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High-dose Alemtuzumab-Cyclosporine vs Tacrolimus-Methotrexate-Sirolimus for Chronic Graft-versus-Host Disease Prevention

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Abstract:
Chronic graft-versus-host disease (cGVHD) remains a significant problem for patients after allogeneic hematopoietic stem cell transplants (allo-HSCT). While in vivo lymphodepletion by antibodies for cGVHD prophylaxis has been explored in the myeloablative setting, its effects after reduced intensity conditioning (RIC) are not well described. Patients (n=83) with hematologic malignancies underwent targeted lymphodepletion chemotherapy followed by a RIC allo-HSCT using peripheral blood stem cells from unrelated donors. Patients were randomized to two GVHD prophylaxis arms: high-dose alemtuzumab/cyclosporine (AC, n=44) and tacrolimus/methotrexate/sirolimus (TMS, n=39) with the primary endpoint of cumulative incidence of severe cGVHD. The incidence of severe cGVHD was lower with AC vs TMS prophylaxis at 1- and 5-years (0% vs 10.3% and 4.5% vs 28.5%, overall p=0.0002), as well as any grade (p=0.003) and moderate-severe (p<0.0001) cGVHD. AC was associated with higher rates of grade III-IV infections (p=0.02) and relapse (52% vs 21%, p=0.003) with a shorter 5-year PFS (18% vs 41%, p=0.01) and no difference in 5-year GRFS, OS, or NRM. AC severely depleted naïve T-cells reconstitution, resulting in reduced TCR repertoire diversity, smaller populations of CD4 Treg and CD8 Tscm, but a higher ratio of Treg to naïve T-cells at 6 months. In summary, an alemtuzumab-based regimen successfully reduced the rate and severity of cGVHD after RIC allo-HSCT and resulted in a distinct immunomodulatory profile which may have reduced cGVHD incidence and severity. However, increased infections and relapse resulted in a lack of survival benefit after long-term follow-up. ClinicalTrials.gov identifier: NCT00520130

Conflict of interest: COI declared - see note

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Key Points:

- High-dose alemtuzumab led to effective suppression of chronic GVHD.
- Lymphodepletion delays Tn recovery resulting in lower TCR repertoire and higher Treg:Tn.

Abstract:

Chronic graft-versus-host disease (cGVHD) remains a significant problem for patients after allogeneic hematopoietic stem cell transplants (allo-HSCT). While *in vivo* lymphodepletion by antibodies for cGVHD prophylaxis has been explored in the myeloablative setting, its effects after reduced intensity conditioning (RIC) are not well described. Patients (n=83) with hematologic malignancies underwent targeted lymphodepletion chemotherapy followed by a RIC allo-HSCT using peripheral blood stem cells from unrelated donors. Patients were randomized to two GVHD prophylaxis arms: high-dose alemtuzumab/cyclosporine (AC, n=44) and tacrolimus/methotrexate/sirolimus (TMS, n=39) with the primary endpoint of cumulative incidence of severe cGVHD. The incidence of severe cGVHD was lower with AC vs TMS prophylaxis at 1- and 5-years (0% vs 10.3% and 4.5% vs 28.5%, overall p=0.0002), as well as any grade (p=0.003) and moderate-severe (p<0.0001) cGVHD. AC was associated with higher rates of grade III-IV infections (p=0.02) and relapse (52% vs 21%, p=0.003) with a shorter 5-year PFS (18% vs 41%, p=0.01) and no difference in 5-year GRFS, OS, or NRM. AC severely depleted naïve T-cells reconstitution, resulting in reduced TCR repertoire diversity, smaller populations of CD4 Treg and CD8 Tscm, but a higher ratio of Treg to naïve T-cells at 6 months. In summary, an alemtuzumab-based regimen successfully reduced the rate and severity of cGVHD after RIC allo-HSCT and resulted in a distinct immunomodulatory profile which may have reduced cGVHD incidence and severity. However, increased infections and relapse resulted in a lack of survival benefit after long-term follow-up. ClinicalTrials.gov identifier: NCT00520130

Introduction

For patients with high-risk hematologic malignancies, allogeneic hematopoietic stem cell transplantation (allo-HSCT) can be a curative therapy, mediated through immunological graft-versus-tumor (GVT) effects. Chronic GVHD (cGVHD) is an important late complication of transplant and a major cause of non-relapse morbidity, mortality, and chronic disability, also associated with fewer malignancy relapses.[1] Since current systemic treatments for patients with cGVHD are unsatisfactory, developing effective prevention strategies are of major interest.[2-3] Patients with severe forms of cGVHD[4] are those who bear the highest mortality and functional disability and should be the primary target for prevention. Therefore, a better understanding of the *in vivo* immunobiology associated with the development of clinical cGVHD is an important clinical research goal.

Methods for pharmacological prevention of GVHD are based on combinations of a calcineurin inhibitor (cyclosporine or tacrolimus) plus another agent, most commonly low-dose methotrexate, or use of high-dose cyclophosphamide post-transplant (PT-Cy).[5] With the recognized role of donor T-cells in GVHD, many studies of GVHD prevention focus on either *in vivo* depletion of donor T-cells by systemic administration of polyclonal or monoclonal antibodies (anti-thymocyte globulin, ATG, and anti-CD52, alemtuzumab, respectively)[6-12] or PT-Cy;[13-15] or *ex vivo* graft manipulation via CD34+ selection[16-19] or αβT-cell depletion.[20-21] Antibodies such as ATG and alemtuzumab exert variable effects based on dosage, timing of administration, and formulation.[22-27] Early studies employed high doses of alemtuzumab (a total dose of 100 mg).[38,51] However, in today's treatment landscape, safe de-escalation to total doses ranging between 30-60 mg is achievable without compromising clinical outcomes.[52,53] While higher doses have a strong protective effect against GVHD,[7-8,28-30] this often comes with increased rates of relapse, graft rejection, infections,[31-32] and Epstein-Barr virus-associated lymphoproliferative disease.[33] Further, timing alemtuzumab dosing during conditioning, proximally or distally, can affect outcomes such as achievement of full donor chimerism, relapse and GVHD.[61-62]

Benefits of *in vivo* lymphodepletion, including the reduction in incidence of both acute and chronic GVHD, for allo-HSCT with myeloablative conditioning (MAC) using matched unrelated donors (MUD) have been well established through several large, randomized controlled trials

using ATG.[7-8,10,34] However, older patients cannot tolerate MAC due to frailty or comorbidity and undergo transplants with reduced intensity conditioning (RIC) regimens in which efficacy is primarily dependent on GVT effects from the functional lymphocytes infused within the graft. Additionally, the risk of cGVHD is increased with older recipient age, transplantation from unrelated donors, and the use of peripheral blood stem cell (PBSC) source – the latter two of which are the predominant stem cell source of RIC allografts for allo-HSCT,[35] emphasizing the need for improved understanding and development of cGVHD prevention strategies in the RIC setting. While the introduction of PT-Cy is adopted as the standard for haploidentical (haplo) allo-HSCT, it is also moving to the forefront of cGVHD prevention for RIC allo-HSCT using fully matched donors due to recently presented randomized data[36] that showed effective decrease in cGVHD and improved GVHD-free, relapse-free survival (GRFS). Though this did not translate into a difference in OS, and long-term effects on organ toxicity, infections, and relapse are yet to be seen. Therefore, exploring alternative approaches for cGVHD prevention and elucidating the immunological mechanisms driving development of cGVHD, or lack thereof, remains an important goal for the field.

In this randomized, single-center, open label, phase 2 prospective trial we directly compared the clinical and immunological consequences of two strategies to prevent GVHD: post-HSCT *in vivo* depletion of donor lymphocytes using high-dose alemtuzumab[37-38] versus pharmacological suppression[39] of immune activation in the setting of reduced-intensity PBSC transplants. We further describe novel immunobiological changes associated with post-HSCT immune reconstitution in the context of these two approaches, as well as that associated with development of cGVHD in this prospective, randomized setting.

Methods

Study details

The study protocol was approved by the National Cancer Institute (NCI) Institutional Review Board and all patients signed an informed consent to participate (ClinicalTrials.gov identifier: NCT00520130). Patients were enrolled between September 2007-May 2014. Details on eligibility criteria and donor selection are reported in the **Supplemental Methods**.

Study design:

Patients first received disease-specific induction chemotherapy cycles (range 1-3 cycles)[40-41] for host lymphodepletion aiming for an absolute CD4+ T-cell target <100/ µL prior to transplant to

accelerate the achievement of full donor chimerism[42-43] (Supplemental Methods, **Supplementary Figure 1**). All patients received an identical conditioning regimen consisting of concurrent fludarabine (30 mg/m^2/d for 4 days) and cyclophosphamide (1200 mg/m^2/d for 4 days),[43] followed by infusion of a G-CSF-mobilized T-cell replete peripheral blood allograft from an HLA-matched or mismatched unrelated donor procured via the National Marrow Donor Program. Patients with hematologic malignancy were randomly assigned to receive either of the following two GVHD prophylaxis regimens: (a) high-dose alemtuzumab and cyclosporine (AC)[37-38] or (b) tacrolimus, sirolimus, and methotrexate (TMS).[39] The primary objective was to determine the cumulative incidence of severe cGVHD per NIH global severity score in the two arms.[44] Secondary objectives were to determine the incidence, organ severity and overall severity of cGVHD,[44] and acute GVHD (grades II-IV, III-IV).[45] Statistical methods, endpoints and immunologic correlate analyses are described in the **Supplemental Methods.**

Treatment arms

Tacrolimus, sirolimus, and methotrexate (TMS) arm: Tacrolimus was initiated on day -3 before stem cell infusion, administered by continuous intravenous (IV) infusion at a dose of 0.02 mg/kg/day with a target serum level of 5-10 ng/mL and converted to an equivalent oral dose given every 12 hours prior to discharge. Sirolimus was initiated on day –3, administered by mouth (PO) with an initial loading dose of 12 mg, and subsequently, 4 mg PO each day, with goal trough levels of 3-12 ng/mL. Both were continued and reduced by 1/3 at days +63, +119 (+/- 2 days), and then completely discontinued by day +182 (6 months) if there were no signs of GVHD. Methotrexate 5 mg/m^2 IV was given over 15 minutes on days +1,+3,+6, and +11.

Alemtuzumab and Cyclosporine (AC) arm: Alemtuzumab (CAMPATH-1H, Campath®, humanized monoclonal anti-CD52 antibody) was administered at 20 mg/day by IV infusion over 8 hours on days −8 to −4 (total dose of 100 mg, defined as high-dose alemtuzumab throughout this manuscript) with pre-medication per standard of care. Cyclosporine (CsA) was initiated on day –1 before HSCT and administered by IV infusion at 2 mg/kg/dose every 12 hours, with target serum range of 175–250 µg/mL. CsA was converted to an equivalent oral dose given every 12 hours and continued until day +100, and then was tapered as long as the severity of GVHD was ≤grade 2. CsA was tapered by reducing the dose by approximately 10% from the last dose administered each week to a dose of 25 mg/day and completely discontinued by 6 months post-HSCT if there were no signs of GVHD.

The study protocol was approved by the National Cancer Institute (NCI) Institutional Review Board and all patients signed an informed consent to participate (ClinicalTrials.gov identifier: NCT00520130).

Results

Patient characteristics

Ninety-two patients were enrolled, 89 patients were randomized, and 83 (AC=44, TMS=39) were ultimately transplanted (**Fig.1**). Patient and disease characteristics of the two arms were similar except for a higher number of prior therapies in the AC arm (p=0.016), and higher CD34+ cells/kg infused dose in the AC arm (p=0.034) (**Table 1)**.

Engraftment and hematologic recovery

Median time to neutrophil recovery was shorter in the AC than in the TMS arm [9 (range, 7-36) vs. 11 (3-19) days, p=0.02]; time to platelet recovery[46] was similar: [14 (1-431) vs. 19 (1-99) days, p=0.96]. In the AC arm, primary graft failure[46] occurred in two patients (one with myeloma, one with myelofibrosis), with the former patient successfully engrafting after a second transplant. High donor chimerism (>95%) was achieved early on days +14 and +28 post-transplant and were similar in both arms (**Supplementary Fig.2**).

Acute and Chronic GVHD

The cumulative incidence of acute GVHD (aGVHD) was similar between both arms for both grade II-IV (32% in AC vs 31% in TMS, p=0.75, **Fig.2A**) and grade III-IV aGVHD (18% in AC vs 10% in TMS, p=0.52, **Fig.2B**) at 100- and 180-days post-transplant (**Table 2**). Organ-specific staging of aGVHD (**Supplementary Table 1)** revealed that Stage 3-4 aGVHD most commonly involved the GI tract (AC, n=7; TMS, n=5), followed by liver (AC, n=4; TMS, n=2). Stage 3 skin involvement was seen in 2 patients on each arm, with no cases of stage 4 skin aGVHD.

The cumulative incidence of severe cGVHD was significantly lower with AC than with TMS at 1-, 2-, and 5-years (overall p=0.0002): 0% vs. 10% (95% CI 3.2-22.3%), 4.5% (0.8-13.9%) vs. 25.6% (13.1-40.3%) and 4.5% (0.8-13.9%) vs. 28.5% (23.0-53.7%), respectively **(Fig.2E; Table 2**)**.** Additionally, significantly lower cumulative incidences of *any grade* cGVHD (p=0.003; **Fig.2C**) and *moderate-severe* cGVHD (p<0.0001, **Fig.2D**) was seen with AC compared to TMS prophylaxis.

Among those who developed cGVHD, the median time to initial onset of cGVHD was similar, 316 (182-1031) in AC vs. 301 (72-870) days in TMS arms. The maximum NIH cGVHD global severity was mild, moderate, and severe in 5 (11%), 4 (9%), and 3 (7%) patients in the AC arm, and in 1 (3%), 9 (23%), and 14 (36%) patients in TMS arm, respectively. Most patients had cGVHD of the skin (AC, n=9 [22%] vs. TMS, n=21 [54%]), followed by oral (AC, n=3 [7%] vs. TMS, n=17 [44%]), and ocular involvement (AC, n=3 [7%] vs. n=14 [36%]) (**Supplementary Table 2**). By multivariable Cox regression, the only prognostic factor for development of severe cGVHD was treatment with TMS (HR, 7.4 [95%CI, 1.7-32.5]; p=0.008; **Supplementary Tables 4-5**). Late onset of cGVHD beyond 2-years post-HSCT occurred in 3 patients at a median of 2.4 years (range 2.3-2.9) with only one having a clear trigger with receipt of DLIs for relapse.

For patients who developed any GVHD, the cumulative incidence of immunosuppression discontinuation at 3 years, competing with death or progression, was similar between both arms (AC, 22.7% vs. TMS, 17.9%; overall p=0.55; **Fig.2F**). At the time of analysis, 7 patients in each arm were permanently off systemic immunosuppression.

Toxicity and transplant-related mortality

The most common grade 3-4 adverse events within 100 days post-transplant were infectious, seen in 80% vs. 54% of patients in AC vs. TMS arms, p=0.019 (**Supplementary Table 3**). More grade 3-4 bacterial infections were seen with AC (p=0.004), with bacteremia and catheter-related infections being the most common. In terms of viral infections, a higher incidence of CMV reactivation was seen in the AC arm by D+100: 55% (95%CI, 39-68) vs. 21% (95%CI, 10-34), overall p=0.030 (**Supplementary Fig.3).** One grade 3 infusion reaction related to alemtuzumab occurred.

TRM at day 100 was 6.8% (95%CI, 1.7-16.9) with AC vs 10.3% (95% CI, 3.2-22.2) with TMS prophylaxis, p=0.33. TRM in patients ≥60 years of age was 0% with AC vs 33.3% (95% CI 9.3-60.1%) with TMS, p=0.26. Factors associated with increased TRM included higher age at transplant (≥60 vs. <60 years; HR, 2.81 [95%CI,1.30-6.11]; p=0.009), positive recipient CMV status (HR, 3.16 [1.44-6.93]; p=0.004) and HCT-CI score of ≥2 (HR, 7.73 [2.29-26.08]; p=0.001; **Supplementary Tables 6-7**). Non-relapse mortality at 2- and 5-years was similar in both arms (p=0.33; **Fig.3E; Table 2**). Causes of late NRM included: pneumonia/respiratory failure (n=4), metastatic secondary cancer (n=2), TMA/respiratory failure due to cGVHD (n=1), GI obstruction complication (n=1), and coronary artery disease/myocardial infarction (n=1). Patients who experienced late NRM had lower absolute CD3 T-cells at 1-year (567 vs 1057 cells/μL, p=

0.004) vs non-late NRM patients, respectively; as well as lower total CD4- (194 vs 372 cells/μL, p=0.02) and CD8- (269 vs 624 cells/μL, p=0.01) T-cell subsets.

Subsequent cancers post-transplant developed in 13 patients (n=7 in AC, n=6 in TMS, p=0.50) at a median of 4.5 months (range, 1-69) post-HSCT. These included cases of PTLD, non-melanoma skin cancers, melanoma, thyroid cancer, lung cancer and a donor-derived myeloid neoplasm.

Survival and relapse outcomes

With a median follow-up of 9.8 years (range, 5.2-12.6), no statistically significant differences were found in 5-year OS (36% vs 46%; p=0.30) or GRFS (14 vs 8%; p=0.71) between both treatment arms (**Fig.3A,3C, Table 2**). There was a trend towards higher moderate-severe cGVHD-free survival (CFS) in the AC arm, 27% (15-49) vs 8% (1-19), p=0.06 (**Fig.3D**). By Cox model, adjusting for treatment arm (HR=0.77; 95%CI: 0.45-1.33; p=0.35), age ≥ 60 (HR=2.27; 95%CI; 1.26-4.07; p=0.0062), HCT-CI score 2-4 vs. 0-1 (HR=2.71; 95%CI: 1.38-5.34; p=0.0038), and positive recipient CMV status (HR=2.06; 95%CI: 1.17-3.63; p=0.013) were all jointly associated with poorer OS (**Supplementary Tables 8-9**). A higher HCT-CI score (2-4) was also associated with poorer GRFS (HR=2.08; 95%CI: 1.25-3.47; p=0.005) and moderate-severe CFS (HR=1.90; 95%CI: 1.11-3.26; p=0.02) after adjusting for treatment arm and CD34 cells/kg, (**Supplementary Tables 10-13**). Recipient female sex was found to be associated with improved moderate-severe CFS (HR=0.48; 95%CI 0.28-0.81, p=0.006, **Supplementary Table 13**)**.**

Cumulative incidence of relapse competing with death at both 2- and 5- years was twofold higher in the AC arm vs TMS arm [48% (32-62) vs 21% (10-35), and 52% (36-66) vs 21% (10-35), respectively, p=0.0027] (**Fig.3F**). Sixteen (19.3%) patients received donor lymphocyte infusions (median 1 infusion, range 1-5) for relapsed disease, of which 13 (81%) were on AC arm and 3 (19%) were on TMS arm. By multivariable analysis, factors associated with higher incidence of relapse included AC treatment arm [HR 2.60 (1.22-6.10), p=0.022], ≥3 prior treatment regimens [HR=2.70 (1.19-6.13), p=0.018], CD34 >8.1 [HR 2.90 (1.39-6.07), p=0.0046], and intermediate-high Kahl Relapse risk score [HR=2.32 (1.13-4.79), p=0.023] (**Supplementary Tables 14-15**). Late relapse (>2 years post-HCT) occurred in 4% (n=3) in AC arm at a median of 3.9 years (range, 2.1-9.2) post-HCT; No relapses were seen beyond 1-year on the TMS arm. Higher relapse-related mortality was associated with older donor age, ≥35 years vs 18-34 years [HR=2.54 (1.04-6.20), p=0.041] after adjusting for treatment arm and

number of prior treatments (**Supplementary Tables 16-17**). Median PFS was longer in TMS arm vs AC, 41.1 months (6-NE) vs 10.9 months (3.3-17.9), p=0.014 (**Fig.3B**).

Effect of baseline ALC on outcomes in AC arm

Among patients randomized to AC (n=44), median ALC (day -8 pre-HSCT, first day of alemtuzumab infusion) and pre-transplant CD4+ count was 2.85 $x10^9$/L (0-37.1) and 0.09 $x10^9$/L (0.01-0.9), respectively. The high ALC (HiALC, n=30, ALC ≥2 $x10^9$/L) and low ALC (LoALC, n=14, ALC<2 $x10^9$/L) groups were similar in all examined baseline variables. After a median follow-up of 1.4 years (0.1-11.5), estimated median OS was 1.4 years (95% CI, 0.7-7.3). Subjects in the HiALC group had improved OS compared to LoALC group (3.2 years [1.6-NE] vs 0.7 years [0.5-NE], p=0.001) with 1-, 2- and 5-year OS of 70 vs 29%, 63 vs 14% and 47 vs 14%, respectively (**Supplementary Fig.7A**). Median GRFS was 0.5 years (0.2-1.5) and 0.3 (0.1-3.9) for HiALC and LoALC group (p=0.08), respectively **(Supplementary Fig.7B**).

Immune reconstitution

Absolute lymphocyte count recovery to 500/µl was significantly delayed in the AC arm (median 76 vs. 16 days, p<0.0001). NK-cell numbers were significantly reduced in AC versus TMS during the first month (p<0.0001), but comparable by 100 days (**Fig.4A**). B-cell reconstitution was delayed in both arms, but comparable after day +180. Delays in T-cell repopulation in the AC arm were profound at 6 months, with 24 months required for repopulation of CD4+ and CD8+ T-cells to levels comparable to TMS (**Fig.4A**). Global T-cell receptor (TCR) repertoires post-transplant had significantly less diversity than those of their donors, consistent with early peripheral expansion of a limited TCR repertoire. Furthermore, the early repertoire diversity of most AC patients was more skewed and oligoclonal than in most TMS patients.

Analyses of T subset reconstitution revealed that the percentages of Treg cells within the CD4 population did not differ between the arms at any timepoint (**Fig.4D, Supplemental Fig.6**). Because of the marked early disparity in CD4+ T-cell numbers/µl, however, the number of Treg/µl in AC was significantly lower than that in TMS patients at 6- (p<0.0001) and 12-months (p=0.003), and became equivalent by 24 months (**Fig.4D, Supplemental Fig.5B**).

Both the percentages and the total number/µl of recent thymic emigrant (RTE) and naïve Treg and non-Treg CD4+, and naïve CD8+ T-cells were significantly lower in AC than in TMS (all p<0.0001) at 6 months. (**Fig.4E**; **Supplemental Fig.5C,6**). By 24 months, however, AC naïve populations became comparable to TMS through renewed thymopoiesis, as indicated by 10-to-

100-fold increases in median RTE and naïve T-cells/µl. In the TMS population, in contrast, no significant increase in naïve T-cells occurred from 6 to 24 months.

While the AC arm protocol resulted in severe quantitative reductions in both Treg and Tnaïve populations, the ratio of Treg cells/µl to non-Treg CD4naïve cells/µl, or of Treg/µl to CD8naïve/µl was significantly higher in AC than in TMS (p=0.0001 each for CD4 and CD8 naïve cells) (**Fig.4F**). Median ratios of Treg to naïve T-cells were almost 10-fold higher in AC than in the TMS arms. Onset of most cGVHD in both arms occurred between 6 and 12 months, that is, in the months following the greatest disparity in the ratios of Treg and Tnaïve cells.

At 6 months, the naïve CD8 population in AC patients were mainly memory stem T-cells (Tscm, CD45RA+CCR7dimCD95+) (**Fig.4C**), but the overall numbers of CD8 naïve and hence CD8 Tscm/µl were very low (**Fig.4H**). In the TMS arm, in contrast, the Tscm cells were not only present as a visible component within the total naïve CD8 cell populations (**Fig.4G),** but also persisted at increasing frequency and higher numbers during the main period of cGVHD onset (**Fig.4G, 4H**). Finally, when the individual AC and TMS patients at 6 months were plotted comparing the ratio of Treg to CD8+ naïve cells versus the number of CD8+ Tscm, the two T-cell population parameters were strongly correlated (ρ=-0.72, p<0.0001), but the two patient protocol arms were sharply distinct (**Fig.4I**).

Cytokines

Upon evaluation of post-HSCT cytokine levels, patients in the AC arm had higher levels of TNF-α and IL-6 at 3- and 6- months posttransplant with no differences between arms at any timepoint in levels of ST2, CXCL9, CXCL10 or BAFF (**Fig.5A**). Patients who developed aGVHD within 180 days posttransplant, excluding patients with prior relapse/progression, had significantly higher levels of ST2 at 6- and 9-months (with a trend at 3 months p=0.07)(**Fig.5B**). A trend towards higher CXCL10 at 9-months was associated with moderate-to-severe cGVHD (**Fig.5C**). No other difference was found in levels of cytokines, including BAFF and CXCL9, for patients who developed moderate-to-severe CGVHD (**Supplementary Figure 8**). Higher levels of IL-6 and TNF-α at 6-months were strongly associated with worse OS (**Fig.5D**) after adjusting for other important factors including treatment arm, age ≥60, recipient CMV status, and HCT-CI≥2 (**Supplementary Table 18**).

Discussion

Chronic GVHD remains a major barrier to successful allo-HSCT despite progress in preventive and treatment strategies over the last two decades.[1,3] This randomized study using a RIC transplant platform demonstrates high and clinically significant potency of a lymphodepleting high-dose alemtuzumab-based regimen in prevention of severe cGVHD. The described post-HCT immune reconstitution with alemtuzumab-based lymphodepletion could potentially inform design of improved future GVHD prophylaxis platforms. Differences in T-cell repopulation post-transplant may have been key factors in the greater incidence, severity, and persistence of cGVHD observed in the TMS compared to the AC arm. The profound depletion of T-cells, particularly naïve cells, in the AC arm limited the numbers of immunocompetent T-cells and early TCR repertoire diversity. Depletion of naïve T-cells also resulted in 5-10-fold higher median ratios of both Treg to naïve non-Treg CD4 cells/μl, and of Treg to naïve CD8 cells/μl in AC than in TMS at 6 months. The high Treg:Tn ratio in AC may preclude escape of allo-reactive T-cells from Treg control,[54] while permitting it in TMS. Finally, a large CD8+ Tscm population emerged in TMS by 6 months, at the end of immunosuppression, but prior to most cGVHD onset. These Tscm expanded and persisted in many cGVHD patients, consistent with growing evidence that Tscm play a role in cGVHD.[55-56] Thus, disparities in repopulation of naïve, regulatory and Tscm T-cells may have mediated the differential cGVHD outcome in this trial.

While no differences were seen in aGVHD between arms, the incidence of any grade, moderate-severe and severe cGVHD were significantly lower with lymphodepletion by alemtuzumab. Despite the significant prevention of cGVHD seen with AC, the benefit was offset by increased relapse, as demonstrated by the longer PFS in the TMS cohort, as well as increased infection. Though these did not translate into any difference in OS, GRFS or NRM between both arms. In terms of infections, this study was conducted in the era prior to regular use of letermovir prophylactic therapy against CMV,[47] and whether the same pattern of CMV reactivation would be replicated in the current era would be of interest. In terms of relapse rate, it is noteworthy that the study population was defined by high-risk characteristics (with 51% having HCT-CI ≥3, 19% KPS 60-80, and 40% Kahl high-risk) and diverse underlying hematologic malignancies. If viral reactivation and relapsed disease risk can be mitigated, then AC would offer a clear advantage in terms of cGVHD protection. In transplant settings where relapse is not of concern, such as non-malignant conditions, alemtuzumab is increasingly being used for this reason.[48] The rate of permanent IST discontinuation at 3-years (with relapse and death as competing risks) was 22.7% and 17.9% for AC and TMS arms, respectively, which is

comparable to that reported from similar LD-focused trials of 3-year IST-free survival of 17%.[8] Transplant success should be weighed heavily upon maximizing this IST-free survival endpoint.

Patients with aGVHD II-IV had higher median levels of ST2, while those with moderate-to-severe cGVHD had higher median levels of CXCL10. While elevated ST2 has been previously associated with aGVHD and NRM,[57] cytokine analyses revealed that only patients from the AC arm who developed aGVHD II-IV and moderate-to-severe cGVHD showed a statistical association with higher ST2 levels at 6-months and at 9-months, respectively.
Despite prior described associations of cGVHD diagnosis or severity with cytokines such as BAFF and CXCL9,[58] we did not find any predictive value or difference among these cytokine levels in this prospective longitudinal study in patients who did or did not develop cGVHD, which speaks to the continued challenge of identifying reliable predictive biomarkers for cGVHD. Finally, when adjusted for other variables associated with OS (recipient CMV status, HCT-CI≥2, and age≥60), higher levels of IL-6 and TNF-α at 6-months for the whole cohort were associated with worse OS – a finding that warrants further larger scale validation. While these correlative cytokine results may not be extrapolatable to other lymphodepletive strategies such as PT-Cy, they may serve as a source of hypothesis generation for future studies of biomarker development.

This study's strength as a single-center trial was that it allowed for a cohesive methodology in a well-controlled setting. Further, though cGVHD incidence was our primary endpoint, we report an extended duration of follow-up, which allows definitive assessment of the impact of cGVHDon other late effects on critical clinical outcomes This provides valuable insight on the long-term sequelae related to cGVHD which are crucial to the definitive success of transplant for survivors, including surveillance and prevention of late NRM, late relapse, and subsequent malignancies. Thirteen patients developed subsequent malignancies, including 4 cases of PTLD (3 in AC arm, 1 in TMS) which has a well-known association with lymphodepletion. [33] Notably, 2 of these second cancer cases developed into metastatic disease and were an important cause of late NRM, which emphasizes the need for close monitoring of our HSCT survivors for subsequent malignancies. Most cases of late NRM, defined as NRM beyond 2-years post-HSCT, occurred in patients on the TMS arm (n=9, vs n=2 in AC arm), and most were due to pneumonia or respiratory failure.

Limitations of this study include that about 20% of patients received grafts from 7/8 mMUD which can increase risk of GVHD; however, this factor was balanced among arms. Additionally,

the distribution of hematologic malignancy indication for transplant in this study included more lymphoma patients than would be expected to in our contemporary transplant landscape, which is predominantly myeloid malignancies, and thus, may influence treatment outcomes, as lymphoid diseases are more salvageable to DLI and adoptive cell therapies. However, this study was conducted in the pre-CAR-T era. Notably, we did not systematically collect data on pre-HSCT measurable residual disease yet acknowledge that this should be included in future studies as it can significantly affect relapse risk in the RIC setting.[59-60]

Another limitation is that alemtuzumab PK/PD studies were not incorporated into this study. Compared to proximal dosing, administering alemtuzumab more distally during transplant conditioning has also been shown to lead to less mixed chimerism and higher event-free survival in transplant studies for non-malignant indications.[61-62] However, since our primary study goal was cGVHD prevention, we elected a more proximal approach to maximize the effect on T cells in the graft rather than in the recipient. Our report provides novel and important immunological takeaways of the effect lymphodepletion as a method of cGVHD prevention, with the consideration that some of the toxicity seen (higher infections and relapse) may be attributed to the high exposure of alemtuzumab, which could be further optimized with dosing adjustments either based on weight or other variables such as ALC. This conclusion is also strengthened by our ALC-subgroup analysis regarding lower ALC ($<2.0 \times 10^9$/L) at time of alemtuzumab infusion associated with inferior survival in the AC arm than those with higher baseline ALC ($\geq 2.0 \times 10^9$/L). This finding is consistent with other reports[10,49-50] of ALC at time of conditioning administration influencing transplant outcomes and warrants further study of ALC-based dosing for alemtuzumab.

In terms of optimal *in vivo* lymphodepletion strategy, much enthusiasm has accompanied the tremendous success of PT-Cy, which has allowed for safe haplo HSCTs with improved rates of GVHD and survival, and has become standard of care in this haplo setting.[13,61-63] Studies have expanded its use to HLA-matched related (MRD) or matched/mismatched unrelated donors. PT-Cy compared to ATG in a study using MUDs led to similar rates of aGVHD, extensive cGVHD, 2-year OS, and relapse among both cohorts;[64] while a study using MRD[65] showed lower rates of cGVHD among the ATG group and no difference in other outcomes. No studies have been reported comparing PT-Cy with alemtuzumab to-date. Three recently reported randomized studies[36,66-67] evaluated PT-Cy compared to other standard prophylaxis after RIC allo-HSCT using a MUD/MRD, in which PT-Cy led to decreased rates of cGVHD, improved GRFS, but no difference in OS. PT-Cy has therefore moved to the forefront of current practice in cGVHD

prevention for RIC allo-HCT, though more data will be needed to better understand the long-term effects on organ toxicity, relapse, secondary malignancies, and, ultimately, overall survival.

In summary, an alemtuzumab-based platform after RIC transplant using MUD PBSCs robustly reduced the rate and severity of cGVHD compared to standard pharmacologic prophylaxis. A distinct immunomodulatory profile after AC may have caused reduced cGVHD incidence and severity, however, increased infections and relapsed malignancy resulted in a lack of survival benefit. Improved strategies to mitigate the associated risks of relapse and infectious toxicities in RIC allo-HSCT setting are needed. Future studies should focus on developing better cGVHD-risk stratification systems to identify those patients that may most benefit from antibody lymphodepletion versus other prevention approaches.

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Table 1: Patient baseline characteristics

Characteristic (n, % unless noted otherwise)	All, n=83	AC, n=44	TMS, n=39	p-value
Age at transplant (median, range)	50.3 (34.8-60.9)	51.3 (22.2-69.9)	49.5 (20.9-71.1)	0.87
Sex				
Male	53 (64)	29 (66)	24 (62)	0.68
Female	30 (36)	15 (34)	15 (38)	
Ethnicity				
Asian	3 (4)	1 (2)	2 (5)	
Black	7 (8)	4 (9)	3 (8)	0.95
Hispanic	4 (5)	2 (5)	2 (5)	
White	68 (82)	36 (82)	32 (82)	
Other	1 (1)	1 (2)	0	
Disease				
ALL	4 (5)	2 (5)	2 (5)	
AML	5 (6)	2 (5)	3 (8)	
MDS	5 (6)	3 (7)	2 (5)	
CLL	18 (22)	9 (20)	9 (23)	0.74
CML	3 (4)	0	3 (8)	
Hodgkin Lymphoma	8 (10)	5 (11)	3 (8)	
Non-Hodgkin Lymphoma	29 (35)	16 (36)	13 (33)	
Other	11 (13)	7 (16)	4 (10)	
Number of Prior Treatment Regimens				
0-2	39 (47)	15 (34)	24 (62)	**0.016**
3-5	32 (39)	19 (43)	13 (33)	
≥6	12 (14)	10 (23)	2 (5)	
Prior Transplants				
Yes				
Autologous	15 (18)	9 (20)	6 (15)	
Allogeneic	1 (1)	1 (2)	0	0.19[a]
Syngeneic	1 (1)	1 (2)	0	
Autologous and Allogeneic	1 (1)	1 (2)	0	
No	65 (79)	32	33	
Disease Status at Time of Transplant				
No Disease	29 (35)	16 (33)	13 (33)	
Active	38 (45)	21 (48)	17 (44)	0.69[b]
Minimal Residual Disease	13 (16)	5 (11)	8 (21)	
Not Evaluable	3 (4)	2 (5)	1 (2)	
Disease Chemotherapy Sensitivity				
Chemo-sensitive	49 (59)	27 (61)	22 (56)	0.51[c]
Chemo-resistant	30 (36)	14 (32)	16 (41)	
Not Evaluable	4 (5)	3 (7)	1 (2)	

HLA Match				
7/8	17 (20)	9 (20)	8 (21)	1.00
8/8	66 (80)	35 (80)	31 (79)	
Cycles of Induction Chemotherapy				
None	32 (39)	17 (39)	15 (39)	
EPOCH-F(R)	37 (45)	21 (48)	16 (41)	
Cycles (median, range)	2 (1-3)	2 (1-3)	1.5 (1-3)	0.52[d]
FLAG	13 (16)	5 (11)	8 (21)	
Cycles (median, range)	1 (1-3)	1 (1-2)	1.5 (1-3)	
Pentostatin/Cytoxan	1 (1)	1 (2)	0	
Baseline CD4 Count* (median, range)	86 (6-520)	86 (10-388)	82 (6-520)	0.75
Infusion Cell Dose (median, range)				
CD34+ (x10^6 cells/kg)	7.15 (2.03-11.1)	7.46 (4.09-10)	6.4 (2.03-11.1)	**0.034**
CD3+ (x10^8 cells/kg)	2.40 (0.80-8.10)	2.29 (1.07-5.32)	2.44 (0.80-8.10)	0.75
CMV Serology				
Both Seropositive	35 (42)	19 (43)	16 (41)	
Both Seronegative	25 (30)	13 (30)	12 (31)	0.54[e]
Seronegative Donor, Seropositive Recipient	12 (14)	8 (18)	4 (10)	
Seropositive Donor, Seronegative Recipient	11 (13)	4 (9)	7 (18)	
Donor and Recipient Sex				
Same Sex	49 (59)	29 (66)	20 (51)	0.35[f]
Female Donor, Male Recipient	13 (16)	5 (11)	8 (21)	
Male Donor, Female Recipient	21 (25)	10 (23)	11 (28)	
ABO Mismatch				
None	47 (57)	26 (59)	21 (54)	
Major	12 (14)	6 (14)	6 (15)	0.93
Minor	17 (20)	9 (20)	8 (21)	
Bidirectional	7 (8)	3 (7)	4 (10)	
HCT-CI Score[69]				
Median (range)	3 (0-7)	3 (0-6)	2 (0-7)	
0	18 (22)	11 (25)	7 (18)	0.86
1	7 (8)	3 (7)	4 (10)	
2	16 (19)	7 (16)	9 (23)	
3+	42 (51)	23 (52)	19 (49)	
Karnofsky Performance Status				
60-80	16 (19)	11 (25)	5 (13)	0.71
90	54 (65)	25 (57)	29 (74)	
100	13 (16)	8 (18)	5 (13)	
Kahl Relapse Risk[70]				
Low	27 (32)	13 (30)	14 (36)	0.37
Intermediate/Standard	23 (28)	11 (25)	12 (31)	
High	33 (40)	20 (45)	13 (33)	

Abbreviations: ALL – acute lymphoblastic leukemia, AML – acute myeloid leukemia, CLL – chronic lymphocytic leukemia, CML – chronic myelogenous leukemia, HCT-CI – hematopoietic cell transplant-comorbidity index, MDS – myelodysplastic syndrome

Continuous parameters were compared between arms using a t-test, categorical parameters were compared using a chi-squared test.

[a] comparison of prior transplant vs. no prior transplant by arm

[b] comparison includes 'Not Evaluable'; p=0.54 if 'Not Evaluable' excluded

[c] comparison includes 'Not Evaluable'; p=0.47 if 'Not Evaluable' excluded

[d] comparison of no induction chemotherapy vs. EPOCH-F(R) vs. FLAG

[e] global p-value; p=0.93 for match vs. mismatch

[f] global p-value; p=0.18 for match vs. mismatch; p=0.25 for female donor, male recipient vs. other

Table 2: Clinical outcomes post-transplant, per arm. Time to event analyses estimating cumulative incidence with competing risks: * cumulative incidence of clinical outcome competing with death, relapse, graft failure, cGVHD without prior aGVHD (for aGVHD only) ** cumulative incidence competing with relapse, *** cumulative incidence competing with death, graft failure.

Outcome	AC Estimate (95% CI)	TMS Estimate (95% CI)	p-value
Grade II-IV acute GVHD*			
100 days	0.32 (0.19-0.46)	0.31 (0.17-0.46)	0.75
180 days	0.39 (0.24-0.53)	0.41 (0.25-0.56)	
Grade III-IV acute GVHD*			
100 days	0.18 (0.08-0.31)	0.10 (0.03-0.22)	0.52
180 days	0.23 (0.12-0.36)	0.16 (0.06-0.29)	
cGVHD of any grade*			
2 years	0.25 (0.13-0.39)	0.54 (0.37-0.68)	**0.0029**
5 years	0.25 (0.13-0.39)	0.59 (0.41-0.79)	
Moderate-severe cGVHD*			
2 years	0.11 (0.04-0.23)	0.51 (0.34-0.66)	**<0.0001**
5 years	0.11 (0.04-0.23)	0.54 (0.37-0.68)	
Severe cGVHD*			
2 years	0.05 (0.008-0.14)	0.26 (0.13-0.40)	**0.0002**
5 years	0.05 (0.008-0.14)	0.29 (0.23-0.54)	
Moderate-severe cGVHD-free survival			
2 years	0.39 (0.25-0.54)	0.18 (0.08-0.31)	**0.06**
5 years	0.27 (0.15-0.49)	0.08 (0.02-0.19)	
GVHD relapse-free survival			
2 years	0.18 (0.09-0.31)	0.18 (0.08-0.31)	0.71
5 years	0.14 (0.06-0.25)	0.08 (0.02-0.19)	
Overall survival			
2 years	0.48 (0.33-0.62)	0.72 (0.55-0.83)	0.30
5 years	0.36 (0.23-0.50)	0.46 (0.30-0.61)	
Progression-free survival			
2 years	0.25 (0.14-0.38)	0.59 (0.42-0.73)	**0.014**
5 years	0.18 (0.09-0.31)	0.41 (0.26-0.56)	
Non-relapse mortality**			
2 years	0.27 (0.15-0.41)	0.21 (0.10-0.35)	0.33
5 years	0.30 (0.17-0.44)	0.39 (0.23-0.54)	
Relapse***			
2 years	0.48 (0.32-0.62)	0.21 (0.10-0.35)	**0.0027**
5 years	0.52 (0.36-0.66)	0.21 (0.10-0.35)	

Figure 1: CONSORT diagram

Figure 2: A. Cumulative incidence (CI) of grade II-IV acute GVHD; **B.** CI of grade III-IV acute GVHD; **C.** CI of any cGVHD; **D**. CI of moderate-severe GVHD. **E.** CI of severe cGVHD. **F.** CI of being taken off immunosuppressive therapy (IST); *all CIs reported are competing with death, relapse or graft failure. For acute GVHD, competing risks also include chronic GVHD (without prior acute). *p-values for differences in cumulative incidence obtained using Gray's method

Figure 3: A Overall survival; **B** Progression-free survival; **C** GVHD-free relapse-free survival; **D** Moderate-severe cGVHD-free survival; **E** Cumulative incidence of non-relapse mortality competing with relapse; **F** Cumulative incidence of relapse competing with death. *p-values for differences in survival estimates obtained from log-rank test, p-values for differences in cumulative incidence obtained using Gray's method

Figure 4. Immune Reconstitution. A. Lymphocyte repopulation. Time course of lymphocyte subsets in AC (blue) and TMS (red) treatment arm patients at 0.5, 1, 3, 6,12 and 24 months. Medians are indicated as circles, 25th and 75th quartiles are shown as error bars. Circles placed below the X axis indicate medians of 0 cells. For patient numbers and statistical disparity between arms at each T-cell time point refer to **Supplemental Figure 5A**. **B. Time course of changes in post-transplant TCR repertoire skewing.** Repertoire skewing (oligoclonality) indices (RSI) determined for individual AC (blue circles) and TMS (red circles) patients at 1-, 3-, 6- and 12-months were compared between arms (top statistics), and to the RSI of their own allo transplant donors (black triangles) (bottom statistics). Both CD4 (left graph) and CD8 cells (right graph) in the AC arm had more repertoire skewing (more oligoclonality) than those in most patients in the TMS arm or in their donor's original T-cells at 1-, 3-, and 6-months in CD4 and at 3- and 6-months in CD8 T-cells. **C. Flow cytometry gating profiles of T lymphocytes at 6 months in the AC and TMS arms**. CD4 cells were gated to distinguish Treg (thick outline) from non-Treg (conventional) CD4 T-cells, based on Treg characterization as CD127-CD25++ CD4 cells. The non-Treg and Treg CD4 and the CD8 cells were then gated to assess naïve (CD45RA+CCR7+; thick outline), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-) and TEMRA (CD45RA+CCR7-) subsets. **D.** Box and whisker plots of the percentage of Treg cells within the CD4 population (left graph) and the total number of Treg/µl (right graph) in AC (white box) and TMS (gray box) patients at 6-, 12- and 24-months post-transplant. **E**. Box and whisker plots of the numbers of naïve CD4 T-cells/µl (left graph) and of naïve CD8 T-cells/µl (right graph) at 6-, 12- and 24-months. **F.** Box and whisker plots of the ratios of the numbers of Treg to the number of naïve CD4 (left graph) or to the number of naïve CD8 T-cells (right graph) at 6-, 12- and 24-months. **G. Flow cytometry gating profiles of CD8 Tscm populations.** TMS patient flow cytometry panels identifying naïve (CD45RA+ CCR7+) CD8 cells (thick line box) at 6-, 9- and 12-months (left column), showing gating on the CD8naïve (arrows) to identify CCR7dimCD95+ Tscm at 3 sequential time points (right column). The patient shown developed moderate CGVHD at 9 months. **H.** Box and whiskers plot comparing the number of CD8 Tscm/µl in AC (white box) and TMS (gray box) at 6-, 12- and 24-months. **I.** Scatter plot of individual AC (blue circle) and TMS (red circle) patients as assessed for the ratio of Treg/µl to CD8naïve/µl versus the number of Tscm/µl. In all box and whisker plots, the number of patients assayed at each timepoint in each arm is shown in **Supplemental Figure 5C**. In all graphs, AC arm patients are shown in white box and TMS in gray box, boxes define the median, 25^{th} and 75^{th} quartiles and whiskers show minimum and maximum values. Mann-Whitney unpaired nonparametric statistics were performed to compare AC and TMS lymphocyte numbers, RSI, and lymphocyte subpopulations and ratios; shown as stars: **** p <0.0001, *** p<0.001, ** p<.01*. p<0.05.

Figure 5. **A.** Trends in cytokine levels compared between arms AC (blue circles) vs. TMS (red circles) at 3-, 6-, 9-, and 12-months post-HSCT. **B.** Trend and differences between ST2 levels post-HSCT among patients who did (orange circles) or did not (gray circles) develop grade 2-4 acute GVHD; and did (purple circles) or did not (gray circles) develop moderate to severe chronic GVHD. **C.** Trend and differences between CXCL10 levels post-HSCT among patients who did (orange circles) or did not (gray circles) develop grade 2-4 acute GVHD, and did (purple circles) or did not (gray circles) develop moderate to severe chronic GVHD. **D.** Higher levels of TNF-α and IL-6 at 6-months were associated with worse overall survival. TNF-α median: 2.7 pg/mL; IL-6 quartiles: Q1 <0.84 pg/mL, Q2 0.84-1.3 pg/mL, Q3 >1.3-2.2 pg/mL, Q4 >2.2 pg/mL. #: p-value <0.05, q-value >0.05.

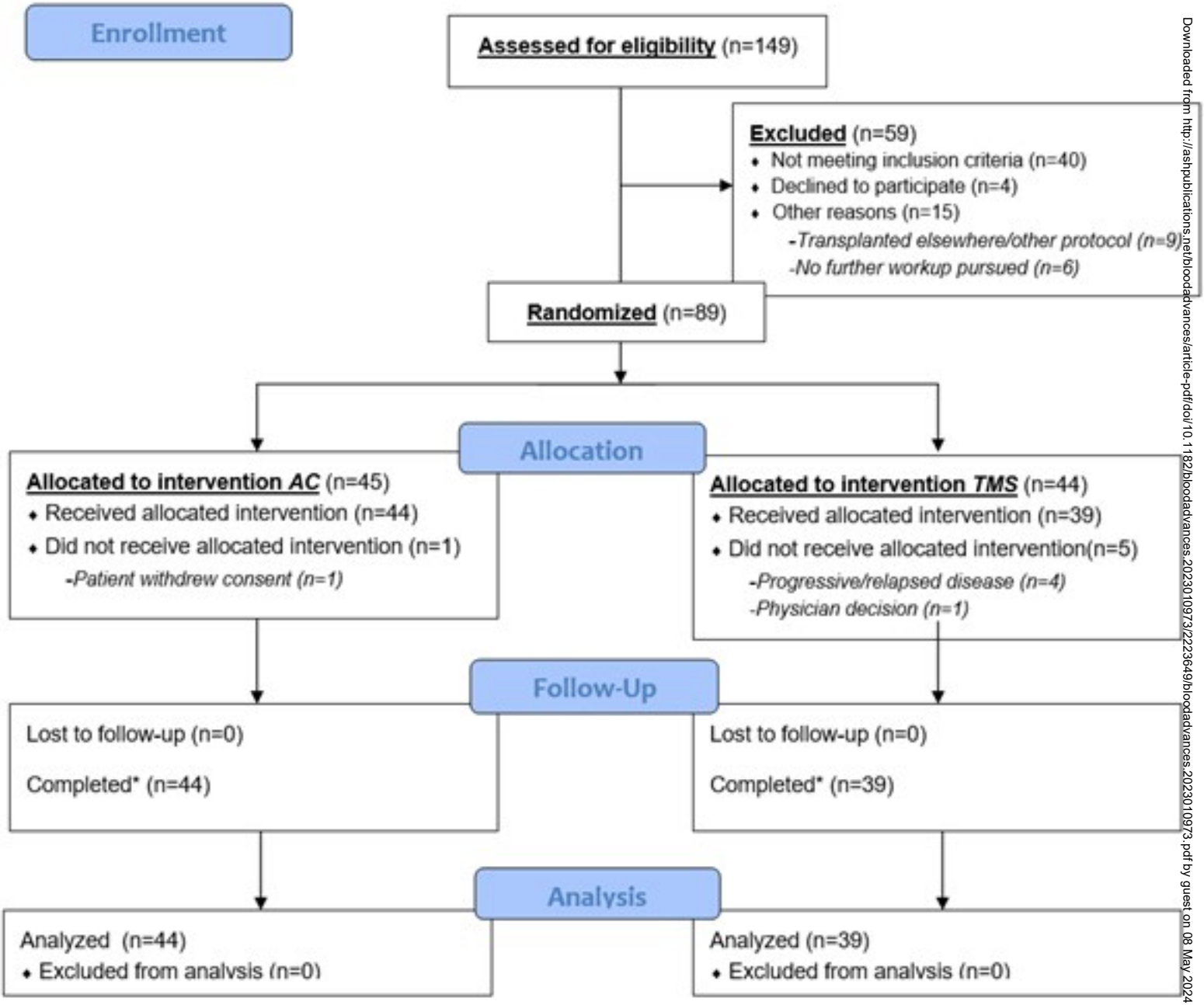
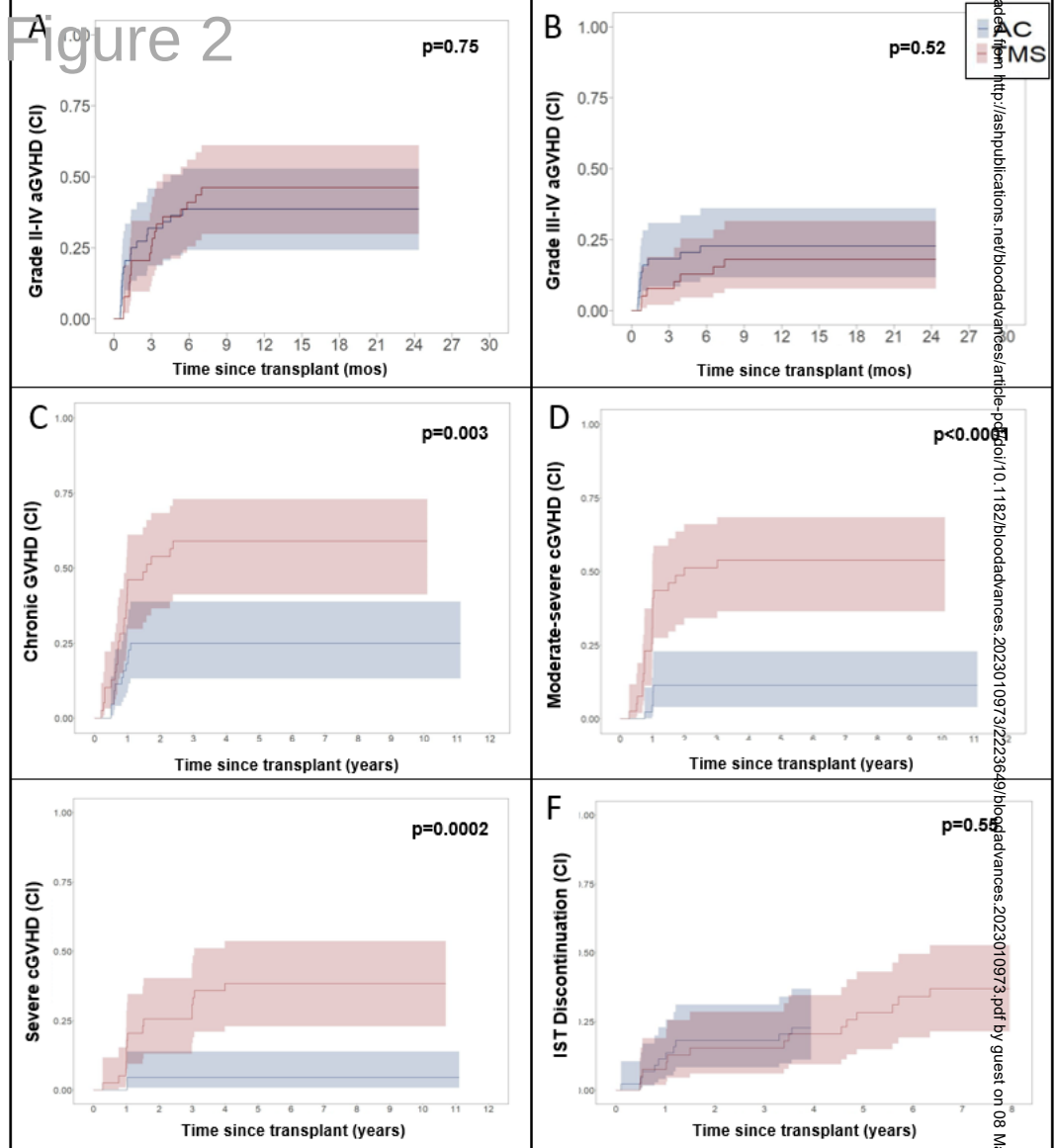
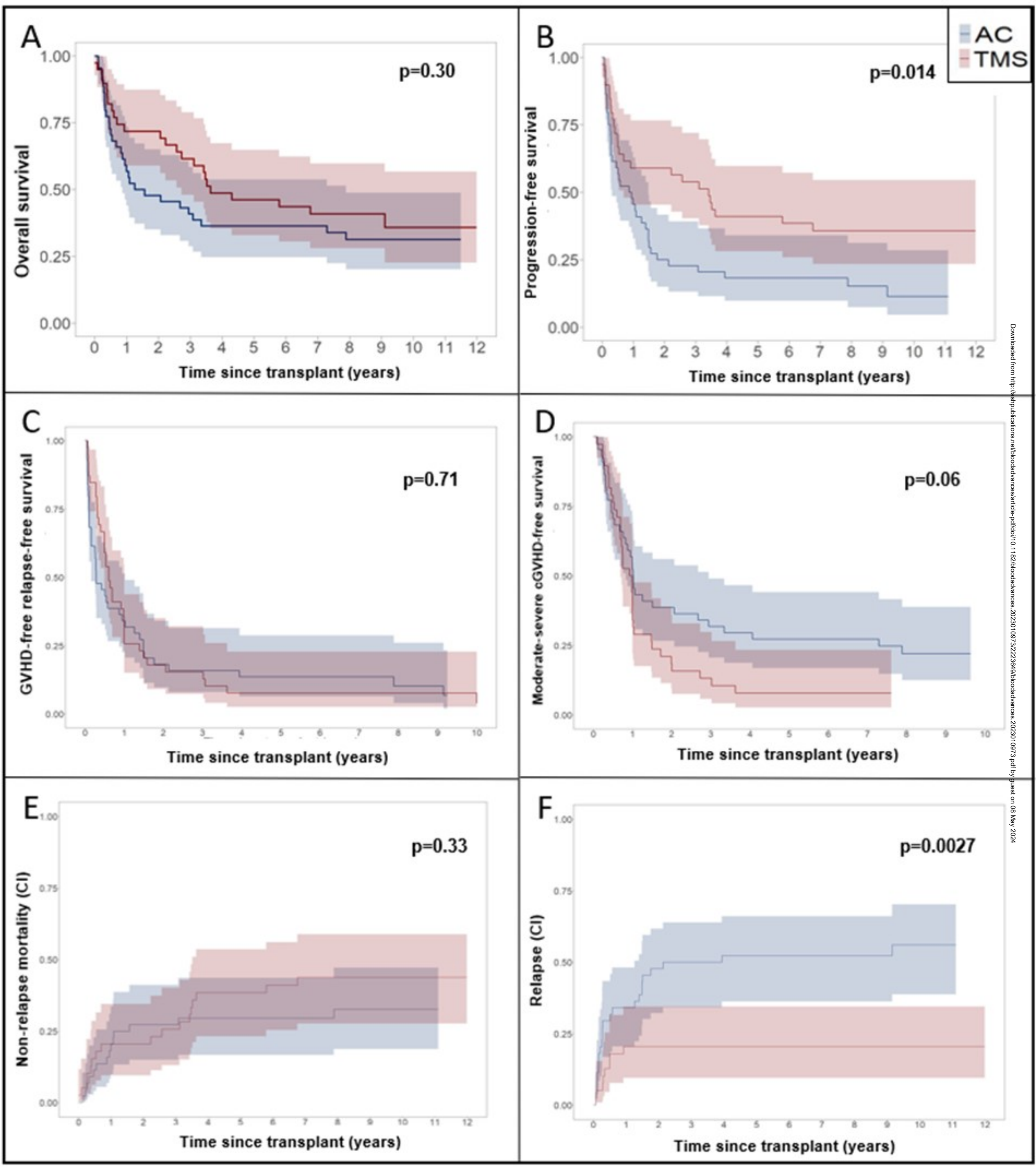
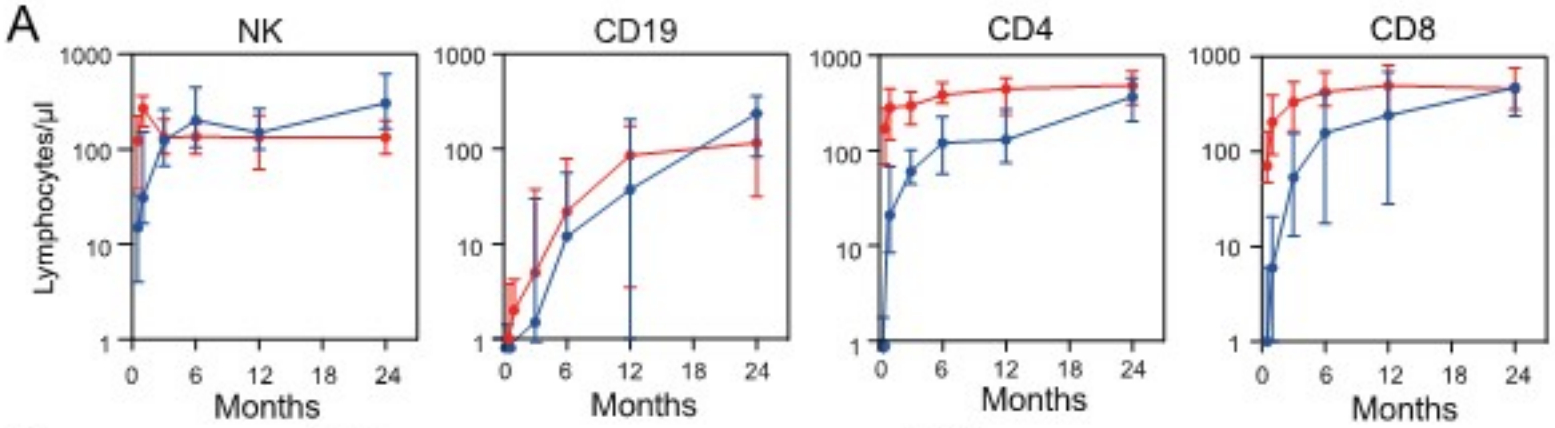
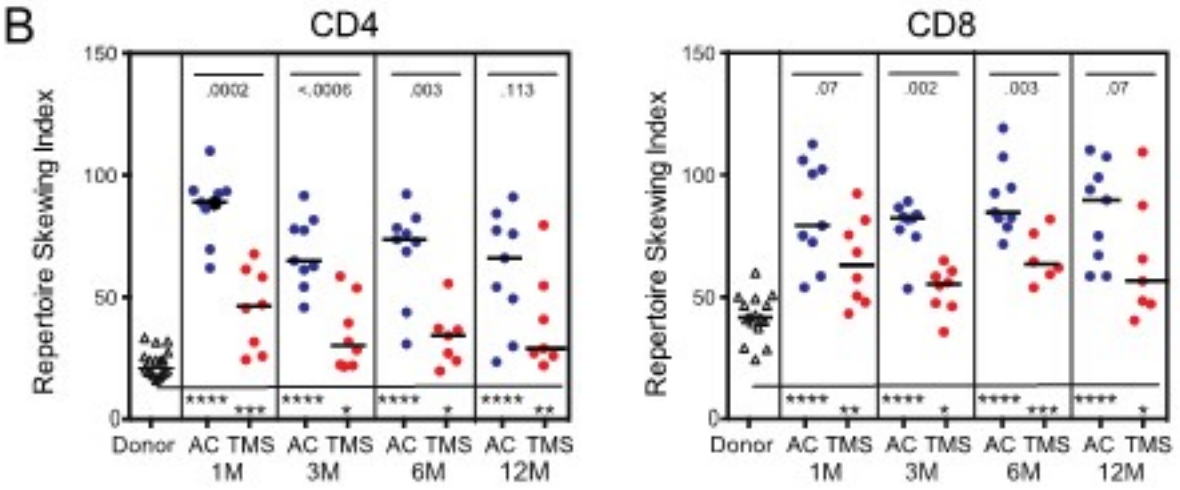


Figure 2









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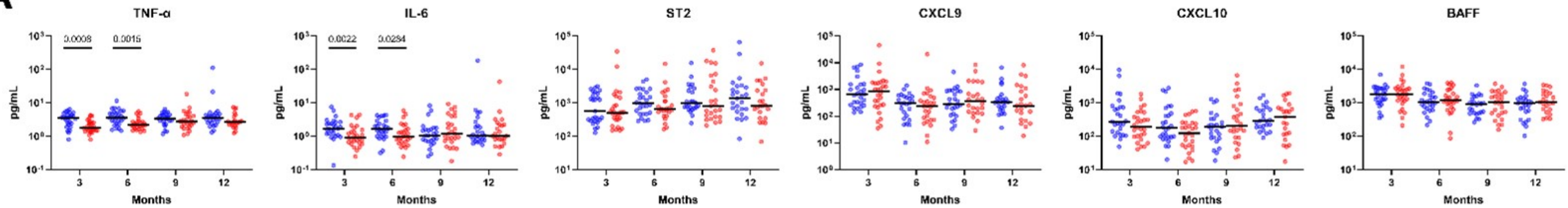

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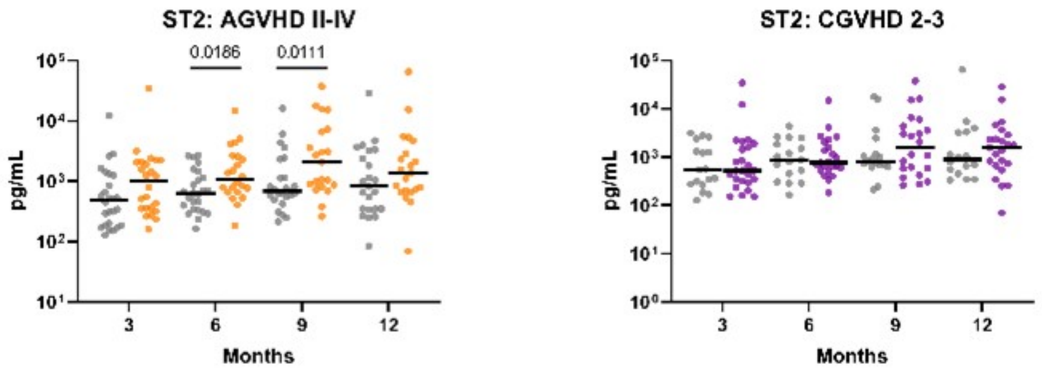

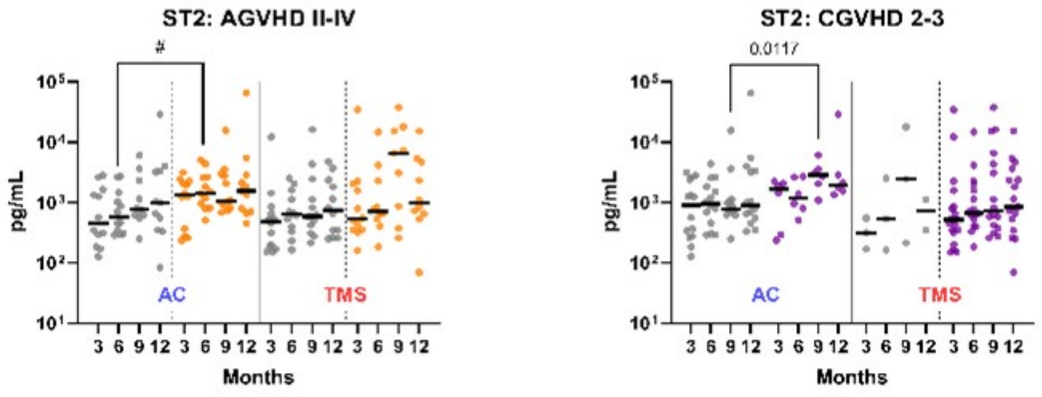

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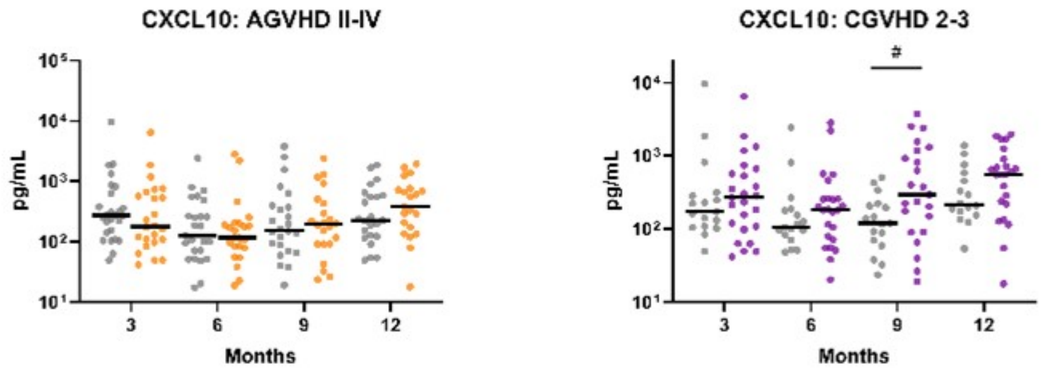

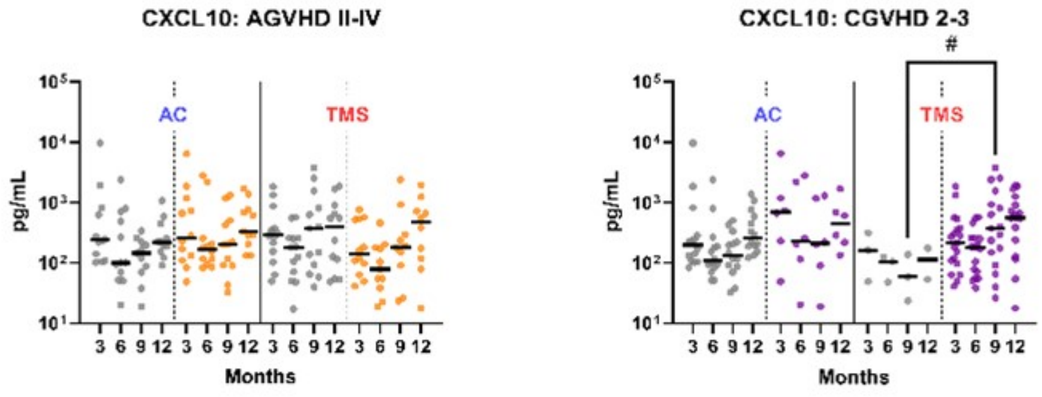

D