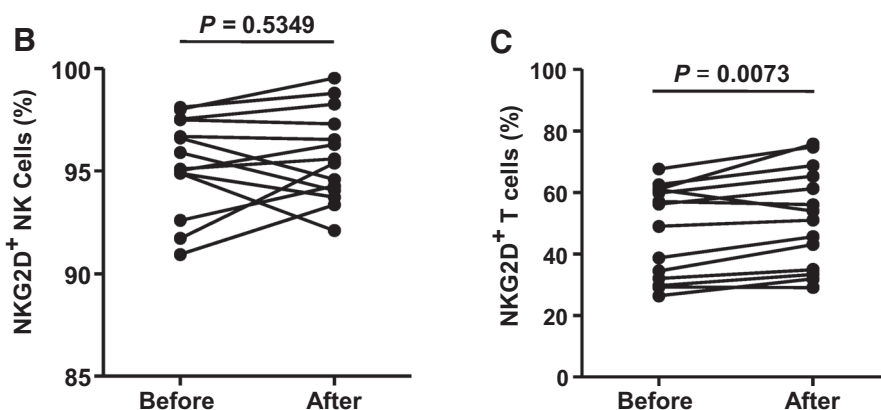


Figure 1. NKG2D expression on NK and T cells in patient peripheral blood. The percentage of NKG2D⁺ NK cells (B) or T cells (C) was analyzed by flow cytometry through lymphogating of patient PBMCs. Gating strategy and representative FACS dot plots are presented (A). The percentage of NKG2D⁺ NK cells (B) or T cells (C) was compared before and after allogeneic NK-cell therapy (day 28 at step 1 or day 35 at step 2, respectively; $n = 18$).



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 β 1 was observed in cohort 6 ($P = 0.0115$; Fig. 2C). Based on the negative effect of TGF β 1 on the activation of effector cells (25), a reduction of TGF β 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 β 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 MG401 was followed by reduced Treg and MDSC populations, a decrease in serum TGF β 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 α , MIP-1 β , RANTES, G-CSF, and IL8. G-CSF and IL8 concentrations did not significantly change (data not shown). In contrast, MIG, MCP-1, MIP-1 β , and RANTES increased after MG4101 injections in all cohort 6 patients (Fig. 4). In the case of MIP-1 α , 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

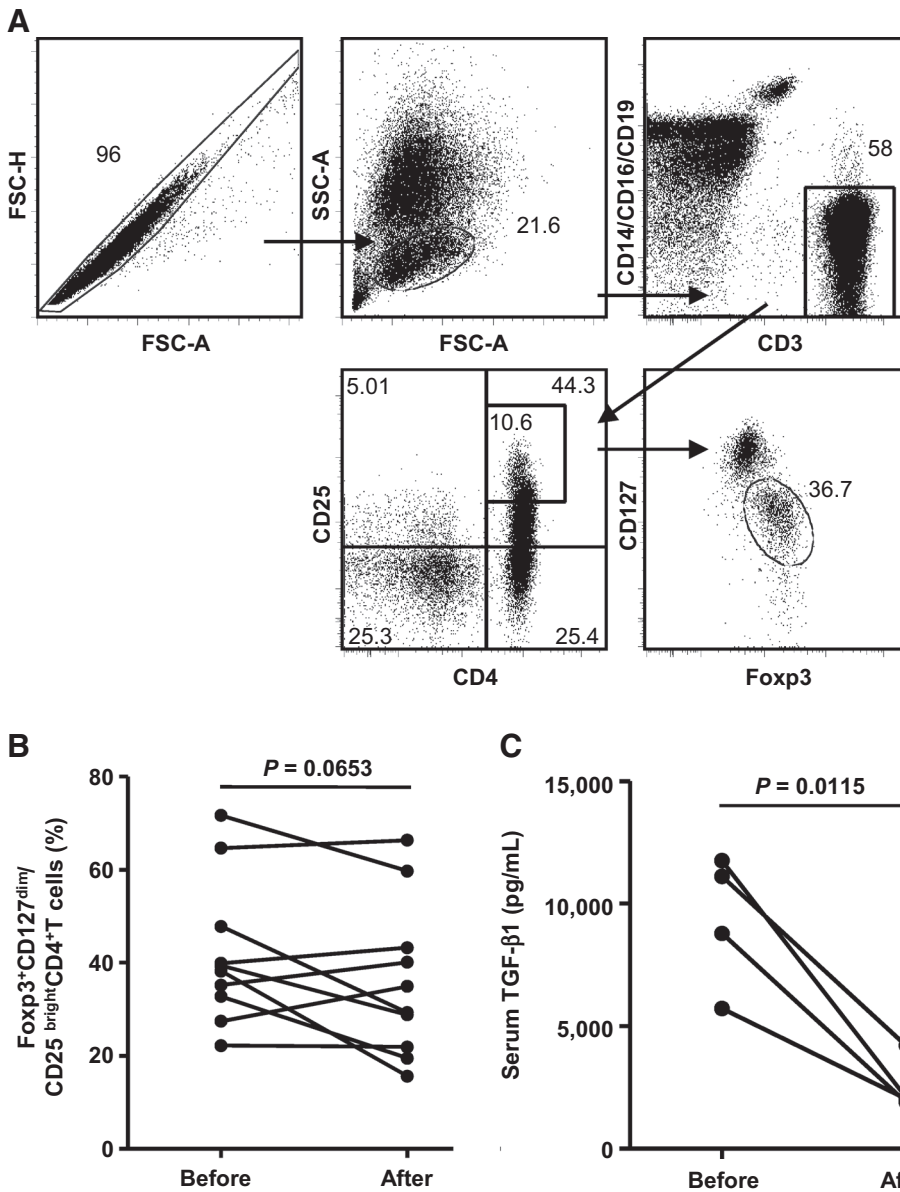
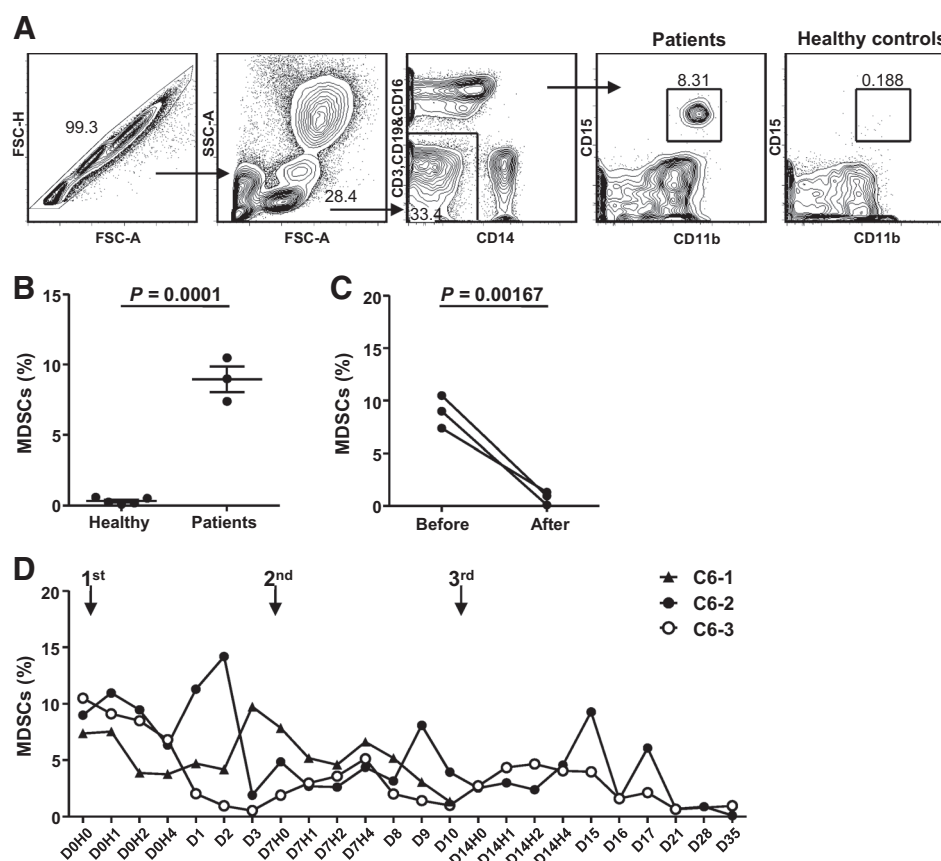


Figure 2. Analysis of Tregs and TGFβ1 levels from patient peripheral blood. A, the percentage of Fxp3⁺CD127^{dim} Tregs was analyzed by flow cytometry after lymphogating on CD25^{bright}CD4⁺CD3⁺ T cells. Gating strategy and representative FACS dot plots are presented. B, the percentage of Tregs from 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.

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 3×10^7 cells/kg/dose up to 6×10^9 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 nonspe-

cific 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 administered 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



donor-derived NK cells (11). In the second study by this group, total-body irradiation was added to the 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 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 IFN γ in a variety of cell types (38–40). We suggest that early IFN γ 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 NKG2D activation marker. We have not characterized the nature of NKG2D⁺ 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 NKG2D⁺ 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 NKG2D⁺ 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 TGF β , a negative immune regulator (41). In the current study, we found that treatment with MG4101 reduced Treg and MDSC populations as well as TGF β 1 secretion. The effects of NK cells against MDSCs have been studied in the context

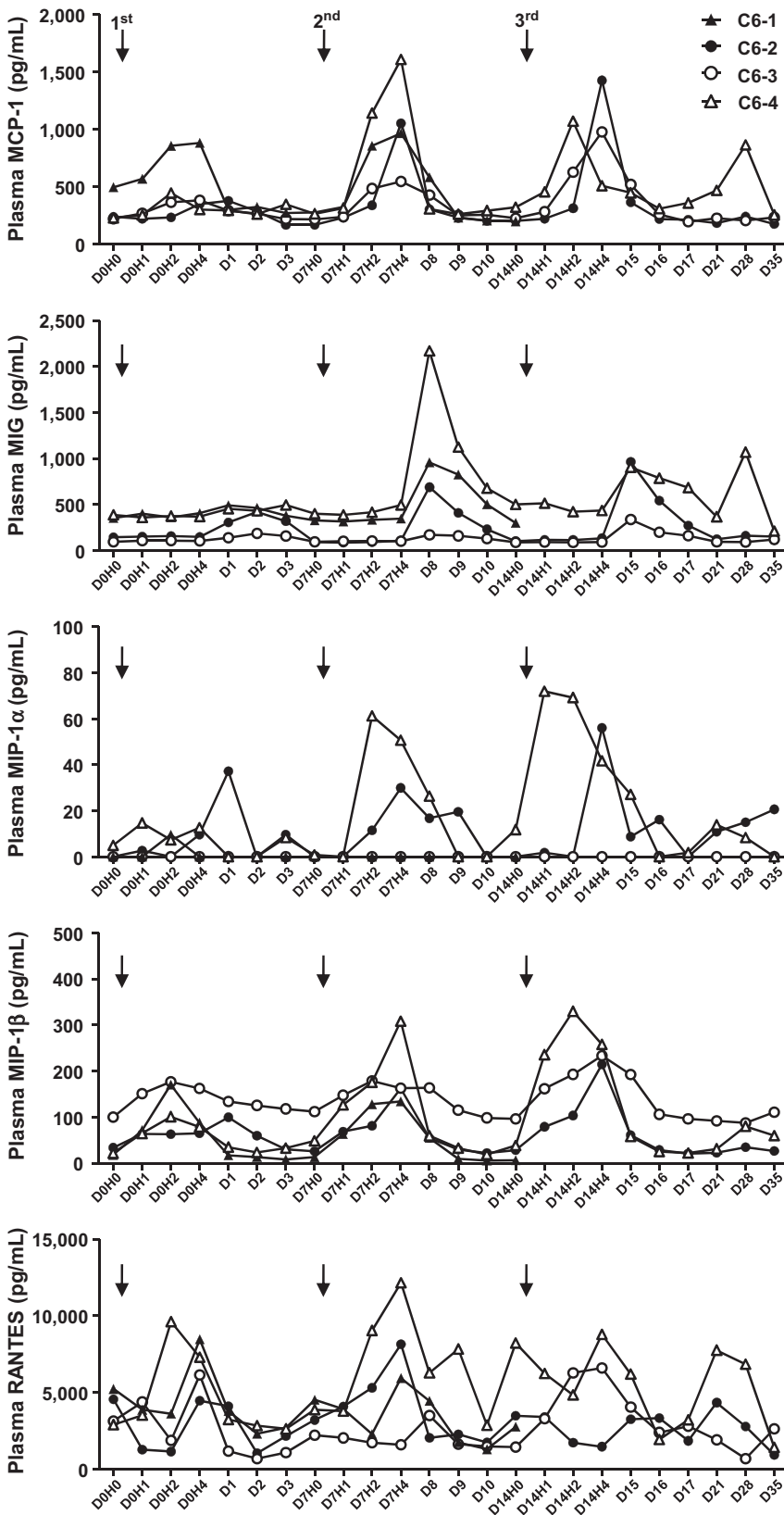


Figure 4. Analysis of plasma chemokines in patient peripheral blood. The blood concentrations of MCP-1, MIG, MIP-1 α , MIP-1 β , and RANTES from cohort 6 were monitored for 5 weeks after MG4101 injection ($n = 4$). The concentration of each chemokine was determined by cytometric bead-based assay.

Downloaded from <http://aacrjournals.org/cancerimmunolres/article-pdf/4/3/215/2350083/215.pdf> by guest on 07 October 2024

of cancer by Sato and colleagues (42), who found that the frequency of MDSC 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 FcR γ III (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 anticancer 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 IL12 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-1 β , 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 lymphodepleting 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

- Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. *Blood* 1990;76:2421–38.
- Farag SS, Fehniger T, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cells: biology and application in stem-cell transplantation. *Cytotherapy* 2002;4:445–6.
- Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999;94:333–9.
- Leung W. Infusions of allogeneic natural killer cells as cancer therapy. *Clin Cancer Res* 2014;20:3390–400.
- Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood* 2003;102:814–9.
- Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. *Blood* 2011;118:3273–9.
- Iliopoulou EG, Kountourakis P, Karamouzis MV, Doufexis D, Ardavanis A, Baxevanis CN, et al. A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer. *Cancer Immunol Immunother* 2010;59:1781–9.
- Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 2007;110:433–40.
- Geller MA, Miller JS. Use of allogeneic NK cells for cancer immunotherapy. *Immunotherapy* 2011;3:1445–59.
- Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* 2009;182:4572–80.
- Miller JS, Soignier Y, Panoskaltis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005;105:3051–7.
- Lim O, Lee Y, Chung H, Her JH, Kang SM, Jung MY, et al. GMP-compliant, large-scale expanded allogeneic natural killer cells have potent cytolytic activity against cancer cells in vitro and in vivo. *PLoS One* 2013;8:e53611.
- Grieco A, Long CJ. Investigation of the Karnofsky Performance Status as a measure of quality of life. *Health Psychol* 1984;3:129–42.
- Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982;5:649–55.
- Trotti A, Colevas AD, Setzer A, Rusch V, Jaques D, Budach V, et al. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin Radiat Oncol* 2003;13:176–81.
- Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009;45:228–47.
- Cheson BD, Pfisterer B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. *J Clin Oncol* 2007;25:579–86.

18. Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 2009;58:652–62.
19. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res* 2005;65:3044–8.
20. Bein G, Glaser R, Kirchner H. Rapid HLA-DRB1 genotyping by nested PCR amplification. *Tissue Antigens* 1992;39:68–73.
21. Karimi MA, Bryson JL, Richman LP, Fesnak AD, Leichner TM, Satake A, et al. NKG2D expression by CD8+ T cells contributes to GVHD and GVT effects in a murine model of allogeneic HSCT. *Blood* 2015;125:3655–63.
22. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, et al. NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. *Blood* 2009;113:6593–602.
23. Barber LD, Madrigal JA. Exploiting beneficial alloreactive T cells. *Vox Sang* 2006;91:20–7.
24. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001;22:633–40.
25. Wright GP, Ehrenstein MR, Stauss HJ. Regulatory T-cell adoptive immunotherapy: potential for treatment of autoimmunity. *Expert Rev Clin Immunol* 2011;7:213–25.
26. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162–74.
27. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaehri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 2001;97:3146–51.
28. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson WE 3rd. Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. *Cancer Res* 2006;66:517–26.
29. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br J Haematol* 2008;143:641–53.
30. 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 cell transplantation (HCT). *Immunol Rev* 2014;258:45–63.
31. Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U, et al. NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. *Trends Immunol* 2003;24:603–9.
32. Nannmark U, Hokland ME, Agger R, Christiansen M, Kjaergaard J, Goldfarb RH, et al. Tumor blood supply and tumor localization by adoptively transferred IL-2 activated natural killer cells. *In Vivo* 2000;14:651–8.
33. Bruno A, Ferlazzo G, Albini A, Noonan DM. A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis. *J Natl Cancer Inst* 2014;106:dju200.
34. Morrison BE, Park SJ, Mooney JM, Mehrad B. Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. *J Clin Invest* 2003;112:1862–70.
35. Khan IA, Thomas SY, Moretto MM, Lee FS, Islam SA, Combe C, et al. CCR5 is essential for NK cell trafficking and host survival following *Toxoplasma gondii* infection. *PLoS Pathog* 2006;2:e49.
36. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 2004;5:1260–5.
37. Huang D, Shi FD, Jung S, Pien GC, Wang J, Salazar-Mather TP, et al. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB J* 2006;20:896–905.
38. Liu C, Luo D, Reynolds BA, Meher G, Katritzky AR, Lu B, et al. Chemokine receptor CXCR3 promotes growth of glioma. *Carcinogenesis* 2011;32:129–37.
39. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, et al. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* 1998;187:2009–21.
40. Luster AD, Ravetch JV. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 1987;166:1084–97.
41. Pedroza-Pacheco I, Madrigal A, Saudemont A. Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. *Cell Mol Immunol* 2013;10:222–9.
42. Sato Y, Shimizu K, Shinga J, Hidaka M, Kawano F, Kakimi K, et al. Characterization of the myeloid-derived suppressor cell subset regulated by NK cells in malignant lymphoma. *Oncoimmunology* 2015;4:e995541.
43. Gleason MK, Ross JA, Warlick ED, Lund TC, Verneris MR, Wiernik A, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. *Blood* 2014;123:3016–26.
44. Fehniger TA, Herbein G, Yu H, Para MI, Bernstein ZP, O'Brien WA, et al. Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection. *J Immunol* 1998;161:6433–8.
45. Arase N, Arase H, Hirano S, Yokosuka T, Sakurai D, Saito T. IgE-mediated activation of NK cells through Fc gamma RIII. *J Immunol* 2003;170:3054–8.
46. Carson WE, Parihar R, Lindemann MJ, Personeni N, Dierksheide J, Meropol NJ, et al. Interleukin-2 enhances the natural killer cell response to Herceptin-coated Her2/neu-positive breast cancer cells. *Eur J Immunol* 2001;31:3016–25.