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Rabbit Antithymocyte Globulin Serum Levels: Factors Impacting the Levels and Clinical Outcomes Impacted by the Levels

Kareem Jamani*, Rosy Dabas, Shahbal B. Kangarloo, Nicole L. Prokopishyn, Joanne Luider, Poonam Dharmani-Khan, Faisal M. Khan, Andrew Daly, Jan Storek

Alberta Blood and Marrow Transplant Program, University of Calgary, Calgary, Alberta, Canada

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Antithymocyte globulin (ATG) levels and clearance vary significantly among patients receiving the same weight-based dose of ATG. To date, ATG area under the curve (AUC), its determinants, and its impact on clinical outcomes have been examined in pediatric hematopoietic cell transplant (HCT) and adult nonmyeloablative HCT. Here we set out to examine ATG AUC in 219 uniformly treated adults undergoing myeloablative allogeneic HCT at our institution. Sera were collected for the determination of pre- or post-HCT ATG AUC. The lowest quintiles of pre- and post-HCT AUC were associated with inferior chronic graft-versus-host disease (GVHD) and relapse-free survival (cGRFS) and a higher risk of acute GVHD, respectively. The highest pre- or post-HCT ATG AUC quintiles were not associated with risk of death, nonrelapse mortality, or relapse. Factors most strongly associated with AUC were day −2 recipient absolute lymphocyte count, body mass index (BMI), and graft lymphocyte content. To achieve ideal pre-HCT AUC (avoiding low AUC to maximize cGRFS) in this HCT setting, ATG dosing will need to take into consideration recipient weight, BMI, and blood and graft lymphocyte counts. Further studies are required to develop a modern ATG dosing schema and to demonstrate that adjusting ATG dose to target a particular AUC is feasible and leads to improved outcomes.

INTRODUCTION

Graft-versus-host disease (GVHD) remains a significant challenge after allogeneic hematopoietic cell transplant (allo-HCT). Rabbit anti–T cell globulin (ATG), a polyclonal antibody produced by immunizing rabbits with human thymocytes (Thymoglobulin; Sanofi, Gentilly, France) or Jurkat T lymphoblastoid cells (Grafalon; Neovii Biotech, Lexington, MA) and extracting IgG from their sera, has been used as prophylaxis against GVHD. As reviewed [1], 6 randomized studies on adult patients have reported a reduced incidence of both acute GVHD (aGVHD) and chronic GVHD (cGVHD) and 2 have reported improved quality of life after prophylaxis with rabbit ATG [2–9]. However, adverse outcomes with ATG prophylaxis have been reported; for example, 1 of the 6 reported randomized trials found a trend toward an increased risk of post-transplant lymphoproliferative disorder in those receiving ATG [4], 1 found inferior progression-free and overall survival in those receiving ATG with total body irradiation (TBI)-based conditioning due to more graft failure and a trend toward more relapse [2], and other nonrandomized studies have found an increased risk of relapse in those receiving reduced-intensity conditioning with ATG [10,11]. ATG dosing and timing (early or late in conditioning) vary significantly across studies and could contribute to these differences in outcomes. ATG dosing by weight has been chosen empirically, and only in recent years have the pharmacokinetic (PK) properties of weight-based ATG dosing been examined.

Early PK studies revealed that ATG levels and clearance vary significantly among patients receiving the same weight-based dose of ATG [12,13]. Beyond this, ATG levels at specific time points have been associated with clinical outcomes: Higher pre-HCT levels of ATG have been associated with a reduced risk of aGVHD [14,15] and cGVHD [16], whereas higher post-HCT levels have been associated with a reduced risk of aGVHD/cGVHD but also an increased risk of viral infections, particularly post-transplant lymphoproliferative disorder [16–18]. However, ATG exposure (area under the curve [AUC]) before and after HCT may provide a more robust assessment of ATG PK and pharmacodynamics as compared with ATG levels at single time points. In addition, the patient-specific modulators of ATG PK are beginning to be elucidated: recipient lymphocyte count before receiving ATG has been described as a modulator of both ATG PK and clinical outcomes after ATG-conditioned HCT [11,18,19].

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* Correspondence and reprint requests: Kareem Jamani, MD, University of Calgary, Hematology and Hematologic Malignancies, Room 603 South Tower, Foothills Hospital, 1403 29 Street NW, Calgary AB, Canada T2N 2T9.

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To date, ATG AUC, the factors that affect it, and their impact on clinical outcomes have been examined in pediatric HCT and adult nonmyeloablative HCT [19,20]. Here we set out to examine these aspects of ATG PK in a uniformly treated adult population undergoing predominantly myeloablative HCT at our institution.

METHODS

Patients and Transplantation

Of 419 patients receiving first allo-HCT in Calgary from February 24, 2011 to June 30, 2017, 219 agreed to have blood drawn and had a minimum set of sera collected for the determination of pre- or post-HCT ATG AUC. For cohort details, including which patients were included into which parts of this study, see Figure 1. Follow-up for all patients was until April 14, 2018. The study was approved by the Health Research Ethics Board of Alberta, and all patients provided written consent.

For patient characteristics, see Table 1. Conditioning was typically with fludarabine (250 mg/m^2) starting on day −6, busulfan (approximately 12.8 mg/kg, PK-adjusted to target AUC of 15,000 μMol/min) starting on day −5, and TBI (4 Gy) starting on day −1 [21]. GVHD prophylaxis was with Thymoglobulin .5 mg/kg on day −2, 2.0 mg/kg on day −1, and 2.0 mg/kg on day 0 (before graft infusion); methotrexate 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11; and cyclosporine targeting trough levels of 200 to 400 until day 56 and then tapered and discontinued on approximately day 84 unless used as treatment for GVHD. aGVHD was graded according to consensus criteria [22], and cGVHD was diagnosed and scored according to National Institutes of Health criteria [23]. Supportive care included ursodiol until 3 months, fluconazole until 1 month, sulfamethoxazole-trimethoprim until 6 months, and valacyclovir until 2 years post-transplant.

Measurement of ATG Levels

Blood was collected from patients at the following time points: end of the last (third) ATG infusion (C_{max}), pregraft infusion (pregraft), 30 minutes after finishing graft infusion (postgraft) because by 30 minutes an equilibrium appeared to be established between serum ATG and other ATG (extravascular or bound to intravascular leukocytes) in a preliminary experiment (see Supplementary Figure 1), and on days 7, 14 and 28 post-HCT. Serum was separated from the blood and stored at −80°C in tightly sealed vials until ATG level determination.

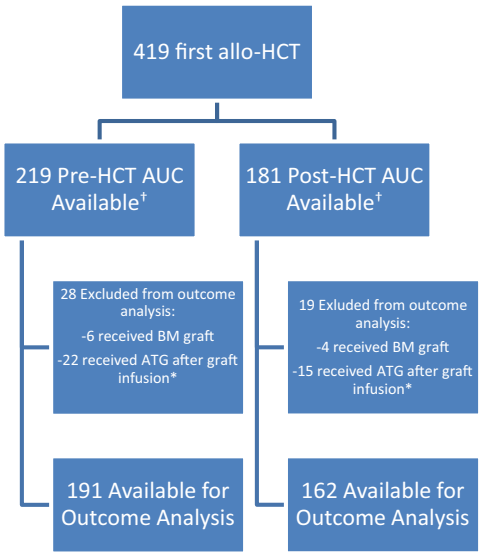

Figure 1. Cohort development. † "Pre-HCT AUC Available" means that pregraft infusion serum was available (pre-HCT AUC was estimated based on the pregraft infusion ATG level). "Post-HCT AUC Available" means that sera from all the following time points were available: pregraft infusion, 30 minutes postgraft infusion, day 7, and day 28 (post-HCT AUC was estimated based on the ATG levels at these time points). All patients with a post-HCT AUC available also had a pre-AUC available. The 219 or 181 patients were used for the analysis of factors influencing ATG levels/AUCs, but only a subset of these patients (191 or 162) was used for the analysis of whether an ATG level/AUC is associated with a clinical outcome ("Outcome Analysis").
* Received a different dosing or timing of ATG than our standard of .5 mg/kg on day −2, 2.0 mg/kg on day −1, and 2.0 mg/kg on day 0 before graft infusion; in particular, some ATG was administered after graft infusion.

Level (concentration) of "functional" ATG (capable of binding to human lymphocytes) was determined using the flow cytometry–based assay developed by Kakhniashvilli et al. [13] with minor modifications. Briefly, standards of known ATG concentrations, ranging from 20 to .0098 mg/L, were prepared by serial 2-fold dilution. Peripheral blood mononuclear cells, drawn from 1 healthy volunteer, were separated from heparinized blood using density gradient centrifugation (Lympholyte; Cedarlane Labs, Burlington, Canada), cryopreserved using 10% dimethylsulfoxide, and stored in liquid nitrogen until use. Mononuclear cells were thawed, washed, and suspended in PBS at a concentration of .5 × 10^6/100 μL. Mononuclear cells were incubated with patient serum or ATG standards. The ATG coated cells were then labeled with PE-conjugated F(ab')$_2$-donkey-anti-rabbit IgG (ThermoFisher Scientific, Waltham, MA). Flow cytometric analysis was then performed on FACSAria IIIu (BD Biosciences, San Jose, CA). Lymphocytes were gated by forward- and side-scatter characteristics. PE fluorescence was measured for each standard and each patient serum sample included in the run. The unknown concentration of ATG in patient samples was extrapolated using a standard curve generated by plotting ATG levels of standard versus PE fluorescence (see Supplementary Figure 2). All patient serum samples were run in triplicates, except pregraft samples which were run in quadruplicates.

The coefficient of variation for the low and high ends of the standard curve was calculated based on 10 experiment repeats using sera with low and high ATG levels. The coefficients of variation for the low and high ATG level sera were .17 and .13, respectively.

Pre- and Post-HCT AUC Calculation, Estimation of Unknown ATG Levels, and Calculation of ATG Clearance

PK parameters were calculated by noncompartmental analysis. Area under the time versus ATG concentration curve as an indicator of ATG exposure was calculated by the linear-log trapezoidal rule [24]. Specifically, the linear trapezoidal rule was used as ATG concentrations were increasing (from the start of the first ATG infusion until the end of the third ATG infusion), and the logarithmic trapezoidal rule was used as ATG concentrations were decreasing (end of third ATG infusion through day 28). Pre-HCT AUC was calculated as the sum of the triangle from the start of the first ATG infusion to the end of the third ATG infusion (C_{max}) and the "trapezoid" from the end of the third ATG infusion (C_{max}) to the start of graft infusion. Post-HCT AUC was calculated as the sum of the following "trapezoids": from the start of graft infusion to 30 minutes after the end of the graft infusion, from 30 minutes postgraft infusion to day 7, from day 7 to day 14, and from day 14 to day 28. The tail end of the time-concentration curve was added to the post-HCT AUC calculation. The tail was calculated by C_{d28}/λ_z, where C_{d28} is the ATG concentration on day 28 and λ_z is the elimination rate constant (the negative slope of ATG concentration between days 7 and 28). AUCs were split into quintiles for analysis.

Not all patients had sera available for measurement of C_{max} or day 14 levels because these sera began to be collected after 2011. C_{max} was extrapolated from the median population log/linear distribution phase slope of C_{max} to graft infusion time. The day 14 ATG level was extrapolated from the median population log/linear elimination phase slopes of days 7 to 14 and days 14 to 28. The mean of these 2 extrapolations was taken as the estimated day 14 ATG level. The estimated C_{max} and day 14 levels were then used for all patients for analysis. One hindered two of 181 patients had a measured C_{max} level available. The Spearman rank correlation between the estimated and the measured values was .67 (see Supplementary Figure 3). Of 181 patients, 141 had a measured day 14 level available. The Spearman rank correlation between the estimated and the measured values was .84 (see Supplementary Figure 4). All levels were split into quintiles for analysis.

Clearance of ATG was calculated by the following formula: total dose of ATG / total ATG AUC, where total ATG AUC is the sum of pre- and post-HCT ATG AUCs.

Statistical Analyses

Univariate Spearman rank correlation was initially used to analyze associations between factors suspected to influence ATG levels/AUCs and the ATG levels/AUCs. To account for multiple comparisons, $P \leq .01$ was considered statistically significant. Statistically significant factors by univariate analysis were included in a multiple linear regression model with backward stepwise selection. The suspected factors included preconditioning IgG levels, preconditioning weight, body mass index (BMI) or body surface area (BSA); day −2 total WBC, lymphocyte, monocyte, or neutrophil counts; graft content of total nucleated cells or lymphocytes; and time between the end of the third (last) ATG infusion and the start of graft infusion. Univariate Spearman rank correlation was also used to analyze the association of recipient weight, BMI, BSA, or day −2 lymphocyte count with clearance of ATG. Statistically significant ($P \leq .01$) factors by univariate analysis were included in a multiple linear regression model. Because of collinearity between weight, BMI, and BSA, a regression model was fitted for each of these variables separately.

Table 1
Patient Characteristics (N = 219)

Characteristic	Subcategory	Value
Median age, yr (interquartile range)		53 (41-60)
Underlying disease	Acute Leukemia	139 (63)
	MDS	22 (10)
	CLL/lymphoma	29 (13)
	CML	11 (5)
	MPN	14 (6)
	Aplastic anemia	4 (2)
Disease risk*	High risk	102 (47)
	Standard risk	117 (53)
Donor	8/8 HLA matched related†	79 (36)
	8/8 HLA matched unrelated	97 (44)
	HLA mismatched unrelated‡	43 (20)
CMV serostatus donor–recipient	Negative–positive	34 (15)
	All other	183 (84)
	Unknown	2 (1)
Stem cell source	Peripheral blood	213 (97)
	Bone marrow	6 (3)
Conditioning	Flu+Bu	11 (5)
	Flu+Bu+TBI§	203 (93)
	Other¶	5 (2)
GVHD prophylaxis‖	Cyclosporine + MTX	219 (100)
aGVHD	None	114 (52)
	I	48 (22)
	II	37 (17)
	III	18 (8)
	IV	2 (1)
cGVHD	None	154 (70)
	Mild	20 (9)
	Moderate-severe	45 (21)
Median hours between ATG end and graft infusion start (interquartile range)		3.2 (2.4-4.2)

Values are n (%) unless otherwise defined. MDS indicates myelodysplastic syndrome; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; MPN, myeloproliferative neoplasm; Flu, fludarabine; Bu, busulfan; CMV, cytomegalovirus; MTX, methotrexate.

* Standard risk disease was defined as primary acute leukemia (AML, ALL, biphenotypic) in first remission, CML in first chronic or accelerated phase, myelodysplasia with <5% marrow blasts or aplastic anemia. All other diseases/disease stages were considered poor risk.

† Including two 7/8 HLA-matched related donors.

‡ Including forty-two 7/8 and one 6/8 HLA-matched donors.

§ FluBuATG conditioning consisted of fludarabine 50 mg/m^2/day for 5 days, busulfan 3.2 mg/kg/day for 4 days pharmacokinetically adjusted, and rabbit ATG total 4.5 mg/kg. TBI was 4 Gy delivered in 2 fractions.

¶ Including 3 fludarabine, cyclophosphamide, rabbit ATG; 1 fludarabine, cyclophosphamide, rabbit ATG and TBI, and 1 fludarabine, thiotepa and rabbit ATG.

‖ In addition to ATG.

Significance of associations between ATG levels/AUCs and survival or cGVHD- and relapse-free survival (cGRFS) was tested using Cox regression. Significance of associations between ATG levels/AUCs and cause-specific outcomes (cumulative incidence of relapse, nonrelapse mortality [NRM], GVHD-associated NRM [NRM occurring any time after the occurrence of grades II to IV aGVHD or moderate to severe cGVHD], or non GVHD-associated NRM [NRM in the absence of preceding grades II to IV aGVHD or moderate to severe cGVHD]) was tested using competing risk regression according to Fine and Gray. Covariates and competing risks used in the regression analyses are detailed in Supplementary Table 1. For most analyses patients were split into quintiles according to ATG levels or AUCs, and patients in quintile 1 were compared with those in quintile 2, 3, 4 or 5. The minimum, median, and maximum ATG levels/AUCs for each quintile are given in Supplementary Table 2. All statistical analyses were performed using STATA 14 (StataCorp, College Station, TX).

RESULTS

Association between ATG AUCs/Levels and Clinical Outcomes

Associations between quintiles of pre-HCT AUC, post-HCT AUC and C_{max} and clinical outcomes are depicted in Figure 2. The first quintile serves as the comparator group for all analyses. For pre- AUC overall survival was similar for all 5 quintiles, whereas cGRFS was the best for quintiles 3 (hazard ratio [HR], 1.92; P = .04) and 4 (HR, 2.22; P = .02). The reason for the high cGRFS in quintiles 3 and 4 was probably the low incidence of moderate to severe cGVHD in these quintiles (subhazard ratio [SHR] .35 [P = .05] for quintile 3 and .42 [P = .06] for quintile 4). Regarding grades II to IV aGVHD, there was a trend toward the lowest incidence for quintiles 3 (SHR, .40; P = .06) and 5 (SHR, .46; P = .09). Pre-AUC did not appear to be associated with cumulative incidence of relapse or NRM.

For post-AUC overall survival was the best for quintile 2 (HR, 19.48; P = .004), whereas there appeared to be no significant association between post-AUC and cGRFS, cGVHD, or relapse. Grades II to IV aGVHD incidence was the lowest for quintiles 4 (SHR, .16; P = .001) and 5 (SHR, .24; P = .013). Grades III to IV aGVHD incidence was also the lowest for quintiles 4 and 5 (data not shown); however, meaningful statistical analysis could not be performed because of low numbers of patients with grades III to IV aGVHD. NRM was the lowest for quintile 2, although the estimate was imprecise (SHR, 7.30×10^{-8}; $P < .001$); this may have contributed to the high overall survival in quintile 2. The incidence of GVHD-associated NRM appeared to be highest in quintile 1, whereas non-GVHD–associated NRM appeared to be highest in quintiles 4 and 5 (data not shown), although meaningful statistical analyses could not be performed because of the low numbers of patients with NRM.

Regarding associations between C_{max} or pre-HCT ATG levels and outcomes, analogous to pre-AUC, C_{max} and pre-AUC had similar associations with mortality, cGRFS, cGVHD, relapse, and NRM. However, C_{max} but not pre-AUC was significantly associated with grades II to IV aGVHD with significantly lower incidence in all quintiles compared with quintile 1 (quintile 2 SHR, .33 [P = .01]; quintile 3 SHR, .46 [P = .04]; quintile 4 SHR, .48 [P = .04]; quintile 5 SHR, .18 [P = .002]). Single post-HCT

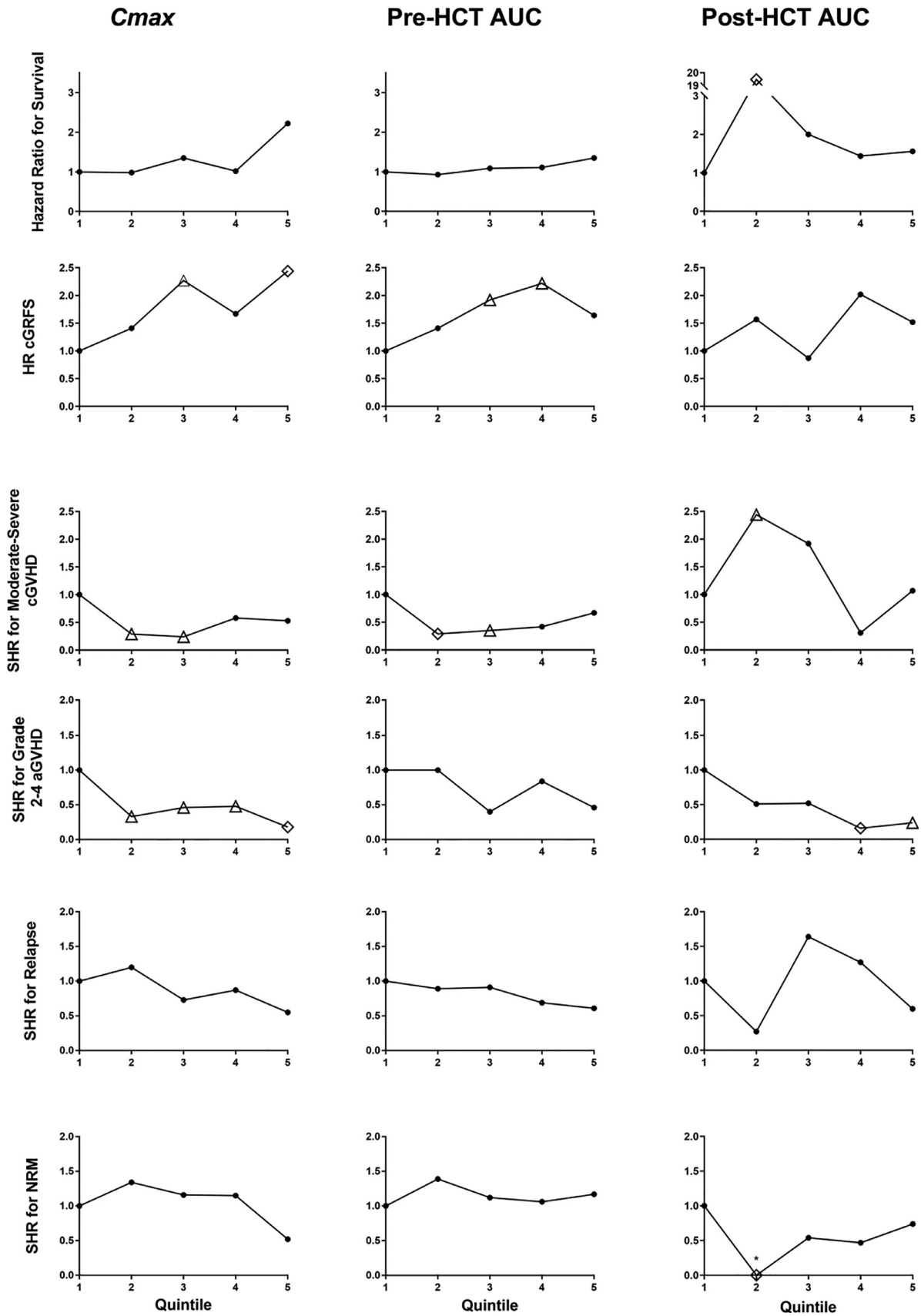

Figure 2. Association between ATG levels at the end of the last ATG infusion (C_{max}) and clinical outcomes or between pre-/post-HCT AUC and clinical outcomes. The median and ranges of each quintile of C_{max}, pre-HCT AUC, and post-HCT AUC are detailed in Supplementary Table 2. *Closed circle* indicates not significantly different from quintile 1; *open triangle*, significantly different from quintile 1 at $P < .05$; *open diamond*, significantly different from quintile 1 at $P < .01$. HR for death, relapse or moderate to severe cGVHD is the inverse of cGRFS.
*HR 7.3×10^{-8}.

levels (30 minutes postgraft, day 7, and day 28) and post-AUC had similar associations with all outcomes (Figure 2 and Supplementary Figure 5).

Drop in ATG Levels and Risk of aGVHD

Of all the outcomes evaluated, the most significant associations with an ATG level at a single time point or with an AUC were noted for aGVHD: Quintile 1 of C_{max}, pregraft, day 7, day 28, and post-AUC were associated with a significantly higher incidence of grades II to IV aGVHD than at least 1 of quintiles 2 to 5 (Figure 2 and Supplementary Figure 5). The high incidence of aGVHD could be due to the low ATG levels per se, which could be improved by a higher pre-HCT dose or an additional post-HCT dose, or due to rapid post-HCT ATG clearance, which could be a surrogate of proliferation of allo-reactive T cells and might not be amenable to a change in ATG dosing. To evaluate the latter we determined whether there was an association between the relative drop in ATG level from postgraft to day 28 (postgraft level divided by day 28 level) and aGVHD. A greater than median relative drop was associated with a higher cumulative incidence of both grades II to IV (SHR, 2.9; $P = .002$) and grades III to IV aGVHD (SHR, 4.6; $P = .04$). Thus, low day 28 ATG level or low post-AUC in patients with aGVHD may be due at least in part to a high post-transplant ATG clearance in these patients.

Factors Influencing ATG AUCs/Levels and Clearance of ATG

As shown in Table 2, on univariate analysis the factors showing the strongest correlation with both pre-AUC and post-AUC (thus assumed to show the most important influence on the pre- and post-AUC) were the absolute lymphocyte count (ALC) on day −2 (Figure 3) and the BMI. For post-AUC, graft lymphocyte content per kilogram of recipient body weight was also important. When the overshadowing AUC variability due to day −2 ALC was minimized by excluding patients with ALC > .2/nL, a correlation between time from the end of the last ATG infusion to the start of the graft infusion and pre-AUC as well as a correlation between IgG level and post-AUC became apparent. On multivariate analysis day −2 ALC, BMI, and time between end of last ATG infusion and start of graft infusion were significantly associated with pre-AUC, whereas day −2 ALC, IgG level, and graft lymphocyte content per kilogram of recipient body weight were significantly associated with post-AUC.

As shown in Supplementary Table 3, correlations between the factors suspected to influence C_{max} or pregraft ATG levels and these levels were similar as for pre-AUC. Analogously, correlations between the factors and ATG levels 30 minutes postgraft, day 7, or day 28 were similar as for post-AUC. Recipient body weight and BSA showed positive correlations with AUCs and levels but with lower R values and higher P values than BMI (data not shown).

Median clearance of ATG was 0.25 L/hour (interquartile range 0.19–0.32 L/hour). On univariate analysis, all of weight (r = 0.29, $P = 0.0004$), BSA (r = 0.29, $P = 0.0004$) and day −2 lymphocyte count (r = 0.37, $P < 0.0001$) were significantly associated with clearance. BMI was not significantly associated with clearance (r = 0.19, $P = 0.02$). On multivariate analysis, day −2 lymphocyte count ($P < 0.001$) and weight ($P < 0.001$) produced the best model fit (R-squared 0.20) as compared to the model containing day −2 lymphocyte count ($P < 0.001$) and BSA ($P = 0.001$, R-squared 0.18).

Do the Factors Influencing ATG AUCs/Levels Impact Outcomes?

The factors most significantly affecting ATG AUCs/levels, specifically day −2 ALC, BMI, and graft lymphocyte content, were evaluated for whether they are associated with clinical outcomes (mortality, cGRFS, grades II to IV aGVHD, moderate to severe cGVHD, relapse, NRM). Here we present only significant associations. Higher day −2 ALC (>.2/nL) was associated with an increased risk of grades II to IV aGVHD (Figure 4A; SHR, 3.84; $P < .01$). Higher BMI (higher than median) was associated with improved cGRFS (Figure 4B; HR, .67; $P = .02$). The improved cGRFS in those with above median BMI may have been a result of a combination of nonstatistically significant reductions in risks of moderate to severe cGVHD (SHR, .87; $P = .59$), relapse (SHR, .73; $P = .27$), and death (HR, .80; $P = .35$). Higher graft lymphocyte content per kilogram of recipient body weight (higher than median) was associated with a higher risk of moderate to severe cGVHD (Figure 4C; SHR, 1.99; $P = .02$).

DISCUSSION

Here we have examined the effect of pre- and post-HCT ATG AUC and levels on clinical outcomes after allo-HCT in a large uniformly treated cohort with myeloablative conditioning. We found that the lowest quintiles of pre- and post-HCT AUC were associated with inferior cGRFS and a higher risk of aGVHD, respectively. We did not find the highest pre- or post-HCT ATG AUC quintiles to be associated with adverse outcomes; particularly, we did not find a significant effect of ATG levels or AUCs on risk of death, NRM, or relapse. Importantly,

Table 2
Factors Influencing Pre- and Post-HCT ATG AUC

	All Patients (Univariate)		Excluding Patients with Day −2 ALC > .2/nL (Univariate)		Multivariate Analysis
	R	*P*	R	*P*	*P*
Pre-HCT AUC					
Day −2 ALC	−.38	<.01			.037
BMI	.22	<.01	.19	.01	.022
Time between ATG end and graft infusion start			.19	.01	<.001
Post-HCT AUC					
Day −2 ALC	−.32	<.01			.015
IgG			−.24	<.01	.041
BMI	.21	<.01			NS
Graft lymphocyte content/kg*	−.23	<.01	−.28	<.01	.008

For univariate analysis, only significant associations ($P \leq .01$) are shown. NS indicates not significant (ie, not included in final regression model).
* Per kilogram recipient body weight.

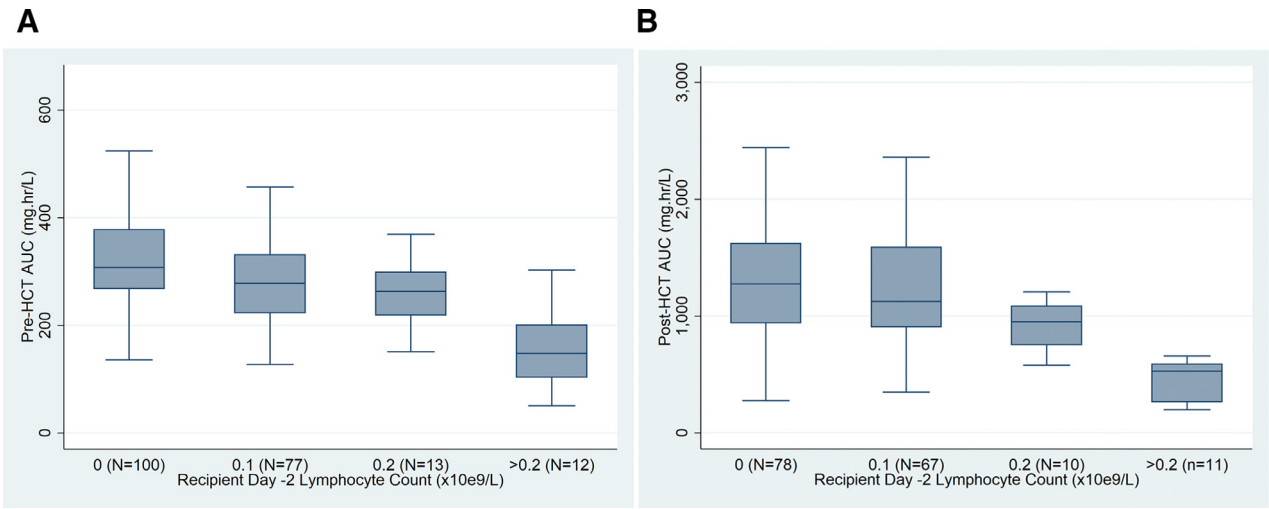

Figure 3. (A) Distribution of pre-HCT AUC in recipients with day −2 lymphocyte counts of 0, .1, .2, or $>.2 \times 10^{e9}$/L. Mean pre-HCT AUC is significantly different between those with lymphocytes > .2 versus .2 ($P = .04$), .1 ($P < .001$), and 0 ($P < .001$) and between those with lymphocytes 0 versus .1 ($P = .002$) and .2 ($P = .02$). (B) Distribution of post-HCT AUC in recipients with day −2 lymphocyte counts of 0, .1, .2, or $>.2 \times 10^{9}$/L. Mean post-HCT AUC is significantly different between those with lymphocytes > .2 versus .1 ($P < .0001$) and 0 ($P < .0001$).

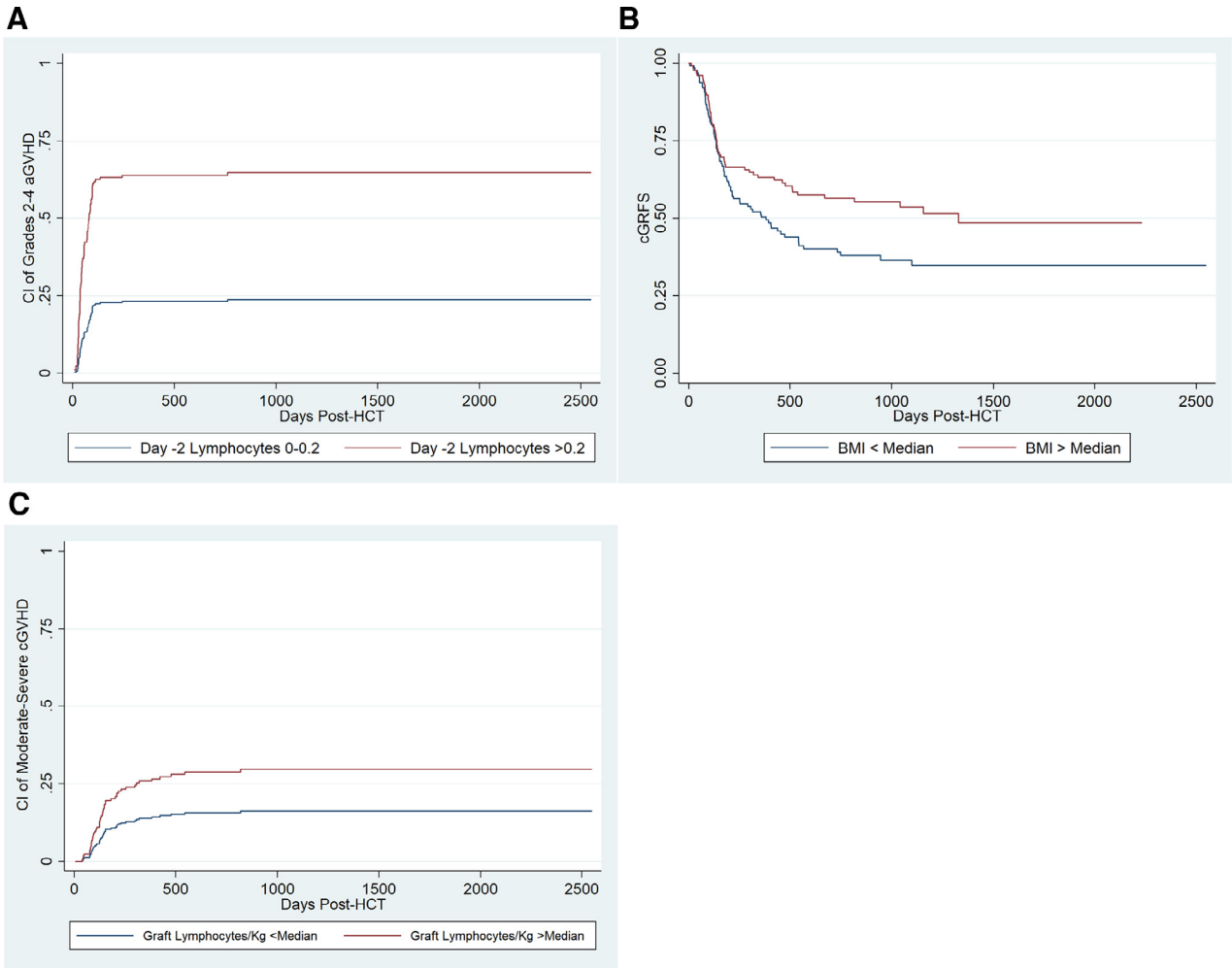

Figure 4. (A) Cumulative incidence of grades II to IV aGVHD in recipients with day −2 lymphocyte counts 0 to .2/nL versus >.2/nL. (B) Cumulative incidence of cGRFS in recipients with BMI less than versus greater than median (median BMI in the cohort is 26.3 kg/m^2 [interquartile range, 23.2 to 29.7]). (C) Cumulative incidence of moderate to severe cGVHD in recipients who received grafts with less than versus greater than median graft lymphocytes per kilogram recipient body weight (median graft lymphocytes/kg in the cohort is 0.36/nL [interquartile range, .27 to .5).

we found that the factors most strongly associated with AUC were day −2 recipient ALC, BMI, and graft lymphocyte content.

The examination of ATG AUC rather than single time point levels is, to our knowledge, limited to only 2 other reports. In contrast to our findings, a recent analysis in adults undergoing nonmyeloablative conditioned-HCT by Admiraal et al. [19] described an optimal post-HCT ATG AUC, with AUCs above and below optimal associated with inferior survival. Above-optimal post-HCT AUC was associated with relapse-related mortality and below-optimal post-HCT AUC with NRM. Pre-HCT ATG AUC was not associated with clinical outcomes, although pre-HCT ATG levels were only available for some of the study population. There are important differences with respect to conditioning technique between this report and ours: Recipients in our study received myeloablative conditioning with 4.5 mg/kg of ATG (Thymoglobulin) starting on day −2 when most patients were already severely lymphopenic, whereas in the aforementioned study recipients received 8 mg/kg ATG before starting nonmyeloablative conditioning chemotherapy (before severe lymphopenia). Admiraal et al. [20] also described ATG (Thymoglobulin) AUC in children undergoing predominantly myeloablative conditioning with various dosing schedules of ATG. Similar to our findings, a higher pre-HCT AUC was associated with a lower risk of aGVHD and cGVHD. Thus, the intensity of conditioning, dose of ATG, and timing of ATG administration pre-HCT likely modulate ATG PKs and their effect on clinical outcomes.

In recent years reports of recipient-specific modulators of outcome of ATG-conditioned HCT have emerged, suggesting that a personalized approach to ATG dosing may optimize outcomes. Here we have described 2 such recipient-specific modulators: pre-ATG recipient lymphocyte count and IgG level and 1 graft-specific modulator, that is, graft lymphocyte count. The best described of these modulators is recipient lymphocyte count before ATG administration. For example, in a recent randomized trial, patients with lower lymphocyte count before ATG and TBI-based conditioning experienced inferior overall and progression-free survival [2]. Similarly, in a recent retrospective study, dose of ATG interacted with recipient lymphocyte count to predict survival [25]. To our knowledge only 1 prior study has directly examined the interaction between lymphocyte count and ATG AUC in adults: In the PK study of Admiraal et al. [19], recipient lymphocyte count was the main driver of ATG clearance but weight was not, leading to the suggestion that optimal ATG AUC could be achieved by dosing ATG simply based on lymphocyte count. Similarly, we found that a higher lymphocyte count was associated with lower pre- and post-HCT ATG AUC and with inferior cGRFS and a higher incidence of aGVHD. We did not, however, find lower lymphocyte counts to be associated with inferior survival; this may be due to inadequate statistical power, because most patients in our study were severely lymphopenic before ATG ($\leq .2 \times 10^9$), and/or because we used lower doses of ATG than the above-described studies, possibly limiting the ATG AUC for those with lower lymphocyte counts. In contrast to the Admiraal study, we found that both weight and lymphocyte count were associated with ATG clearance, perhaps suggesting that in the presence of severe lymphopenia weight plays an important role in ATG PK. Possibly because ATG is not expected to distribute into body fat, we particularly found higher BMI to be associated with higher ATG AUC.

Our study is the first to our knowledge to demonstrate that recipient pre-HCT IgG level is inversely correlated with post-HCT ATG AUC. We hypothesize that rabbit IgG may compete with recipient IgG for binding to FcRn receptors. Thus, in the setting of lower pre-HCT recipient IgG levels there may be enhanced binding of rabbit IgG to FcRn receptors and consequently prolonged rabbit IgG/ATG half-life.

With respect to graft-specific modulators, we found that higher graft lymphocyte content was associated with lower post-HCT ATG AUC and a higher risk of grades II to IV aGVHD and moderate to severe cGVHD. This finding is consistent with the hypothesis that lymphocytes provide targets for ATG, with higher quantities of lymphocytes leading to lower levels of residual ATG in the recipient. In the same vein, in the pediatric Admiraal et al. study [20], all quartiles of ATG AUC beyond the first were associated with impaired immune reconstitution in cord blood recipients, whereas moderate AUCs were better tolerated in bone marrow and peripheral blood graft recipients; the low lymphocyte content of cord blood grafts was likely a contributor to this finding. However, Admiraal et al. did not find a significant association between graft lymphocyte count and ATG AUCs. This may be because these investigators did not collect recipient blood at prespecified time points before and after graft infusion and rather relied on mathematical modeling based on samples collected at various time points peri-HCT. The limitation of this approach is that a sudden drop, such as the 1 occurring during graft infusion, may be missed unless the sampling is very frequent and in most patients includes immediate pregraft and early ($\leq$1 hour) postgraft infusion sampling. Finally, it is important to note that higher graft lymphocyte content has been associated with a higher risk of aGVHD and cGVHD even in the absence of therapy with ATG [26,27].

With respect to ATG dosing, our data suggest that weight cannot be ignored, although adjustments upward are likely required for higher recipient lymphocyte counts. We are unable to determine an appropriate adjustment for lymphocyte count because of the scarcity of recipients with lymphocytes $> .2 \times 10^9$ before starting ATG in our cohort. In addition, ATG dose adjustment upward may be required for those receiving a graft with higher lymphocyte content, although from a logistical perspective adjusting pre-HCT dosing of ATG for graft content would be difficult. However, it is yet to be demonstrated that dose adjusting ATG will lead to a desired pre- or post-HCT ATG AUC. Finally, it is important to note that our findings are specific to adult HCT recipients receiving 4.5 mg/kg of Thymoglobulin at the end of myeloablative conditioning. As discussed above, varying doses of ATG, varying timing of ATG administration, and varying conditioning regimens will likely lead to different ATG PK scenarios.

Our study has limitations. First, because we did not have end of ATG infusion levels (C_{max}) and day 14 levels for all patients, we estimated these values. Although we demonstrated that estimation was reasonably accurate, these estimated values were used to calculate pre- and post-HCT AUC, possibly compounding the element of inaccuracy. Second, we used a noncompartmental model to estimate ATG AUC, which may lead to inaccuracy, particularly in the setting of the study of antibody with nonlinear clearance. We attempted to minimize inaccuracy by applying the logarithmic rather than linear trapezoidal rule during the decline in ATG levels and by adding the end AUC to the post-HCT AUC calculation to avoid truncation at the end of the time-concentration curve. Reassuringly, we replicate the findings of other investigators who have used compartmental analysis. Similarly, our calculation of ATG clearance may not be entirely accurate as it assumes that ATG is eliminated with first-order PKs and in only 1 compartment, which is probably an oversimplification. Clearance of ATG is likely dynamic; for example, significant clearance may occur

after the first infusion of ATG when recipient lymphocytes are present or immediately after graft infusion (target-mediated disposition), whereas there is likely little clearance between the end of the last ATG infusion and beginning of graft infusion when virtually no lymphocytes are available for ATG binding. To model this dynamic clearance would require very frequent blood sampling for ATG level determination. Here we present the average clearance derived from the total AUC, recognizing the limited applicability of our reported ATG clearance values to other allo-HCT scenarios (ie, different conditioning regimens, graft source, or lymphocyte count before ATG initiation). Third, the low incidence of grades III to IV aGVHD and NRM in our cohort did not allow for a statistically robust examination of these outcomes. Finally, we did not determine the effect of ATG AUC, particularly post-HCT AUC, on immune reconstitution and infection. However, our group has previously described that subjects with higher ATG levels on days 7 and 28 post-HCT (thus, presumably higher post-HCT AUC) face transient suppression of B cells and CD8 T cells as well as prolonged suppression of most CD4 T cell subsets [28]. These findings highlight the need for herpes virus monitoring strategies and prophylaxis after conditioning with the protocol that we have described [29–31].

In summary, after myeloablative conditioning in adults, higher pre-HCT ATG AUC was associated with improved cGRFS, whereas in those in the lowest pre-HCT AUC quintile experienced inferior cGRFS. ATG dosing in this HCT setting will need to take into consideration recipient weight, BMI, and lymphocyte and graft lymphocyte counts. Further studies are required to develop a modern ATG dosing schema and to demonstrate that adjusting ATG dose to target a particular AUC is feasible and leads to improved outcomes.

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SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.12.065.

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