# Immune reconstitution dynamics after unrelated allogeneic transplantation with post-transplant cyclophosphamide compared to classical immunosuppression with anti-thymocyte globulin: a prospective cohort study

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**Supplemental material**

**Evaluations and treatments**

As per protocol in our service, during pre-transplant evaluation, all patients underwent serological assessment for HIV, hepatitis A, B, and C, syphilis, Chagas disease, toxoplasmosis, HTLV, and herpesviruses, including CMV, EBV, herpes simplex, and varicella-zoster. Post-transplant viral monitoring was conducted as follows: quantitative polymerase chain reaction (PCR) for CMV twice a week, quantitative PCR for adenovirus weekly, and quantitative PCR for EBV every two weeks, while the patient was under immunosuppression. Quantitative PCR for polyomavirus in urine (BKV) and human herpesvirus 6 (HHV-6) were performed only in cases of clinical suspicion.

All patients received antimicrobial prophylaxis with trimethoprim-sulfamethoxazole until day -2. This antibiotic was resumed after hematological reconstitution. Intravenous pentamidine, fluconazole 400mg/day or micafungin 100mg/day was used as antifungal prophylaxis and, in cases of high risk for filamentous fungi infection, voriconazole 200mg every 12 hours was used at least until day +75 post-transplant. High-risk cases were considered when patients had high-risk acute myeloid leukemia (AML) or refractory disease, prolonged neutropenia, elderly patients, or previous filamentous fungal infection. Levofloxacin 500mg/day was used as bacterial prophylaxis until neutrophil recovery, and acyclovir 500mg/$m^2$ two to three times a day (intravenously) or 400mg orally every 12 hours or valacyclovir 500mg/day was used as viral prophylaxis for at least 6 months post-transplant. No patient used CMV prophylaxis with letermovir.

CMV infection was defined as any detection of CMV DNA in plasma. Recurrent CMV infection was defined as a new CMV infection in a patient with previous evidence of CMV infection without virus detection for 4 weeks during active surveillance. CMV disease was defined as the presence of symptoms and/or clinical signs related to the involved organ, with documented CMV in tissue (22). In cases of CMV infection, ganciclovir 5mg/Kg 12/12h until 2 negative CMV PCR tests or at least 3 weeks in case of CMV diasese was the first-line therapy. In cases of cytopenias or ganciclovir toxicity, foscarnet 90mg/Kg 12/12h was used. If EBV viremia exceeded 10,000 copies/mL, treatment with rituximab 375mg/$m^2$, four injections weekly, was initiated.

Hemorrhagic cystitis caused by BK virus was defined according to the European Conference on Infections in Leukemia (ECIL)(23) as: signs or clinical symptoms of cystitis, hematuria grade 2 or higher, and BKV viremia loads > 7 log 10 copies/mL. Cidofovir 3-5mg/Kg weekly with probenecid was the treatment of choice in cases of refractory and severe disease.

Neutrophil engraftment was defined as the first of three consecutive days with neutrophil count >0.5x$10^9$/L. Platelet engraftment was defined as seven consecutive days with platelet count above 20,000/uL, in the absence of platelet transfusion.

Regarding the intensity of the conditioning regimens used, myeloablative conditioning consisted of busulfan with an area under the curve (AUC) of 4000 to 5000 for 4 days (on days D-6 to D-3) and fludarabine 40mg/$m^2$ (D-6 to D-3) or cyclophosphamide 60mg/kg (D-5 and D-4) and total body irradiation (TBI) with 1200cGy (divided into D-3 to D-1) or 990 to 1200cGy divided on the days D-8, D-7, and D-6. Fludarabine 30mg/m² was given on days D-5, D-4, D-3, and D-2. Reduced intensity conditioning regimen consisted of busulfan AUC 5000 for 2 days (D-4 to D-3) and

fludarabine 40mg/$m^2$ (D-6 to D-3) or total marrow irradiation (TMI) 6Gy (D-8 and D-7), fludarabine 30mg/$m^2$ (D-6 to D-2) with busulfan AUC 4800 (D-5 and D-4).

GVHD prophylaxis consisted of cyclophosphamide 50mg/kg/day on D+3 and D+4; tacrolimus on D+5 (target concentration 5-15 ng/mL) and mycophenolic acid 15mg/kg/day orally from D+5 to D+35. In cases receiving ATG, prophylaxis consisted of rabbit ATG 3-7.5mg/kg (Sanofi Genzyme), methotrexate 5mg/m2 on D+1, D+3, D+6, and D+11, and initiation of calcineurin inhibitor on D-2. Calcineurin inhibitor was maintained until D+120 in both groups, when we started tapering immunosuppression if there were no GVHD.

**Antibodies used in the Immune reconstitution**

The antibodies used were CD19-FITC (clone:4G7), CD56-PE (clone:REA196), CD3-PE-CF594 (clone:UCHT1), CD314/NKG2D-PE-Cy7 (clone 1D11), CD45-PerCP-Cy5.5 (clone: 2D1), CD16-APC (clone:B73.1), CD4-APC-Cy7 (clone: SK3), CD8-Alexa1700 (clone:RPA-T8), CD11b-BV421 (clone:CBRM1/5), CD14-BV510 (clone: MfP9), CD62L-FITC (clone: SK11), CD45RO-PE (clone:UCHL1), CD127-PE-Cy7 (clone: HIL-7R-M21), CD25-APC (clone: M-A251), CD45RA-BV421 (clone: HI100), CD197/CCR7-BV510 (clone: 3D12), CD27-BV605 (clone: L128), CD28-BV650 (clone: L-293), CD45 and a violet viable dye - BUV393 (BD Horizon Fixable Viability Stain 440UV). All antibodies were provided by BD Biosciences, BD Pharmingen, BD Horizon, and Miltenyi. For the control of unspecific staining, fluorescence minus one (FMO) was used.

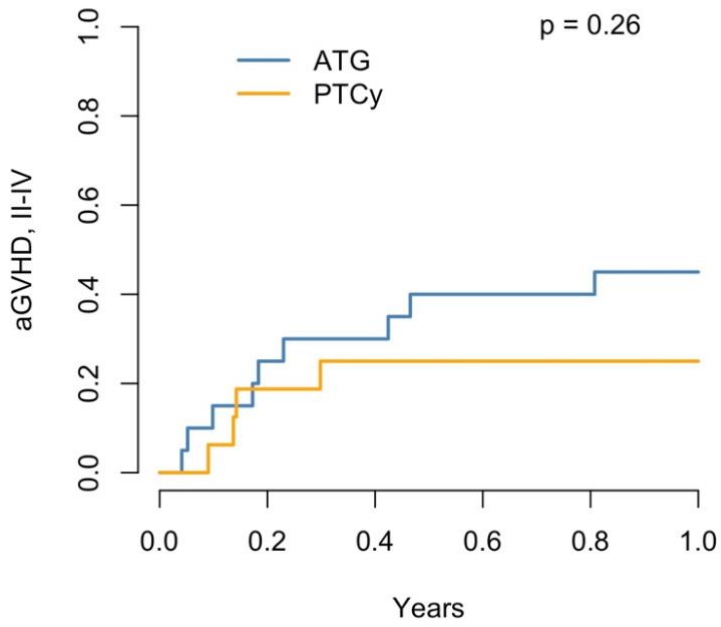
The samples acquisitions were performed using BD LSRFortessa Flow Cytometer (BD Biosciences). All data analysis was performed using the software FlowJo (BD Biosciences).

**Clinical outcomes**

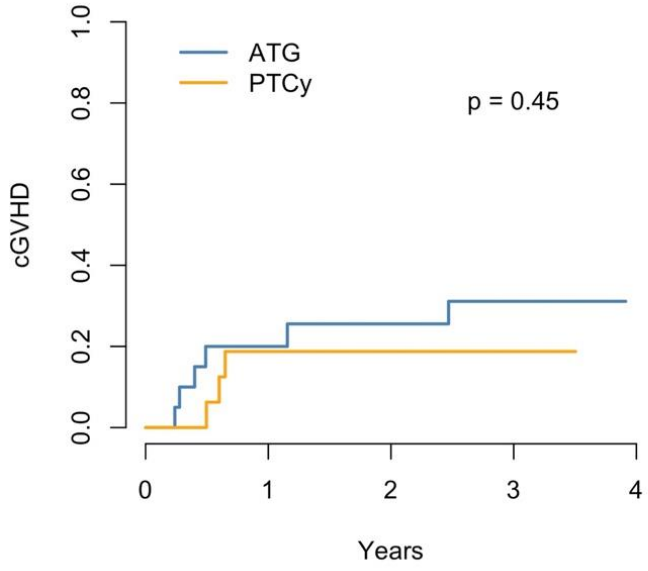
In the ATG group, 4 patients experienced non-relapse mortality. One patient died due to respiratory failure secondary to an infectious complication, another to septic shock before achieving neutrophil engraftment, and two died due to COVID-19 infection (31% at 3 years). In the PTCy group, there were 2 cases of non-relapse mortality (1 due to septic shock secondary to secondary graft failure and 1 due to acute gastrointestinal GVHD) (13% at 3 years) (p=0.30). In the ATG group, there were 5 deaths related to relapse of the underlying disease (25%), while in the PTCy group, there were 2 deaths related to relapse (12.5%).

Regarding other viral reactivations, in the ATG group, the second most frequent reactivation was EBV, which occurred in 7/20 (35%) patients, in a median of 60 days with Rituximab. Hemorrhagic cystitis by BK virus occurred in only 1/20 patient (5%) at 31 days post-transplant. Two patients (10%) had adenovirus infections, with one of them presenting with cystitis and viremia at 48 days post-transplant, and the other presenting with viremia, cystitis, and pulmonary involvement at 21 days post-transplant. In the cyclophosphamide group, 3/16 (18.7%) of the patients had hemorrhagic cystitis caused by BK virus, in a median of 36 days post-transplant. One patient had adenovirus viremia at 47 days post-transplant, and no patient had EBV-related viremia.

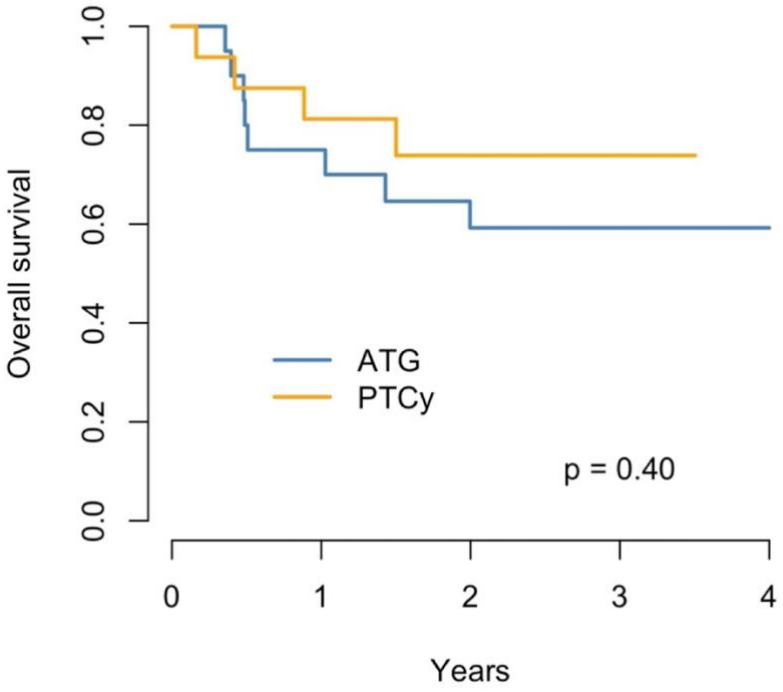
**Supplementary Figure 1. Acute GVHD grades II to IV in patients who used ATG (blue line) or cyclophosphamide (orange line).**



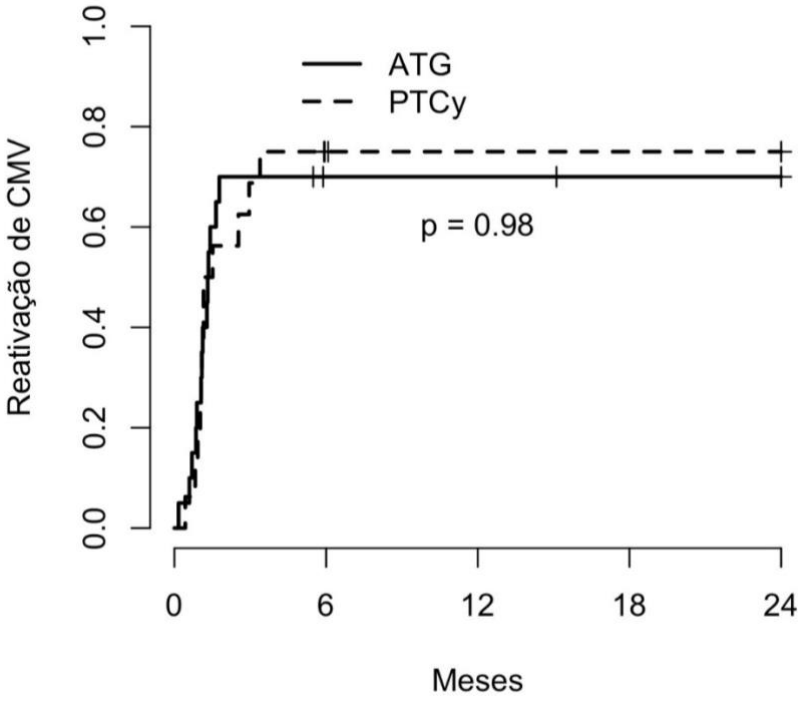

**Supplementary Figure 2. Chronic GVHD in patients who used ATG (blue line) or cyclophosphamide (orange line)**



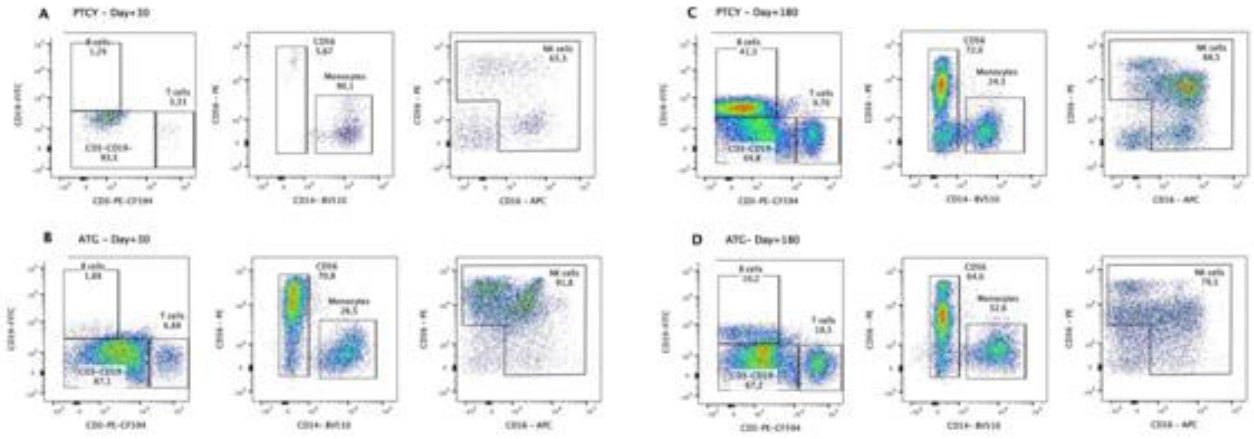
**Supplementary Figure 3. Overall survival in patients who used ATG (blue line) or cyclophosphamide (orange line**).



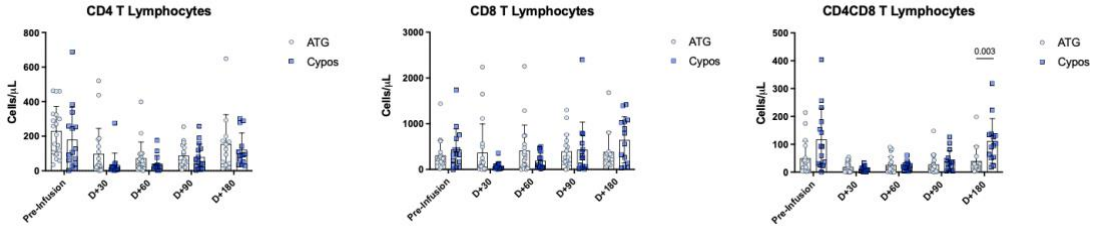

**Supplementary Figure 4. CMV Reactivation in patients who used ATG (solid line) or cyclophosphamide (dashed line).**



**Supplementary Figure 5.** Flow cytometry results of patients undergoing hematopoietic stem cell transplantation (HTSC) according to the type of immunosupression used: anti-thymocyte globulin (ATG) or post-transplant cyclophosphamide (PTCy) on 30 (D+30), panel A, or 180 (D+180) days after transplant, panel B

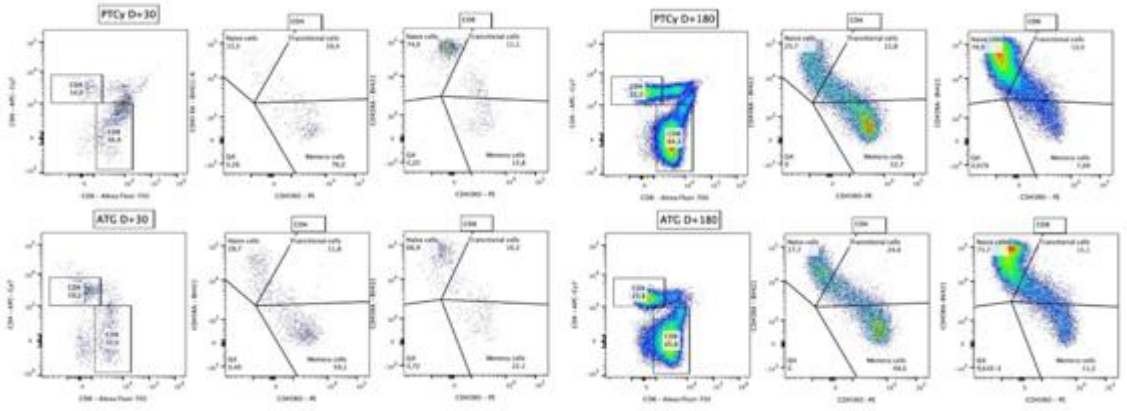


**Supplementary Figure 6 . Distribution of T CD4+, CD8+ and CD4+CD8+ lymphocytes by type of prophylatic immunosupression used in patients undergoing hematopoietic stem cell transplantation (HTSC): anti-thymocyte globulin (ATG) or post-transplant cyclophosphamide (PTCy) for pre transplant and 30, 60, 90 and 180 days**
There is an inversion of the CD4/CD8 ratio in both groups and a higher number of DP cells in the cyclophosphamide group on D+180



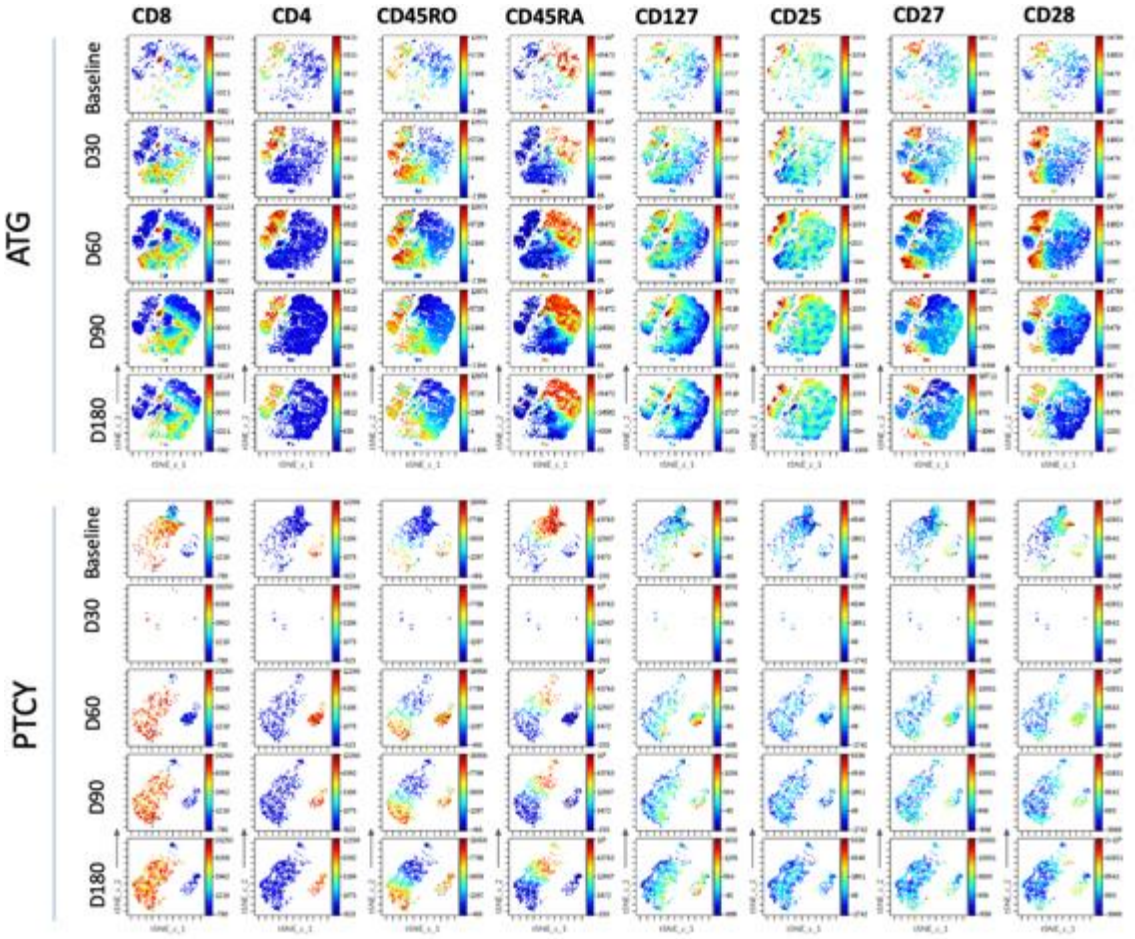

**Supplementary Figure 7. Flow cytometry results for T lymphocytes of patients undergoing hematopoietic stem cell transplantation (HTSC) according to the type of immunosupression used: anti-thymocyte globulin (ATG) or post-transplant cyclophosphamide (PTCy) on 30 (D+30) or 180 (D+180) days after transplant**

Cases of T lymphocyte profiling cytometry from patients who received PTCy or ATG demonstrate a lower number of T lymphocytes in both groups on D+30 compared to D+180, with a predominance of memory CD4+ T lymphocytes and a higher number of naive CD8+ T lymphocytes in both groups.




**Supplementary Figure 8. Cell clusters from the Cytobank platform showing differences between 2 patients, one who received anti-thymocyte globulin (ATG) and another who received post-transplant cyclophosphamide (PTCy) regarding markers of subsets of T cells on the days + 30, 60, 90 and 180**

There is a predominance of CD8+ T lymphocytes in the early stages up to D+180, with a predominance of CD4+ cells with memory characteristics (CD45RO+) and higher number of transition and naive cells (CD45RA+) in CD8+ cells



**Supplementary Table 1**. T lymphocytes subsets

| Variable | | ATG | PTCy | P |
|---|---|---|---|---|
| | | **Cells/uL - mean [range]** | **Cells/uL - mean [range]** | |
| **Pre** | CD4+ memory | 114[77-148] | 66[29-253] | 0.579 |
| | CD4+ naive | 40[16-95] | 27[5-50] | 0.119 |
| | CD4+ transitional | 31[18-47] | 31[14-72] | 0.972 |
| | CD8+ memory | 47[35-82] | 37[20-171] | 0.703 |
| | CD8+ naive | 128[77-242] | 139[59-204] | 0.652 |
| | CD8+ transitional | 44 [38-110] | 81[81-131] | 0.945 |
| **D+30** | CD4+ memory | 100[36.2-157] | 78[21-168] | 0.795 |
| | CD4+ naive | 3[0.7-13] | 3[0.8-5] | 0.728 |
| | CD4+ transitional | 9[2-31] | 8[4-25] | 0.972 |
| | CD8+ memory | 108[79-233] | 120[34-315] | 0.959 |
| | CD8+ naive | 103[38-145] | 41[25-100] | 0.188 |
| | CD8+ transitional | 54[28-90] | 79[23-129] | 0.488 |
| **D+60** | CD4+ memory | 61[43-82] | 42[24-167] | 0.945 |
| | CD4+ naive | 3[2-15] | 5[0.6-10] | 0.781 |
| | CD4+ transitional | 8[4-17] | 18[4-36] | 0.386 |
| | CD8+ memory | 98[62-165] | 97[60-257] | 0.677 |
| | CD8+ naive | 108[48-209] | 77[39-153] | 0.405 |
| | CD8+ transitional | 106[84-162] | 108[42-241] | 0.945 |
| **D+90** | CD4+ memory | 98[73-119] | 91[34-172] | 0.970 |
| | CD4+ naive | 5[0.7-21] | 7[1-12] | 0.623 |
| | CD4+ transitional | 12[6-35] | 18[9-50] | 0.650 |
| | CD8+memoy | 97[53-148] | 67[53-166] | 0.880 |
| | CD8+ naive | 106[73-259] | 82[52-154] | 0.257 |
| | CD8+ transitional | 114[78-130] | 167[74-214] | 0.257 |
| **D+180** | CD4+ memory | 122[48-160] | 57[25-120] | 0.320 |
| | CD4+ naive | 12[0.8-27] | 7[2-15] | 0.698 |
| | CD4+ transitional | 8[4-27] | 12[7-18] | 1.000 |
| | CD8+ memory | 63[46-124] | 108[38-243] | 0.512 |
| | CD8+ naive | 133[72-408] | 117[66-152] | 0.903 |
| | CD8+ transitional | 112[69-167] | 93[33-254] | 0.903 |

ATG: anti-thymocyte globulin; PTCy: post-transplant cyclophosphamide.

**Supplementary Table 2**. Effects of acute GVHD on immune reconstitution

| Variable | Without DECH Cells/uL (mean) | With DECH Cells/uL (mean) | p-value |
|---|---|---|---|
| **Lymphocytes (D+60)** | 939.95 | 476.0 | **0.009** |
| **T cells (D+60)** | 307.0 | 186.0 | 0.157 |
| **CD4 (D+60)** | 34.0 | 33.0 | 0.715 |
| **CD8 (D+60)** | 246.0 | 50.0 | 0.135 |
| **CD4CD8 (D+60)** | 18.0 | 18.0 | 0.64 |
| **B cells (D+60)** | 44.0 | 10.0 | 0.104 |
| **NK cells (D+60)** | 260.0 | 163.0 | 0.268 |
| **CD56high (D+60)** | 124.0 | 118.0 | 0.903 |
| **CD56dim (D+60)** | 74.0 | 49.0 | 0.066 |
| **CD4 memory (D+60)** | 31.0 | 26.0 | 0.715 |
| **CD4 transitional (D+60)** | 4.0 | 11.0 | 0.448 |
| **CD4 naive (D+60)** | 2.0 | 3.0 | 0.94 |
| **CD8 memory (D+60)** | 78.0 | 11.0 | 0.157 |
| **CD8 transitional (D+60)** | 50.0 | 18.0 | 0.19 |
| **CD8 naive (D+60)** | 36.0 | 24.0 | 419 |
| **Lymphocytes (D+90)** | 1155.6 | 739.8 | 0.19 |
| **T cells (D+90)** | 404.0 | 289.0 | 0.237 |
| **CD4 (D+90)** | 74.0 | 44.0 | 0.124 |
| **CD8 (D+90)** | 280.0 | 164.0 | 0.295 |
| **CD4CD8 (D+90)** | 28.0 | 5.0 | 0.061 |
| **B cells (D+90)** | 79.0 | 20.0 | **0.037** |
| **NK cells (D+90)** | 267.0 | 259.0 | 1.0 |
| **CD56 (D+90)** | 90.0 | 147.0 | 0.508 |
| **CD56dim (D+90)** | 88.0 | 79.0 | 0.623 |
| **CD56 neg (D+90)** | 22.0 | 12.0 | 0.064 |
| **Monocytes (D+90)** | 170.0 | 180.0 | 0.404 |
| **CD4 memory (D+90)** | 76.0 | 37.0 | **0.026** |
| **CD4 naive (D+90)** | 4.0 | 1.0 | 0.057 |
| **CD4 transitional (D+90)** | 12.0 | 3.0 | 0.057 |
| **CD8 memory (D+90)** | 67.0 | 51.0 | 0.508 |
| **CD8 transitional (D+90)** | 90.0 | 45.0 | 0.273 |
| **CD8 naive (D+90)** | 93.0 | 53.0 | 0.404 |

GVHD: graft versus host disease