RESEARCH ARTICLE

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Abnormalities by Multicolor Flow Cytometry for Detection of Minimal Residual Disease in Recipients of Allo-HSCT Originating from Donors: A Cohort Study

Allo-KHN Alıcılarında Minimal Kalıntı Hastalık Tespitindeki çok Renkli Akım Sitometri ile Saptanan Verici Kaynaklı Anormallikler: Bir Kohort Çalışması

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Abstract

Objective: In minimal residual disease (MRD) analysis after allogeneic hematopoietic stem cell transplantation (allo-HSCT), abnormal immunophenotyping is commonly considered as evidence of a secondary recurrence or complications, leading to overtreatment. We aimed to confirm whether such phenotypic abnormality might originate from donors using multicolor flow cytometry (MFC).

Materials and Methods: The MRD of bone marrow specimens of 3395 patients who had received allo-HSCT were analyzed using the conventional two-tube, eight-color MFC panel. The frequencies of abnormal immunophenotypes were also evaluated in three groups of patients without malignancies.

Results: The frequency of new abnormal polymorphisms was 0.088% (3/3395) among patients who received allo-HSCT. The abnormal cells seen in three patients in complete remission were Fcγ receptor IIIB (FcγRIIIB) gene deletion ($CD16^-$ neutrophils), $CD2^-CD159a^-CD159c^+$ natural killer (NK) cells, and monoclonal B lymphocytosis (MBL), respectively. In addition, abnormal T-cells ($CD4^+CD8^+$) were detected in one donor before allo-HSCT. Identical abnormalities were found in the peripheral blood of the corresponding donors of the three patients via MFC. Among the individuals without malignancies, the incidence of FcγRIIIB deletion was 0.2% (11/5256), that of NK cells with the absence of CD2 and single-positive CD159c was 0.05% (1/2000), that of monoclonal CD4/CD8 double-positive T-cells was 0.05% (1/2000), and that of MBL was 1.3% (14/1100). The frequency of NK cells with the absence of CD2 was 1.3% (1/79) and with $CD8^{dim}$ was 14% (11/79) in NK cell lymphoma. The following abnormalities could be identified by the two-tube, eight-color MFC panel: cκ/cλ/CD19/CD5/CD20/CD38/CD45/CD56 (adding CD10 and CD34 as the ninth and tenth colors) and $CD16^+$CD56/CD5/CD3/CD7/CD4/CD8/CD2/CD45 (adding CD117 as the ninth color).

Öz

Amaç: Allojeneik hematopoetik kök hücre nakli (allo-KHN) sonrası minimal kalıntı hastalık (MKH) analizinde anormal immünofenotiplendirme sıklıkla sekonder rekürrensin ya da komplikasyonların kanıtıdır, neticede fazla tedaviye yol açar. Bu tarz fenotipik anormalliklerin çok renkli akım sitometri (ASM) kullanarak verici kökenli olabileceğini göstermek ve bunu doğrulamayı hedefledik.

Gereç ve Yöntemler: Allo-KHN olmuş 3395 hastanın kemik iliği örneklerinde konvansiyonel iki tüp sekiz renkli ASM paneli ile MKH bakıldı. Anormal immünfenotiplenmenin sıklıkları da malignitesi olmayan üç grup hastada değerlendirildi.

Bulgular: Allo-KHN olan hastalarda yeni anormal polimorfizmlerin sıklığı %0,088 (3/3395) idi. Tam remisyondaki üç hastada mevcut anormal hücreler sırasıyla Fcγ reseptörü IIIB (FcγRIIIB) gen delesyonu ($CD16^-$ nötrofil), $CD2^-CD159a^-CD159c^+$ doğal öldürücü (NK) hücreleri ve monoclonal B lenfositoz (MBL) idi. Ilaveten, bir vericide allo-KHN öncesinde anormal T-hücreleri ($CD4^+CD8^+$) tespit edilmişti. Benzer anormallikler üç hastanın vericilerinin periferik kanlarında ASM ile bulunmuştur. Malignitesi olmayan bireylerde FcγRIIIB delesyonu insidansı %0,2 (11/5256), CD2 si olmayan NK hücreleri ve tek CD159c pozitif olanlar %0,05 (1/2000), monoclonal CD4/CD8 çift pozitif T-hücreleri %0,05 (1/2000), MBL %1,3 (14/1100) bulundu. NK hücreli lenfomada CD2 negatif NK hücreleri sıklığı %1,3 (1/79) ve $CD8^{dim}$ olanlar %14 (11/79) idi. Sıradaki anormallikler çift tüp sekiz renkli ASM paneli ile tanımlanmıştır: cκ/cλ/CD19/CD5/CD20/CD38/CD45/CD56 (CD10 ve CD34 dokuzuncu ve onuncu renk olarak eklenmiştir) ve $CD16^+$CD56/CD5/CD3/CD7/CD4/CD8/CD2/CD45(CD117 dokuzuncu renk olarak eklenmiştir).

Sonuç: Allo-KHN alıcılarında MKH ile tespit edilen anormallikler vericilerinden kaynaklanabilir. Verici örneklerinin çift tüp sekiz-on

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Abstract

Conclusion: Abnormalities in recipients of allo-HSCT detected by MRD analysis may originate from their donors. Screening of donor specimens with a suitable two-tube, eight- to ten-color MFC panel may be a promising method for minimizing misdiagnoses.

Keywords: Multicolor flow cytometry, Allogeneic hematopoietic stem cell transplantation, FcγRIIIB deletion, Monoclonal B lymphocytosis, Monoclonal $CD4^+CD8^+$ T cells, $CD2^-CD159c^+$ natural killer cells

Öz

renkli ASM paneli ile taranması yanlış tanıları en aza indirmek için ümit veren bir yöntemdir.

Anahtar Sözcükler: Çok renkli akım sitometri, Allojeneik hematopoetik kök hücre nakli, FcγRIIIB delesyonu, Monoklonal B lenfositoz, Monoklonal $CD4^+CD8^+$ T-hücreleri, $CD2^-CD159c^+$ Doğal öldürücü hücreler

Introduction

When abnormal cells associated with the primary disease are observed in recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT), the first consideration is minimal residual disease (MRD) or relapse, followed by the possibility of a second tumor or post-transplant lymphoproliferative disorder (PTLD) [1,2,3,4]. In rare cases, a benign phenotypic polymorphism of the donor may be the cause [5]. The effect of abnormal cells from donors on the health of recipients is unfavorable. For example, leukemia was previously discovered in donors [1,2,3,4]. The most important consequence is that it may result in misdiagnosis as it is challenging to distinguish monoclonal B lymphocytosis (MBL) from early-phase PTLD in patients after allo-HSCT. The detection of abnormal cells in the donor is therefore of significant importance. Multicolor flow cytometry (MFC) has been adopted as an effective method for the detection of MRD with detection sensitivity of 85% [6,7]. To minimize the effect of the donor on the prognosis of the patient, the screening of donor blood specimens by MFC before allo-HSCT may be an applicable and highly cost-efficient method [8].

The aim of this study was to improve our understanding of the importance of the detection of abnormal cells in donor specimens by MFC to reduce the probability of the misdiagnosis of various abnormalities detected in post-transplant patients.

Materials and Methods

Patients

This study was approved by the Hebei Yanda Lu Daopei Hospital Ethics Committee and informed consent was obtained from all participants before any study procedures were conducted. It was organized as a retrospective review study and Figure 1 illustrates the research design.

A total of 3395 patients who had received allo-HSCT from January 2013 to December 2019 in this hospital were identified. The MRD of these patients was analyzed by two-tube, eight-color MFC panel.

Three groups of patients who underwent bone marrow (BM) or peripheral blood (PB) immunophenotyping for non-malignant diseases at Hebei Yanda Lu Daopei Hospital were also identified. The first group included 5256 participants and the presence of CD16 expression in granulocytes was determined. The second group included 2000 participants who were screened for natural killer (NK) or T-cells for further immunophenotype analysis of NK lymphoma. The third group included 1100 participants over 35 years of age with non-neoplastic diseases who underwent screening of plasma and B cells.

Multicolor Flow Cytometry

The donor's PB and the MRD of the recipient's BM were evaluated by MFC. The panel, sources of antibodies, and other flow cytometric parameters used in our study are shown in Table 1. MRD and lymphocytes of the samples were analyzed with an eight-color immunophenotyping panel using a BD FACSCanto II cytometer (Becton Dickinson, San Jose, CA, USA), and a four-color immunophenotyping panel (CD16/CD13/CD45/CD11b) was used to screen the neutrophils of samples with a BD FACSCalibur cytometer (Becton Dickinson). The instrument set-up and compensation matrix were established using CS&T and FACS CompBeads according to the manufacturer's recommendations (Becton Dickinson). Samples were processed using standard lyse/wash surface or surface/cytoplasm staining with FACS Lysing Solution or Fix/Perm Kits according to the manufacturer's protocol (Becton Dickinson).

Other Laboratory Examinations

All patients with acute leukemia (AL) underwent BM examinations at the time of initial diagnosis, including evaluations of morphology, chromosome G banding, 33 fusion genes commonly seen in AL as determined by polymerase chain reaction (PCR), and 58 gene mutations commonly seen in AL as determined by next-generation sequencing. All recipients of allo-HSCT underwent regular BM examinations for morphology, chromosome G banding, donor and recipient chimerism rates, and positive fusion genes by PCR. All BM samples from donors were examined for morphology and chromosome G banding before allo-HSCT. PB samples from recipients and donors underwent PCR screening for the detection of Epstein-Barr virus (EBV) and cytomegalovirus (CMV).

Table 1. Combinations of antibodies and fluorochromes used in the panel.								
NK/T-lymphocyte screening panel								
Fluorescein	FITC	PE	PerCp-Cy5.5	PE-CY7	APC	APC-CY7	BV421	V500
Antigen	CD2	CD117 or CD5	CD3	CD4	CD56	CD8	CD7#	CD45
Clone no.	S5.2	104D2/L17F12	SK7	SK3	NCAM16.2	SK1	M-T701	2D1
Cat. no.	652817	652806/652832	340949	663493	663503	663521	562635	662912
B-lymphocyte or plasma cell screening panel								
Fluorescein	FITC	PE	PerCp-Cy5.5	PE-CY7	APC	APC-CY7	BV421	V500
Antigen	cκ*	cλ*	/	CD19	CD38	CD20	CD56	CD45
Clone no.	Rb Fab'2	Rb Fab'2		SJ25C1	HB7	L27	NCAM16.2	2D1
Cat. no.	F043401	R043701		341113	345807	335829	664148	662912
Neutrophil screening panel								
Fluorescein	FITC	PE	PerCp-Cy5.5	APC				
Antigen	CD16#	CD13	CD45	CD11b				
Clone no.	3G8	L138	2D1	D12				
Cat. no.	556618	652820	663499	652819				
MRD panel for B-ALL								
Fluorescein	FITC	PE	PerCp-Cy5.5	PE-CY7	APC	APC-CY7	BV421	V500
Antigen	CD38	CD10	CD34	CD19	CD13#+CD33	CD20	/	CD45
Clone no.	HB7	HI10a	8G12	SJ25C1	P67.6/WM15	L27		2D1
Cat. no.	340909	662854	347203	341113	652807/557454	335829		662912
Antigen	TdT*	CD10	CD34	CD19	cCD79a	/	/	CD45
Clone no.	HT-6	HI10a	8G12	SJ25C1	HM47			2D1
Cat. no.	F713950	662854	347203	341113	340578			662912
MRD panel for T-ALL								
Fluorescein	FITC	PE	PerCp-Cy5.5	PE-CY7	APC	APC-CY7	BV421	V500
Antigen	TdT*	CD34	CD3	CD4	CD5	CD8	CD7#	CD45
Clone no.	HT-6	8G12	SK7	SK3	L17F12	SK1	M-T701	2D1
Cat. no.	F713950	652802	340949	663493	652840	663521	562635	662912
Antigen	CD99#	CD1a	CD45	/	CD2	/	/	/
Clone no.	TÜ12	SK9	2D1		S5.2			
Cat. no.	555688	652818	663499		341024			
Antigen	CD16#	cCD3#	CD3	/	CD56	/	/	/
Clone no.	3G8	OKT3	SK7		NCAM16.2			
Cat. no.	556618	566683	340949		663503			

*: Antibodies were purchased from Dako (Agilent, Santa Clara, CA, USA); #: antibodies were purchased from Becton Dickinson Pharmingen (Becton Dickinson, San Jose, CA, USA). All other antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The minimal residual disease and lymphocytes of the samples were analyzed with an eight-color immunophenotyping panel using a FACSCanto II cytometer (Becton Dickinson) and a four-color immunophenotyping panel was used to screen the neutrophils of the samples using a FACSCalibur cytometer (Becton Dickinson). FACSDiva, CellQuest, and Kaluza were used to analyze the results. MRD: Minimal residual disease; B-ALL: B-cell acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia.

Results

Prevalence of Abnormal Immunophenotyping of Allo-HSCT Recipients Originating from Donors

Among the 3395 patients who received allo-HSCT, three engrafted patients had gained new abnormal cells of the same lineage as the primary tumor but of a different nature at the first or fourth month after the transplant. Detailed information about these three recipients with abnormal cells and four donors is provided in Table 2 and in the following text.

One patient with myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN) progressing to acute myeloid leukemia (AML, Case 1, Table 2) received allo-HSCT from her mother

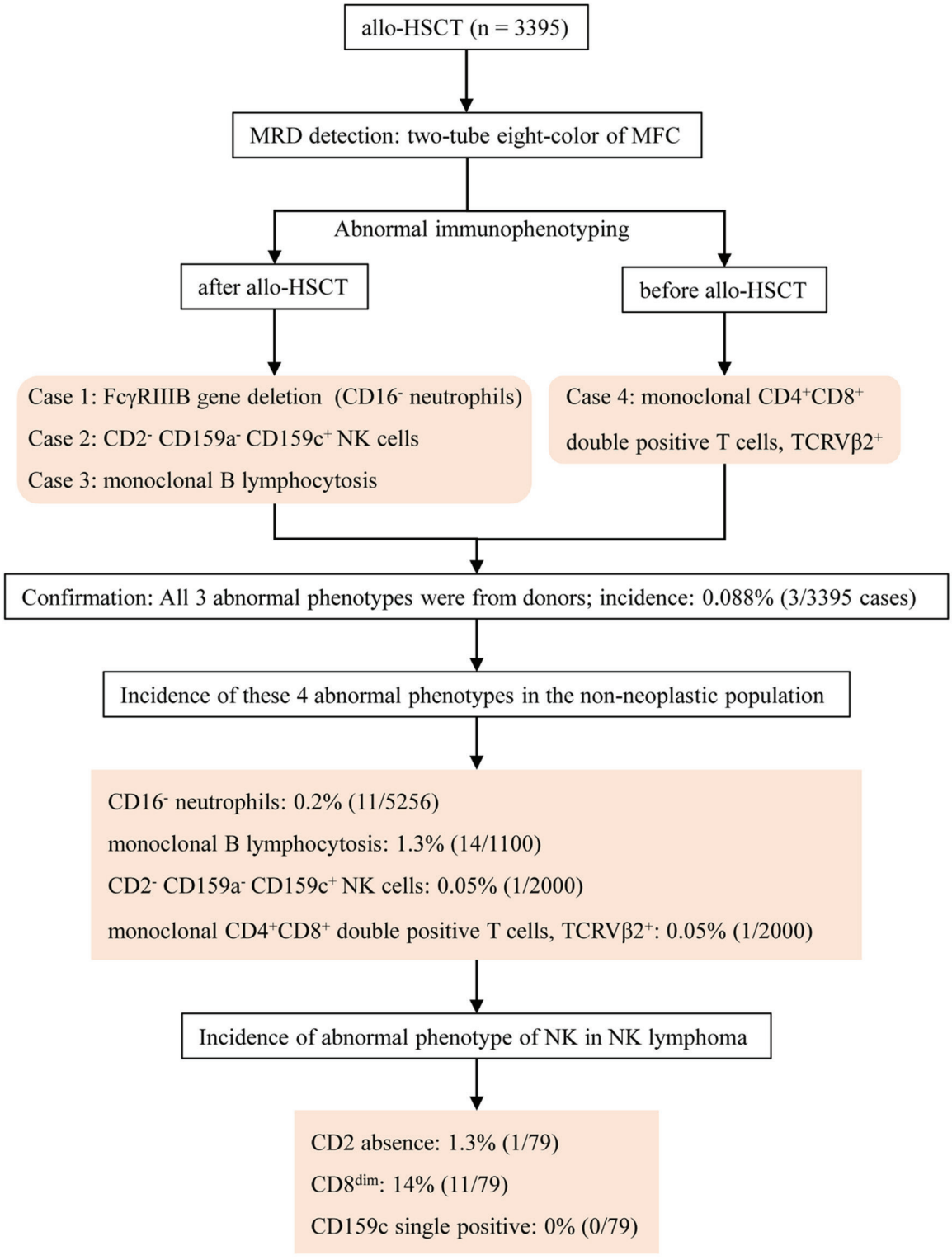

Figure 1. Flow chart of experimental design.

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; MRD: minimal residual disease; MFC: multicolor flow cytometry; NK: natural killer.

in September 2014 with a haploidentical donor type. Before the allo-HSCT, the MFC results of BM MRD showed that the granulocytic development pattern was abnormal, but CD16 was still partially expressed (Figure 2a). However, 1 month after allo-HSCT, CD16 was completely absent in granulocytes but expressed in NK cells. The dot plots of CD13/CD11b showed normal maturational patterns as $CD13^{+}CD11b^{+}$ myelocytes made up 65% of the granulocytes (Figure 2b). No abnormalities were found in the other myeloid blasts or cells in the developmental stage. Morphological and chromosomal tests

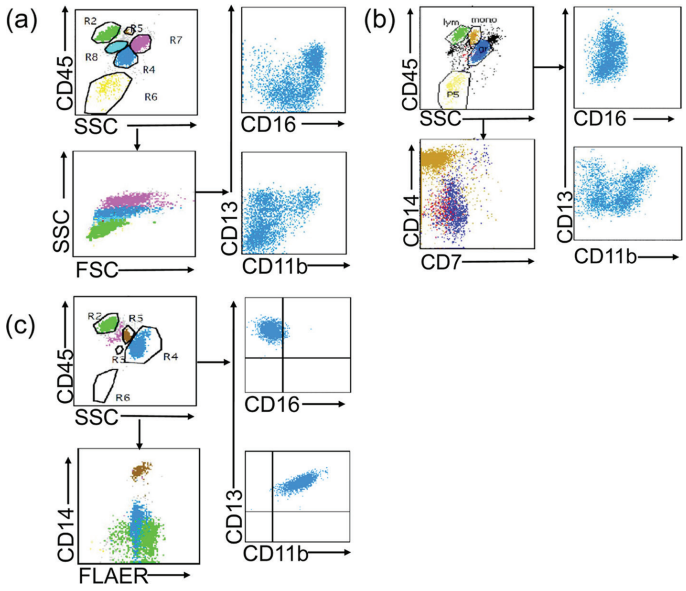

Figure 2. CD16 expression in the bone marrow (BM) of a patient with myelodysplastic syndrome/myeloproliferative neoplasms progressing to acute myeloid leukemia (AML) (Case 1) and the peripheral blood (PB) of the donor. Green, dark blue, pink, and light blue dots represent lymphocytes, granulocytes in the differentiation phase, eosinophils, and basophilic granulocytes, respectively. (a) BM immunophenotyping of the patient with AML before allogeneic hematopoietic stem cell transplantation (allo-HSCT) and (b) after allo-HSCT. (c) Immunophenotyping of the donor's PB.

BM: Bone marrow; MDS/MPN: myelodysplastic syndrome/myeloproliferative-neoplasms; AML: acute myeloid leukemia; allo-HSCT: allogeneic hematopoietic stem cell transplantation; PB: peripheral blood.

indicated complete remission (CR) and the donor chimerism was 100%. The glycosylphosphatidylinositol-related markers CD14, CD55, and CD59 were normally expressed. Subsequently, the PB of the donor was evaluated in the fifth month after allo-HSCT. In parallel, CD16 was expressed in NK cells but not in granulocytes, CD14 and fluorescein-labeled proaerolysin (FLAER) were normally expressed, and the positive expression of $CD13^{+}CD11b^{+}$ suggested that the granulocytes were in the mature stage (Figure 2c). Therefore, considering that this patient that received allo-HSCT and the donor had similar abnormal CD16 expression in granulocytes, and after excluding MDS and paroxysmal nocturnal hemoglobinuria (PNH), we attributed the abnormal CD16 expression in the granulocytes of the patient to the donor and postulated that the donor could have the previously reported FcγRIIIB gene deletion.

In the patient with T-cell acute lymphoblastic leukemia (T-ALL, Case 2, Table 2), before allo-HSCT, BM MRD detection indicated CR status, and CD2 was expressed on NK cells while malignant naive cells were not observed (Figure 3a). This patient had received allo-HSCT from his older sister in May 2015 and the donor chimerism was 100%. In the first 10 months following allo-HSCT, no malignant naive cells were observed. However, 10

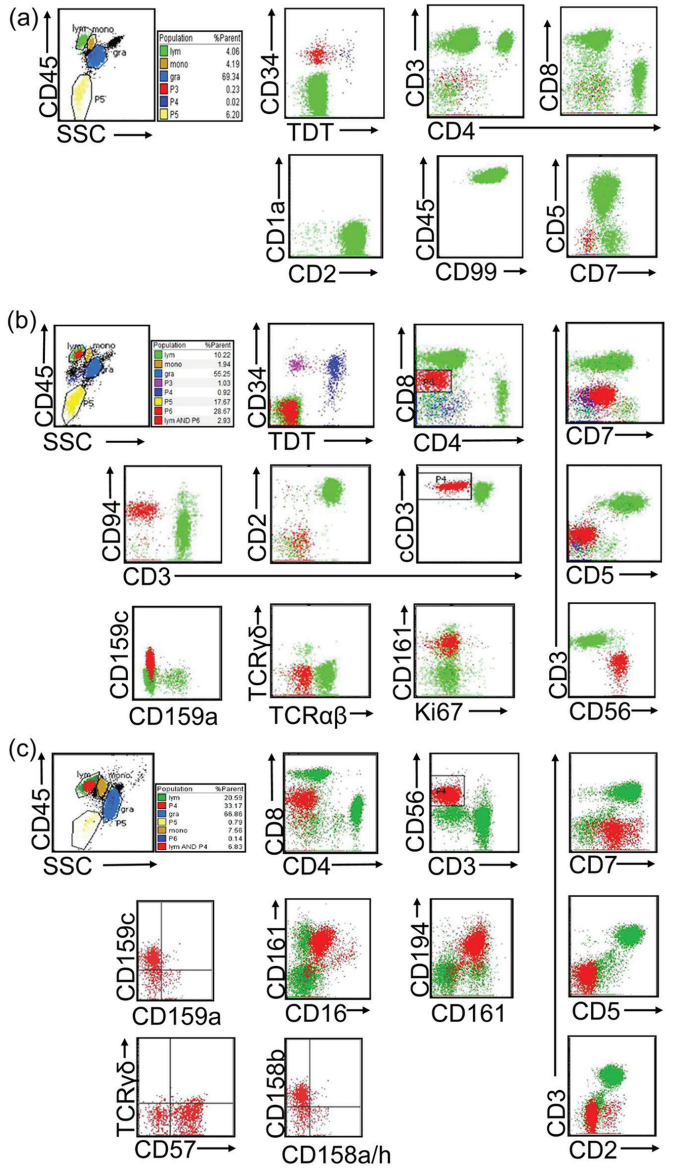

Figure 3. Immunophenotyping of abnormal natural killer (NK) cells in the bone marrow (BM) of a patient with T-cell acute lymphoblastic leukemia (T-ALL) (Case 2) and the donor's peripheral blood (PB). Green and red dots represent mature lymphocytes and abnormal NK cells, respectively. (a) BM immunophenotyping of the patient with T-ALL before allogeneic hematopoietic stem cell transplantation (allo-HSCT) and (b) after allo-HSCT. (c) Immunophenotyping of the donor's PB.

BM: Bone marrow; PB: peripheral blood; AML: acute myeloid leukemia; allo-HSCT: allogeneic hematopoietic stem cell transplantation.

months after allo-HSCT, abnormal NK cells were detected in the BM MRD of the patient by MFC. The abnormal NK cells accounted for 2%-4% of nucleated cells (20%-30% of lymphocytes) with positive expression of $CD8^{dim}$, cCD3, $CD7^{dim}$, CD16, CD56, CD161, and CD94 and negative expression of CD159c, CD2, CD5, CD3, CD4, TCRαβ, TCRγδ, ki67, TdT, CD34, CD1a, and CD159a (Figure 3b). At 18 months after allo-HSCT, the PB immunophenotype of the donor was determined and a similar abnormal NK cell

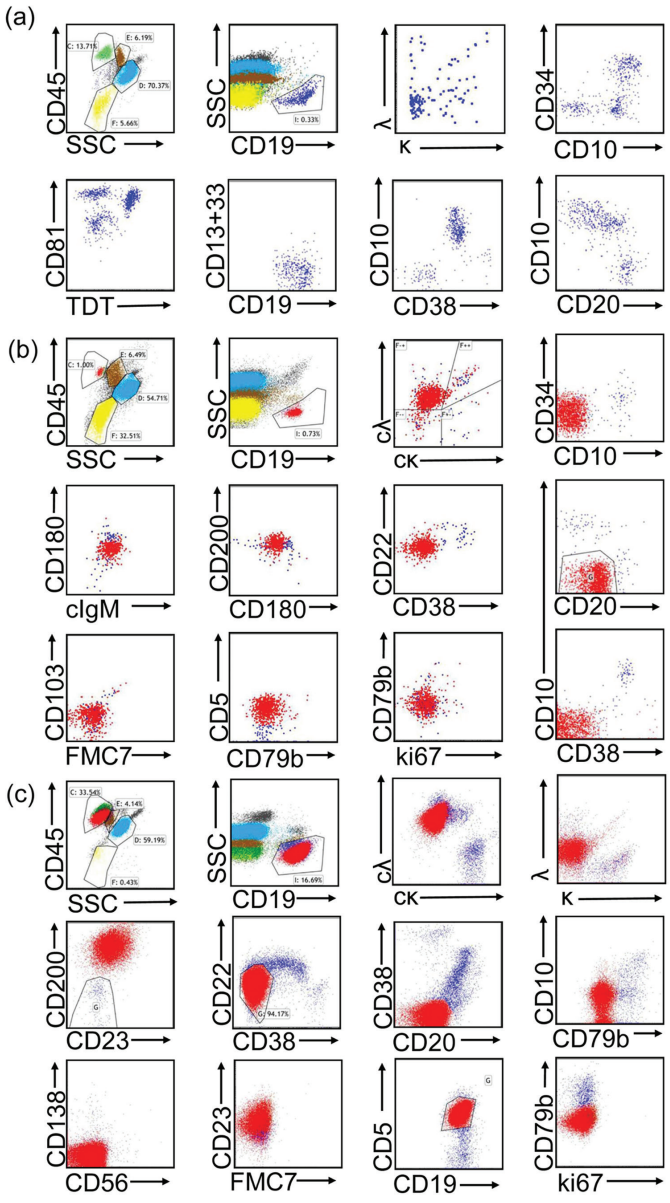

Figure 4. Immunophenotyping of the bone marrow (BM) of a patient with monoclonal B lymphocytosis (MBL) (Case 3) and the donor's peripheral blood (PB). Red, dark blue, light blue, brown, and green dots represent monoclonal B cells, normal B cells, granulocytes, monocytes, and other lymphocytes, respectively. (a) BM immunophenotyping of the patient with MBL before allogeneic hematopoietic stem cell transplantation (allo-HSCT) and (b) after allo-HSCT. (c) Immunophenotyping of the donor's PB.

MBL: Monoclonal B lymphocytosis; BM: bone marrow; allo-HSCT: allogeneic hematopoietic stem cell transplantation.

phenotype was observed by MFC, which accounted for 6.83% of nucleated cells and 33.17% of lymphocytes (Figure 3c). Therefore, given that both the recipient and donor had similar abnormal NK cell phenotypes, we concluded that the abnormal NK cell phenotype in the patient who was diagnosed with T-ALL originated from the donor.

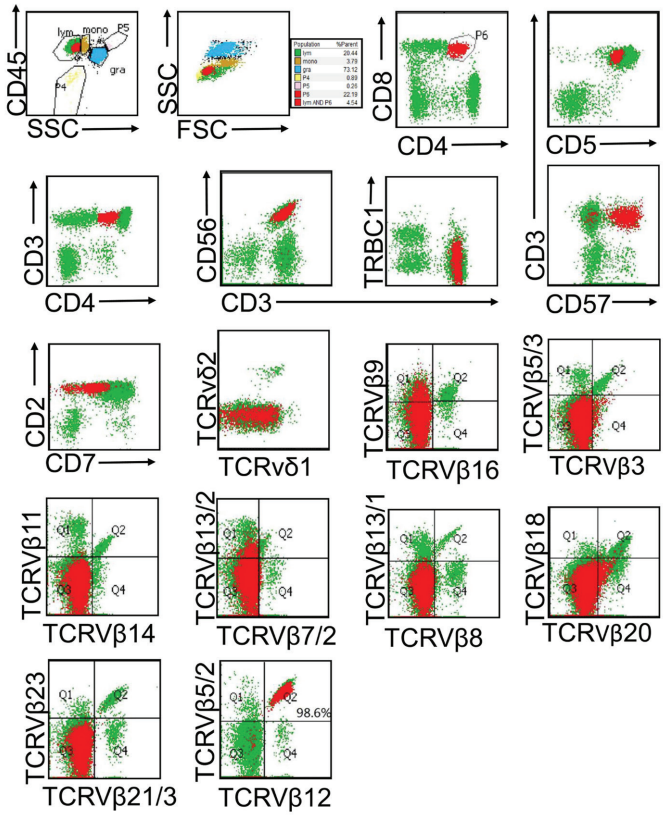

Figure 5. Immunophenotyping of abnormal T-cells from the fourth donor (Case 4). Red, blue, brown, and green dots represent abnormal T-cells, granulocytes, monocytes, and other lymphocytes, respectively.

The BM MRD of the patient with B-cell acute lymphoblastic leukemia (B-ALL, Case 3, Table 2) was normal before allo-HSCT, with predominantly pro-B and few mature B cells, and no monoclonal B cells (Figure 4a). This patient had received haploidentical allo-HSCT from his father in April 2018. At the first BM MRD examination by MFC performed for the recipient 1 month after allo-HSCT, MBL was observed. Mature B cells were prominent, being positive for CD19, CD20^{dim}, CD81^{dim}, cCD79a, cλ, CD22^{dim}, CD180, and CD200 and negative for CD38, CD10, CD34, CD13^{+}33, TdT, CD138, Ki67, CD79b^{dim}, CD5, FMC7, CD103, CD25, CD9, CD97, CD11c, cIgM, and cκ (Figure 4b). Monoclonal B cells accounted for 0.72% of the nucleated cells and no malignant naive B cells were observed. At 2 months after allo-HSCT, the donor's PB was analyzed and the same monoclonal B cell phenotype was revealed (Figure 4c). Consequently, we concluded that the MBL phenotype in the patient was attributable to the donor.

Abnormal T-cells were also discovered in the PB of one donor (Case 4, Table 2) before allo-HSCT. These T-cells were positive for CD3, CD8, CD4^{dim}, CD5, CD2, CD7^{dim}, TRBC1, CD57, and monoclonal TCRVβ2 (98.6%) and negative for CD56, TCRvδ1, TCRvδ2, and other 23 TCRVβ repertoire subsets, accounting for 4.54% of the nucleated cells (Figure 5).

Table 2. Clinical information of the patients and corresponding donors.

Clinical information of the patients					Clinical information of the donors				Abnormal immunophenotyping
Case no.	Age	Sex	Diagnosis	Time to discovery of abnormal cells, months after allo-HSCT	Relationship between donor and recipient	Age	Sex	Detection time, months after allo-HSCT	
1	15	F	MDS/MPN progressing to AML	1	Mother and daughter	41	F	5	$CD16^-$ neutrophils
2	25	M	T-ALL	4	Older sister and brother	29	F	18	$CD2^-CD159a^-CD159c^+$ NK cells
3	36	M	B-ALL	1.5	Father and son	60	M	2	Monoclonal B lymphocytosis (MBL)
4	10	M	Aplastic anemia	Before allo-HSCT	Mother and son	36	F	Before allo-HSCT	Monoclonal $CD4^+CD8^+$ double-positive T-cells, $TCRV\beta2^+$

Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; MDS/MPN: myelodysplastic syndrome/myeloproliferative neoplasms; AML: acute myeloid leukemia; T-ALL: T-cell acute lymphoblastic leukemia; B-ALL: B-cell acute lymphoblastic leukemia.

Prevalence of Same Abnormal Immunophenotypes in Patients Without Malignancies

The incidence of the above three abnormal immunophenotypes or polymorphisms derived from donors accounted for 0.088% (3/3395 cases) of the allo-HSCT cases at Hebei Yanda Lu Daopei Hospital. To evaluate the frequency of abnormal phenotypic cells or polymorphisms in donors without malignancies, we examined three groups of individuals who underwent BM or PB immunophenotyping for non-malignant diseases in the same hospital.

In the first group (n=5256), after excluding MDS and PNH, 11/5256 (0.2%) samples had CD16-negative granulocytes, which were diagnosed with FcγRIIIB gene deletion. The clinical diagnoses of these 11 patients included aplastic anemia in four cases, leukopenia in three cases, and idiopathic eosinophilia, nutritional anemia, and idiopathic thrombocytopenia in one case each. The remaining sample was from a healthy individual (Case 1, Table 2).

In the second group (n=2000) with NK/T-cell immunotyping, aplastic anemia was identified in an individual with CD159c single-positive NK cells, and Case 2 was accompanied by CD2 loss (0.05%, 1/2000). Moreover, although healthy participants may present with small numbers of $CD4^+CD8^+$ T-cells, only the donor of Case 4 presented with more than 1% (0.05%, 1/2000), and the $CD4^+CD8^+$ T-cells were identified as monoclonal T-cells based on detection of the expression of the TCRVβ repertoire (TRBC1 and TCRVβ2). Two donors with abnormal NK and monoclonal T-cells were positive for EBV and CMV.

In the third group (n=1100) of non-clinical lymphoma and/or plasma cell tumors, the MFC screening results for monoclonal B and plasma cells revealed 14 cases (1.3%, 14/1100) of MBL.

Finally, a two-tube, eight-color panel was applied and successfully evaluated these abnormalities, including cκ/cλ/CD19/CD5/CD20/CD38/CD45/CD56 (with optional addition of CD10 and CD34 for a nine- or ten-color panel) and $CD16^+$CD56/CD5/CD3/CD7/CD4/CD8/CD2/CD45 (with optional addition of CD117 for a nine-color panel).

Incidence of Abnormal NK Cell Immunophenotypes in NK Lymphoma

In Case 1, the immunophenotype was similar to that of NK lymphoma. However, because CD159c NK lymphoma is very rare, the phenotype of 79 patients with NK lymphoma in the second group (n=2000) was considered. The results are shown in Table 3 and the incidence of CD2 absence was 1.3% (1/79), $CD8^{dim}$ was 14% (11/79), and homogeneous CD159c positivity was 0% (0/79).

Discussion

Prevalence of Abnormal Immunophenotypes of Allo-HSCT Recipients Derived from Donors

At present, it is unknown whether donor-derived abnormal cells have an impact on the health of the donor and the recipient. Importantly, however, donor-derived abnormal cells may result in misdiagnoses for the recipients after allo-HSCT. For example, as revealed by Case 1 in this study, MDS entails not only the abnormal proliferation of blasT-cells, but also the abnormal development of granulocytes, and in the chimeric state there is abnormal CD16/CD13 developmental patterns. In Case 2 ($CD2^-$ $CD159a^-CD159c^+$ NK cells), without further patient information, we could assume that the patient was misdiagnosed or that there were secondary tumor changes. In Case 3, the emergence of monoclonal B cells after allo-HSCT could have been misdiagnosed as PTLD; however, donor-derived MBL was confirmed. In this study, a two-tube, eight-color MFC panel protocol was applied, which covered the four observed abnormalities, and three of the four (0.088%, 3/3395) abnormalities seen in these allo-HSCT recipients may have originated from the donors. The fourth may

Table 3. Immunophenotyping of 79 cases of natural killer (NK) lymphoma.

	Case number	Negative	Positive	Strongly positive	Dimly positive	Positivity rate (%)	Frequency of abnormal expression (%)
CD2	77	1	76			98.70	1.30
CD5	79	76	3			3.80	3.80
CD7	79	14	65		12	82.28	32.91
CD8	79	67	12	1	11	15.19	100
CD56	79	6	73	18		92.40	30.38
CD57	53	48	5			9.43	100
CD16	71	45	26			36.62	63.38
CD161	78	30	48			61.54	38.46
CD94	79	6	73	21		92.41	34.18
Ki67	69	34	35			50.72	50.72
CD30	57	45	12			21.05	21.05
CD159a	39	5	34			87.18	100
CD159c*	39	33	6			15.38	100
CD158#	70	63	7			10.00	100

*: Six cases of CD159c positivity were all CD159a/CD159c double-positive. #: CD158 included CD158a/h, CD158b, and CD158e.

have been hereditary. Thus, screening the PB of donors using MFC before allo-HSCT may be a feasible and promising method to avoid the difficulties described here.

Although relevant research is seldom reported, donor-to-recipient transmission-induced abnormalities have been described [9], which supports our conclusion that donor-derived abnormal cells may cause misdiagnoses in recipients after allo-HSCT. For example, in an endocrinological study of 402 patients receiving allo-HSCT with 10 years of follow-up, it was suggested that misdiagnosed donor-derived abnormalities may be responsible for endocrine abnormalities observed in patients after allo-HSCT and that this possibility merits deeper evaluation [10]. Cook et al. demonstrated that donor-originated T-cells with human T-lymphotropic virus type 1 resulted in the infection of three allo-HSCT recipients [11]. Similarly, studies have shown that a small number of patients exhibit donor-derived T-cell leukemia/lymphoma after receiving allo-HSCT from donors positive for human T-cell lymphotropic virus type 1 [12,13,14]. Moreover, memory-like NK cells could also be transplanted from donor to recipient, as evidenced by several studies [15,16]. According to a case report, donor-originated PTLD could have been transmitted to the haploidentical HSCT recipient; although the donor was hematologically normal at the time of donation, diffuse large B-cell lymphoma developed 380 days after donation [17]. In short, these previous studies all support our conclusion that abnormal phenotypes in healthy donor populations can be a source of risk for abnormal phenotypes in allo-HSCT recipients. Although this risk may be small, it could still lead to abnormalities in allo-HSCT recipients.

It is worth noting that although the four abnormalities detected by MFC in the donors of this study before allo-HSCT have very low rates, identifying them will not only reduce the misdiagnosis of MRD among recipients but can also prevent misdiagnosis and secondary tumors in the absence of patient immunophenotyping information. To determine whether these four abnormalities have an impact on patient outcomes, long-term observation is required, but in the meantime, the misdiagnosis of these abnormalities as other abnormalities leading to overtreatment should be avoided. It is hoped that this study will encourage clinicians to consider donor abnormalities.

Prevalence of the Same Abnormal Immunophenotypes in Patients Without Malignancies

To further assess the necessity and feasibility of screening donor specimens with the MFC panel, we conducted a related study of patients with non-neoplastic diseases. In that population, the incidence of FcγRIIIB gene deletion was 0.2% (11/5256) as determined by the detection of CD16/CD13/CD11b expression on neutrophils in the BM. Similar to the incidence rate obtained in this work, a previous study showed that the frequency of FcγRIIIB gene deletion in Caucasians was 0.1% and that 3% of those cases were heterozygous [18,19]. The CD16 antigen is human Fc receptor III, which is encoded by Fcγ receptor IIIA (FcγRIIIA) and FcγRIIIB [18,19,20,21]. FcγRIIIA is primarily expressed in NK cells and macrophages [21], and FcγRIIIB is expressed in granulocytes and normally expressed in NK cells [18]. Therefore, we characterized FcγRIIIB gene deletion by the complete loss of CD16 expression in granulocytes and normal expression in NK cells. This incidence of FcγRIIIB gene deletion

in individuals without tumors demonstrates the effectiveness of MFC in screening donor specimens.

MBL incidence increases with age and occurs more often in Western countries. It was reported that the overall prevalence of MBL in people over the age of 45 years is 7.1% [22,23] and a rate as high as 14% was reported in Uganda [24]. Unusually, according to the 2008 World Health Organization criteria, the incidence of MBL was 64% higher in Alberta, Canada, compared to rates obtained with previous criteria [25]. Using the 2008 IWCLL criteria, the age- and sex-adjusted incidence of MBL was 3.5 per 100,000 in Minnesota [26]. There was no reported incidence of MBL in China before the present study and we have determined MBL incidence of 1.3% (14/1100) in people over 35 years of age. This large variation may be related to the geographical regions in which the research was performed and the testing methods. Many patients with MBL are diagnosed incidentally and these patients do not require deliberate treatment, which reduces the statistical incidence to a large extent. This also suggests that there is still much room for revisions in the current epidemiological understanding The low incidence rate of MBL among patients without tumors in this study not only demonstrates the effectiveness of MFC in screening donor specimens; it also suggests the necessity of donor testing before allo-HSCT to exclude abnormal phenotypes.

Normal NK cells express CD7, CD161, and CD94 and do not express CD4, CD3, CD5, TCR$\alpha\beta$, or TCR$\gamma\delta$. At the same time, NK cells may demonstrate low expression of CD8 and cCD3, and the CD159a, CD159c, and CD158 series (generally represented by CD158a, CD158b, and CD158e) is distributed in some subsets [27,28,29,30]. CD2 was partially expressed in adult NK cells (76.97$\pm$12.89%) in previous research and higher values were found for children and adolescents [27,28]. Reactive NK cells had high expression of CD5 and cCD3, low expression of CD2, and upregulation of CD159a or CD159c, but the complete loss of CD2 in NK cells has not been previously reported [29]. In our study, the incidence of CD2 loss and single-positive CD159c in NK cells was less common at a rate of only 1.3% (1/79). T lymphocytes show various immunophenotypes that are stimulated by certain autoimmune diseases or infections, but it is rare to find monoclonal CD4/CD8 double-positive T-cells accounting for higher percentages such as 4.54% [31,32,33]. In our study, the abnormalities observed in lymphoma were plausible, but none of the 79 patients displayed CD159c. Therefore, caution should be exercised when there is a small probability of occurrence.

Conclusion

The two-tube, eight-color MFC panel could detect the abnormalities in the cases evaluated in this study. Therefore, this MFC panel may be a feasible and promising method for screening for abnormalities in donor blood samples before allo-HSCT.

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Authorship Contributions

Surgical and Medical Practices: A.W., M.C., Data Collection or Processing: M.G., X.W., J.Z., Y.L.; Analysis or Interpretation: M.G., X.W., J.Z., Y.L.; Literature Search: H.W., M.C., M.G., X.W., J.Z., Y.L.; Writing: H.W. A.W., M.C.

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