Introduction

Based on genetic risk allogeneic stem cell transplantation (allo-SCT) is the only curative treatment for some forms of acute myeloid leukemia (AML). However, post-transplantation relapse remains a frequent cause of transplantation failure. Natural killer (NK) cells and CD8+ T cells are important effector lymphocytes with pivotal roles in tumor surveillance and anti-tumor immune response. In this study, a 14-year-old female patient with AML was treated with allogeneic peripheral blood stem cell transplantation (allo-PBSCT). Bone marrow relapse was found 6 months later. Thereafter, the patient was treated with DAE (daunorubicin, cytosine arabinoside, and etoposide), followed by IAE (idarubicin, cytosine arabinoside, and etoposide), and then MA (mitoxantrone and cytosine arabinoside) regimens. A series of experiments including Wright-Giemsa stain analyses, cytogenetic analysis and flow cytometry were conducted to investigate the characteristic of the patient. Although there was a short remission after the DAE regimen, the patient experienced another relapse after finishing the MA regimen. The CD56+CD3− cells in the bone marrow showed severely impaired activation and anti-tumor function, while extraordinarily increased CD33 expression. Moreover, the proportion of multifunctional effector CD8+ T cells remained stable, though they had high PD-1 expression. These findings revealed the dysfunction of abnormal CD56+CD3− cells with high CD33 expression, which might be targetable and related to the relapsed/refractory AML after allo-SCT.

Keywords: Case report; natural killer (NK) cells; allogeneic stem cell transplantation (allo-SCT); acute myeloid leukemia (AML); CD33

Case Report

Extraordinarily elevated CD33 expression in CD56+CD3− cells in the bone marrow of a patient with relapsed acute myeloid leukemia: a case report

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Abstract: Based on genetic risk allogeneic stem cell transplantation (allo-SCT) is the only curative treatment for some forms of acute myeloid leukemia (AML). However, post-transplantation relapse remains a frequent cause of transplantation failure. Natural killer (NK) cells and CD8+ T cells are important effector lymphocytes with pivotal roles in tumor surveillance and anti-tumor immune response. In this study, a 14-year-old female patient with AML was treated with allogeneic peripheral blood stem cell transplantation (allo-PBSCT). Bone marrow relapse was found 6 months later. Thereafter, the patient was treated with DAE (daunorubicin, cytosine arabinoside, and etoposide), followed by IAE (idarubicin, cytosine arabinoside, and etoposide), and then MA (mitoxantrone and cytosine arabinoside) regimens. A series of experiments including Wright-Giemsa stain analyses, cytogenetic analysis and flow cytometry were conducted to investigate the characteristic of the patient. Although there was a short remission after the DAE regimen, the patient experienced another relapse after finishing the MA regimen. The CD56+CD3− cells in the bone marrow showed severely impaired activation and anti-tumor function, while extraordinarily increased CD33 expression. Moreover, the proportion of multifunctional effector CD8+ T cells remained stable, though they had high PD-1 expression. These findings revealed the dysfunction of abnormal CD56+CD3− cells with high CD33 expression, which might be targetable and related to the relapsed/refractory AML after allo-SCT.

Keywords: Case report; natural killer (NK) cells; allogeneic stem cell transplantation (allo-SCT); acute myeloid leukemia (AML); CD33

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cytometry in patients with AML has been widely used to guide clinical management. The expression of CD33, a member of the sialic acid-binding immunoglobulin-like lectin family and assumed to be restricted to the myeloid lineage of immune cells, is generally used in MRD detection (4,5). Natural killer (NK) cells originate from hematopoietic stem cells (HSCs), primarily in the bone marrow, and play crucial roles in tumor surveillance and controlling tumor invasion (6,7). The NK cell function is finely tuned by activating and inhibitory receptors, allowing NK cells to discriminate between normal and aberrant cells (8). NK cells can kill AML directly via cytotoxicity and cytokines, such as IFN-γ, TNF-α, and CD107a. Therefore, it is important to maintain function of NK cells. A recent study reported that CD33 expression on NK cells could be a potential confounder for MRD detection, although the percentage of CD33+ NK cells was low (4). However, little is known about the interrelationships between extraordinarily high CD33 expression on CD56+CD3− cells and the impaired anti-tumor function of CD56+CD3− cells, as well as other immune characteristics in the bone marrow of patients with relapsed/refractory AML.

Here, we examined the characteristics and the impaired function of CD56+CD3− cells and demonstrated the abnormal proportion of CD33+ CD56+CD3− cells in the bone marrow of a patient with relapsed AML after allo-HSCT and remission induction, to ascertain whether abnormal CD56+CD3− cells are related to relapse.

We present the following article in accordance with the CARE reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-733).

**Case presentation**

The case report was approved by the institutional review board of the First Affiliated Hospital of University of Science and Technology of China (2021-N(H)-120). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration (as revised in 2013). Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal. Patient data were collected via retrospective chart review, which included clinical characteristics; comprehensive hematopathologic examination, such as Wright-Giemsa stain analyses, cytogenetic analysis and flow cytometry (9) (Appendix 1); and clinical outcome.

The characteristics of the 14-year-old girl were shown in (Table S1). The girl was diagnosed with AML-M1 in the First Affiliated Hospital of Anhui Medical University, in May 2019. Her karyotype was 46 XX, t(2; 17) (q31; q25), +8, t(16; 21) (p11; q22). PML/RARA and AML1/ETO fusion genes were not present. Then she received an allogeneic peripheral blood stem cell transplant (allo-PBSCT) from the matched sibling donor (MSD) in Children’s Hospital of Nanjing Medical University (15/November/2019). Relapse was confirmed in the bone marrow 6 months later. Thereafter, remission was induced using the DAE (daunorubicin, cytosine arabinoside, and etoposide) regimen once a month. This patient received daunorubicin (20 mg/m²/day) for 3 days (day 1–3), etoposide (VP-16, 120 mg/m²/day) for 5 days (day 1–5), and cytarabine (100 mg/m²/d) twice a day for 7 days (day 1–7). On 21/June/2020, MRD was found to be negative and the patient achieved complete remission (CR). The patient was then given the IAE (idarubicin, etoposide, and cytosine arabinoside) regimen: idarubicin (40 mg/m²/day) for 3 consecutive days, then etoposide (120 mg/m²/day) for 5 consecutive days, followed by cytarabine (100 mg/m²/d) twice a day for 7 consecutive days. On 29/July/2020, a secondary breast tumor was found. Thereafter, the patient was treated with the MA (mitoxantrone and cytosine arabinoside) regimen once a month for 2 months: mitoxantrone (10 mg/m²/day) for 3 consecutive days and cytarabine (100 mg/m²/d) twice a day for 7 consecutive days. During the MA regimen, the patient also received radiotherapy 12 times. Relapse was confirmed again on 14/October/2020 in bone marrow after the last radiotherapy with the treatment of anti-CD38 antibody (Figure 1).

Through morphologic analysis of the leukemia cells in the bone marrow, we found that primitive cells accounted for 74% of the nucleated cells (Figure 2A). Thereafter, to investigate the immunological characteristics and the possible mechanism underlying the AML relapse, we isolated mononuclear cells from the bone marrow and systematically analyzed molecular expression related to NK cell function. The percentage of CD56+CD3− cells was nearly 90% when gated from CD45+ cells. Furthermore, CD33, generally assumed to be restricted to the myeloid lineage of immune cells [and not to erythrocytes, platelets, T cells, B cells, or NK cells (4)] was extraordinarily highly expressed on the CD56+CD3− cells (Figure 2B). The antibody used in this study were shown in (Table S2). As
the activation of the CD56^+CD3^- cells was limited, we then analyzed CD69, CD38, NKG2D and NKp30 expression and found that their expressions were low (Figure 2C, 2D). We characterized four populations of CD56^+CD3^- cells defined by CD11b and CD27, which represent the distinct stages of human NK cells from different tissues (10). The majority of CD56^+CD3^- cells had the CD11b^-CD27^- phenotype, which is reported to be an immature phenotype (11). Additionally, the CD56^+CD3^- cells had low expression of NKG2A, KLRG1, and PD-1 (Figure 2E). Moreover, human NK cells can be classified into two main classic subsets dependent on CD56 and CD16: CD56^{bright}CD16^- and CD56^{dim}CD16^- NK cells (12). The former is considered efficient cytokine producers. The proportion of these cells was nearly 99% (Figure 2E). Then, to demonstrate the effector functions of the NK cells, we stimulated the mononuclear cells with PMA and ionomycin in the presence of monensin for 4 h. We found that the CD56^+CD3^- cells from the bone marrow exhibited an extremely low percentage (<0.1%) of polyfunctional effector IFN-\(\gamma\) TNF-\(\alpha\), IFN-\(\gamma\) CD107a, and IFN-\(\gamma\) GranzymeB cells (Figure 2F). CD8^+ T cells also play an important anti-tumor role, here we demonstrated that the proportion of polyfunctional effector IFN-\(\gamma\) TNF-\(\alpha\), IFN-\(\gamma\) CD107a and IFN-\(\gamma\) GranzymeB^+ CD8^+ T cells was normal (Figure 2G). This implied that the anti-tumor effect was heavily impaired in CD56^+CD3^- cells but not in CD8^+ T cells. Furthermore, we demonstrated that Ki67 was highly expressed on these abnormal CD56^+CD3^- cells, but not on T cells (Figure 2H). Moreover, we verified the low expression of the early-activation marker CD69 on T cells (Figure 2I) and the high expression of PD-1 on CD8^+ T cells (Figure 2J).

**Discussion**

Recently, Eckel et al. reported that CD33 expression on NK cells in AML was a potential confounder for MRD detection, and nearly 18% of MRD^+ patients were CD33 positive. Furthermore, the CD33^+ NK population accounted for a mean of 11.4% of NK cells (median 11.9%, range,
Figure 2 Extraordinarily increased CD33+CD56−CD3− cells with poor function in bone marrow at relapse. Mononuclear cells were derived from the bone marrow of the patient with relapsed acute myeloid leukemia (AML). (A) Wright-Giemsa stain analyses of the leukemia cells in bone marrow smears before (left) and at (right) AML relapse. Scale bar = 20 µm. (B) Flow cytometry gating strategy for CD56+CD3−, CD8+T, and CD4+T cells, derived from bone marrow samples. (C) Density plots of CD122, CD69, and CD38 expression in gated CD56+CD3− cells. (D) Density plots of NKP30 and NKG2D expression in gated CD45+ cells. (E) Density plots of CD11b, CD16, NKG2A, KLRG1, and PD-1 expression in gated CD56+CD3− cells. (F) Intracellular cytokine staining of IFN-γ, TNF-α, and Granzyme B in gated NK cells. Mononuclear cells from the bone marrow were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma) and ionomycin (1 µg/mL; Calbiochem) in the presence of monensin (10 µg/mL; Sigma) for 4 h. (G) Intracellular cytokine staining of IFN-γ, TNF-α, and Granzyme B in gated CD8+T cells. Mononuclear cells from bone marrow were stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL) in the presence of monensin (10 µg/mL) for 4 h. (H) Density plots of Ki67 expression in gated NK cells and CD4+ T cells, CD8+ T cells, and double negative T (DNT) cells (CD4−CD8−). (I) Density plot of CD69 expression in gated CD3+ T cells. (J) Density plot of PD-1 expression in gated CD8+ T cells.
Here, we demonstrated that CD33 might not only be a potential confounder for MRD detection, but also a targetable molecule in our relapsed/refractory AML patient. We verified that the CD33^+CD56^-CD3^- population accounted for 93.8% of the CD56^-CD3^- cells. Additionally, we found low expression of the activating receptors Nkp30 and NKG2D. Importantly, we observed that CD56^-CD3^- cells had downregulated granzyme B, TNF-α, CD107a, and IFN-γ, suggesting impaired functionality. In addition, we speculated that these markers might be useful for predicting immune states in the tumor microenvironment. Given that several CD33-targeted therapies have been used in AML, and that they can mediate killing of AML blasts by CD56^-CD3^- cells (13-15), more attention should be paid to the influence of CD56^-CD3^- cells expressing CD33.

Taken together, these results showed that CD56^-CD3^- cells in the bone marrow of this patient with relapsed AML had abnormally high CD33 expression and severely impaired anti-tumor function, however, the immune function of CD8^+ T cells in the bone marrow seemed to be normal. This study provides new perspectives on the mechanism underlying relapse after allo-SCT, and treatment with anti-CD33 monoclonal antibody may be worthy of attention.

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**Footnote**

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**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The case report was approved by the institutional review board of the First Affiliated Hospital of University of Science and Technology of China (2021-N(H)-120). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration (as revised in 2013). Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal.

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Appendix 1 Wright-Giemsa stain analyses and flow cytometry

Bone marrow aspirates from the patient were treated with Wright-Giemsa stain (cat. no. G1020; Solarbio) according to the manufacturer's protocol and the morphological changes were observed under an optical microscope. Mononuclear cells were firstly derived from the bone marrow and the mononuclear cell suspensions were then treated with mouse serum to block the binding to non-specific Fc receptors. Next, to assess the surface and intracellular markers, the cells were stained with anti-human monoclonal antibodies, from BD Biosciences and BioLegend, in accordance with the manufacturer's instructions. Intracellular cytokine staining involved 4 h of stimulation of the cells with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma) and ionomycin (1 µg/mL; Calbiochem) in the presence of monensin (10 µg/mL; Sigma). Data were collected using an FCM LSR II flow cytometer (BD Biosciences, USA) and analyzed using FlowJo software (Tree Star, USA).

| Characteristic                        | Value          |
|---------------------------------------|----------------|
| Gender (M/F)                          | F              |
| Age, years                            | 14.0           |
| White blood cell count, \( \times 10^9/L \) | 26.8           |
| Red blood cell count, \( \times 10^12/L \) | 2.95           |
| Nucleated red blood cell count, \( \times 10^9/L \) | 0.02           |
| Nucleated red blood cell (%)          | 0.10           |
| Immature reticulocytes (%)            | 29.4           |
| Platelet count, \( \times 10^9/L \)   | 55.0           |
| Lymphocyte count, \( \times 10^9/L \)  | 3.19           |
| Lymphocytes (%)                       | 11.9           |
| Neutrophil count, \( \times 10^9/L \)  | 3.54           |
| Neutrophils (%)                       | 13.2           |
| Monocyte count, \( \times 10^9/L \)   | 3.54           |
| Monocytes (%)                         | 74.7           |
| Eosinophil count, \( \times 10^9/L \)  | 0.02           |
| Eosinophils (%)                       | 0.10           |
| Basophil count, \( \times 10^9/L \)   | 0.03           |
| Basophils (%)                         | 0.10           |
| Hemoglobin (g/L)                      | 87.0           |
| Antibody                                      | Brand          | Cat. No.                  |
|----------------------------------------------|----------------|--------------------------|
| APC-CY7 Mouse Anti-Human CD3                 | BD Bioscience  | Cat# 557832, RRID:AB_396890 |
| FITC Mouse Anti-Human CD16                   | BD Bioscience  | Cat# 555406, RRID:AB_395806 |
| FITC Mouse Anti-Human CD27                   | BD Bioscience  | Cat# 555440, RRID:AB_395833 |
| APC-Cy7 Mouse Anti-Human CD14                | BD Bioscience  | Cat# 557831               |
| APC Mouse Anti-Human CD107a                  | BD Bioscience  | Cat# 560664, RRID:AB_396135 |
| PerCP-CY5.5 Mouse Anti-Human CD38            | BD Bioscience  | Cat# 551400, RRID:AB_394184 |
| FITC Mouse Anti-Human CD4                    | BD Pharmingen  | Cat# 555346, RRID:AB_395751 |
| PE-Cy7 Mouse Anti-Human CD8                  | BD Pharmingen  | Cat# 557746, RRID:AB_396852 |
| FITC Mouse Anti-Human IFN-γ                   | BD Bioscience  | Cat# 554700, RRID:AB_395517 |
| PE Mouse Anti-Human CD69                     | BD Bioscience  | Cat# 555531, RRID:AB_395916 |
| PE Mouse Anti-Human PD-1                     | BD Bioscience  | Cat# 560795               |
| 7-AAD                                        | BD Bioscience  | Cat# 559925               |
| APC Mouse Anti-Human HLA-DR                  | BD Bioscience  | Cat# 559866, RRID:AB_398674 |
| PerCP-CY5.5 Mouse Anti-Human CD123           | BD Bioscience  | Cat# 558714, RRID:AB_891359 |
| FITC Mouse anti-human Lineage Cocktail       | BD Bioscience  | Cat# 348801, RRID:AB_10612570 |
| APC-CY7 Mouse Anti-Human CD11c               | Biolegend      | Cat# 337218               |
| PE Mouse Anti-Human NKG2D                    | BD Bioscience  | Cat# 557940               |
| PE Mouse Anti-Human TNF-α                    | BD Bioscience  | Cat# 559321, RRID:AB_397219 |
| Alexa Fluor® 647 Mouse Anti-Human NKP30      | BD Bioscience  | Cat# 558408, RRID:AB_398454 |
| FITC Mouse IgG1, κ                           | BD             | Cat# 555748, RRID:AB_396090 |
| PE Mouse IgG1, κ                             | BD             | Cat# 55749, RRID:AB_396091 |
| PerCP-Cy5.5 Mouse IgG1, κ                    | BD             | Cat# 552834, RRID:AB_394484 |
| PE-Cy7 Mouse IgG1, κ                         | BD             | Cat# 557872, RRID:AB_396914 |
| Alexa Fluor 647 Mouse IgG1, κ                | BD             | Cat# 557714, RRID:AB_396823 |
| APC-Cy7 Mouse IgG1, κ                        | BD             | Cat# 557873, RRID:AB_396915 |
| APC-Cy7 Mouse IgG1, κx                       | Biolegend      | Cat# 400161, RRID:AB_11125373 |
| PE Mouse IgG2a, κ                            | BD             | Cat# 555574, RRID:AB_395953 |
| PerCP-Cy5.5 Mouse IgG2a, κ                   | BD             | Cat# 558020, RRID:AB_396989 |
| PE-Cy7 Mouse IgG2b, κ                        | Biolegend      | Cat# 400325               |

No., number; Cat., catalog; RRID, Research Resource Identifier.