Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies

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Prophylaxis of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HCT) remains challenging. Because prospective randomized trials of in vivo T cell depletion using anti-T-lymphocyte globulin (ATLG) in addition to a calcineurin inhibitor and methotrexate (MTX) led to conflicting outcome results, we evaluated the impact of ATLG on clinical outcome, lymphocyte- and immune reconstitution survival models. In total, 1500 consecutive patients with hematologic malignancies received matched unrelated donor (MUD) HCT with cyclosporin and MTX (N = 723, 48%) or with additional ATLG (N = 777, 52%). In the ATLG cohort, grades III-IV acute (12% vs 23%) and extensive chronic GVHD (18% vs 34%) incidences were significantly reduced (P < .0001). Nonrelapse mortality (27% vs 45%) and relapse (30% vs 22%) differed also significantly. Event-free and overall survival estimates at 10 years were 44% and 51% with ATLG and 33% and 35% without ATLG (P < .002 and <.0001). A dose-dependent ATLG effect on lymphocyte- and neutrophil reconstitution was observed. At ATLG exposure, lymphocyte counts and survival associated through a logarithmically increasing function. In this survival model, the lymphocyte count optimum range at exposure was between 0.4 and 1.45/nL (P = .001). This study supports additional ATLG immune prophylaxis and is the first study to associate optimal lymphocyte counts with survival after MUD-HCT.

KEYWORDS
bone marrow/hematopoietic stem cell transplantation, clinical research/practice, flow cytometry, graft-versus-host disease (GVHD), graft-versus-leukemia (GVL)/graft versus tumor, hematology/oncology, immunosuppressant – polyclonal preparations: rabbit antithymocyte globulin, immunosuppression/immune modulation, mathematical model, translational research/science

Abbreviations: aGVHD, acute graft-versus-host disease; ALC, absolute lymphocyte count; AML, acute myeloid leukemia; aSCT, allogeneic stem cell transplantation; ATG, antithymocyte-globulin; ATLG, anti-T-lymphocyte-globulin; CI, confidence interval; CML, chronic myeloid leukemia; CMV, cytomegalovirus; CSP, cyclosporin; CTCAE, common terminology criteria for adverse events; EBV, Epstein-Barr virus; EFS, event-free survival; FACS, fluorescence-activated cell sorting; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen; HR, hazard ratio; MDS, myelodysplastic syndromes; MMF, mycophenolate-mofetil; MUD, matched unrelated donors; NRM, nonrelapse mortality; OS, overall survival; PBSC, peripheral blood stem cells; RIC, reduced intensity conditioning.

[Correction added on February 6, 2019, after first online publication: The term globulin has been corrected in the title and throughout the article.]
1 | INTRODUCTION

Effective prophylaxis of acute- and chronic graft-versus-host disease (GVHD) remains an unmet medical need after matched unrelated donor (MUD) allogeneic hematopoietic stem cell transplantation (HCT). Both grades III-IV acute GVHD (aGVHD) and severe chronic GVHD (cGVHD) associate with substantial morbidity and nonrelapse mortality (NRM) after HCT. Standard prophylactic immunosuppressive regimens combining a calcineurin inhibitor, such as cyclosporin (CSP) with short-course MTX have shown limited efficacy to prevent grades III-IV aGVHD and severe cGVHD. In several clinical studies, additional in-vivo T cell depletion has reduced the incidence of both aGVHD and cGVHD as a consequence it has become standard of care for MUD HCT in many transplant centers. There are different polyclonal T cell depleting globulins: Rabbit anti-T-lymphocyte globulin (ATLG) and antithymocyte globulin (ATG) and polyclonal horse ATG. In-vivo T cell depletion is equally used as a preparatory regimen prior to solid organ transplantation, in particular in patients with positive crossmatch or alloantibodies. Two prospective randomized trials, which investigated in-vivo T cell depletion using ATLG as part of immune prophylaxis in myeloablative MUD HCT recipients, led to inconsistent outcome results concerning relapse incidence and its impact on overall survival (OS). As a consequence, a number of questions have been discussed relating to study design, ATLG in different malignancies, optimal ATLG dosage/kg body weight, ATG-area under the curve (AUC)-dependent dosage, lymphocyte-ATG dosage ratio and immune reconstitution.

In other HCT settings, such as myeloablative HCT from sibling donors, additional ATLG reduced the incidence of cGVHD without affecting OS. In myeloablative and non-myeloablative MUD HCT trials ATG reduced aGVHD without increasing risk of relapse. After reduced intensity conditioning (RIC), ATG reduced GVHD, increased relapse, and was associated with a comparable OS. Large, retrospective registry analyses associated ATG with either reduced OS or reported similar risk of relapse and OS in MUD patients. Again, the recently presented innovative analyses analyzing the timing of ATG exposure and its AUC serum levels may help to solve the controversies concerning ATG and leukemic relapse.

In order to comprehend conflicting published results and overcome limitations of study design we hypothesized that homogenous data of a large single-center clinical cohort combined with studies of engraftment, immune reconstitution, and optimized ATLG dosage models should contribute to clarify the role of additional ATLG as immune prophylaxis. We evaluated the impact of ATLG in a large cohort of MUD HCT patients with hematologic malignancies and a long-term observation of up to 10 years. Due to center policy and data of previous randomized trials, exclusively ATLG was used.

2 | PATIENTS AND METHODS

2.1 | Patients

Between June 1991 and July 2016, a total of 1500 consecutive patients with hematologic malignancies underwent MUD-HCT with a uniform GVHD prophylaxis in combination with ATLG (N = 777, 52%) or without ATLG (N = 723, 48%) in the Department of Bone Marrow Transplantation of the West-German Cancer Center at University Hospital Essen. HLA-mismatch between patients and donors was allowed, but limited to a maximum of one single antigen or allele difference at the HLA loci A, B, C, DRB1, or DQB1 (9/10 match). HLA-DPB1 was not considered for donor-recipient matching. Assignment to ATLG-prophylaxis was based on standardized clinical treatment protocols of the center. Early supportive and follow-up care was identical for all patients. All data on baseline patient, donor, HCT characteristics and HCT outcome were documented prospectively in electronic forms. Clinical characteristics and laboratory parameters of patients after allogeneic stem cell transplantation were retrospectively analyzed. OS was calculated from transplantation to last follow-up visit or death of any cause. Patients were followed for up to 10 years after transplantation. Patients with longer survival were censored at maximum follow-up.

2.2 | Treatment

Patients of the no-ATLG cohort received a uniform pharmacologic GVHD prophylaxis with 3 mg/kg body weight CSP starting from day −1 before HCT in combination with 15 mg/m² MTX on day +1 and 10 mg/m² MTX on days +3, +6, and +11 after HCT. Patients of the ATLG cohort received additional polyclonal rabbit-ATLG (anti-Jurkat-T lymphocyte globulin; formerly ATG-Fresenius®, now Grafalon® Neovii, Neovii Biotech, Lexington, MA) at a dosage of 10 mg/kg body weight on days −4, −3, and −2 (cumulative dosage: ATLG 30 mg/kg) or at a dosage of 20 mg/kg body weight on days −4, −3, and −2 (cumulative dosage: ATLG 60 mg/kg). A total of 49 patients (6%) with ATLG dosages other than 30 or 60 mg/kg were excluded from multivariate ATLG analysis.

2.3 | Assessments

For inpatients, daily clinical assessment and standard laboratory parameters, such as peripheral blood cell parameters for hematologic regeneration, were obtained. Standard laboratory procedures were performed at the central laboratory of the University Hospital Essen. Hematologic regeneration was assessed daily for inpatients during the first 28 days after transplantation and weekly for outpatients during the following 2 months. Further outpatient follow-up intervals were sequentially extended, depending on clinical performance and transplant-associated complications. Transplant engraftment was defined as time from transplantation (day 0) to the first of 3 consecutive days with a measured leukocyte count of ≥1,000/µL, a neutrophil count of ≥500/µL, a lymphocyte count of ≥500/µL, and platelets of ≥20,000/µL, respectively. Acute GVHD (aGVHD) was clinically assessed and classified according to published criteria for aGVHD. Staging data of aGVHD maximum organ involvement (skin, gut, or liver) and the date of first aGVHD diagnosis were prospectively collected in the institutional clinical database. Chronic GVHD (cGVHD) was diagnosed after day +100.
based on characteristic clinical signs and symptoms according to published criteria for cGVHD. For prospective documentation of cGVHD before 2014, the Seattle criteria were applied whereas the National Institutes of Health criteria were used for more recent diagnoses. For purpose of consistency, only the Seattle terminology was used throughout this manuscript. OS was calculated from day of transplantation to death of any cause. Event-free survival (EFS) was calculated from day of transplantation to diagnosis of relapse, persistence, or death of any cause. Patients’ EFS was censored at last follow-up. For patients without relapse or persisting hematologic malignancy, nonrelapse mortality (NRM) was calculated from day of transplantation to death. Cumulative relapse incidence (CRI) was calculated from day of transplantation to diagnosis of relapse or persistence of malignancy. NRM and CRI were considered as respective competing risks.

2.4 | Ethics

The study and data acquisition were conducted in accordance with German legislation, the revised Helsinki Declaration, evaluated and approved by the ethics committee of the University of Duisburg-Essen (Protocol No. 18-8299-BQ). All patients have given written consent on collection, electronic storage, and scientific analysis of anonymized transplant-specific patient data. We confirm that no patient can be identified because of anonymized patient data.

2.5 | Statistical analysis

Statistical methods and software for clinical data analysis and predictive absolute lymphocyte count (ALC) models are detailed in the Appendix S1.

3 | RESULTS

3.1 | Patient characteristics

A total of 1500 consecutive patients with hematologic malignancies underwent MUD-HCT with a uniform immune prophylaxis of CSP and MTX alone (N = 723, 48%) or in combination with ATLG (N = 777, 52%). Baseline demographic characteristics including underlying hematologic disease, conditioning regimen, patient and donor HLA, and gender constellation are detailed in Table 1. Acute myeloid leukemia (AML) was the predominant disease in both cohorts (43% vs 38%). Established GVHD risk factors, such as HLA mismatch (9% vs 10%) or female donor to male recipient gender matching, were evenly distributed between cohorts. Differences between cohorts involved diagnosis of myelodysplastic syndromes (MDS, 12% vs 5%), chronic myeloid leukemia (CML, 9% vs 31%), myeloablative conditioning regimen (31% vs 73%), high-risk leukemia (60% vs 86%), peripheral blood stem cells as transplant source (95% vs 71%), median age at allogeneic HCT (54 vs 44 years), and the median year of allogeneic HCT (2012 vs 2004).

3.2 | Therapy and response

The addition of ATLG to standard immune prophylaxis with CSP and MTX resulted in significant clinical effects. After a median follow-up of 7.6 years from HCT, the OS estimate at 10 years was 51% for patients with ATLG and 35% for patients without ATLG (P < .002; Figure 1). The corresponding EFS was 44% with ATLG and 33% without ATLG (P < .0001). Non-relapse mortality (NRM) was significantly reduced in the ATLG cohorts. The 10-year cumulative incidence (CumIn) of NRM was 27% with ATLG and 45% without ATLG (P < .0001). Patients’ GVHD characteristics are detailed in Table 2. Detailed analysis revealed in particular a reduction of aGVHD grades III-IV to 11% in ATLG patients compared to 22% in the no-ATLG cohort (P < .0001; Figure 2B). The use of ATLG resulted in a relative downgrading through all observed aGVHD grades, compared to the no-ATLG cohort (Table 2). A similar effect of relative downgrading was also observed with regards to cGVHD. The overall cGVHD incidence was 59% with ATLG and 62% without ATLG (P = .04; Table 2). For limited and extensive cGVHD the cohorts separated clearly: the Cumln of limited cGVHD was 41% with ATLG opposed to 26% without ATLG (P < .0001; Figure 2C). In contrast, extensive cGVHD was 18% with ATLG and 35% in the no-ATLG cohort (P < .0001; Figure 2D). Thus, the significant difference of NRM between patient cohorts with and without prophylactic ATLG appeared primarily attributable to significantly lower grades III-IV aGVHD and extensive cGVHD rates in patients with prophylactic ATLG.

As expected, the relapse rate was higher in the ATLG cohorts. The 10-year Cumln of hematologic relapse was 30% with and 22% without ATLG (P < .0008). Still, OS remained significantly higher in the ATLG cohort (Figure 1), because relapse-related mortality did not increase after prophylactic ATLG (22%; 95% confidence interval [95% CI], 19%-25% and 20% without ATLG; 95% CI, 17%-23%). EFS was also higher for the ATLG patient cohorts with a probability at 10 years of 44% (95% CI, 36%-52%) after 60 mg/kg ATLG, 39% (95% CI, 34%-45%) after 30 mg/kg ATLG, and 33% (95% CI, 30%-37%) in patients without prophylactic ATLG (P < .002). Systematic review of replicative viral infections in a subset of 470 patients associated the administration of ATLG only with a significantly higher incidence of replicative cytomegalovirus infections as compared to no-ATLG (49%; 95% CI, 43%-55% vs 28%; 95% CI, 23%-33%; P < .0001).

3.3 | Multivariate analysis

Multivariate analysis (Table 3) confirmed observed differences with regard to OS. Both ATLG dosages associated with reduced mortality (hazard ratio [HR] 0.64; 95% CI 0.55-0.75 and HR 0.54; 95% CI, 0.42-0.70 for 30 and 60 mg/kg ATLG, respectively). As expected, the incidence of grades III-IV aGVHD (HR 2.17; 95% CI, 1.84-2.56) and of extensive cGVHD (HR 3.62; 95% CI, 3.04-4.32) associated with substantially increased mortality. High-risk disease stages also associated with increased mortality (HR 1.38, 95% CI 1.12-1.70, P < .0005;
In concordance with previously published studies, both ATLG dosages of 30 mg/kg (HR 0.52; 95% CI, 0.38-0.73) and 60 mg/kg (HR 0.29; 95% CI, 0.17-0.53) were associated with reduced incidence of grades III-IV aGVHD. In addition, aGVHD (HR 0.49; 95% CI, 0.32-0.74) and cGVHD were associated with reduced relapse incidence (Table 3) as were myeloablative conditioning regimens (HR...
In high-risk disease stages relapse incidence was not significantly influenced by prophylactic ATLG (HR 1.17; 95% CI, 0.87‐1.57; Table S1). Multivariate analysis was also performed for the largest subgroup of patients with acute leukemia (n = 766). Within this subgroup, both ATLG dosages also associated with significantly reduced hazards with respect to grades II‐IV and III‐IV aGVHD, extensive cGVHD, and thereby significantly improved OS as compared to the no-ATLG cohort (Table 4). The additional comparison of patient subsets of different disease categories (leukemias, other myeloid malignancies, and other lymphoid malignancies) with prophylactic 30 or 60 mg/kg ATLG revealed no significant difference in the incidence of grades II‐IV aGVHD (Table S4).

Significantly differing patient characteristics between ATLG and no-ATLG cohorts were analyzed with Cox regression analysis (Table S1). The median year of transplantation of all 1500 patients was 2009. Although both cohorts diverged with regards to the transplantation period, this variance did not associate with significant differences in OS analysis (P = .29; Table S1). Further, multivariate analysis revealed that the observed OS benefit associated with ATLG would have been even larger in absence of bias. Elements of bias in the no-ATLG cohort, which were associated with longer survival, were lower age, myeloablative conditioning regimen, and the diagnosis of CML. In a subgroup analysis excluding all CML patients from both cohorts, high-risk disease was well balanced between the ATLG and no-ATLG cohort (398 vs 394 patients). Within this patient subset, the use of ATLG was also associated with improved OS (HR 0.70; 95% CI, 0.56-0.88, P < .0005; Table S1).

### 3.4 Correlative studies, lymphocyte and leukocyte dynamics models

In order to analyze the interaction of ATLG and absolute lymphocyte counts (ALC) before HCT, we first performed a categorical Cox regression analysis. Lymphocyte counts >0.1/nL at day −5 (before ATLG exposure) significantly correlated with higher OS (HR 0.68; 95% CI, 0.47-1.00, P = .05). We then constructed a noncategorical Cox regression model by adopting the natural logarithm of ALC on day −5 as continuous variable stratified for ATLG dosages. The result was a logarithmically increasing power function for lymphocytes >0.1/nL, meaning that patients with very low ALC (<0.1/nL) and patients with ALC > 1.45/nL both associated with increased hazard (Figure S1 and Table S2). The ALC optimum for patients with ATLG was between 0.4 and 1.45/nL at day −5. These effects were not reproducible in the no-ATLG cohort, where ALC did not correlate with OS at all (Table S2, Figure S2).

### TABLE 2 Acute and chronic GVHD incidence by prophylactic ATLG administration

| ATLG (%) | No ATLG (%) | P    |
|-----------|-------------|------|
| Acute GVHD grades | | | |
| 0         | 21          | 15   | .002 |
| I         | 41          | 34   | <.02   |
| II        | 26          | 25   | n.s.   |
| III       | 6           | 12   | <.0001 |
| IV        | 6           | 13   | <.0001 |
| II ‐ IV   | 37          | 49   | <.0001 |
| III ‐ IV  | 11          | 22   | <.0001 |
| Number of organ involvements with acute GVHD | | | |
| 1         | 47          | 46   | n.s.   |
| 2         | 25          | 21   | n.s.   |
| 3         | 5           | 15   | <.0001 |
| Chronic GVHD grading | | | |
| Total incidence | 59 | 62 | .04 |
| Limited cGVHD | 41 | 26 | <.0001 |
| Extensive cGVHD | 18 | 35 | <.0001 |

cGVHD, chronic graft-versus-host disease; GVHD, graft-versus-host disease; n.s., not significant.
After HCT, leukocyte and lymphocyte recovery significantly differed between no-ATLG and ATLG cohorts. Furthermore, we detected significant differences in leukocyte recovery dynamics between the ATLG dosages of 30 and 60 mg/kg (Figure 3). Overall leukocyte recovery was significantly delayed in both ATLG cohorts as compared to the no-ATLG cohort ($P < .0001$). Remarkably, neutrophil recovery did not differ between the no-ATLG and 30 mg/kg ATLG cohorts but was significantly delayed with the use of 60 mg/kg ATLG (Figure 3B). Median time to platelet recovery ($\geq 50,000/\mu L$) was 22 days in the no-ATLG cohort (range, 10-99) compared to 23 days in the ATLG 30 mg/kg subgroup (range, 10-92) and 27 days (range, 10-96) in the ATLG 60 mg/kg subgroup. Clinical endpoints (OS, EFS, NRM) did not significantly differ between the cumulative ATLG dosages of either 30 mg/kg ($N = 567$) or 60 mg/kg ($N = 161$). Median time to lymphocyte recovery ($>500/\mu L$) was 21 days in the no-ATLG cohort (range, 10-92) compared to 24 days (range, 12-93) in the ATLG 30 mg/kg subgroup and 32 days (range, 14-98) in the ATLG 60 mg/kg subgroup. Interestingly, lymphocyte recovery dynamics did not only significantly differ between the no-ATLG and both ATLG cohorts ($P < .0001$) but also between each of the 30 and 60 mg/kg ATLG dosage subgroups ($P < .0001$; Figure 3C).

Cellular immune reconstitution after HCT revealed differences and similarities of ATLG and no-ATLG cohorts (Figure 4). T helper cell immune reconstitution was significantly faster in patients without ATLG ($P < .001$). This difference was more pronounced for naïve T helper cells than for memory T helper cells. B cell and NK cell recovery appeared faster in the ATLG cohort, but these differences were not significant. Cytotoxic T cell recovery was comparable between both cohorts (Figure 4C). Of notice, early T helper cell reconstitution showed a significant dose-dependent delay for patients with 30 and 60 mg/kg ATLG at month 3 after HCT (Table S3), which parallels the observed dose dependency with regard to neutrophil and total lymphocyte regeneration. Further, the ALC optimum model was associated with significantly higher T helper counts in patients within the optimum range before ATLG exposure as compared to patients with ATLG exposure outside the optimum ALC range ($P = .002$; Table S3). A normalized description (Appendix S1) of T cell recovery illustrated a decreasing T cell count variation with time after HCT (Figure S3). The large range of both average T cell counts and standard deviations at month 3 and 6 after HCT decreased to no difference at month 12. Maximum T cell counts were reached around 15 months after HCT.

**FIGURE 2** Time-dependent cumulative incidence of graft-versus-host disease. Time-dependent cumulative incidence (95% CI shaded) of acute GVHD grades II$^-$IV$^+$ (A) and III$^+$ - IV$^+$ (B). Cumulative incidence of limited (C) and extensive (D) chronic GVHD. Significance levels of cause-specific risk functions were tested according to Fine und Gray. A: No ATLG vs ATLG, $P = .0001$. B: No ATLG vs ATLG, $P < .0001$. C: No ATLG vs ATLG, $P < .0001$; 30 vs 60 mg/kg ATLG, n.s.; no ATLG vs 30 mg/kg ATLG, $P < .0001$, no ATLG vs 60 mg/kg ATLG, $P < .0009$. D: No ATLG vs ATLG, $P < .0001$; 30 vs 60 mg/kg ATLG, n.s.; no ATLG vs 30 mg/kg ATLG, $P < .0001$; no ATLG vs 60 mg/kg ATLG, $P < .0005$ [Color figure can be viewed at wileyonlinelibrary.com]
The normalized analysis revealed similar T cell recovery kinetics across major (eg, CD4+ and CD8+) T cell subsets (Figure S3). T cell recovery after HCT started at 60% of its maximum value in the ATLG cohort and at 45% of its maximum value in the no-ATLG cohort, which may in part be explained by the more frequent use of myeloablative conditioning.

4 | DISCUSSION

The addition of ATLG to a standard immune prophylactic regimen improved both OS and EFS. In the ATLG cohort both aGVHD and cGVHD severity were significantly downgraded resulting in reduced incidence of grades III-IV aGVHD and extensive cGVHD. This finding is consistent with previous randomized ATLG trials and several retrospective registry analyses, which associated in vivo T cell depletion with reduced aGVHD and cGVHD. In contrast to previous studies, our data revealed an optimum ALC at ATLG exposure that significantly correlated with OS. Its correlate may be the observed ATLG-dose-dependency in overall leukocyte recovery (Figure 3) as well as in early immune reconstitution of helper T cells (Table S3).

Currently, the impact of ATLG on relapse incidence and relapse-associated mortality in the MUD setting is controversial. In line with previous reports, the relapse rate was significantly higher in the ATLG cohort (30% vs 20%). Soiffer et al reported a cumulative 2-year relapse incidence in the ATLG and no-ATLG cohorts of 32% vs 21%. Finke et al observed no significant difference between both arms at 2 years posttransplant (28.9% vs 23.6%). Other studies suggested that in vivo T cell depletion might be more efficient in patients with RIC conditioning regimens. Although the relative
proportion of RIC patients was also larger in our ATLG cohort than in the no-ATLG cohort (69% vs 27%, P < .0001), multivariate analysis identified RIC as an independent risk factor associated with reduced OS. Patient age in the ATLG cohort was also significantly higher than in the two aforementioned prospective trials. Similar to reports from other transplant centers,11 median patient age in our study steadily increased over the total observation period of 25 years, and resulted in differences of median age (54 vs 44 years) and RIC rates between ATLG and no-ATLG cohorts. Thus, the administration of ATLG at least partially compensated for the adverse bias of patient age >45 years and RIC on OS.

Exposure to T cell depleting agents has previously been associated with delayed hematological engraftment31 or immune reconstitution.32,33 Our data showed a dose-dependent difference in both lymphocyte and neutrophil regeneration between the 30 and 60 mg/kg ATLG cohorts (Figure 3). Interestingly, neutrophil engraftment did not differ between the 30 mg/kg ATLG subgroup and the no-ATLG cohort. In a recent analysis of immune reconstitution involving patients with or without ATLG, higher absolute numbers of CD3+, CD4+, naïve- and regulatory T cells as well as B cells associated with improved OS and reduced NRM.15 We also found significantly lower T helper cell counts in the ATLG cohort after HCT (Figure 4), but no significant differences in cytotoxic T cell and B cell reconstitution. Our data showed an ATLG dose dependency in early T helper cell reconstitution (Table 3). The normalized analysis of immune reconstitution (Figure 3) revealed similar regeneration kinetics across different T cell subsets.

### TABLE 4 Cox regression analysis in patients with acute leukemias (n = 766)

| Predictor                        | HR     | 95% CI          | P     |
|---------------------------------|--------|-----------------|-------|
| Overall survival                 |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 0.621  | 0.497-0.776     | <.0001|
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 0.634  | 0.464-0.867     | .004  |
| Grade II-IV aGVHD                |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 0.735  | 0.551-0.982     | .0374 |
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 0.617  | 0.425-0.897     | .0115 |
| ATLG (n = 422) vs no ATLG (n = 344) | 0.694  | 0.536-0.899     | .0056 |
| Grade III-IV aGVHD               |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 0.570  | 0.351-0.926     | .0231 |
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 0.340  | 0.169-0.684     | .0025 |
| ATLG (n = 422) vs no ATLG (n = 344) | 0.486  | 0.320-0.739     | .0007 |
| Chronic GVHD (all stages)        |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 0.757  | 0.590-0.971     | .0282 |
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 0.687  | 0.510-0.925     | .0133 |
| ATLG (n = 422) vs no ATLG (n = 344) | 0.723  | 0.579-0.903     | .0043 |
| Limited cGVHD                    |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 1.714  | 1.203-2.443     | .0029 |
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 1.775  | 1.186-2.658     | .0053 |
| ATLG (n = 422) vs no ATLG (n = 344) | 1.693  | 1.224-2.341     | .0015 |
| Extensive cGVHD                  |        |                 |       |
| 30 mg/kg ATLG (n = 304) vs no ATLG (n = 344) | 0.374  | 0.250-0.559     | <.0001|
| 60 mg/kg ATLG (n = 105) vs no ATLG (n = 344) | 0.310  | 0.179-0.538     | <.0001|
| ATLG (n = 422) vs no ATLG (n = 344) | 0.361  | 0.255-0.511     | <.0001|

aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; CI, confidence interval; HR, hazard ratio; P, significance as P value.
The observed differences to other published studies might also relate to direct or indirect drug interactions of standard immunosuppressive regimens and ATLG. Whereas Bacigalupo et al, Finke et al, Kröger et al, and the present study exclusively used short-course MTX and CSP, patients in Soiffer et al received tacrolimus in combination with short-course MTX or in Baron et al tacrolimus was combined with mycophenolate mofetil. ATLG may be administered at different cumulative dosages (eg, 30 or 60 mg/kg body weight). The question of optimal ATLG dosage has been previously discussed by several authors and is still inconclusive. Whereas in both prospective, randomized ATLG trials, the ATLG dose was 60 mg/kg, other previous studies successfully applied 30 mg/kg resulting in a similar relapse risk compared to placebo. The great majority of patients (72%) in our analysis received a cumulative dosage of 30 mg/kg, with sustained beneficial effect on OS along with a significant reduction of NRM, which was most probably related to a reduction of grades III-IV aGVHD and extensive cGVHD. Thus, the different ATLG doses may in part explain differences of clinical results compared to previous trials.

Beyond conventional dose categories, we investigated optimal ATLG dosing with respect to patient’s ALC before administration of ATLG. Our data confirmed Soiffer et al, who had shown increased hazard in a categorical analysis of lymphocyte counts < 0.1/nL. Still, the association of lymphocyte counts and hazard rate was more complex. It followed a logarithmically increasing power function above lymphocyte counts of > 0.1/nL, which showed an optimum range between lymphocytes of 0.4 and 1.45/nL (Table S1, Figure S1). Patients within this ALC range and ATLG exposure had a significantly improved OS as compared to patients outside this range (Table S2). As a consequence, both patients with very low ALC and those with higher ALC associated with reduced OS, when exposed to ATLG. In our hypothesis, the biological correlate of this model would relate to free excess ATLG due to a low binding capacity of residual recipient T cells at the time of ATLG administration, which in turn leads to more vigorous in-vivo donor T cell depletion after HCT (ALC < 0.4/nL). Conversely, ATLG might extensively be absorbed by recipient T cells, resulting in less effective donor T cell depletion after HCT (ALC > 1.45/nL). Patients within the optimum

**FIGURE 3** Time-dependent cumulative incidence of cellular regeneration. Engraftment dynamics are significantly delayed in high-dosed ATLG. Daily change of hematologic cell number was evaluated. Time-dependent cumulative incidence (95% confidence intervals shaded) of leukocyte engraftment ≥ 1,000/μL (A), neutrophil engraftment ≥ 500/μL (B), lymphocyte engraftment ≥ 500/μL (C), and lymphocyte regeneration ≥ 1,000/μL (D). Significance levels of cause-specific risk functions were tested according to Fine und Gray. A: All tests with \( P < .0001 \). B: No ATLG vs ATLG, \( P < .001 \); 30 vs 60 mg/kg ATLG, \( P < .004 \); no ATLG vs 30 mg/kg ATLG, n.s.; no ATLG vs 60 mg/kg ATLG, \( P < .001 \). C: All tests, \( P < .0001 \). D: No ATLG vs ATLG, \( P < .0001 \); 30 vs 60 mg/kg ATLG, \( P < .006 \); no ATLG vs 30 mg/kg ATLG, \( P < .0001 \); no ATLG vs 60 mg/kg ATLG, \( P < .0001 \) [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 4  Comparison of cellular immune reconstitution between the ALTG and no-ATLG cohort. Lymphocyte subsets in the peripheral blood were measured by flow cytometry after HCT. Specific cell subsets within the CD45+ lymphocyte gate were characterized as follows: (A) T cells, CD3+; (B) T helper cells, CD3+/CD4+; (C) cytotoxic T cells, CD3+/CD8+; (D) activated T cells, CD3+/HLA-DR+; (E) naive T helper cells, CD3+/CD4+/CD45RA+; (F) memory T helper cells, CD3+/CD4+/CD45RO+; (G) B cells, CD19+; (H) natural killer (NK) cells, CD16+/56+; (I) αβ T cells, CD3+/αβ-T+; (J) γδ T Cells, CD3+/γδ-T+. Absolute cell numbers after transplantation were analyzed by the two-sample t test. ATLG cohort around 3 mo (n = 221), 6- (n = 119), 9- (n = 105), 12- (n = 111), 15- (n = 93), and 18 mo (n = 80); no-ATLG cohort around 3 (n = 124), 6 (n = 64), 9 (n = 62), 12 (n = 48), 15 (n = 50), and 18 mo (n = 48). Mean values and the standard error of the mean are shown. A P < .05 was considered statistically significant and indicated in the figure with an asterisk, P < .001 was indicated with two asterisks.
ALC range also had significantly improved early immune reconstitution compared to patients who received ATLG outside this range (Table S3). Together with our data on the ATLG dose dependency of leukocyte recovery dynamics, this is the first study to associate optimal ALC with OS and to support the concept of individualized ATLG dosing. Previous reports aiming for optimal ATG dosing using AUC-ATG dosage\textsuperscript{13} or patient’s lymphocyte count at the start of the preparative regimen\textsuperscript{14} provided important evidence on individualized T cell depletion using ATG and supported these new dosage approaches. Maybe due to smaller sample size these data did not associate ALC and OS. Future prospective clinical trials are thus clearly warranted, which should investigate the concept of individualized ATLG or ATG dosage.

This study has a number of limitations due to its retrospective nature, a long recruiting period, the inclusion of different hematologic malignancies and conditioning regimens. We have included all possible elements of bias in multivariate and subgroup analyses and described the impact of each element of bias within both ATLG and no-ATLG cohorts. Despite the observed differences, multivariate analysis supported the study’s conclusions. Furthermore, we have provided a large subgroup analysis of patients with acute leukemia, whose results are consistent with the findings in the entire study cohort.

In the face of two conflicting prospective clinical trials without upcoming further prospective studies on ATLG, large retrospective analyses may help to clarify its prophylactic role and thereby provide important hints for its use in clinical practice. Our single-center data, which is delineated from one of the largest ATLG patient cohorts, supports the addition of ATLG to the short-course MTX and CSP regimen. The use of ATLG effectively reduced grades III-IV aGVHD and extensive cGVHD translating into improved long-term EFS and OS after MUD-HCT. In addition, this study supports the concept of individualized dosing of ATLG. Within the ALC optimum of 0.4 and 1.45/ml, our data suggest an ATLG dosage of 30 mg/kg. Our data on the ATLG dose-dependency of leukocyte and T helper cell recovery dynamics and on the association of optimum ALC with improved OS provide a new perspective on in-vivo T cell depleting GVHD prophylaxis that should be pursued in future studies.

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DISCLOSURE

The authors of this manuscript have potential conflicts of interest to disclose as described by the American Journal of Transplantation. ATT: Consultancy for MSD, JAZZ, CSL. Travel subsidies from Neovii Biotech outside the submitted work. VK received travel subsidies from Gilead and JAZZ, NKS received travel subsidies from MSD and DWB received travel subsidies from Medac, all outside the submitted work. The other authors declare no competing financial interests within the submitted work.

AUTHOR CONTRIBUTIONS

ATT and DWB designed the study. VK performed data collection; RT, HO, NKS, MD, LK, TL, and MK participated in clinical data acquisition. EB and DWB performed statistical analysis. EB and ATT developed the ALC model, and ATT, DWB, and EB interpreted data. VK, SL, and NTM participated in data analysis. ATT and DWB wrote the manuscript. VK, EB, LK, MK, and KF contributed to write the manuscript. KF corrected the manuscript. All the authors had access to primary clinical trial data, read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

Data are available on reasonable request due to privacy/ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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