Improvement of NK Cell Cytotoxicity in Reconstituting NK Cells after Allogeneic Stem Cell Transplantation by Blocking NKG2A Checkpoint

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Abstract

Background: One cause of tumor relapse after allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the alteration of the graft-versus-tumor effect of early reconstituting natural killer (NK) cells due to overexpression of the NKG2A inhibitory receptor. This study aims to determine the effect of Monalizumab, an anti-NKG2A receptor, on the effector functions of reconstituting NK cells after allo-HSCT.

Method: In this prospective cohort study, 18 patients with hematological malignancies were divided into three groups: dose 1 group (0.1 mg/kg, n = 5), dose 2 group (0.5 mg/kg, n = 8), and dose 3 group (1 mg/kg, n = 5), and followed up for six months. Blood samples were taken directly before the administration of Monalizumab and at different time points post-treatment. Reconstituting NK cells were phenotypically and functionally assessed by flow cytometry.

Results: Our results showed a more pronounced increase in the expression of activating NK receptors (NKG2D, NKp30, NKp46) on the reconstituting CD56dim NK cells of patients receiving 1 mg/kg of Monalizumab compared with other participants. Additionally, we observed that patients treated with dose 3 of Monalizumab had the highest levels of degranulation compared with other patients and controls. Moreover, we noticed that CD56dim NK cells of dose 2- and dose 3-related patients produced significant levels of perforin, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) in response to K562 stimulation post-Monalizumab treatment compared with controls and dose 1-treated patients.

Conclusion: We suggest that using Monalizumab improves the phenotype and cytotoxicity of reconstituting NK cells after allo-HSCT.

Keywords: Natural killer cells, Monalizumab, NKG2A, Cell cytotoxicity, Allogeneic hematopoietic stem cell transplantation

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a promising alternative to
chemotherapy for the treatment of hematological malignancies. However, it is associated with serious complications such as tumor relapse, infections, and graft-versus-host disease.\textsuperscript{1-3} The immune system plays a critical role in tumor surveillance, and the performance of the patient's immune system after transplantation is a key determinant in allo-HSCT outcomes. One type of cell that plays a role in this process is natural killer (NK) cells, which are lymphocytes of the innate immune system that can recognize tumors and virus-infected cells without prior sensitization.\textsuperscript{4,5} The function of NK cells is regulated by the expression of various inhibitory and activating receptors.\textsuperscript{4,5} Additionally, NK cells exhibit antitumor activity through direct cytotoxic function and by regulating other immune cells through cytokine secretion. It has been observed that NK cells are the first lymphocytes to reconstitute following transplantation, and their rapid recovery is associated with lower relapse rates and improved survival.\textsuperscript{6-8} However, early reconstituting NK cells are characterized by a high percentage of CD56\textsuperscript{bright} immature NK cells, higher expression of the inhibitory receptor NKG2A, and variable expression of activating receptors, resulting in impaired cytotoxic function.\textsuperscript{9-12}

NKG2A is an inhibitory receptor that belongs to the C-type lectin receptor family and recognizes non-classical HLA class I molecule, HLA-E.\textsuperscript{13,14} It is expressed in association with CD94 on nearly 50% of the circulating NK cells. Furthermore, many types of tumor cells can evade NK cell immunity by upregulating HLA-E expression and increasing the expression of NKG2A on the surface of NK cells.\textsuperscript{15,16} Therefore, blocking the NKG2A receptor with a monoclonal antibody could enhance the antitumor activity of reconstituted NK cells after hematopoietic stem cell transplantation (HSCT) and improve the cytotoxicity of NK cells in cancer patients.\textsuperscript{16,17}

Monalizumab (previously known as IPH2201) is a humanized monoclonal antibody that targets the NKG2A inhibitory receptor.\textsuperscript{18} Numerous studies have shown that the use of Monalizumab can restore and enhance the antitumor activity of NK cells in vitro and in humanized mouse

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**Figure 1.** Time frame of the study: Patients suffering from hematological malignancies received allo-HSCT after achieving complete remission (CR) through chemotherapy. Monalizumab was infused into patients using an escalation dose regimen (3 doses). Peripheral blood samples were collected from both patients and controls at different time points. Subsequently, phenotype and functional assays were performed on the reconstituting NK cells.

HLA: Human leukocyte antigen; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; NK: Natural killer
models. Moreover, several ongoing clinical trials are evaluating the safety and efficacy of Monalizumab in patients with different tumor types.

In the current study, three different doses of Monalizumab were administered to patients two months after allo-HSCT for hematological malignancies. Patients were monitored before and at various time points following Monalizumab administration to evaluate its effect on the functional activity of reconstituting NK cells.

**Methods and Materials**

**Study design and patients**

This is a prospective cohort study conducted at the Institute Paoli-Calmettes (IPC) in Marseille, France. The study adhered to the Helsinki Declaration criteria and received approval from the Ethics Committee of the Paoli – Calmettes Institutional Review Board under the ethical code of PIRAT-IPC 2019-018. A total of 18 patients with hematological malignancies were enrolled in the study. The sample size was calculated using...
the formula for comparing means, with a confidence level of 95%, test power of 80%, and an effect size of approximately 0.8. All participants provided written informed consent in accordance with the Declaration of Helsinki.

The inclusion criteria were patients with hematological malignancies, including acute myeloid leukemia, acute lymphoblastic leukemia, multiple myeloma, chronic lymphoid leukemia, chronic myeloid leukemia, Hodgkin lymphoma or non-Hodgkin lymphoma, who had received allo-HSCT from HLA-matched (10/10) donors. The median age of the patients was 59 years (range: 39-68 years). Additionally, all patients received a reduced-intensity conditioning regimen consisting of fludarabine (30 mg/m²/day), busulfan (3.2 mg/kg/day), and Thymoglobuline (5 mg/kg) for 2 days. They underwent HLA-identical allo-HSCT using granulocyte colony-stimulating factor-mobilized peripheral blood stem cells.

After 60 days post-transplantation, patients received intravenous Monalizumab according to a protocol approved by the ethical review boards at IPC. At day 60, Monalizumab was administered intravenously to patients in three dose groups: dose 1 group (0.1 mg/kg, 5 patients), dose 2 group (0.5 mg/kg, 8 patients), and dose 3 group (1 mg/kg, 5 patients). Peripheral blood samples were collected from patients at various intervals over a period of 6 months and cryopreserved for later

Figure 3. Activating NK cell receptors expression on reconstituting CD56dim NK cells after Monalizumab treatment PBMCs were obtained from patients, stained with specific mAbs and analyzed by flow cytometry at different time points. The CD56dim NK subset was identified within CD3-CD56+ NK cells. (A-C) Bar graphs display the mean ± SD of the frequencies of positive CD56dim NK cells for Nkp30, Nkp46, and NKG2D receptors in PB of patients treated with dose 1 (n = 5, gray columns), dose 2 (n = 8, black columns), and dose 3 (n = 5, blue columns), as well as controls (red columns), before and after different time points of Monalizumab treatment. Comparisons between each pair of groups at the same time point were performed using a non-parametric Mann-Whitney U test (*P ≤ 0.05, **P < 0.01, ***P < 0.001).

NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; mAbs: Monoclonal antibodies; PB: Peripheral blood
Figure 4. Effector function of reconstituting CD56dim NK cells after Monalizumab treatment PBMCs from patients were collected at various time points and allowed to rest without cytokine activation. Resting cells were then cultured for 4 hours at 37°C, either alone or with K562 cells (E/T ratio, 10:1). After stimulation, the cells were stained for surface markers and intracellular molecules, and analyzed by flow cytometry. The CD56dim NK subset was identified within the CD4+CD56+ NK cell population. (A, C, E, G) Bar graphs show the frequencies (mean ± SD) of CD107a+CD56dim NK cells (A), Perforin+CD56dim cells (C), IFN-γ+CD56dim cells (E), and TNF-α+CD56dim NK cells (G) in the dose 1 group (n=5, gray columns), dose 2 group (n=8, black columns), dose 3 group (n=5, blue columns), as well as controls (red columns), before and after different time points of Monalizumab treatment. (B, D, F, H) Displaying the changes in frequencies of CD107a+CD56dim NK cells (B), Perforin+CD56dim cells (D), IFN-γ+CD56dim cells (F), and TNF-α+CD56dim NK cells (H) from day 1 to month 6 in dose 1 recipients (gray lines), dose 2 recipients (black lines), dose 3 recipients (blue lines), and controls (red lines). Comparisons between each two groups at the same time point were performed using the Wilcoxon non-parametric t-test. Comparisons between each two groups at the same time point were performed using the non-parametric Mann-Whitney U test (*P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

NK: Natural killer; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor alpha
use. The samples were obtained directly before Monalizumab treatment (Day 0, baseline) and at month 1, month 3, and month 6 after Monalizumab treatment (Figure 1). Genoidentical controls were used, and peripheral blood samples corresponding to the patients’ time frame samples were utilized. Patients with a history of grade ≥ II acute graft-versus-host disease (GVHD), a history of another malignancy, abnormal cardiac status, previous allo-HSCT or solid organ transplantation, or ongoing use of systemic corticosteroids were excluded from the study. Additionally, five HLA-genoidentical transplanted patients were used as controls.

Flow cytometry

After thawing, the cells were immunostained with fluorochrome-labeled antibodies from phenotypic panels listed in table 1. Acquisition was performed using a FACS LSR II (BD Biosciences), and analysis was done using FlowJo v10 software (LLC, Ashland, Oregon). The gating strategy was based on the elimination of doublets using FSC-A/FSC-H parameters, followed by the removal of dead cells using a cell viability marker. The NK cell population was defined as CD$^3$–CD$^{56^+}$ cells within the lymphocyte gate. The phenotypic data are represented as the percentage of cells positive for a given marker.

NK cell functions

PBMCs at all time points were thawed and rested overnight at 37°C in RPMI 1640 medium supplemented with 10% FCS (complete medium) without interleukins (IL-2 or IL-15). Cytokine production and degranulation capacity were determined by stimulating NK cells with the erythroleukemia cell line K562. Briefly, 1x10⁶ PBMCs were incubated with K562 cells (ratio 10:1) at 37°C and 5% CO₂ for 4 hours in the presence of GolgiPlug® (BD Biosciences, #555029) and FITC-conjugated anti-human CD107a (BD Biosciences, clone H4A3). After 4 hours of incubation, the cells were collected, washed, and immunostained for surface markers with fluorochrome-labeled antibodies listed in table 1. For intracellular staining, cells were fixed and permeabilized using the Cytotox/Cytoperm® kit (BD Biosciences) according to the manufacturer’s instructions, and then immunostained with fluorochrome-conjugated anti-cytokine antibodies listed in table 1. Finally, the cells were prepared for flow cytometry analysis using a FACS LSR II (BD Biosciences) and FlowJo v10 software (LLC, Ashland, Oregon).

Statistical analysis

Graphics were generated using GraphPad Prism software (San Diego, CA, USA). Statistical analyses were performed using GraphPad Prism software. Comparisons between two different groups were conducted using the Mann-Whitney test, while comparisons between the same individual in each group were carried out using the Wilcoxon matched pairs T test. A significance level of $P \leq 0.05$ was considered statistically significant.

Results

Normal pattern of NKG2A expression on the reconstituting CD$^{56^+}$ NK cells following Monalizumab treatment

To determine the expression of NKG2A on reconstituting CD$^{56^+}$ NK cells following Monalizumab treatment, peripheral blood mononuclear cells (PBMCs) of patients before and after relevant time points of Monalizumab usage were stained with specific monoclonal antibodies (mAbs) and analyzed by cytometry. NKG2A expression was also assessed among control patients at the same time frames as the treated patients.

Firstly, NK cells were identified within PBMCs as CD$^3$–CD$^{56^+}$ cells among viable lymphocytes (Figure 2A). Since early reconstituted NK cells display an immature phenotype characterized by high expression of NKG2A, our results showed an increase in the frequencies of NKG2A+CD$^{56^+}$ NK cells at day 0 and day 1 in all patients and controls, compared with healthy individuals. However, when compared with day 0, the frequencies of NKG2A+CD$^{56^+}$ NK cells began to significantly decline throughout the study period. At month 6, the frequencies of NKG2A+CD$^{56^+}$ NK cells were significantly lower compared with day 0 (controls: 45% at month 6 versus 78% at day 0, $P < 0.01$; dose 1:
48% at month 6 versus 80% at day 0, \( P < 0.01 \); dose 2: 46% at month 6 versus 77% at day 0, \( P < 0.01 \); dose 3: 45% at month 6 versus 79% at day 0, \( P < 0.01 \) (Figure 2B).

Furthermore, we did not observe any variations in NKG2A expression between Monalizumab recipients and the controls at a given time point. The study population exhibited a similar NKG2A expression profile at the same time point (\( P > 0.05 \)). These findings suggest that the use of Monalizumab did not alter the normal pattern of NKG2A expression on reconstituting CD\(^{56\text{dim}}\) NK cells.

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**Monalizumab increases the expression of activating NK cell receptors on reconstituting CD\(^{56\text{dim}}\) NK cells**

Next, we investigated whether the use of Monalizumab affected the expression of NK cell activating receptors by measuring the frequencies of NKp30, NKp46, and NKG2D activating receptors on the reconstituting CD\(^{56\text{dim}}\) NK cells in the study population at different time points. Our results showed that there were no changes in the expression of NK cell activating receptors among study participants before using Monalizumab and at day 1 of Monalizumab use (\( P > 0.05 \)) (Figure 3A, B, and C). Additionally, the expression of NKp30, NKp46, and NKG2D remained consistent among the controls and patients in the dose 1 group throughout the study period (\( P > 0.05 \)).

Interestingly, the frequency of NKp30\(^+\) CD\(^{56\text{dim}}\), NKp46\(^+\) CD\(^{56\text{dim}}\), and NKG2D\(^+\) CD\(^{56\text{dim}}\) NK cells significantly increased after one month of using Monalizumab in patients who received dose 2 and dose 3 of Monalizumab compared with baseline, as well as control subjects (\( P < 0.05 \)). Moreover, the increase was more pronounced after 6 months of using Monalizumab among patients treated with dose 3 of Monalizumab compared with baseline, as well as control subjects (\( P < 0.05 \)).

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However, we did not find a significant change in the expression of activating NK cell receptors between patients in the dose 2 and dose 3 groups
at the corresponding time points \( (P > 0.05) \). Taken together, these findings suggest that blocking NKG2A receptors using Monalizumab could upregulate the expression of NK cell activating receptors on the reconstituting CD\(^{56\text{dim}}\) NK cells. **Blocking NKG2A enhances the cytolytic function of reconstituting CD\(^{56\text{dim}}\) NK cells**

In this study, we aimed to assess the impact of NKG2A blockade using Monalizumab on the effector function of reconstituting CD\(^{56\text{dim}}\) NK cells. We evaluated this by monitoring the cell surface expression of CD\(^{107\text{a}}\), perforin secretion, and cytokine production (interferon gamma, IFN-\(\gamma\), and tumor necrosis factor alpha, TNF-\(\alpha\)) of reconstituting CD\(^{56\text{dim}}\) NK cells co-cultured with K562 target cells expressing HLA-E. Our results revealed that patients treated with doses 2 and 3 of Monalizumab exhibited higher levels of degranulation in reconstituting CD\(^{56\text{dim}}\) NK cells compared with patients treated with dose 1 and controls.

Moreover, the increase in degranulation levels was particularly significant in patients treated with dose 3, especially after 6 months of Monalizumab use. At month 6, the percentage of CD\(^{107\text{a}+}\) CD\(^{56\text{dim}}\) NK cells was 23.5% in dose 3-treated patients, whereas it was 19.2% in dose 2-treated patients \( (P < 0.05) \), 14.4% in dose 1-treated patients \( (P < 0.0001) \), and 10.1% in controls \( (P < 0.0001) \) (Figure 4 A, B). Similarly, Monalizumab treatment resulted in increased perforin secretion by reconstituting CD\(^{56\text{dim}}\) NK cells, primarily in patients treated with dose 3. At month 6, the percentage of perforin \(^{+}\) CD\(^{56\text{dim}}\) NK cells was 20.3% in dose 3-treated patients, while it was 18% in dose 2-treated patients \( (P < 0.01) \), 12% in dose 1-treated patients \( (P < 0.0001) \), and 9.6% in controls \( (P < 0.0001) \) (Figure 4 C, D).

Regarding cytokine production, CD\(^{56\text{dim}}\) NK cells from patients treated with doses 2 and 3 exhibited significant levels of IFN-\(\gamma\) and TNF-\(\alpha\) cytokines in response to K562 stimulation post-Monalizumab treatment, compared with controls and dose 1-treated patients. This change was particularly pronounced after 6 months of Monalizumab use (Figure 4 E-H). Interestingly, we observed that patients receiving dose 3 of Monalizumab had higher frequencies of IFN-\(\gamma\)-CD\(^{56\text{dim}}\) and TNF-\(\alpha\)-CD\(^{56\text{dim}}\) NK cells compared with patients receiving dose 2, especially in the 6\(^{th}\) month of treatment \( (% \text{IFN-} \gamma\text{-CD}^{56\text{dim}}\text{cells at month 6 in dose 3-treated patients was 14.3% compared with 10.2% in dose 2-treated patients, }P < 0.01,\) and \( (% \text{TNF-} \alpha\text{-CD}^{56\text{dim}}\text{NK cells at month 6 in dose 3-treated patients was 16.2% compared with 12.7% in dose 2-treated patients, }P < 0.05)\). Taken together, these findings suggest that blocking NKG2A receptors using Monalizumab after allo-HSCT may enhance the cytotoxic activity of reconstituting CD\(^{56\text{dim}}\) NK cells.

**Discussion**

In the current study, we discovered that blocking the inhibitory function of NKG2A using Monalizumab after allo-HSCT led to improved NK cell restoration and increased expression of NK cell activating receptors on the reconstituting CD\(^{56\text{dim}}\) NK cells. Consequently, the effector function of these cells was enhanced. This improvement in both the morphological and functional properties of the reconstituting NK cells was particularly evident among patients treated with dose 3 of Monalizumab. HSCT patients undergo an aplastic period which can lead to infections and tumor relapse in immune-compromised recipients. Therefore, it is crucial for donor-derived HSCs to engraft rapidly and for immune cells to recover quickly in order to achieve positive clinical outcomes from HSCT.\(^{20}\)

NK cells are the first donor-derived lymphocytes to regenerate following HSCT, serving as the initial protective line in immune-compromised recipients.\(^{21,22}\) They are cytotoxic lymphocytes involved in innate immune responses, recognizing and eliminating virus-infected and tumor cells without prior stimulation. Clinical studies have demonstrated that NK cell-based immunotherapy represents a promising strategy for potent antitumor effects against hematological malignancies.\(^{23-27}\) However, it has been shown that early reconstituting NK cells after HSCT have an immature phenotype
characterized by high expression of NKG2A, which is associated with diminished cytotoxic activity. This is because the balance between activating and inhibiting receptors precisely regulates NK cell behavior. Furthermore, certain types of cancer highly express HLA-E, a specific ligand of NKG2A inhibitory receptors. As a result, these phenotypic abnormalities can hinder the effector functions of reconstituting NK cells, increasing the risk of tumor relapse and mortality.

In this study, we aimed to improve the functional activity of reconstituting NK cells after allo-HSCT in patients with hematological malignancies by blocking the NKG2A checkpoint using Monalizumab. Immune checkpoint inhibitors have generally had a positive impact on the management of many types of cancer; however, the efficacy of many of these inhibitors still needs to be further improved. Moreover, there have been very limited studies evaluating the effect of Monalizumab on the immune system and cancer cells. Unfortunately, we did not find any studies that assessed the effect of Monalizumab on the biology of NK cells. Nevertheless, our findings demonstrated that the maturation pattern and expression of activating receptors were improved in reconstituting NK cells. After six months of Monalizumab treatment, patients receiving dose 3 of Monalizumab experienced a significant decrease in the expression of NKG2A receptors and a remarkable increase in the expression of NKp30+, NKp46+, and NKG2D+ CD56dim NK cells compared with baseline and other study participants at the relevant time periods.

The exact mechanism by which Monalizumab upregulates the expression of NK cell activating receptors is not well known. However, we believe that blocking NKG2A receptors diminishes the inhibitory signals that may be involved in inhibiting the gene transcription of the activating NK cell receptors. Additionally, it reduces the secretion of certain cytokines that may downregulate the expression of activating NK cell receptors.

Previous studies have reported that tumor cells can evade NK cell immunosurveillance by altering the phenotypic properties of NK cells. This is achieved through upregulation of NKG2A receptors and ligands, downregulation of NK cell activating receptors and their ligands, as well as the secretion of cytokines by tumor cells. Therefore, we hypothesize that by inhibiting the primary inhibitory NK cell receptors (NKG2A) and enhancing the expression of NK cell activating receptors, Monalizumab can potentially prevent tumor progression and relapse.

Based on the aforementioned progression in morphological features of early developing NK cells, we evaluated whether these changes would affect the functional activity of NK cells. Additionally, it has been reported that early reconstituting NK cells after HSCT have diminished cytotoxic function. Our findings revealed that peripheral CD56dim NK cells from patients receiving higher doses of Monalizumab displayed a higher degree of degranulation and greater proinflammatory cytokine secretion in response to K562 cell stimulation than CD56dim NK cells from patients receiving dose 1 of Monalizumab or from controls. Notably, the increase was more pronounced for patients treated with dose 3 of Monalizumab. In agreement with our results, Andre et al. found that blockade of NKG2A in vitro with Monalizumab promoted the expression of CD107a and increased cytokine secretion by NK cells in response to target cell stimulation. Additionally, they showed that Monalizumab improved NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) when combined with cetuximab, an anti-epidermal growth factor receptor. On the other hand, Tinker and his colleagues found that Monalizumab monotherapy had very little clinical activity in patients with gynecologic cancers. However, using Monalizumab in combination with anti-PD-L1 in vivo enhanced the antitumor activity of CD8+ T cells in a murine model of cancer, suggesting that using Monalizumab in combination with checkpoint inhibitors could provide synergistic anti-tumor effectiveness. Given that prior studies have shown mature CD56dim NK cells to be cytotoxic cells and the
major source of proinflammatory cytokines and chemokines after target cell stimulation, we believe that the functional activity improvement of reconstituting CD $^{56}$dim NK cells may be attributed to the blockade of NKG2A inhibitory function, as well as the significant recovery of NK cell maturity following Monalizumab treatment. Moreover, the upregulation of activating receptor expression on circulating CD $^{56}$dim NK cells may also contribute to the improved functional activity of reconstituting CD $^{56}$dim NK cells after treatment with Monalizumab. 38, 39

Our study has some limitations, such as a small sample population, a short follow-up period, and the fact that we did not consider NK cell infiltration into the tumor microenvironment or interactions with other immune cells that might alter the activity of Monalizumab. Additionally, it is essential to evaluate the expression of NK cell activation markers, as well as exhaustion markers, on the reconstituting NK cells before and after using Monalizumab.

Conclusion

In conclusion, we report here the benefits of using Monalizumab as a therapeutic antibody that enhances the antitumor activities of reconstituting CD $^{56}$dim NK cells by blocking the inhibitory function of NKG2A, as well as increasing the expression of NK cell activating receptors. Therefore, anti-NKG2A mAb is a promising checkpoint inhibitor that promotes antitumor immunity by enhancing the cytotoxic activity of NK cells. These results suggest that additional clinical trials using Monalizumab as mono-therapy or in combination with other immune agents are necessary.

Acknowledgement

The authors would like to thank the local tumor biobank directed by Prof. Chabannon for providing access to patients' samples, Ms. N. Cherouat from the local blood bank for HLA and KIR genotyping, and the Transplantation Department and the Bio-pathology Department for their assistance with patient characteristics. The authors also acknowledge the CRCM cytomtery core facility and the immunomonitoring platform for their support.

Conflict of Interest

None declared.

References


