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CD56-enriched donor cell infusion after post-transplantation cyclophosphamide for haploidentical transplantation of advanced myeloid malignancies is associated with prompt reconstitution of mature natural killer cells and regulatory T cells with reduced incidence of acute graft versus host disease: A pilot study

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Abstract

We conducted a pilot study on the feasibility of CD56-enriched donor cell infusion after post-transplantation cyclophosphamide (PTCy) for 10 patients with advanced myeloid malignancies undergoing haploidentical peripheral blood stem cell transplantation with cyclosporine alone as graft-versus-host disease (GVHD) prophylaxis and compared the outcome and immune reconstitution with a control group of 20 patients undergoing the same without CD56-enriched donor cell infusion. An early and rapid surge of mature NK cells as well as $CD4^+$ T cells and regulatory T cells (Tregs) was noted compared with the control group. KIR of donor phenotype reconstituted as early as day 30 with expression of $CD56^{dim}CD16^+NKG2A^-KIR^+$ phenotype. None experienced viral or fungal infections, and non-relapse mortality was 10% only. The incidence of grade 2–4 acute GVHD was 50% in the control group with none in the CD56 group ($P = 0.01$). Only two had *de novo* chronic GVHD in each group. Relapse occurred in five patients in CD56 group with a median follow-up of 12 months, similar to the control group. Our preliminary data show that $CD56^+$ donor cell infusion after PTCy and short-course cyclosporine is feasible with prompt engraftment, rapid reconstitution of $CD4^+$T cells, Tregs and NK cells and reduced incidence of acute GVHD.

Key Words: *acute myeloid leukemia, AML, donor lymphocyte infusion, DLI, haploidentical, NK cell, post-transplantation cyclophosphamide*

Introduction

Post-transplantation cyclophosphamide (PTCy) has greatly broadened the scope of haploidentical hematopoietic stem cell transplantation (HSCT) across the entire spectrum of hematological malignancies [1]. The hallmark of this approach has been a low incidence of severe acute and chronic graft-versus-host disease (GVHD) despite the use of unmanipulated grafts either from bone marrow or mobilized peripheral blood stem cells (PBSCs) [2]. On the other hand, relapse has been extremely high in patients with advanced disease [3]. This could be partly attributable to the reduced-intensity conditioning employed in these studies. Employment of myeloablative conditioning (MAC) resulted in reduction of relapse in those with advanced leukemia across all age groups [4–6]. A limitation, however, is the inability to use MAC in all such patients, due either to age or comorbidities.

We demonstrated in an earlier study that the use of early donor lymphocyte infusion (DLI) after MAC, but not reduced-intensity conditioning, improves progression-free survival in patients with refractory/relapsed acute myeloid leukemia [7]. In this study, patients who did not receive DLI, a natural killer (NK) cell alloreactive donor, had a reduced incidence of relapse [7].

On the basis of these findings from our previous study, we hypothesized that infusion of NK cells following less intensive conditioning might augment the

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graft-versus-leukemia (GVL) effect and reduce GVHD when cyclosporine alone is used as GVHD prophylaxis after PTCy. To exploit the GVL effect of any form of immunotherapy, the optimal window is probably in the aplastic phase. In a preclinical model, it was demonstrated that the timing of infusion of NK cells was critical for both reduction of GVHD and tumor progression [8]. Although infusion of NK cells with a T-cell deplete graft reduced GVHD after DLI on day 4, NK-cell infusion with or before the graft was not beneficial after a T-cell-replete graft. The anti-GVHD and anti-GVL effect were both maximized if NK cells were infused on day 5. When haploidentical NK-cell infusion was carried out after low-dose cyclophosphamide (CY) for relapsed acute myeloid leukemia in a non-transplant setting, the NK cells did not proliferate or survive in the host [9]. However, the same after high-dose CY resulted in marked rise in endogenous interleukin-15 and expansion of donor NK cells. Furthermore, calcineurin inhibitors promote proliferation and cytotoxicity of NK cells compared with mycophenolate and rapamycin [10]. Thus, we designed the study to infuse NK cells 72 h after PTCy and initiation of cyclosporine A (CSA). In addition, we did not encounter an increase in early GVHD in older children and adults after rapid tapering of mycophenolate mofetil (MMF) in our earlier study [7] and hence decided to omit MMF and use alone as GVHD prophylaxis in the present study.

Another major deviation from the previous studies employing NK-cell infusion has been the use of single-step positive selection rather than a two-step process in which CD3 cells are depleted. Only one study in the past had explored single-step CD56 enriched cell infusions following haploidentical HSCT, which showed an improvement in NK and T-cell recovery without an increase in GVHD [11]. We had hypothesized that $CD56^+CD3^+$ cells in the donor cell infusions might attenuate GVHD as described in preclinical models [12].

In the present report, we describe the preliminary outcome of this approach with respect to engraftment, GVHD, infections and immune reconstitution with particular emphasis on T-regulatory cells (Tregs) and KIR reconstitution of the NK-cell subsets vis-à-vis donor phenotype. This was compared with a control group receiving PTCy based haploidentical HSCT without infusion of $CD56^+$ cells. The clinical outcome of the control group has been reported earlier [7] and is cited for comparative purposes only. The early immune reconstitution of both groups was the major focus of this study.

Methods

In a pilot study, from March 2014 to February 2016, patients with refractory hematological malignancies between the ages of 2 and 65 years without a matched family donor were enrolled in this study if they possessed a haploidentical family donor and were not eligible for myeloablative conditioning. "Refractory disease" was defined as blasts >5% at the time of HSCT. Written informed consent was obtained for all patients, and approval was obtained from Institute Review Committee in accordance with the Declaration of Helsinki.

Conditioning regimen and GVHD prophylaxis

The myeloablative conditioning protocol as described earlier for patients undergoing haploidentical HSCT in the control group was based on fludarabine, busulfan and melphalan [7]. Patients who were not deemed suitable for myeloablative conditioning were included in the study group (referred to as CD56 group). They were conditioned with treosulfan at 10 g/m^2 from day −6 to day −4 along with fludarabine 30 mg/m^2 from day −5 to day −2, and 2-Gy TBI was administered as a single dose at 8 AM on day 0. PTCy in all patients was administered 64 h after infusion of the graft at 50 mg/kg twice at 24-h intervals along with Mesna as described previously [6,13]. Another 24 h after completion of CY, intravenous CSA at 3 mg/kg in two divided doses was started. CSA doses were adjusted to maintain a trough level of 100 to 200 ng/mL and tapered over 4 weeks after day 60 in the absence of GVHD. MMF was not used in the CD56 group but was used in the control group at 15 mg/kg every 8 h from day 5 to day 21 as previously reported [7].

Filgrastim was not routinely used post-transplant, and its use was restricted to patients with life-threatening sepsis and those showing no sign of engraftment by day 14.

Stem cell source and harvest

Donors were treated with filgrastim 12 μg/kg/day in divided doses for 4 days before initiation of harvest on the fifth day. On an average, three times the blood volume was processed with an average yield of 200 mL of final PBSC products. The target dose of $CD34^+$ cells was $5–10 \times 10^6$/kg with the minimum cell dose required being 3×10^6/kg.

CD56-enriched donor lymphocyte infusions

Unstimulated mononuclear cells were collected from the donor on the morning of day 7. A total of 10–12 L of blood volume were processed to yield 50–100 mL of final product. The product was immediately transported to the Cell Processing Lab. Positive selection of $CD56^+$ cells was carried out on the CliniMACS^{plus} cell selection system as a single-step enrichment protocol [14]. The product was assessed

at each stage for CD56 and CD3 cells using a single as well as dual platform protocol as previously described. The target cell dose was a minimum of 1×10^6/kg CD56$^+$ cells and $<1 \times 10^6$/kg CD3$^+$CD56$^-$ cells. An aliquot of 3 mL from the final product was preserved for further studies. The final product was infused without dilution on the same day. Only pheniramine maleate was used as a premedication.

Supportive care

All patients were treated in protective isolation rooms provided with high-efficiency particle air filters. Antimicrobial prophylaxis was instituted as per the departmental guidelines. Cytomegalovirus (CMV) prophylaxis was guided by preemptive monitoring of viral CMV load by quantitative polymerase chain reaction (PCR) twice a week until day 100. Viral loads of CMV, Epstein-Barr virus and adenovirus were monitored twice weekly.

Acute GVHD was graded according to modified Glucksberg criteria [15], and chronic GVHD was scored on the basis of the National Institutes of Health global severity criteria [16].

Management of disease progression

In case of relapse after HSCT, CSA was withdrawn immediately. This was followed by conventional DLI, administered as per CD3$^+$ T cells/kg body weight of the patient at 5×10^6/kg every 2 weeks for 2 doses. If there was no response after 4 weeks, a second haploidentical HSCT was planned from a different donor if possible.

HLA typing, NK KIR haplotype assignment and B scores

Patients and donors were typed for alleles at HLA-A, B, C, DRB1, DRB3/4/5 and DQB1 by PCR amplification and oligonucleotide hybridization by molecular methods using commercial kits from Olerup, which achieved intermediate resolution. Both parents, if available, were typed for HLA haplotypes.

Patients and donors were genotyped for 17 NK KIR genes and KIR-HLA ligands at HLA-B and C loci by PCR amplification method using commercial kits from Olerup. B haplotype was defined as presence of at least one of defining loci: KIR2DL5, 2DS1, 2DS2, 2DS3, 2DS5 or 3DS1. Donors possessing the preceding criteria were designated as haplotype Bx. Those lacking the same were assigned as haplotype AA. The KIR genotype was analyzed for the "B content" of KIR genes as proposed by Cooley *et al.*, based on centromeric or telomeric position of the inhibitory and activating genes [17]. Accordingly B scores of 0–4 were assigned depending on the alignment of KIR genes.

NK KIR ligand mismatch

NK alloreactivity was defined as mismatch of NK-KIR ligands C1/C2 or BW4 determined in GVH direction based on the "missing self" hypothesis as described previously [18]. KIR-HLA Ligands C1, C2 and Bw4 were identified by PCR amplification method using commercial kits from Olerup.

Donor selection

NK KIR ligand mismatch (NKLMM) donors were preferentially chosen. In the absence of a NKLMM donor, a donor with B haplotype and a B score of 2 or higher was preferred. Tissue cross-matching was done to rule out clinically significant anti-donor HLA antibodies.

Assessment of immune reconstitution

This assessment was carried out at days 30, 60 and 90 after the HSCT on whole blood sample. Cell surface staining procedure was carried out in 5-mL propylene tube containing 1.5×10^6 cells in 100 μL of the peripheral blood. The NK-cell, KIR receptors and T-cell immunophenotype was carried out by six color flow cytometry in Navios (Beckman Coulter) using the following mouse anti-human mAbs from Beckman Coulter, Immunotech: CD45 (J33), CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), CD56 (N901), CD16 (3G8), CD158a (EB6B), CD158b (GL183), CD158e (Z27.3.7) and CD159a (Z199). The samples were incubated with antibodies was for 20 min at room temperature in the dark followed by incubation with red blood cell lyses buffer Optilise C (Beckman Coulter) for 10 and two washes and resuspension of the pellet in 500 μL of phosphate-buffered saline buffer. Data were analyzed in Kaluza ver 1.3 (Beckman Coulter) in analysis software.

KIR receptor repertoire of the donors were checked on two occasions on peripheral blood samples taken before mobilization and at the time of lymphocyte collection on day 7 post-transplantation and the average value was taken for comparing the KIR expression amongst donors and recipients.

Tregs were analyzed from peripheral blood using mouse anti-human mAbs from BD Biosciences; CD4 (SK3), CD25 (2A3), CD127 (HIL-7R-M21) as described previously [19]. Tregs were defined as the population of lymphocytes expressing CD4$^+$CD25$^+$CD127^{dim} phenotype with expression of FoxP3. Intracellular staining for FoxP3 was carried out with Mouse anti-human FoxP3 (259D/C7; BD Biosciences). The gating strategy for all the lymphocyte

subsets, KIRs and Tregs are described in the supplementary figures.

Statistics

Binary variables were compared between the two groups using chi-square test, and continuous variables were analyzed using independent sample Student's *t*-test taking into account Levenes test for equality of variances. Probabilities of survival were estimated using the Kaplan-Meier product-limit method. The cumulative incidence rates of non-relapse mortality (NRM), disease progression, acute GVHD (aGVHD) and chronic GVHD (cGVHD) were computed to take account of the presence of competing risks. An outcome was determined to be significantly different if the observed P value was <0.05. All analyses were performed using statistical software IBM SPSS Statistics Version 22.

Results

Patient and donor characteristics

Ten patients were enrolled in the CD56 group. The characteristics of individual patients are detailed in Table I. The median age was 36 years (range 2–65 years). The comorbidities prohibiting MAC are detailed in Table I.

The median donor age was 37 years (range 17–58 years). Only three donor-recipient pairs were NKLMM in the GVH direction. Nine donors had KIR B haplotype. The control group was similar in terms of pre-transplant characteristics (Table II).

Graft composition

The grafts were not manipulated for ABO mismatch. The median $CD34^+$ cell count was 8.8×10^6/kg (range 6.2–14.4), and the $CD3^+$ cell count was 25×10^7/kg (range 9.2–110) in the CD56 group, which was similar to the control group (Tables I and II).

CD56-enriched donor cell infusions

Infusion was administered in all patients on day 7, 72 h after completion of PTCy. The median volume infused was 100 mL (50–150 mL). The target cell dose for $CD56^+$ cells was achieved in all the patients with a single procedure. Composition of the $CD56^+$ infusions for individual patients is detailed in Table I. The median $CD56^+CD3^-$ cell count in the final product was 6.7×10^6/kg (range 1.7–17.7) and the median $CD56^+CD3^+$ cell count was 1.15×10^6/kg (range 0.13–5.3). The $CD56^+CD3^+$ cells were predominantly $CD8^+$ (median 89%, range 86.9–92.7%). $CD56^+CD3^+$ $CD4^+$ cells accounted for 2.39–3.47% (median 2.7%) and $CD4^+/CD8^+$ double negative subpopulation of $CD56^+CD3^+$ cells was a median of 8.6% (range 3.26–9.6). The median $CD3^+CD56^-$ cells in the infusion were 1.15×10^5/kg (range 1.13–10.8). There were no infusion-related toxicities.

Engraftment and chimerism

All 10 patients engrafted both neutrophils and platelets at a median of 15 days (range 11–19 days for neutrophils and 9–21 days for platelets), which was 14 days for the control group. All had >95% donor chimerism at day 30 post-transplant. There were no regimen-related toxicities apart from grade 2 mucositis in three patients.

GVHD

None of the patients developed grade 2–4 acute GVHD in the CD56 group compared with a cumulative incidence of 50% in the control group ($P = 0.01$, Figure 1A). In CD56 group, two patients developed mild and self-limiting skin erythema that did not require intervention, and unique patient number (UPN) 4 and 9 developed *de novo* chronic GVHD. UPN 4 developed chronic GVHD involving the skin alone at day 196. This promptly responded to a short course of corticosteroids. UPN 9 developed diarrhea after day 100. The gut biopsy was suggestive of chronic GVHD and improved with treatment. On the National Institutes of Health global severity scale, one was graded as mild and other as moderate. The incidence of *de novo* chronic GVHD in the CD56 group was similar to the control group (32.3% versus 11.2%, $P = 0.7$, Figure 1B).

Two more patients (UPN 2 and 5) in the CD56 group developed GVHD 46 and 95 days after DLI for progressive disease. Both were treated with steroids and sirolimus and were off all immunosuppressive therapy at 1-year follow-up.

Infections and NRM

There were no major post-engraftment infections. Only two patients had CMV reactivation. None reactivated adenovirus, and only one (UPN-2) reactivated Epstein-Barr virus while on cortico-steroids for post-DLI GVHD. There were no infection-related morbidity and mortality. Only UPN 9 succumbed to an unexplained sudden cardiac arrest whilst on tapering dose of steroids for post-DLI GVHD. Thus the NRM was only 10% (95% confidence interval [CI] 0.5–19.5) compared with 17.3% (95% CI 8.1–26.5) in the control group ($P = 0.6$, Figure 1C).

Disease relapse and survival

Relapse was the major cause of treatment failure in both groups. Five patients in CD56 group had disease

Table I. Outcome of patients receiving CD56-enriched donor cell infusion after haploidentical HSCT.

UPN	Patient age/sex	Donor age/sex	Diagnosis	Reason for RIC	NK-KIR ligand mismatch	NK-KIRhaplotype/ B score	Graft composition		CD56 enriched cell infusion		Engraftment (days post-transplant)		Acute GVHD Grade 2–4	Chronic GVHD/NIH Global Severity Score	CMV infection /disease	Relapse/ treatment	Outcome
							CD34 ($\times 10^6$)	CD3 ($\times 10^7$)	$CD56^+3^-$ ($\times 10^6$)	$CD56^+3^+$ ($\times 10^6$)	Neutr >500/μL	Platelet >20 000/μL					
1	35/M	55/M	Relapsed AML	Liver disease	No	Bx/1	8.5	9.1	4.25	1.73	16.0	20.0	No	No	No/no	No	Day 297 Alive in CR
2	65/M	24/F	IMF (MK, CK) transformed	Cardiac disease, poor PS	Yes	AA/0	8.2	12	17.7	1.1	18.0	17.0	No	Yes (post-DLI)/ mild	No/no	Yes Day 63/DLI	Day 670 Alive in CR after DLI
3	37/M	39/M	Ref AML	Liver disease	No	Bx/2	11.4	34	10.7	0.40	11	9	No	No	No/no	No	Day 543 Alive in CR
4	8/F	35/F	Ref AML (MK)	Prior HSCT	No	Bx/1	9.4	16.8	7.1	0.4	13	11	No	Yes/mild	Yes/no	No	Day 345 Alive in CR
5	46/M	17/F	Ref CML-blast crises	Liver dysfunction	No	Bx/1	6.2	18.0	6.3	0.4	17	16	No	Yes (post-DLI)/ moderate	No/no	Yes Day 60/DLI	Day 473 Alive in CR after DLI
6	26/F	48/F	Relapsed AML (CK)	Seizure disorder	No	Bx/3	7.6	18.0	3.57	0.13	12	10	No	No	No/no	Yes Day 150/No response to DLI-second transplant	Day 410 Died relapsed after second transplant
7	59/F	37/F	Ref MPAL (MK, BCR:ABL+)	Rheumatoid arthritis, renal dysfunction	Yes	Bx/2	10.0	34.0	3.02	0.18	19	21	No	No	No/no	Yes Day 56/no response to DLI	Day 354 Died—relapse
8	2/F	37/M	Ref AML (CK)	Relapsed within 3 months of first allograft	Yes	Bx/2	9.6	32	1.70	1.20	13	11	No	No	No/no	Yes Day 35/no response to DLI, second transplant	Day 153 Died in CR after second treanspant
9	28/M	58/F	Ref AML (MK)	Poor PS, liver dysfunction	No	Bx/3	14.4	110	8.20	5.3	14	14	No	Yes/ moderate	Yes/no	No	Day 122 Died of sudden cardiac arrest
10	50/F	32/M	Ref AML (CK)	Inflammatory arthritis, cardiac disease	No	Bx/3	8.4	60	9.7	2.50	17	21	No	No	No/no	No	Day 395 Alive in CR

ABL, Abelson murine leukemia; AML, acute myeloid leukemia; BCR, break-point cluster region; CK, complex karyotype; CML, chronic myeloid leukemia; CR, complete remission; CRGNB, carbapenem resistant Gram-negative bacteria; EBV, Epstein-Barr virus; F, female; HPS, hemophagocytic syndrome; M, male; MK, monosomal karyotype; MPAL, mixed phenotype acute leukemia; NIH, National Institutes of Health; Neutr, neutrophils; PS, performance status; ref, refractory; UPN, unique patient number.

Table II. Patient characteristics and outcome.

	CD56 group (n = 10)	Control group (n = 20)
Median age in years (range)	36 (2–65)	30 (4–56)
Median donor age in years (range)	37 (17–58)	33 (17–66)
Sex (male/female)	5/5	16/4
Donor sex (male/female)	4/6	8/12
Maternal donor	3	10
Adverse prognostic features[a]	8	8
Marrow blasts pre-transplant median (range)	15 (5–35)	14 (6–55)
HCT-CI		
0–1	7	17
>1	3	3
HLA mismatch		
3–4	2	5
5	8	15
NK alloreactive donor	3	11/20
NK cell B score		
0–2	7	12
3–4	3	8
ABO mismatch	3	9/20
Graft composition		
CD34 ($\times 10^6$/kg)	8.8 (6.2–14.4)	7.6 (3.0–12)
CD3 ($\times 10^7$/kg)	25 (9.2–110)	54.5 (9.7–88)
Neutrophils >500/μL, median (range)	15 (11–19)	14 (12–17)
Platelets >20 000/μL, median (range)	15 (9–21)	14.5 (11–30)
CMV infection	2	11
CMV disease	0	1
Fungal infection	0	1

The difference between the groups was not significant ($P > 0.05$) for any of the parameters.
HCT-CI, Hematopoietic Cell Transplantation-Comorbidity Index.
[a]Adverse prognostic features include complex and monosomal karyotype, high-risk translocations and Fms-related tyrosine kinase 3.

progression at a median of 60 days (range 35–150 days) (Table I), compared with 12 of 20 in the control group. In the CD56 group, among the five patients who relapsed, three received DLI and two (UPN 2 and 5) achieved a sustained complete remission (CR). Both had developed GVHD post-DLI. The other patient (UPN 7) who received DLI did not show response and subsequently received low-dose hypomethylating agent and achieved a CR. She subsequently succumbed to relapsed disease. Two other patients (UPN 6 and 8) who relapsed did not respond to DLI and received a second transplantation. UPN 6 succumbed to relapsed disease after a second allograft from another haploidentical donor. UPN 8 developed hyperacute GVHD after the subsequent transplantation from the mother and achieved a CR. However, she succumbed to progressive GVHD and related complications on day 153. Four patients (UPN 1, 3, 4 and 10) in the CD56 group continue to be in CR without GVHD or relapse at a median follow-up of 12 months. UPN 9 died of a sudden cardiac event while in CR.

The cumulative incidences of relapse (Figure 1D) and the overall survival were 52% (95% CI 35.6–68.4) and 50% (31.2–68.8) in the CD56 group compared with 66% (95% CI, 54.5–77.5%; $P = 0.9$) and 35% (95% CI 24.3–45.7%; $P = 0.18$] in the control group, respectively. There was no relationship of disease progression with NKLMM or B score in the CD56 group.

T-cell subset reconstitution

$CD3^+CD4^+$ T cells reconstituted faster in the CD56 group

Reconstitution of $CD3^+$ cells was similar in both the group at all three time points. The median absolute $CD3^+$ cell count was 273 cells/μL at day 30 with a median $CD8^+$ cell count of 206 cells/μL in the CD56 group. The median $CD4^+$ cell count was 110 cells/μL at day 30 in the CD56 group compared with 50 cells/μL in the control group ($P = 0.01$) with this trend continuing to day 90 (Figure 2A). The CD4/CD8 ratio varied from 1.1 to 0.45 at day 30, suggesting a less skewed T-cell subset recovery in favor of $CD4^+$ population.

Rapid recovery of Tregs in the CD56 group

Tregs represented by $CD4^+CD25^+CD127^{dim}$FoxP3 positive cells accounted for 5.1% of $CD4^+$ cells at day 30 compared with 1.3% in the control group ($P = 0.001$) and increased progressively over the next 60 days to a median of 8.65% versus 2.1% in the control group ($P = 0.003$; Figure 2B).

Reconstitution of NK-cell subsets and KIRNK cells reconstituted faster in the CD56 group with predominantly mature phenotype

NK cells represented the dominant population among lymphocytes at day 30 in all patients in the CD56 group. The NK-cell population peaked at day 30 and diminished in proportion as well as absolute numbers at subsequent time points. The absolute NK-cell count at day 30 was 294 cells/μL in the study cohort compared with 79 cells/μL in the control group ($P = 0.004$). However, this was not significantly different at later time points. On further subset analysis, it was found that the $CD56^{dim}$ $CD16^+$ population representing the mature NK cells accounted for the majority of the reconstituted $CD56^+$ cells at all three time points in the CD56 group, increasing from a median of 61% at day 30 to 85% at day 90, contrary to the predominance of $CD56^{bright}$ $CD16^-$ populations in the control group (Figures 3A,B). This trend was maintained throughout the study period indicating a rapid surge

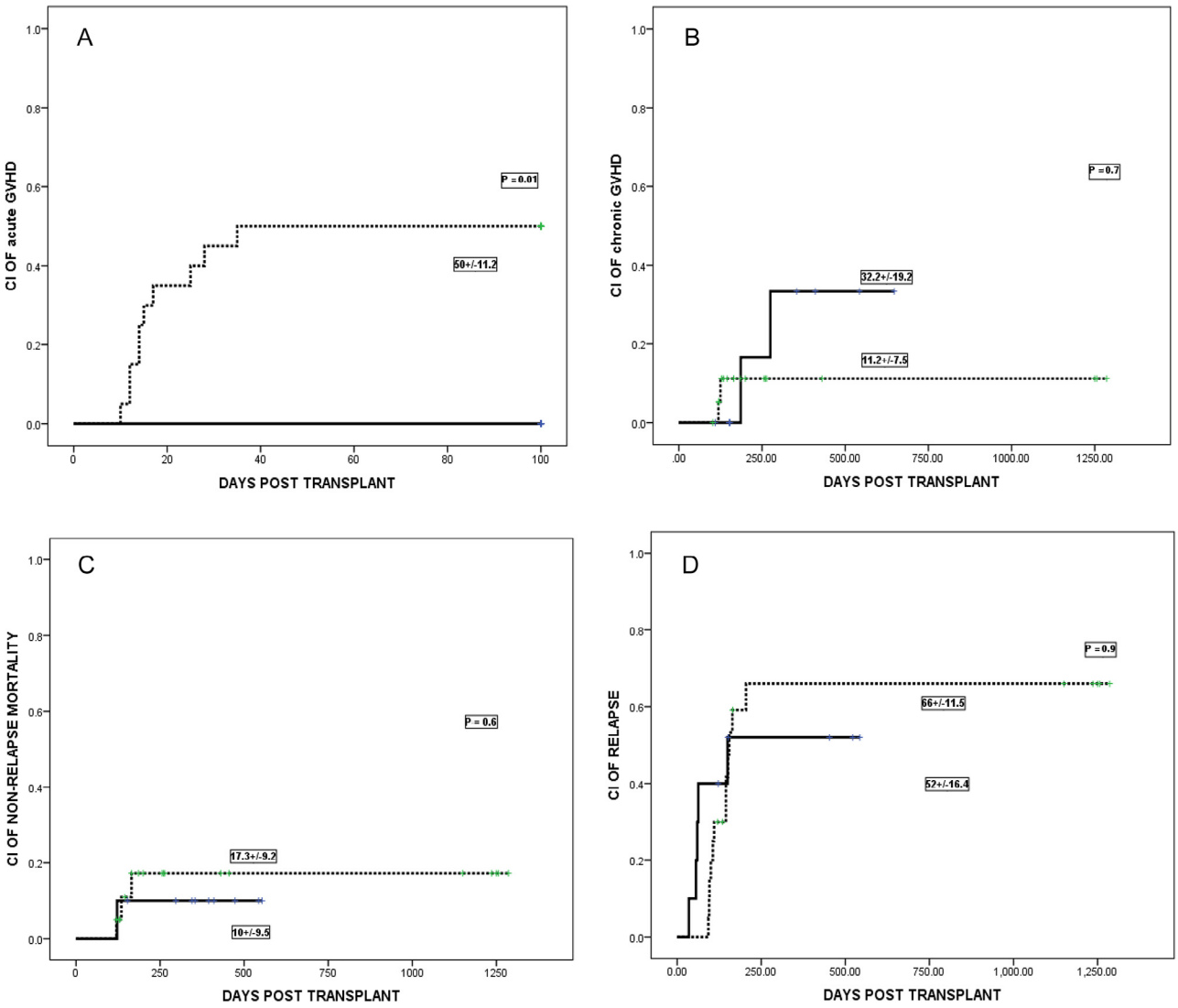

Figure 1. Cumulative incidences of (A) acute GVHD, (B) chronic GVHD, (C) non-relapse mortality and (D) relapse. The solid line represents the CD56 group, and the dashed line represents the control group.

of mature NK cells and its sustenance in the CD56 group.

The comparisons of immune reconstitution of all subsets is detailed in Table III.

Mature NK cells in the CD56 group resembled donor KIR phenotype by Day +30

We studied the KIR expression on both $CD56^{dim}$ and $CD56^{bright}$ NK cells and compared those with the donor phenotypes. The patient:donor ratio of 1 would represent similar expression of a particular KIR receptor, whereas a ratio >1 would mean a higher expression in the patient and vice versa. Among the $CD56^{dim}$ $CD16^{+}$ populations, KIR reconstitution closely resembled the donor phenotype. The patient:donor KIR ratio was 0.9 to 1.2 or greater for 2DL2 and 3DL1 at all three time points, whereas the 2DL1 ratio varied between 0.4 to 0.7. More importantly, the median value of $NKG2A^{+}$ cells was 49.8% at day 30, compared with 34.8% in the donors (Figures 4A–D). Thus 50% of the mature NK cells were licensed to exert cytotoxic effect with high KIR repertoire with $CD56^{dim}CD16^{+}NKG2A^{-}KIR^{+}$ phenotype.

$CD56^{bright}$ $CD16^{-}$ NK cells with high NKG2A expression dominated the NK-cell subset in the control group

Among the $CD56^{bright}$ $CD16^{-}$ population representing the immature NK cells, the reconstituted KIR^{+} patient:donor ratio was similar in all three time points for 2DL2 and 3DL1. The median value for NKG2A in the post-transplant period was 90–95% compared with 70.65% in the donors. There was no correlation

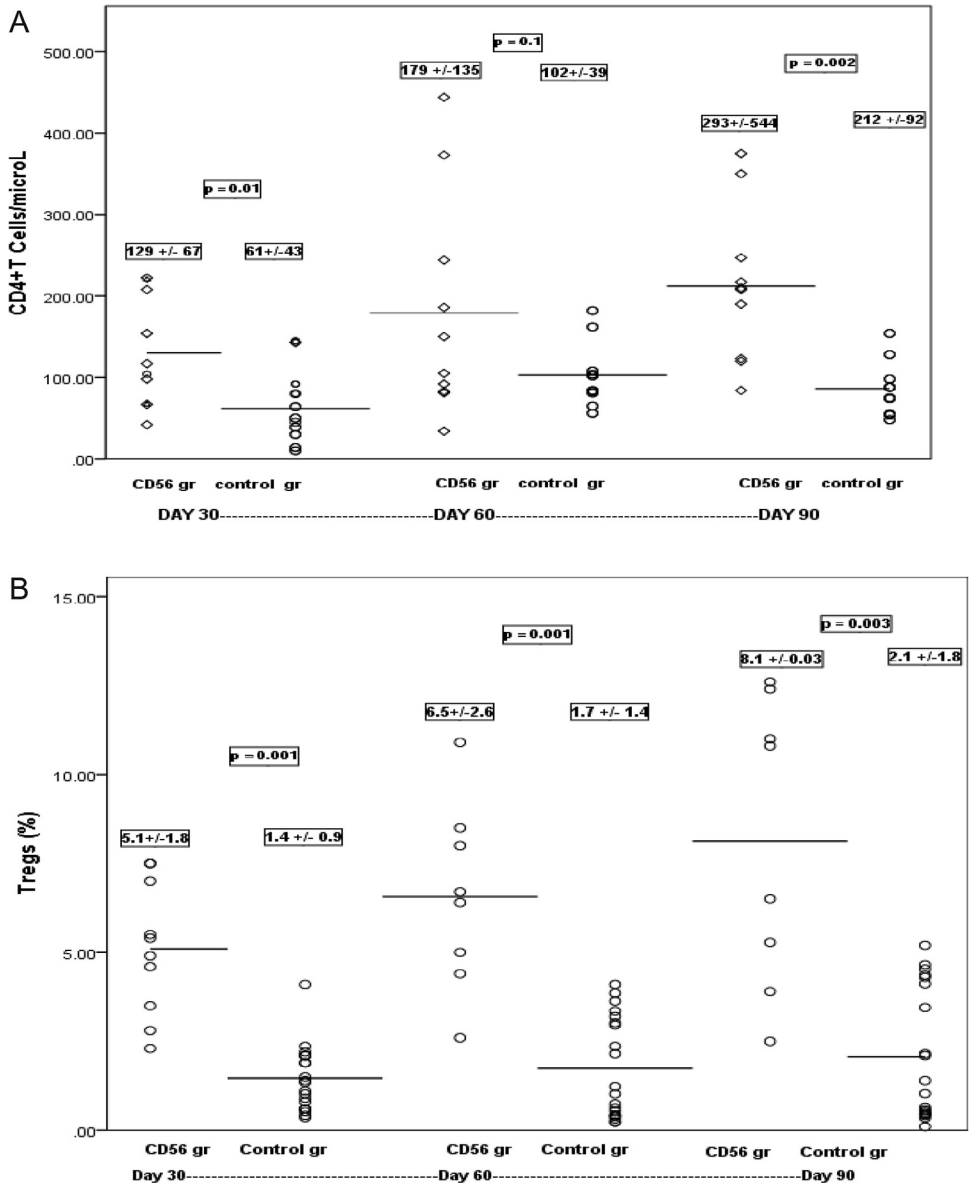

Figure 2. Scatterplots showing immune reconstitution in the CD56 and control groups at days 30, 60 and 90 post-transplantation. Reconstitution of (A) $CD3^{+}CD4^{+}$ and (B) Tregs.

between the pattern of KIR recovery and NKLMM or B scores.

In contrast, KIR expression was weak with >80–90% NKG2A expression in both CD56 bright and dim NK-cell populations in the control group. Although the absolute NK-cell population increased in the control group at day 90, these were predominantly $CD56^{bright}$ $CD16^{-}$ populations with high NKG2A expression.

Discussion

NK-cell infusions have been attempted several times in the past at variable time points after haploidentical HSCT to exploit GVL effect [11,20–24]. Most of these studies employed a two-step selection of $CD3^{+}$ cell depletion followed by $CD56^{+}$ selection to attenuate the risk of GVHD. However, we carried out a single-step enrichment of $CD56^{+}$ cells without depletion of $CD3^{+}$ cells to utilize the tolerizing potential of NKT-cell population. The remarkable finding in our study was the complete absence of acute GVHD as well as severe chronic GVHD despite the use of only a short course of CSA as GVHD prophylaxis, even though the cell infusion was carried out on day 7. The incidence of grade 2–4 GVHD with PTCy has varied between 30% and 50% with marrow or PBSC graft [1] similar to that observed in the control group. Several

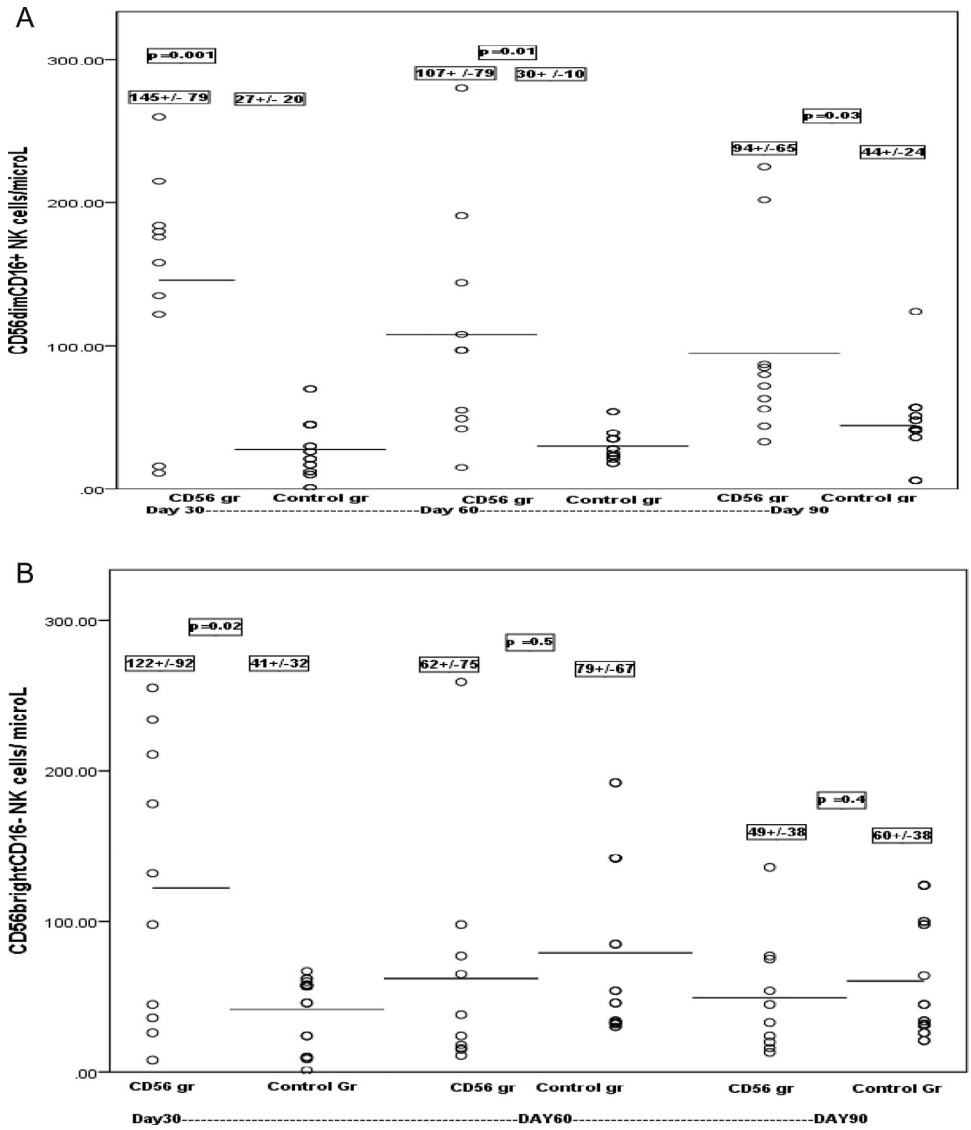

Figure 3. Scatterplots showing immune reconstitution in the CD56 and control groups at days 30, 60 and 90 post-transplantation. Reconstitution of NK cells and NK cell subsets: (A) $CD56^{+}CD16^{+}$ and (B) $CD56^{+}CD16^{-}$.

hypotheses might be offered for this finding, but the most plausible reason could be rapid recovery of Tregs in these patients [12]. This was accompanied by absence of infection related morbidity or mortality. Given that the patients in our cohort were heavily pretreated and had refractory disease, the lack of NRM was also revealing and might be attributed to the early recovery of NK cells as well as $CD4^{+}$ T cells.

The rapid recovery of Tregs could be deemed to have a detrimental effect on relapse. However, the relapse incidence seemed similar between the two groups despite a more rapid and sustained recovery of Tregs in the CD56 group. This might seem counterintuitive on the face of it, but evidence from recent studies suggest that Tregs might not compromise antileukemia effect of other GVL mediators as they do not efficiently home to the marrow due to a lack of CXCR4 receptor [25]. Martelli *et al.* elegantly demonstrated that infusion of Tregs followed by conventional T-cell augmented GVL reduced GVHD [26]. It has been demonstrated that NKT cells produce interleukin-4, which promotes expansion of Tregs [27,28]. Attention could be drawn to $CD56^{+}CD3^{+}$ cells in the infusion, which have not been explored in the past. This population of cells expressing both CD3 and CD56 also expressed CD8 and might represent a specific population of NKT cells. In animal studies, NKT cells with CD8 expression were shown to preserve GVL effect without increase in GVHD [29]. Indeed, both GVL and anti-GVHD properties of NKT cells have been reported previously [12], and a single-step $CD56^{+}$ enrichment might allow deployment of this population, which is otherwise lost with $CD3^{+}$ depletion. One of the limitations of our study was that the NKT

Table III. Comparison of immune reconstitution between the CD56 and control groups.

Cells/μL (mean ± SD)	CD56 Infusion Group (n = 10)	Control Group (n = 20)	*P* value
Day 30			
$CD3^+$	411 ± 314.0	357 ± 555	0.7
$CD4^+$	129 ± 67	61 ± 43	0.01
$CD8^+$	197 ± 94	293 ± 544	0.6
$CD56^+16^+$	145 ± 79	27 ± 20	0.001
$CD56^+CD16^-$	122 ± 92	41 ± 22	0.02
$CD56^+CD3^-$	261 ± 158	72 ± 36	0.04
Tregs	5.1 ± 1.8	1.4 ± 0.9	0.001
Day 60			
CD3	701 ± 820	409 ± 377	0.3
$CD4^+$	179 ± 135	102 ± 39	0.1
$CD8^+$	469 ± 699	303 ± 385	0.4
$CD56^+CD16^+$	107 ± 79	30 ± 10	0.01
$CD56^+CD16^-$	62 ± 75	79 ± 57	0.5
$CD56^+CD3^-$	159 ± 124	100 ± 156	0.8
Tregs	6.5 ± 2.6	1.7 ± 1.4	0.001
Day 90			
CD3	753 ± 399	486 ± 380	0.09
$CD4^+$	293 ± 544	212 ± 92	0.002
$CD8^+$	437 ± 303	374 ± 368	0.6
$CD56+CD16^+$	94 ± 65	44 ± 24	0.03
$CD56+CD16^-$	49 ± 38	60 ± 38	0.4
$CD56+CD3^-$	140 ± 100	97 ± 34	0.2
Tregs	8.1 ± 4.03	2.1 ± 1.8	0.003

subsets were not further defined. It would have been informative to have assessed the invariant NKT-cell subsets in the CD56 enriched cell infusion as well as reconstituted lymphocytes.

NK-cell recovery has been noticeable after a T-cell deplete haploidentical HSCT due to lack of early T-cell recovery [30]. However, on phenotypic analysis, these cells were found to have an immature $CD56^{bright}$ $CD16^-$ phenotype [31]. More importantly, these NK cells expressed high levels of NKG2A, which is a receptor of HLA-E ligand and is ubiquitously expressed. Unless the NK cells mature and lose NKG2A expression, these cells would be unable to exert anti-tumor cytotoxicity [32] despite mismatch of NK-cell ligands. The post-transplant recovery of NK-KIR receptors following matched donor HSCT has revealed muted expression of KIR receptors that attenuate the alloreactivity of NK cells [31,33]. The pattern of NK-cell recovery in our control group of MAC-based standard PTCy haploidentical HSCT recipients mimicked the same pattern.

On the other hand, NK-cell recovery witnessed in the CD56 group was much to the contrary. Early recovery of $CD56^{dim}$ NK cells was associated with rapid loss of NKG2A and acquisition of KIR receptors. This resembled the donor phenotype of $CD56^{dim}CD16^+NKG2A^-KIR^+$, which defines the functionally competent and cytotoxic subset of NK cells

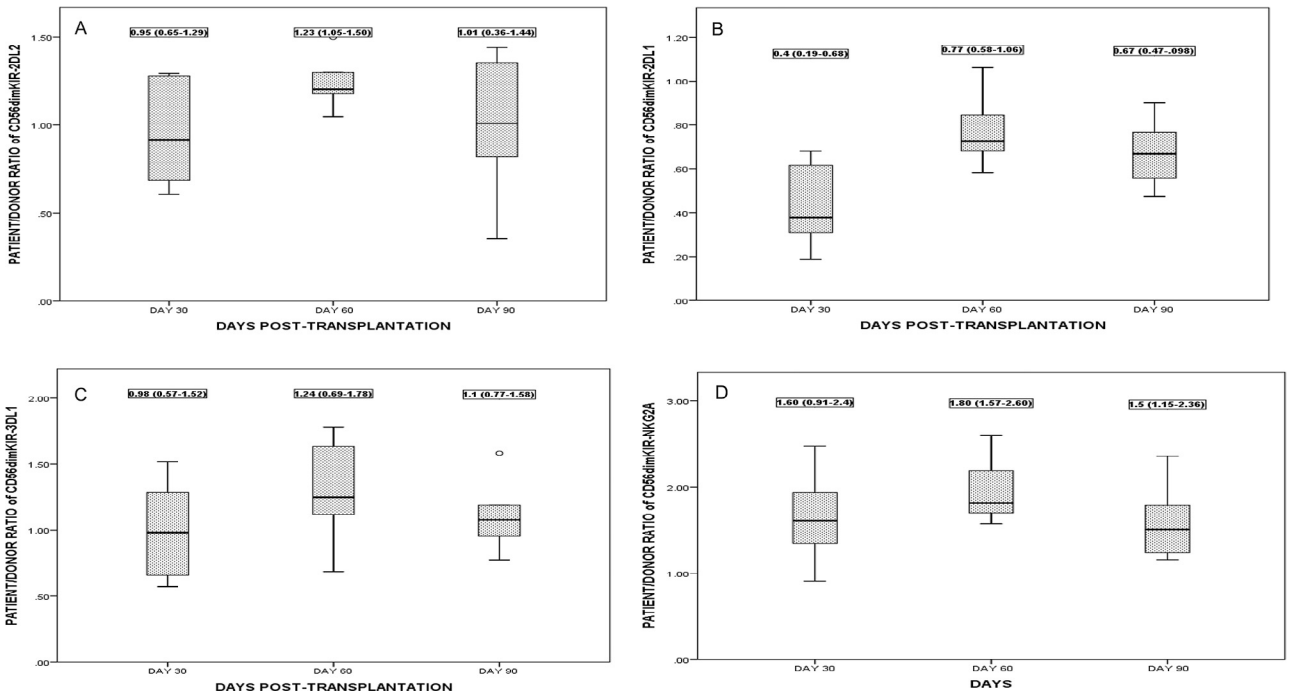

Figure 4. Scatterplots showing reconstitution of KIR-receptor repertoire for $CD56^{dim}$ NK cells in the CD56 and control groups at days 30, 60 and 90 post-transplantation. (A) KIR-2DL2, (B) KIR-2DL1, (C) KIR-3DL1 and (D) NKG2A. The values shown are the ratios of the percentage of KIR expression in the patient and the donor.

[32]. This could be due to an early wave of NK-cell proliferation arising from infused NK cells. The process might have been aided by the absence of MMF and as well as granulocyte colony-stimulating factor in the protocol, both of which probably impair NK-cell proliferation and cytotoxicity [34,35]. It has been shown in a study on HSCT from matched donors that better recovery of CD56 dim NK cells was associated with an improved outcome [36]. The same study showed a correlation between better maturation and functional recovery of NK cells with CMV reactivation, GVHD and survival. The low incidence of CMV reactivation, GVHD and NRM in our cohort perhaps underlines the impact of functional NK-cell maturation on the preceding outcome parameters within the limitation of the small cohort size. More importantly, a significant proportion of reconstituted mature NK cells lost NKG2A early, which possibly enabled the NK-cell cytotoxicity in our cohort, a phenomenon not witnessed in previous studies [31,33,36]. The lack of impact of NKLMM or B scores could be either due to a small sample size or to a lack of tangible benefit of either of these phenomenon in a T-cell-replete HSCT. It is worth mentioning in this context that in a study of 51 patients with refractory acute myeloid leukemia, we witnessed reduction in relapse if they were transplanted from NKLMM donors after MAC [7]. However, the beneficial effect of NKLMM was neutralized if DLI was administered as early as day 21. A previous study employing CD56-enriched cell infusion late after transplant also failed to show any correlation with NKLMM.

Even though the overall survival at a median of 12 months in this group of poor-risk myeloid malignancies was similar to the control group, we believe the follow-up is not long enough to be overly optimistic regarding the moderation of relapse risk due to this intervention. Several questions remain. Whether the encouraging recovery of mature KIR+ NK cells following early CD56-enriched cell infusion could translate to a prominent GVL effect despite attenuated GVHD or whether NKLMM or B haplotype might or might not contribute to an augmented GVL effect remains unclear from this study. It is also possible that more than one such CD56 enriched cell infusion might be necessary early rather than later in the post-transplant period to optimize these benefits.

The preliminary findings of our ongoing study highlight a striking lack of acute GVHD or infections after CD56-enriched cell infusion and the impressive reconstitution of the immune repertoire of T cells, Tregs and NK cells. This might raise interest in using or further refining this approach to improve the outcome after haploidentical HSCT in patients with both early and advanced malignancies.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.12.006.