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CTLA4Ig Primed Donor Lymphocyte Infusion: A Novel Approach to Immunotherapy after Haploidentical Transplantation for Advanced Leukemia

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CTLA4Ig attenuates T cell activation by co-stimulation blockade, but natural killer (NK) cells are not only resistant to CTLA4Ig, they also may demonstrate better antileukemia effect in the presence of CTLA4Ig. To explore this phenomenon we used sequential CTLA4Ig primed donor lymphocyte infusion (DLI) after post-transplant cyclophosphamide–based haploidentical transplantation. Thirty patients (CTLA4Ig-DLI group) with advanced leukemia received CTLA4Ig on day −1 and subsequently on days +7, +21, and +35, followed 12 hours later by DLI of 1 to 10×10^6 $CD3^+$ T cells/kg containing .1 to 3.27×10^6/kg $CD56^+$ NK cells, with low dose cyclosporine for 60 days. The incidences of acute graft-versus-host disease (GVHD), chronic GVHD and nonrelapse mortality (NRM) were 6.7%, 21%, and 4.5 %, respectively, with disease progression of 23.3% and overall survival of 79% at 18 months. Patients without disease progression had a significant early surge in $CD56^{dim}CD16^+$NK cells with lower NKG2A expression. CTLA4Ig primed DLI was associated with an upregulation of CD86 in mature NK cells that was not witnessed with CTLA4Ig administration alone. Thus, CTLA4Ig primed DLI resulted in early proliferation of mature NK cells with cytotoxic potential enabling early institution of adoptive immunotherapy to mitigate the risk of relapse in advanced leukemia with reduced GVHD and NRM.

INTRODUCTION

Early post-transplantation interventions aimed at attenuating the possibility of disease progression (DP) in advanced hematologic malignancies have been limited because of the perceived risk of toxicity. In this context we had demonstrated the feasibility of administering prophylactic unmanipulated donor lymphocyte infusions (DLIs) as early as day +21 [1], as well as $CD56^+$ enriched DLI on day +7 after post-transplantation cyclophosphamide (PTCy)-based haploidentical hematopoietic stem cell transplantation (HSCT) with reduction in DP with the former and significant reduction of acute graft-versus-host disease (GVHD) with the latter approach [2].

CTLA4Ig prevents T cell activation by binding to CD80 and CD86 (B7) ligands on antigen-presenting cells, thus preventing the critical costimulatory pathway of ligation of CD28 receptor on T cells with the B7 ligands. However, natural killer (NK) cells are resistant to CTLA4Ig mediated anergy as shown in both murine and canine models of mismatched transplantation [3,4]. In addition, NK cells were shown to have an augmented antitumor effect in the presence of CTLA4Ig [5]. We had successfully used T cell co-stimulation blockade with CTLA4Ig in children and young adults with both malignant and nonmalignant disorders to combat the problem of early alloreactivity after PTCy-based haploidentical HSCT [6,7].

Based on the above principles we further hypothesized that if DLI is used early and sequentially after CTLA4Ig, the NK cell–mediated antileukemia effect could be exploited without an increase in T cell–mediated alloreactivity. Here, we report on the pilot study using sequential DLI after CTLA4Ig, starting at day +7 of PTCy-based haploidentical HSCT in patients with advanced hematologic malignancies.

METHODS

Between January 2015 and February 2018 patients with advanced hematologic malignancies between the ages of 2 and 65 years without a suitable matched family donor were enrolled if they possessed a haploidentical family donor. Approval was obtained from Institute Review Committee in accordance with the Declaration of Helsinki, and written informed consent was obtained from all patients and donors.

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Study Protocol

The conditioning regimen consisted of fludarabine 30 mg/m^2 for 5 days, i.v. busulfan 9.6 mg/kg over 3 days, and melphalan 140 mg/m^2 as previously published [1]. PTCy was administered 64 hours after infusion of the graft at 50 mg/kg twice at 24-hour intervals along with mesna. In the study protocol MCF0401G patients received CTLA4Ig (Abatacept; BMS, Mumbai, India) at 10 mg/kg on days −1, +7, +21, and +35. Cyclosporine was administered from day +5 at 1.5 mg/kg 12 hours apart to maintain a trough level of 75 to 150 ng/ml.

Donor lymphocytes were collected and cryopreserved as described earlier [1]. This was administered as a fixed dose of CD3$^+$ T cells irrespective of the NK cell content, 12 hours after infusion of CTLA4Ig on day +7. On day +7 the DLI contained 1×10^6/kg CD3$^+$ T cells. In the absence of acute GVHD, this was increased to 5×10^6/kg CD3$^+$ T cells at day +21 and 10×10^6/kg at day +35.

Donor Selection and Mobilization Protocol

The methods followed for HLA typing, killer cell immunoglobulin-like receptor (KIR) genotyping, and defining NK cell alloreactivity were described in our earlier studies [2]. The Bx haplotype was defined as the presence of at least 1 of the defining loci: KIR2DL5, 2DS1, 2DS2, 2DS3, 2DS5, or 3DS1. The KIR genotype was analyzed for the "B content" of KIR genes as proposed by Cooley et al. [8] based on centromeric or telomeric position of the inhibitory and activating genes. NK alloreactivity was defined as mismatch of NK-KIR ligands C1/C2 or BW4 determined in the GVH direction based on the "missing self" hypothesis [9]. The mobilization protocol for HSCs was described in our earlier study [10].

Supportive Care

All patients were treated in protective isolation rooms provided with high-efficiency particle air filters. Antimicrobial prophylaxis was instituted as per departmental guidelines. Cytomegalovirus prophylaxis was guided by preemptive monitoring of viral cytomegalovirus load by quantitative PCR twice a week until day 100. Viral loads of cytomegalovirus, Epstein-Barr virus, and adenovirus were monitored twice weekly. Acute GVHD was graded according to modified Glucksberg criteria [11], and chronic GVHD was scored based on National Institutes of Health global severity criteria [12].

Flow Cytometric Assessment of T and NK Cell Subtypes

This technique was described earlier [2,13]. In brief, flow cytometric assessment was carried out on the donor leukapheresis products and on peripheral blood samples of patients at days + 30, +60, and +90 after HSCT. The NK cell and T cell immunophenotypes were carried out by 8-color flow cytometry in Navios (Beckman Coulter Inc. Indianapolis, Indiana) using the following mouse anti-human mAbs from Beckman Coulter, Immunotech (Marseille, France): CD45 (J33), CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), CD56 (N901), CD16 (3G8), CD80 (MAB104), CD86 (HA5.2B7), CD158a (EB6B), CD158b (GL183), CD158e (Z27.3.7), and CD159a (Z199). Regulatory T cells (Tregs) were analyzed using mouse anti-human mAbs from BD Biosciences (San Jose, CA): CD4 (SK3), CD25 (2A3), and CD127 (HIL-7R-M21). 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). Gating strategies were illustrated previously [2,13] and are shown in Supplementary Figures 1 to 3. The gating strategy for expression of CD80 and CD86 on NK cells was established on healthy donors using fluorescence minus 1 controls (see Supplementary Figure 4).

Statistics

The primary objectives were to study the safety and feasibility of CTLA4Ig primed DLI after PTCy-based haploidentical HSCT in patients with advanced leukemia. The primary endpoints of the study were grades II to IV acute GVHD, nonrelapse mortality (NRM), and DP at 6 and 12 months. The secondary endpoints were chronic GVHD, progression-free survival (PFS), and overall survival at 18 months and immune reconstitution at days +30, +60, and +90. The study was reviewed by the Data Safety Monitoring Committee after completion of the first 10 enrolments. Grades II to IV GVHD or DP exceeding 50% and/or grades III to IV acute GVHD exceeding 25% or NRM greater than 30% at 6 months follow-up were triggers for halting the study. The cut-off values were based on our previous studies with DLI and CD56 enriched DLI [1,2].

T cell and NK cell subsets in the graft and in peripheral blood of the patients at days +30, +60, and +90 were analyzed. Binary variables were compared between the groups using the chi-square test. Continuous variables were analyzed using an independent sample *t*-test considering Levenes test for equality of variances and nonparametric tests (Mann-Whitney U test). Probabilities of survival were estimated using the Kaplan-Meier product-limit method. The cumulative incidence rates of NRM, acute GVHD, chronic GVHD, and DP were computed censuring competing risks. An outcome was determined to be significantly different if $P < .05$. All analyses were performed using statistical software IBM SPSS Statistics version 21 (Armonk, NY).

RESULTS

Patients

Thirty patients (median age, 28 years; range, 4 to 65) with relapsed/refractory leukemia were enrolled in this study (Table 1). Fourteen were transplanted for myeloid malignancies (acute myeloid leukemia, 12; chronic myeloid leukemia in blast crisis, 2) and 16 for acute lymphoblastic leukemia (B cell, 10; T cell, 6). All patients were high/very high risk on the disease risk index [14], with 16 of those having adverse cytogenetics as well. Ten of 14 patients with myeloid leukemia and 12 of 16 with lymphoid leukemia were not in complete morphologic remission at transplantation. The other 8 patients who were in morphologic complete remission were minimal residual disease positive at the time of transplantation. The median percentage of marrow blasts was 5% (range, 1 to 60).

Donor Characteristics

The median age of donors was 36 years (range, 12 to 62). Fourteen were parents, 7 were offspring, and the rest were siblings. Six of 9 sibling donors were non-inherited maternal antigen (NIMA) mismatched. Eight received female to male transplants. Ten donors were NK ligand mismatched with the patient in the GVH direction.

Engraftment and Chimerism

All patients engrafted both neutrophils and platelets by a median of 15 days. Twenty-nine patients without DP had a donor chimerism of over 95% by day +30. There was no major conditioning-related toxicity apart from grades 2 to 3 mucositis in 20 patients, with none developing veno-occlusive disease of liver.

DLI: Target Dose and Achieved Dose

All patients received scheduled doses of DLIs on days +7 and +21. However, 4 patients did not receive the third dose on day +35 because of they developed acute GVHD (n = 2), possible viral parotitis (n = 1), and DP (n = 1). There were no acute toxicities related to CTLA4Ig or DLI administration. All those who received DLIs were administered the planned dose of 5×10^6/kg and 10×10^6/kg CD3$^+$ T cells without de-escalation.

Table 1
Patient and Donor Characteristics in CTLA4Ig-DLI Group in Relation to DP

	No DP (n = 23)	DP (n = 7)	*P*
Median age, yr (range)	27 (4-61)	28 (14-65)	.3
Disease type			
AML/CML-BC	13	1	.08
ALL	10	6	
Disease risk category			1.0
DRI: high/very high	23	7	
Adverse cytogenetics	12	4	
Marrow blasts > 5%	19	3	
MRD positive	4	4	
Median donor age, yr (range)	36 (12-59)	36 (12-62)	.4
Gender, male/female	13/10	5/2	.6
Donor gender, male/female	12/11	6/1	.2
NK alloreactive donor	7	3	.6
NK-KIR B score	2 (0-4)	2 (0-3)	1.0
NK B haplotype	21	3	.03
Median graft composition (range)			
CD34, x10^6/kg	8.2 (6-14)	8.4 (4.2-14.4)	.9
CD56, x10^6/kg	21.35 (3.4-61.3)	18.0 (10-53.1)	.9
CD3, x10^7/kg	15.7 (1.4-52)	18 (9.2-42)	.3

AML indicates acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML-BC, chronic myeloid leukemia-blast crisis; DRI, disease risk index; MRD, minimal residual diseasel.

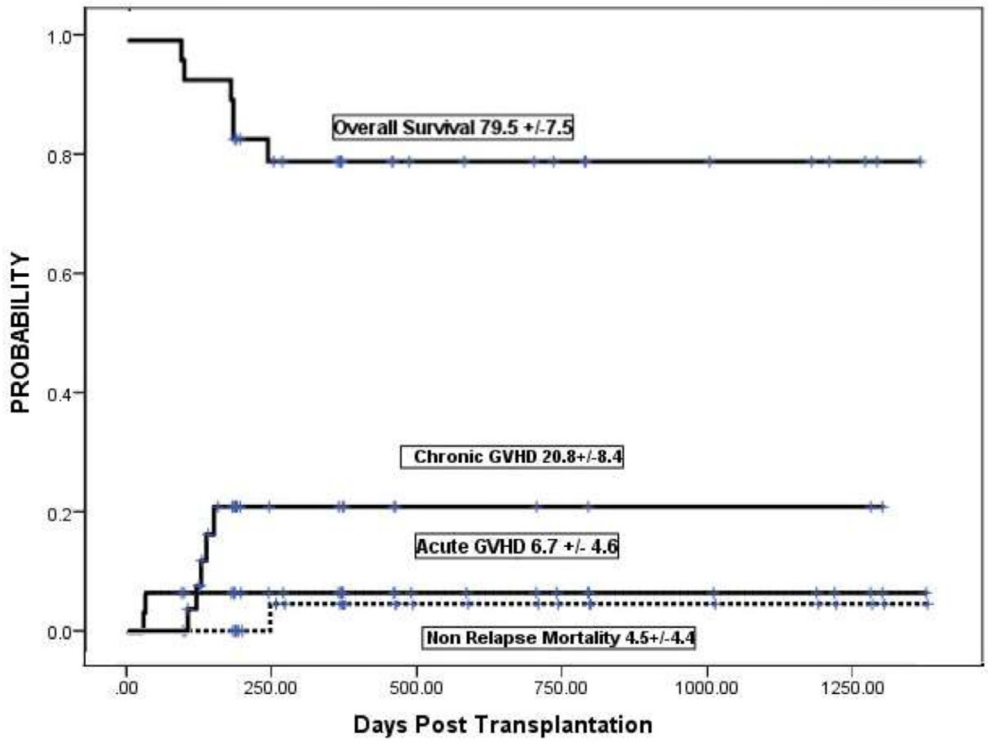

Figure 1. Incidences of acute GVHD, chronic GVHD, NRM, and overall survival in the CTLA4Ig-DLI group (N = 30).

The content of $CD56^{+}3^{-}$NK cells in the DLI ranged from .1 to 3.27×10^{6}/kg.

Acute GVHD

The incidence of acute GVHD grades II to IV was only 6.7% (95% confidence interval [CI], 2.1 to 11.3) (Figure 1). The 2 patients (ages 8 and 12 years) who developed grade III acute GVHD at days +25 and +28 were transplanted for relapsed and refractory T cell acute lymphoblastic leukemia. Both subsequently improved and remained free of GVHD and DP.

Chronic GVHD

The incidence of chronic GVHD was 20.8% (95% CI, 12.4 to 29.2) (Figure 1). Chronic GVHD was limited to skin, oral, or ocular regions without systemic organ involvement, and none was graded as "severe" by National Institutes of Health criteria. Three patients were graded moderate and received systemic immunosuppression. Among those with chronic GVHD, 4 of 5 patients were free of GVHD and off immunosuppression at 1 year.

Nonrelapse Mortality

The incidence of postengraftment infections was extremely low (4.5%; 95% CI, .1 to 8.9) (Figure 1). The only patient in the CTLA4Ig-DLI group who died from nonrelapse causes succumbed to a febrile illness of unknown etiology after she had returned to her home at day +240.

DP and Survival

Seven patients had DP at a median of 90 days (range, 28 to 135). The cumulative incidence of DP was 23.3% (95% CI, 15.6 to 31.0). Among patients with acute lymphoblastic leukemia, the incidence of DP was 37.5% (6/16 patients), with only 1 of 14 patients with myeloid leukemia experiencing DP (cumulative incidence, 7.1%; 95% CI, .2 to 14.0; $P = .05$) (Figure 2). The probabilities of PFS and overall survival were 75.8% (95% CI, 67.8 to 83.8) and 79.5% (95% CI, 72.0 to 88.0), respectively, at a median follow-up of 18 months (range, 11.5 to 44).

Graft composition or the NK cell content of the DLI did not influence DP

There was no difference in the graft composition in terms of $CD34^{+}$ HSCs, $CD3^{+}$ T cells, and $CD56^{+}$ NK cells between those with and without DP (Table 1). The median $CD56^{+}$ NK cells infused in the DLI was $.96 \times 10^{6}$/kg (range, .16 to 3.27) in those without DP compared with 1.12×10^{6}/kg (range, .1 to 2.2) in those with DP ($P = .5$).

NK-KIR B haplotype and not B score or NK-ligand mismatch was associated with lower DP

There was no impact of NK-ligand mismatch or NK-KIR B scores on DP (Table 1). However, only 3 of 24 patients with NK-Bx haplotype relapsed compared with 4 of 6 with AA haplotype ($P = .03$) (Table 1).

Reconstitution of NK and T Cell Subtypes

Failure of early reconstitution $CD56^{dim}$ 16^{+} NK cells was associated with DP

On analysis of NK cell subsets in the CTLA4Ig-DLI group, total NK cell recovery did not differ in relation to DP (Table 2). The median $CD56^{bright}$ 16^{-} NK cells at day +30 were 68 cells/μL (range, 21 to 164) in those with DP compared with 67 cells/μL (range, 7 to 256) in those without DP ($P = .8$). However, the median $CD56^{dim}16^{+}$ NK cells were only 17 cells/μL (range, 4 to 27) in those with DP compared with 78 cells/μL (range, 25 to 579) in those without DP at day +30 ($P = .002$) (Figure 3). This trend was maintained across the study time points (Table 2). There was no correlation with infused NK cells in the DLI. NK cell recovery at day 30 was not influenced by disease type (myeloid versus lymphoid; $P = .3$).

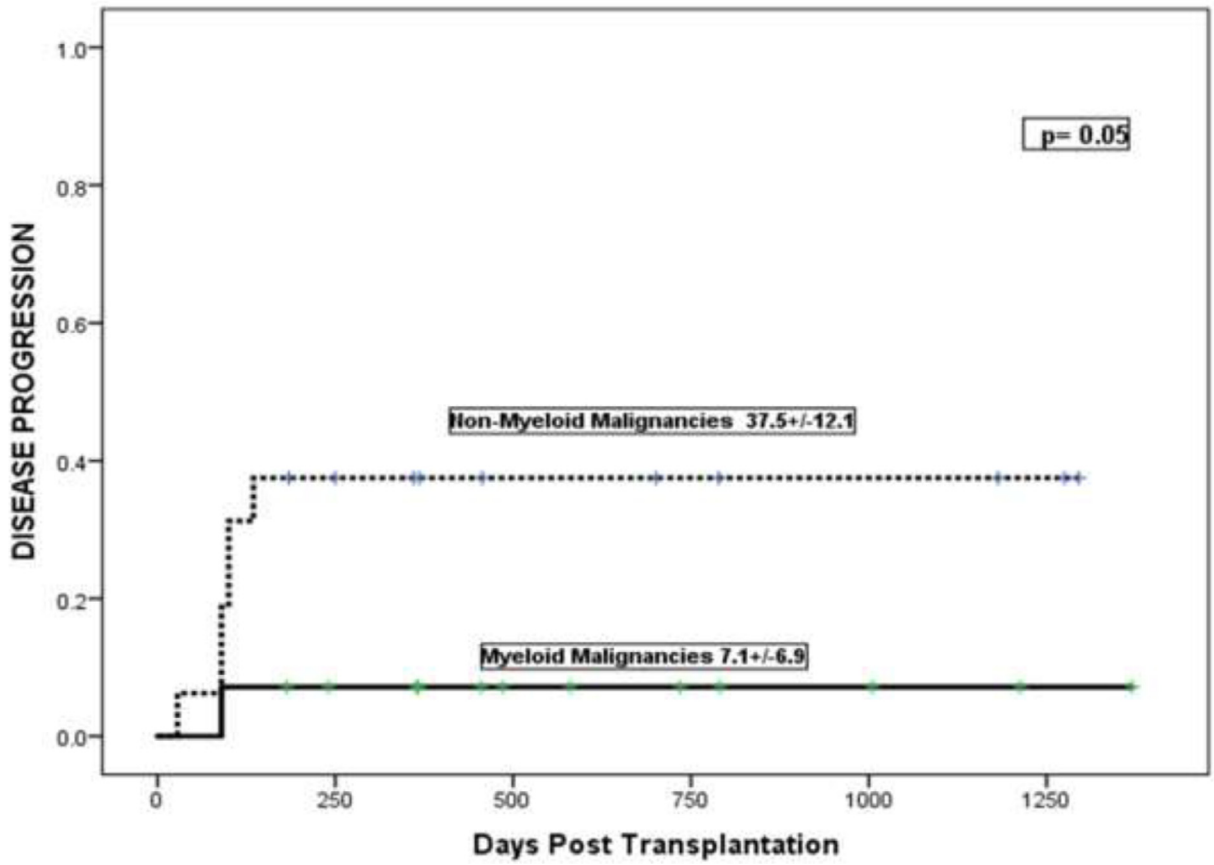

Figure 2. Incidence of DP in patients with myeloid (*solid line*) and nonmyeloid leukemia (*dotted line*) in the CTLA4Ig-DLI group.

NKG2A and KIR expression on CD56^{dim}16^{+} NK cells at days +30 and +60 correlated with DP

The expressions of KIR receptors on both subsets of NK cells were studied in donors and patients at all 3 time points (Table 3). Achievement of donor KIR phenotypes in the reconstituting CD56^{dim}16^{+} NK cells was strongly associated with lower DP (Table 3).

Donor NKG2A expression was not associated with DP (median, 24% [range, 5.5 to 38.6] versus 25.7% [range, 5.4 to 38.4]; $P = .8$). However, a lower expression of NKG2A in the reconstituting mature NK cells at early time points as well as expression of KIRs of donor phenotype were strongly associated with lower DP (Table 3). The median NKG2A expressions in mature NK cells were 28.4% (range, 8 to 74.2) and 32.9% (range, 2 to 72.5) in those without DP at days +30 and +60, respectively, compared with 71% (range, 37.5 to 84.3) and 61.4% (range, 31.8 to 88.5) in those with DP ($P = .01$ in both). KIR or NKG2A ratios at various time points were not influenced by disease type (myeloid versus lymphoid).

Table 2
Immune Reconstitution at Days +30, +60, and +90 Post-Transplantation in the CTLA4Ig-DLI Group in Relation to DP

	No DP (n = 23)	DP (n = 7)	*P*
Day +30			
CD3^{+}	559 ± 425	1481 ± 1288	.2
CD4^{+}	69 ± 48	114 ± 76	.08
CD8^{+}	486 ± 281	1396 ± 1018	.2
CD56^{+}CD3-	174 ± 170	141 ± 118	.6
CD56^{+}CD16^{+}	113 ± 106	16 ± 7	.002
CD56^{+}CD16-	75 ± 69	82 ± 48	.8
CD4^{+}25^{hi}127^{low}FOXP3^{+} (%)	7 ± 5	4 ± 2	.2
Day +60			
CD3^{+}	1038 ± 881	948 ± 821	.8
CD4^{+}	147 ± 81	129 ± 51	.6
CD8^{+}	813 ± 788	770 ± 758	.9
CD56^{+}CD3-	150 ± 102	94 ± 80	.02
CD56^{+}CD16^{+}	98 ± 78	24 ± 14	.001
CD56^{+}CD16-	50 ± 35	40 ± 29	.5
CD4^{+}25^{hi}127^{low}FOXP3^{+} (%)	5 ± 4	5 ± 2	.8
Day +90			
CD3^{+}	1193 ± 1176	981 ± 634	.6
CD4^{+}	259 ± 149	233 ± 77	.7
CD8^{+}	1044 ± 867	578 ± 573	.5
CD56^{+}CD3-	147 ± 78	114 ± 75	.4
CD56^{+}CD16^{+}	107 ± 59	54 ± 56	.1
CD56^{+}CD16-	41 ± 46	32 ± 26	.7
CD4^{+}25^{hi}127^{low}FOXP3^{+} (%)	5 ± 2	3 ± 1	.05

Values are mean cells/μL ± standard deviation.

CD86 was upregulated in CD56^{dim}16^{+} NK cells after CTLA4Ig-DLI but not with CTLA4Ig alone

CD86 expression was analyzed in 5 donors and patients in the CTLA4Ig-DLI group (Figure 4, bottom). This was also assessed in 5 patient–donor pairs receiving CTLA4Ig alone without DLI.

The median CD86 expression on CD56^{dim}16^{+} NK cells was 4.3% (range, 1.1 to 5.9) in the 10 donors assessed. The CD86 expression in patients receiving CTLA4Ig-DLI peaked at day +30 with a median of 16.1% (range, 11.2 to 22.3). This remained overexpressed at days +60 (median, 10.9; range, 5.6 to 14.0) and +90 (median; 12.2; range, 5.4 to 18.3) as well (Figure 5). In those receiving CTLA4Ig alone, CD86 expression was not upregulated (1.9%; range, 1.3 to 3.6) at day +30 and thereafter (Figure 5).

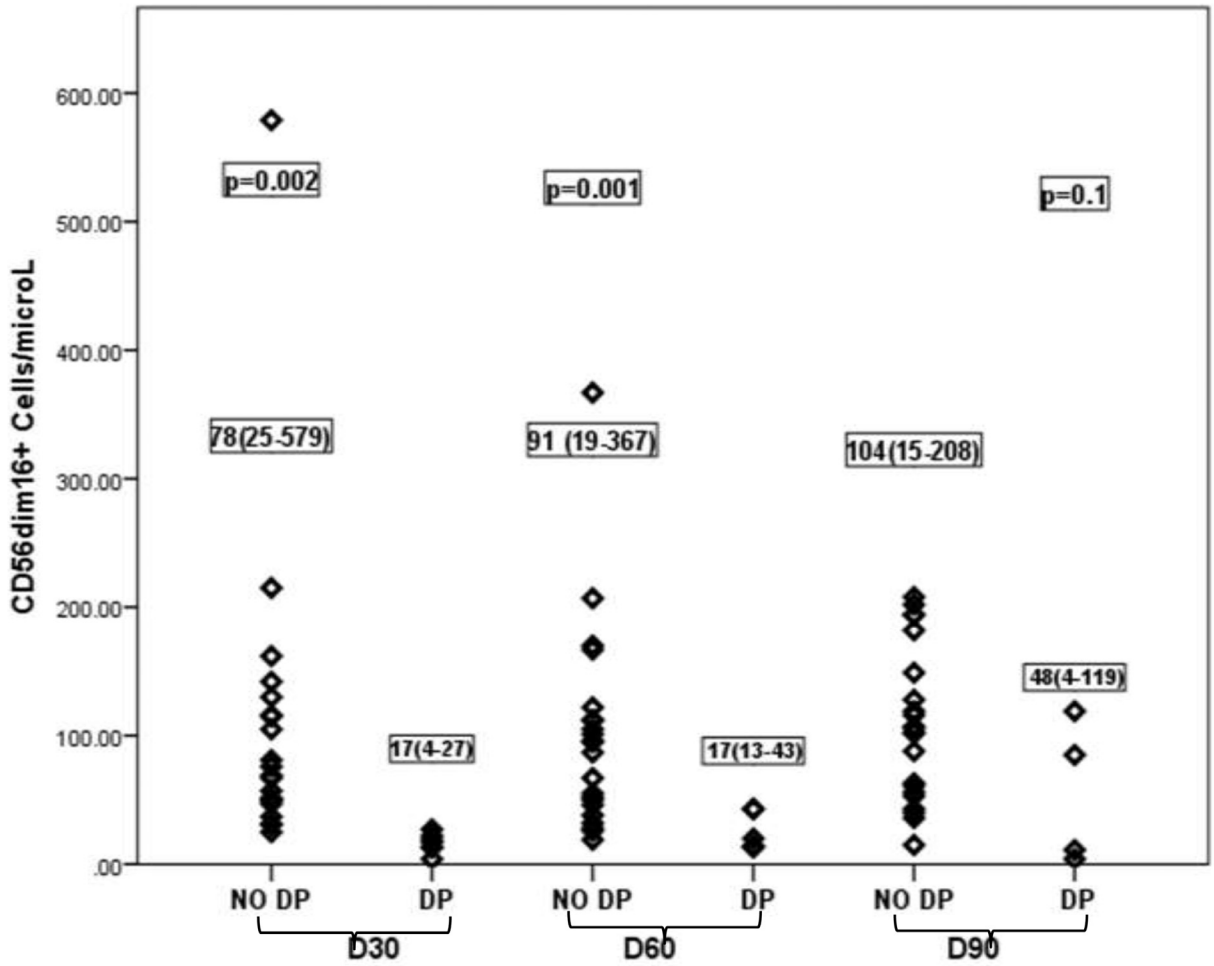

Figure 3. Recovery of $CD56^{dim}16^{+}$ NK cells/μL in peripheral blood on the *y*-axis in patients on days +30, +60, and +90 post-transplantation shown on the *x*-axis among the CTLA4Ig-DLI group who had DP (n = 7) and no DP (n = 23). The values mentioned in the parenthesis for each group represent the median values and the range in cells/μL.

Recovery of Tregs was early and sustained in the CTLA4Ig-DLI group

Tregs represented by $CD4^{+}CD25^{+}CD127^{dim}$FoxP3 positive cells accounted for 7.0% of $CD4^{+}$ cells at day +30. This population was sustained over the next study periods on days +60 and +90. There was no adverse impact of Tregs on DP. There was no difference in reconstitution of other T cell subsets in relation to DP (Table 2). Tregs at day +90 did not influence the development of chronic GVHD beyond this period ($P = .7$).

Table 3
NK-KIR Expression in $CD56^{+}16^{+}$ NK Cells Ratio of Patient-to-Donor at Days +30 and +60 Post-Transplantation in the CTLA4Ig-DLI Group in Relation to DP

	No DP (n = 20)	DP (n = 7)	*P*
Day +30			
2DL1	.77 ± .62	.40 ± .30	.03
2DL2	1.1 ± .72	1.1 ± .98	.66
3DL1	2.1 ± 2.2	.59 ± .54	.01
NKG2A	1.4 ± .96	7.7 ± 3.5	.001
Day +60			
2DL1	1.1 ± 1.1	.28 ± .15	.01
2DL2	1.4 ± .93	1.9 ± 1.7	. 4
3DL1	3.1 ± 2.9	1.09 ± .95	.08
NKG2A	1.8 ± 1.6	7.2 ± 3.3	.001

Values are mean cells/μL ± standard deviation.

DISCUSSION

DP remains the major cause for treatment failure after allogeneic HSCT for advanced leukemia. In addition, several reports suggest that minimal residual disease positivity at HSCT might confer a grim prognosis despite being in morphologic remission [15]. Intensification of conditioning and pharmacologic interventions post-transplantation have their limitations [16]. The success of allogeneic HSCT in prevention of DP hinges predominantly on the generation of a strong and persistent graft-versus-leukemia (GVL) effect, particularly within the first few weeks of transplant [17]. We had successfully used granulocyte colony-stimulating factor–mobilized DLI as early as 21 and 35 days after haploidentical HSCT with advanced myeloid malignancies with 62% PFS [1]. Although this established the feasibility of early adoptive immunotherapy after PTCy in the setting of haploidentical HSCT with an impressive GVL effect, GVHD remained a concern [18,19]. Given the fact that T cell–mediated GVL effect cannot be consistently devoid of significant GVHD, we had explored the infusion of NK cells as early as 7 days post-transplantation to mediate T cell–independent GVL effect if possible [2]. Although the risk of GVHD was markedly minimized after this approach, prevention of DP was not as pronounced as that demonstrated with sequential DLI.

T cell co-stimulation blockade with CTLA4Ig had been demonstrated in preclinical models to effectively induce transplantation

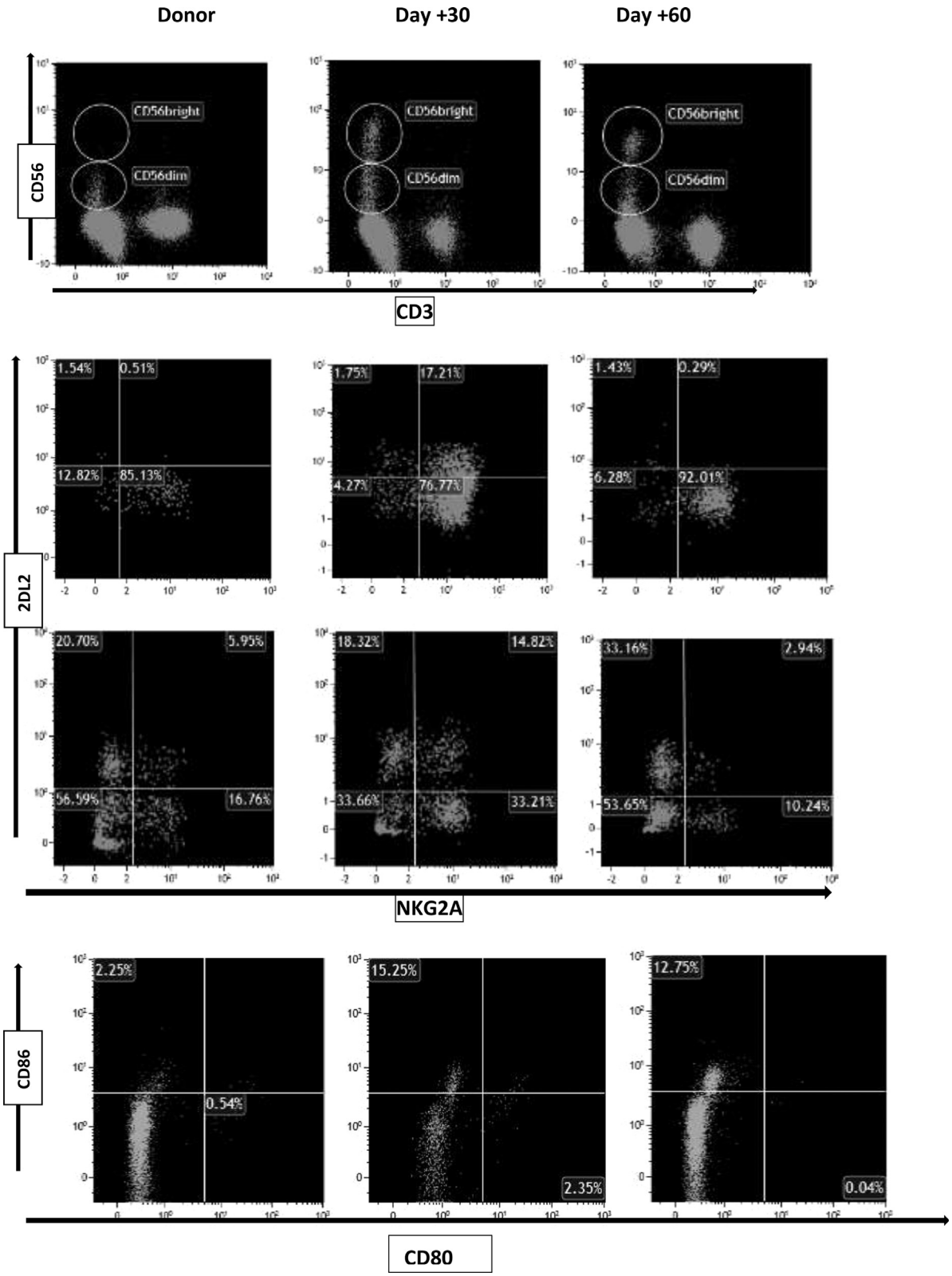

Figure 4. Patterns of NKG2A, KIR-2DL2, and CD86 expression on NK cells in the donor of UPN 30 and in the patient at days +30 and +60 of the CTLA4Ig-DLI group. Top: $CD56^{+}CD3^{-}$ NK cells (both $CD56^{bright}$ and $CD56^{dim}$ populations). Middle: NKG2A and 2DL2 expressions in the $CD56^{bright}$ (*upper panel*) and $CD56^{dim}$ (*lower panel*) subpopulations of NK cells. The expression of NKG2A in the $CD56^{bright}$ population is 85% in the donor and is maintained at a similar level in the patient at days +30 and +60. On the other hand, the expression of NKG2A is much lower in the $CD56^{dim}$ NK cells of the donor (21.71%). The reconstituting $CD56^{dim}$ NK cells maintain low NKG2A expressions (48% and 13.18% at days +30 and +60). The proportion of cells expressing 2DL2 without co-expression of NKG2A in the patient at day +30 (18.3%) and day +60 (33.16%) is similar to that of the donor (20.7%). Bottom: CD86 and CD80 expressions in the $CD56^{dim}$ subpopulations of NK cells. CD80 is unexpressed in the donor as well as the patient. On the other hand, CD86 expression increases from 2.25% in the donor to 15.25% in the patient at day +30. This remained elevated at 12.75% on day +60 as well (see Supplementary Figure 4 for gating strategy).

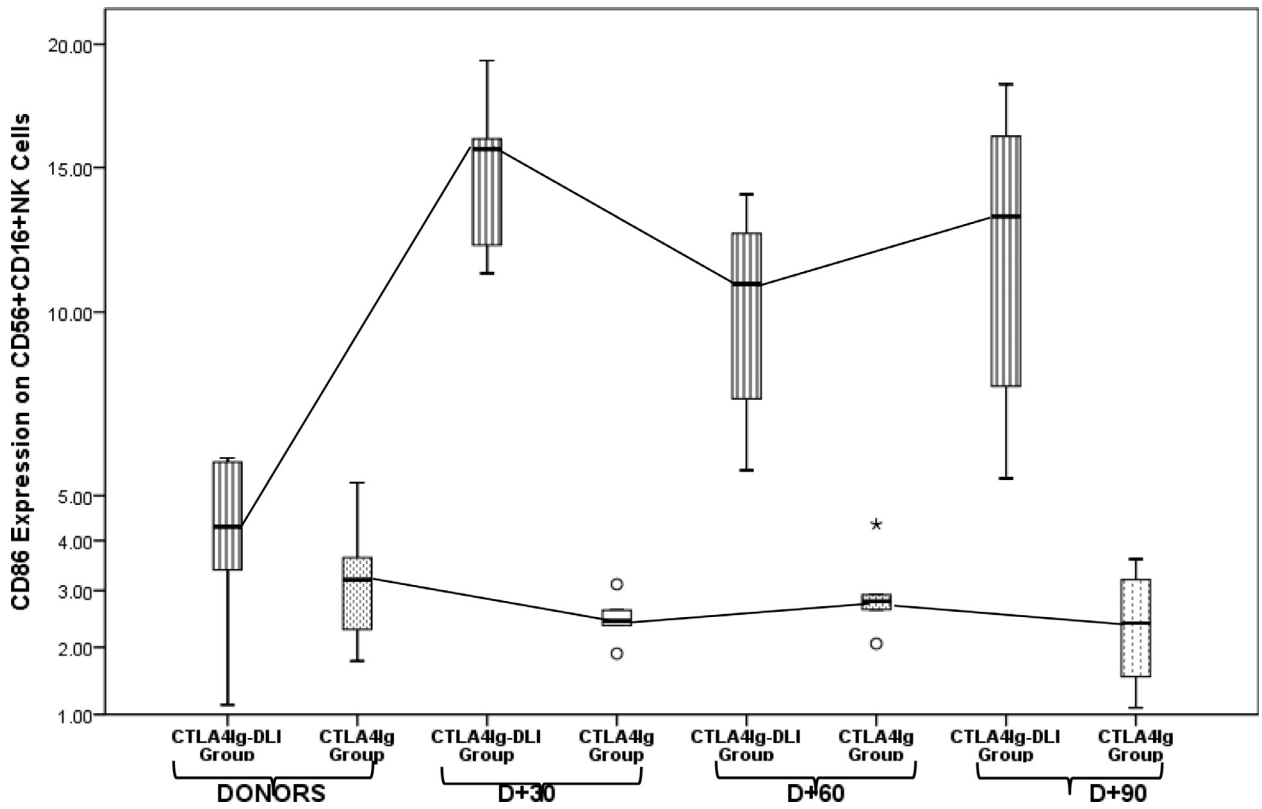

Figure 5. CD86 expression on $CD56^{dim}$ NK cells in the donors and patients at days +30 and +60 post-transplantation among the CTLA4Ig-DLI group (n = 5) and CTLA4Ig group (n = 5). The median values are connected through straight lines in each group. Increased expression of CD86 at days +30 and +60 post-transplant is illustrated in the CTLA4Ig-DLI group compared with baseline donor values which was not observed in the CTLA4Ig group.

tolerance [20]. We have successfully combined PTCy and T cell co-stimulation blockade with sirolimus in nonmalignant diseases and with cyclosporine in malignant diseases for haploidentical HSCT in younger patients with a reduced incidence of early alloreactivity [6,7]. Although used as a co-stimulation blockade for tolerance induction in both canine and murine mismatched transplant models without myeloablation, CTLA4Ig without myeloablation was associated with graft rejections [3,4]. This was identified to be due to host NK cells that were resistant to CTLA4Ig. T cell hyporesponsiveness was adequately achieved as demonstrated on mixed lymphocyte culture (MLC) assays [3,21]. However, NK cell cytotoxicity remained unaffected. In a murine model Kean et al. [4] demonstrated NK cell–mediated alloreactivity in the host-versus-graft direction that was unaffected by CTLA4Ig and in fact might be augmented via the lymphocyte function associated antigen-1 (LFA-1) pathway.

The above seminal studies had established the resistance of NK cells to CTLA4Ig. We conjectured that CTLA4Ig if given before DLI might prevent T cell–mediated GVH reactions and yet allow NK cell–mediated alloreactivity in the GVH direction. The next question that arose was whether CTLA4Ig merely spared the NK cells or it augmented NK cell–mediated cytotoxicity in any way. In a series of experiments, Peng et al. [5] demonstrated increased survival in mice receiving CTLA4Ig in a mouse melanoma model that was neutralized if NK cells were depleted before CTLA4Ig infusion. This was the first preclinical illustration of a possible antitumor effect of CTLA4Ig, mediated via NK cells.

In the current study we modified our DLI protocol to infuse CTLA4Ig 12 hours before the infusion of DLI. Based on the safety and feasibility of NK cell infusion as early as day +7, the first dose of CTLA4Ig-DLI was administered on day +7 and subsequently on days +21 and +35. We had earlier hypothesized based on the study by Miller et al. [22] that high-dose Cy results in marked increase in endogenous IL-15 in the days after its administration, and infusion of NK cells 48 hours after PTCy might result in proliferation of infused NK cells. The same has been further demonstrated in a study on NK cell recovery after PTCy-based haploidentical HSCT [23]. Cyclosporine with low trough levels was used as short-term GVHD prophylaxis with tapering at 60 days post-transplant. The use of cyclosporine along with CTLA4Ig was based on experiments carried out by Comoli et al. [24] where T cell anergy was effectively induced by this combination without affecting antileukemia or antiviral cytotoxic T lymphocyte responses.

Compared with the cohort of patients with advanced myeloid malignancies who had received DLI in an earlier study [1], a significant reduction of acute GVHD with no mortality associated with GVHD or infections was observed in the CTLA4Ig-DLI group (6.7% versus 31%). Despite administration of CTLA4Ig-DLI as early as 7 days after transplant, all patients received the next dose of DLI on day +21. Only 3 of 30 patients missed the third planned dose due to GVHD or infection, compared with 6 of 21 patients who received DLI alone [1]. Chronic GVHD also tended to be lower in the CTLA4Ig-DLI group than in previous cohort (DLI alone, 21% versus 41%) [1] with no significant morbidity associated with it.

To highlight our findings in the right perspective, it is worth noting that the PFS for 372 patients with high and very high disease risk index malignancies after PTCy-based haploidentical HSCT was reported to be 22%, with acute GVHD and NRM of 8% and 6%, respectively, by the Baltimore group [25], which was similar to the validation study reported on 13,131 patients

from the Center for International Blood and Marrow Transplant Research registry [26]. In a much smaller cohort, with early and sequential post-transplant CTLA4Ig-primed DLI, we report a PFS of 75% in patients bearing similar disease risk profile with incidences of NRM and acute GVHD of less than 10%. This probably underscores the favorable impact of this approach on reducing the risk of DP without an increase in toxicity.

Analysis of the T cell and NK cell subset reconstitution revealed a significant surge of $CD56^{dim}$ NK cells in the study group that was persistent through the first 100 days, indicating that CTLA4Ig-DLI resulted in predominant proliferation of NK cells. This pattern of proliferation of mature NK cells was similar to that witnessed after $CD56^{+}$ cell enriched DLI in our earlier study [2]. We further analyzed the role of these populations in the CTLA4Ig-DLI group with respect to DP. It was observed that those who experienced DP failed to proliferate $CD56^{dim}$ NK cells even though the $CD56^{bright}16^{-}$ populations were similar across the first 100 days.

NK cells do reconstitute early after allogeneic HSCT but with an immature phenotype with high NKG2A expression [23,27]. HLA-E is ubiquitously expressed in human cells and is a ligand for NKG2A. This is a natural barrier to NK cell–mediated killing. Although the $CD56^{bright}$ NK cells uniformly express NKG2A, it is essential for NK cells to downregulate NKG2A expression with maturation to be able to generate alloreactivity [27] in an unstimulated milieu. NKG2A expression was significantly downregulated in those without DP along with achievement of KIR expressions of donor phenotype. This phenomenon has been emphasized in a study by Nguyen et al. [28] where the effect of alloreactivity associated with NK ligand mismatch was negated by high expression of NKG2A in the reconstituting $CD56^{dim}$ NK cell population. Russo et al. [23] contributed the lack of NK cell alloreactivity to the PTCy-induced killing of mature NK cells and the predominance of $CD56^{bright}$ NK cells at day 30, similar to that demonstrated in our previous study [2]. Recent studies have shown that a subpopulation of $CD56^{bright}$ NK cells might be endowed with antitumor potential if primed with IL-15 [29,30]. As we have shown in the current study as well as earlier [2], the infusion of NK cells or CTLA4Ig primed DLI at day 7 might utilize the possible IL-15 surge after PTCy and result in enhanced antileukemia potential of NK cells through both CD56 dim and bright subgroups. However, the weight of evidence favors the concept that NK cell cytotoxicity in the post-transplant setting is dependent on proliferation of the mature phenotype. Our study was not designed for further dissection of the effect of individual KIRs on DP. Even though a third of our patients were NK ligand mismatched, this did not impact DP. Neither did we observe any impact of NK-KIR B scores. However, DP tended to be lower in those with KIR-Bx haplotype, a trend that has been reported in both lymphoid and myeloid malignancies in the past [31,32].

Peng et al. [5] had demonstrated that CD86 is an activation receptor for NK cells and is upregulated by ligation with CTLA4Ig with CD86 receptor of NK cells enhancing the antitumor activity. This was negated by anti-CD86 antibodies. Although CTLA4Ig ligates both CD80 and CD86, no effect on CD80 was noted. In keeping with these findings, in our study CD86 was upregulated 2- to 8-fold at 30 days in the CTLA4Ig-DLI group but not the CTLA4Ig group. The expression of CD86 reduced over the next few months but remained above the donor baseline levels even at 90 days, suggesting lasting effect of CTLA4Ig on the primed NK cells and possible persistence of cytotoxic potential of these cells exposed to CTLA4Ig. Functional studies on cytotoxicity of NK cells were not a part of the original protocol in our study and limits the ability of this study to establish functional cytotoxicity of the proliferating NK cells. However, given the above findings we are undertaking functional studies to better understand this phenomenon.

DP tended to be lower in the myeloid malignancies compared with lymphoid leukemia in the CTLA4Ig-DLI group. This probably underscores the more dominant NK cell–mediated GVL effect in myeloid leukemia as suggested by the Perugia group in their earlier studies with CD34 selected haploidentical HSCT [9]. The antileukemia effect of CTLA4Ig-DLI cannot be discounted completely in lymphoid leukemia because the relapse rate was only 37.5% in this extremely high-risk group of patients with acute lymphoblastic leukemia, many of whom had active disease at transplantation. NK cell–mediated killing of both T and B lymphoblasts could be compromised by several factors such as high expression of ligands for inhibitory KIR receptors and lower expression of ligands for activating receptors [33]. Several in vitro studies have suggested preferential killing of myeloid or T lymphoid blasts due to presence of NKG2D or DNAM-1 ligands that might be reduced or absent in B lymphoblasts [34,35]. However, our study was not designed to address these issues, which might need a much larger cohort of patients. On the other hand, human acute myeloid leukemia blasts have been shown to activate the aryl hydrocarbon receptor (AHR) pathway and induce miR-29b expression in NK cells, thereby impairing NK cell maturation and NK cell function [36]. This might be a reason as to why NK cell infusions might not be effective in patients with high leukemia burden. In this context, allogeneic NK cells infused after myeloablative conditioning might provide the optimal platform for the NK cell–mediated antileukemia effect.

Another critical observation that might generate interest in this approach is the lack of adverse effect on $CD4^{+}$ and $CD8^{+}$ T cell recovery. There was no mortality related to infectious complications in the CTLA4Ig-DLI group. This corroborates the experimental findings that the T cells exposed to the combination of CTLA4Ig and CSA retain virus-specific immunity [24]. Tregs recovered rapidly, and the levels were sustained in the CTLA4Ig-DLI group. As we had highlighted before [6], this might be a direct impact of CTLA4Ig on induced Tregs, and more importantly this phenomenon was perhaps responsible for attenuation of early T cell–mediated alloreactivity. However, it is important to note that the younger median age of our cohort might have favorably influenced the outcome, and these results might not be reproducible in older cohorts where myeloablative conditioning is not feasible. Indeed, we believe myeloablative conditioning is an important component of the protocol enabling the NK cell–mediated GVL effect.

Based on the above findings, we could presume that CTLA4Ig primed DLI might be augmenting a GVL effect mediated via NK cells and suggest the following theories regarding the same (Figure 6). First, NK cells exert their optimum antileukemia effect in a T cell–free environment as demonstrated by studies in T cell–depleted haploidentical HSCT, which was not uniformly reproducible after T cell–replete HSCT [9]. By selectively blocking T cell activation, CTLA4Ig might be creating a similar platform for NK cells that are resistant to CTLA4Ig mediated blockade. Second, the mature cytotoxic NK cells delivered with the graft are eliminated effectively by PTCy, as demonstrated by Russo et al. [23]. Hence, CTLA4Ig administered alone would not achieve the proliferation of mature NK cells because most recovering NK cells at day 30 are of immature phenotype. Thus, when DLI primed with CTLA4Ig are

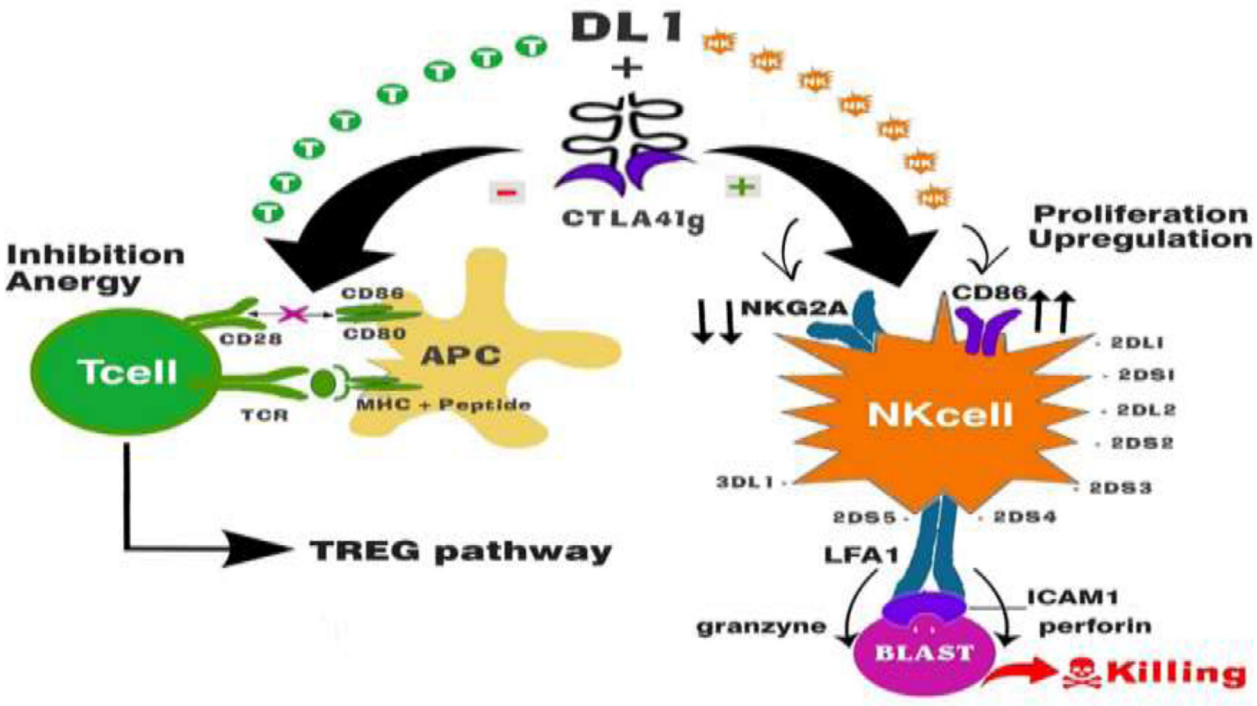

Figure 6. Possible pathways through which CTLA4Ig primed DLI might be exerting NK cell–mediated GVL effect without T cell–mediated alloreactivity. CTLA4Ig administered before DLI binds to CD80 and CD86 receptors on antigen-presenting cells and blocks the costimulatory signal, preventing T cell activation and promoting the Treg pathway. Not only are the NK cells resistant to CTLA4Ig blockade, CTLA4Ig ligation to CD86 on NK cells upregulates CD86 expression, which might act as an activation receptor itself or upregulate other activation receptors with downregulation of NKG2A. CTLA4Ig might also indirectly promote the LFA-1 pathway and augment killing of leukemia blasts.

delivered at quick successions and multiple occasions early post-transplantation, mature NK cells are provided with an opportunity to proliferate in an IL-15 surged environment immediately after PTCy and thereafter without hindrance from T cell–mediated alloreactivity. Finally, CTLA4Ig might be instrumental in augmenting the NK cell–mediated GVL effect via 2 possible pathways. A direct pathway could be via upregulation of CD86, which might aid or augment cytotoxicity, a phenomenon we are currently investigating. In addition, upregulation of other activation receptors such as NKp44 and NKG2D might be involved as well [5]. The other hypothesis concerns an indirect pathway via LFA-1. NK cell–mediated cytotoxicity that was resistant to CTLA4Ig was aborted if the LFA-1 pathway was blocked [4]. It is possible that after blockade of CD80 and CD86 by CTLA4Ig, LFA-1 might be overexpressed as a compensatory mechanism to over-ride the activation blockade and indirectly augmenting NK cell cytotoxicity. Unlike T cells, LFA-1 might be a more potent trigger for target cell killing, and this activity is optimized in $CD56^{dim}$ NK cells [37,38]. This plausible hypothesis is also being investigated by our group.

The concept of CTLA4Ig primed DLI promises to be an innovative method of immunotherapy through promoting mature NK cell proliferation without T cell–mediated early alloreactivity as has been demonstrated in our study. This approach seems to achieve encouraging PFS with reduced incidence of GVHD, infections, and NRM in those with advanced leukemia. We believe this novel approach should be explored further to promote early post-transplant immunotherapy in larger cohort of patients with advanced leukemia to better elucidate the NK cell biology in this context.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2018.12.836.

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