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High Rabbit-Antihuman Thymocyte Globulin Levels Are Associated with Low Likelihood of Graft-vs-Host Disease and High Likelihood of Posttransplant Lymphoproliferative Disorder

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Rabbit-antithymocyte globulin (ATG) given with conditioning has the potential to decrease the likelihood of graft-versus-host disease (GVHD) or graft failure and to increase the likelihood of relapse or infections. After a given ATG dose, serum ATG levels are variable. Here we determined ATG levels on days 7 and 28 in 153 patients whose conditioning included 4.5 mg/kg ATG (thymoglobulin). Median follow-up was 547 days (range: 14-1519, minimum for patients who have not died, relapsed, developed second malignancy, or had graft failure, 365). Both high day 7 levels and high day 28 levels were associated with low likelihoods of grade II-IV acute GVHD and chronic GVHD needing systemic immunosuppressive therapy, and a high likelihood of posttransplant lymphoproliferative disorder (PTLD). Patients with day 7 ATG levels above 0.803 mg/L had 0.52-fold risk of developing chronic GVHD needing systemic therapy ($P = 0.012$) and patients with day 7 ATG levels above 1.436 mg/L had 5.84-fold risk of developing PTLD ($P = 0.001$) compared to patients with lower ATG levels. There was no association of ATG levels with relapse, death, or non-PTLD infections. Association with graft failure could not be evaluated due to only 4 graft failures in the cohort. In conclusion, patients with slow clearance of ATG have a low risk of GVHD, but a high risk of PTLD. The clearance of this relatively low dose of ATG does not impact the likelihood of relapse, death, or non-PTLD infections.

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INTRODUCTION

Rabbit-antithymocyte globulin (ATG) has been increasingly used as a component of allogeneic hematopoietic cell transplant (HCT) conditioning [1-9]. Two prospective randomized studies, comparing either rabbit-antihuman T cell line (Jurkat) globulin (ATG-F®, Fresenius, Phoenix, AZ) versus no ATG [10] or rabbit-antihuman thymocyte globulin (Thymoglobulin®, Genzyme, Phoenix, AZ) versus no ATG [11], showed that ATG results in decreased incidence of acute and chronic graft-versus-host disease (aGVHD, cGVHD) and thus presumably improved quality of life, without having a significant impact on malignancy relapse, nonrelapse mortality (NRM), relapse-free survival (RFS), or overall survival (OS). Of note, no anti-GVHD effect was observed in a prospective randomized study evaluating horse-antihuman thymocyte globulin (Atgam®, Upjohn, Kalamazoo, MI) [12], which is not the subject of this article. On another note, the anti-GVHD activity of rabbit ATG is present if used prophylactically, but may be minimal or absent if used therapeutically [13].

The mechanism of anti-GVHD effect of rabbit ATG is complex and not completely understood [14]. The antibodies within ATG are polyclonal, and target antigens expressed on not only T cells but also other hematolymphatic cells that may be involved in the pathogenesis of aGVHD or cGVHD, like dendritic cells or B cells. The antibodies may kill the targeted immune cells (inducing apoptosis, natural killer [NK] cell-mediated lysis, or complement-mediated lysis) or alter their function (inducing T cell differentiation into regulatory cells, inhibiting

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T cell proliferation, or blocking surface antigens needed for interaction with other cells or chemotaxis) [15-17]. When ATG is given during conditioning, not only recipient but also donor immune cells are depleted or inhibited, as the serum half-life of ATG is 1-6 weeks [16,18-24]. ATG may kill not only immune cells, but also some leukemic cells [25].

Theoretically, ATG should minimize the likelihood of graft failure, as ATG should kill or inhibit recipient T cells that mediate graft rejection and not donor hematopoietic stem cells [25]. However, in the prospective randomized studies the incidence of graft failure appeared similar between the ATG and non-ATG arms, but the number of patients with graft failure was too small for a meaningful analysis [10,11]. In a retrospective study, the incidence of graft failure was lower with versus without thymoglobulin [26].

Theoretically, ATG should also increase the likelihood of infections, as ATG should kill or inhibit donor or recipient pathogen-specific T cells and other immune cells. However, in the randomized study evaluating ATG-F there was no difference in the rates of microbiologically documented infections [10]. There was a trend toward higher rates of presumed (not microbiologically documented) infections in the ATG-F arm compared to the no-ATG arm, and among microbiologically documented infections, there was a trend toward higher rates of herpes simplex virus disease, cytomegalovirus (CMV) reactivation (not CMV disease), and Epstein-Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (PTLD). In the randomized study evaluating thymoglobulin, there was an increased incidence of fatal infections in a subgroup receiving 15 mg/kg, but not in a subgroup receiving 7.5 mg/kg compared to no ATG [11]. The fatal infections were bacterial or fungal. CMV reactivation did not appear to be increased, and no PTLD is mentioned in the article.

Collectively, ATG has been shown to reduce the likelihood of aGVHD and cGVHD; however, its impact on graft failure and infections (including PTLD) is unclear. Here we set out to determine the impact of ATG on clinical outcomes including infections. We took advantage of the fact that there is a marked interpatient variability in ATG clearance (different serum ATG levels are detected after the same dose of ATG) [20,22], and that we were able to study a relatively homogeneous patient group given the same conditioning, including the same dose of ATG.

PATIENTS AND METHODS

Patients and Transplantation

We studied 153 consecutive recipients of allogeneic HCT performed in Calgary who received ATG as a part of their conditioning and consented to donate blood for research on day 7 and day 28. The transplants were performed between December 2004 and September 2008. Patients typically received conditioning with fludarabine (250 mg/m^2), busulfan (approximately 13 mg/kg i.v., pharmacokinetically adjusted) and ATG, and additional GVHD prophylaxis with methotrexate on days 1, 3, 6, and 11 and cyclosporine from day −1 until 3 to 6 months posttransplant (longer in the case of cGVHD) [8]. Conditioning of some patients included total body irradiation (TBI) (4 cGy) [27]. All patients received ATG (thymoglobulin, Genzyme) 0.5 mg/kg on day −2, 2.0 mg/kg on day −1, and 2.0 mg/kg on day 0 (total, 4.5 mg/kg) [8]. Table 1 displays patient and donor characteristics. Supportive care was similar for all patients. All blood products were from CMV seronegative donors and were leukocyte depleted. No antibacterial or antifungal prophylaxis was given routinely (except for trimethoprim-sulfamethoxazole for Pneumocystis prophylaxis). Pneumocystis prophylaxis, typically using trimethoprim-sulfamethoxazole, was given until 6 months posttransplant or longer (in the case of cGVHD needing systemic therapy). Acyclovir, typically 400 mg twice a day orally, was used until 6-12 months posttransplant or longer (in the case of cGVHD needing systemic therapy). Monitoring of EBV DNAemia was not done. Median follow-up was 547 days (range: 14-1519 days; minimum for patients who have not died, relapsed, developed second malignancy or had graft failure, 365 days).

Fourty-eight autologous HCT recipients (who received no ATG) were used as controls for the determination of ATG serum level detection limit.

Determination of ATG Levels

Blood was scheduled to be drawn from patients on approximately day 7 and 28 posttransplant. The actual median day of the day 7 blood draw was day 7 (range: 6-8), and the actual median day of the day 28 blood draw was day 28 (range: 23-34). The "day 7" blood draw was performed on 115 patients and the "day 28" blood draw on 137 patients. Serum was separated from the blood and kept in tightly sealed vials at minus 80°C until ATG level determination.

Level (concentration) of "functional" ATG (capable of binding to human lymphocytes) was determined using the method of Kakhniashvili et al. [22] with minor modifications. To prepare standards of known ATG concentration, ATG (thymoglobulin, Genzyme) was diluted in normal human serum to a concentration of 20 mg/L. This was serially 2-fold diluted to produce a range of ATG standards ranging from 20 to 0.0098 mg/L. Peripheral blood mononuclear cells (drawn from 1 individual at 1 time to minimize assay variability) were separated from heparinized blood using density gradient centrifugation (Lympholyte, Cedarlane

Table 1. Patient Characteristics

N	*153*
Median patient age	*49 (range, 19-66)*
Median donor age	*36 (range, 15-67)*
Patient sex	*91 M, 62 F*
Donor sex	*99 M, 54 F*
*Diagnosis/disease stage at transplant**	
Poor risk	73
Good risk	80
Diagnosis	
AML in first remission	42
AML beyond first remission	20
ALL in first remission	13
ALL beyond second remission	7
CML in first chronic/accelerated phase	10
CML in blast or second chronic/ accelerated phase	2
CMML	5
CLL	10
Non-Hodgkin lymphoma	21
Hodgkin lymphoma	2
Myelodysplastic syndrome/ myelofibrosis	15
Aplastic anemia	3
Other	3
Stem cell source	
Bone marrow	10
Blood stem cells	143
Donor/Recipient CMV serostatus at HCT	
Positive/positive	45
Positive/negative	14
Negative/positive	34
Negative/negative	59
Unknown or indeterminate	1
Donor/Recipient EBV serostatus at HCT	
Positive/positive	131
Positive/negative	5
Negative/positive	9
Negative/negative	0
Unknown or indeterminate	8
Conditioning with TBI	
Yes	96
No	57
Donor type	
HLA-matched sibling	76
Other†	77
Graft failure	
Primary	3
Secondary	1
PTLD	
Yes	14
No	139
Acute GVHD by grade	
None	73
Grade 1	41
Grade 2	23
Grade 3	14
Grade 4	2
Chronic GVHD	
None	58
NNST‡	13
NST‡	54
Not-evaluable (end of FU before day 100)	28

AML indicates acute myelogenous leukemia; ALL, acute lymphoid leukemia; CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; CLL, chronic lymphocytic leukemia; GVHD, graft-versus-host disease; PTLD, posttransplant lymphoproliferative disorder; TBI, total body irradiation; HCT, hematopoietic cell transplant; EBV, Epstein-Barr virus; CMV, cytomegalovirus; FU, follow up.

*Good risk disease was defined as acute leukemia in first remission, chronic myelogenous leukemia in first chronic or accelerated phase, myelodysplastic syndrome with <5% marrow blasts or aplastic anemia. All other diseases/disease stages were considered poor risk.

Labs, Canada). Half a million cells suspended in 100 μL phosphate-buffered saline (PBS) were added to 20 μL patient serum or ATG standards. After a 30-minute incubation at room temperature and 3 washes in PBS, the cells, now coated with ATG, were labeled with phycoerythrin (PE)-conjugated goat-antirabbit IgG (Sigma-Aldrich, St. Louis, MO). To accomplish this, 20 μL PBS containing 0.5 μg of the antibody was added to the cells and incubated at room temperature in the dark. After 2 washes with PBS, flow cytometric analysis was performed on FACSAria (BD Biosciences, San Jose, CA). Lymphocytes were gated by forward and side scatter characteristics. PE fluorescence was measured for each standard (Figure 1A) and for each patient serum included in the run. Titration curve was generated by plotting ATG level versus median channel of PE fluorescence intensity (Figure 1B). Patient ATG levels were extrapolated from the curve, using the equation $y = a \cdot x^b$ .The numbers for a and b were produced by power regression using Microsoft Excel software. All patient serum samples were analyzed twice (2 different runs). The average of the 2 values was used for analysis. The assays were run by P.J.P., who was blinded to the patient outcomes.

Coefficients of variation for the low, middle, and high ends of the standard curve were calculated based on 10 experiment repeats, using sera with low ATG level, intermediate ATG level, and high ATG level. The coefficient of variation for the low level serum was 0.26, for the intermediate level serum 0.22, and for the high level serum 0.15.

Detection limit of the assay was calculated as the 90th percentile of values obtained from measuring ATG levels in day 7 and/or 28 sera from 48 autologous HCT recipients. The detection limit was 0.00131 mg/L. In the allogenic HCT recipients, values under the detection limit were arbitrarily assigned a level of 0.00066 mg/L (half of the detection limit).

Definitions of Outcomes

Relapse, death, nonrelapse death, and graft failure were defined using standard criteria. aGVHD and cGVHD were diagnosed according to historical criteria (aGVHD if onset by day 100, cGVHD if present after day 100). aGVHD was graded according to the 1994 consensus conference [28]. aGVHD-related death was defined as nonrelapse death occurring in the first 100 days in a patient with grade II-IV aGVHD. cGVHD was graded as none, not needing systemic therapy ("cGVHD NNST") or needing

†Matched unrelated donors (n=51), mismatched donors (n=26).

‡NNST indicates "not needing systemic therapy" and NST indicated "needing systemic therapy."

systemic therapy ("cGVHD NST"). Patients were considered treated (rather than prophylaxed) with systemic immunosuppressive drugs if cyclosporine was given beyond 6 months and/or additional immunosuppressive drug(s) was (were) given at any time after 3 months posttransplant for treatment of cGVHD. cGVHD-related death was defined as nonrelapse death occurring after day 100 while the patient was still treated with systemic immunosuppressive drugs for cGVHD.

Definite infection was defined as an illness with symptoms and signs consistent with an infection and microbiological documentation of a pathogen. For zoster, clinical diagnosis was considered sufficient. Microbiological documentation included isolation of the pathogen by culture from a sterile site or a nonsterile site (if from a nonsterile site, the organism had to be clinically judged as pathogenic) or histological/immunohistological evidence. Culture-documented viremia, bacteremia, or fungemia was counted even in the absence of symptoms or signs of infection, except for Micrococcus or non-JK Corynebacterium, unless clinically clearly judged as pathogens.

Presumed infection (without an identified microorganism) was defined as illness with symptoms and signs consistent with an infection. However, presumed oral, gastrointestinal, conjunctival, and respiratory tract infections were discounted because they could not be reliably distinguished from GVHD or allergy. Fever without other symptoms/signs was also discounted as it could not be reliably attributed to an infection. Hemorrhagic cystitis was discounted because it could not be differentiated from conditioning regimen-induced cystitis. Sinusitis and pneumonia were counted only if radiologically documented.

A recurrent infection was counted as multiple infections if the episodes were separated by >4-week asymptomatic period. A chronic infection (with asymptomatic periods lasting ≤4 weeks) was counted as 1 infection. A polymicrobial infection of 1 organ or several adjacent organs was counted as 1 infection. An infection in ≥2 nonadjacent organs because of the same microorganism was counted as 1 infection (disseminated).

Death because of an infection was defined as (1) autopsy findings consistent with an infection and the detection of the pathogen in autopsy specimen, or (2) death that followed an infection that was judged to cause the death either directly (eg, severe pneumonia) or indirectly (eg, sepsis with subsequent adult respiratory distress syndrome).

PTLD was counted as a viral infection. It was defined as an illness with signs or imaging results consistent with PTLD (eg, fever not because of other causes, lymphadenopathy, splenomegaly, or a mass) with EBV DNA above 10,000 copies/μg leukocyte DNA or immunohistological evidence of PTLD.

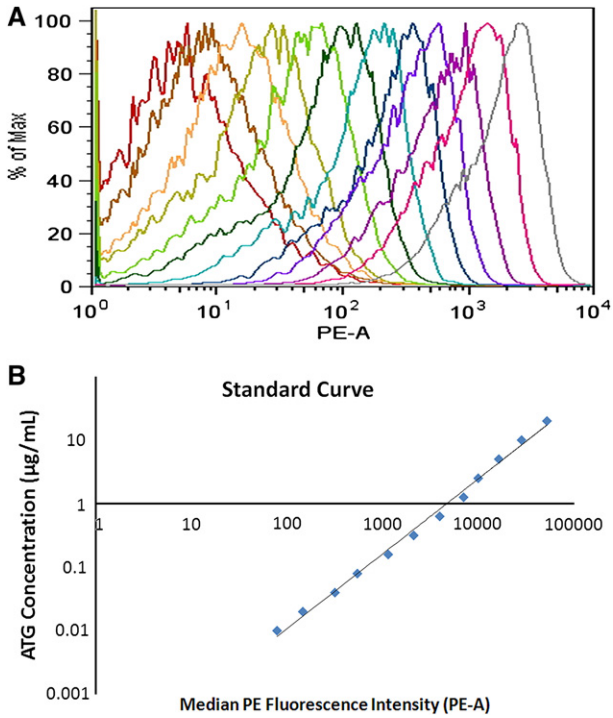

Figure 1. Determination of ATG level. (A) Phycoerythrin fluorescence peaks, each corresponding to a standard ATG concentration. X-axis shows phycoerythrin fluorescence intensity area (PE-A). Y-axis shows the percent maximum cell count (of lymphocytes acquired for each ATG concentration). (B) Example of a standard curve.

PTLD was typically treated with rituximab, with or without taper of immunosuppressive drug(s).

Statistics

ATG levels in patients with versus without a clinical outcome were compared using Mann-Whitney-Wilcoxon test. For outcomes for which ATG levels appeared to be significantly different between patients with versus without that outcome ($P \leq 0.15$), we determined whether patients with higher than cutoff ATG levels had a higher/lower likelihood of the outcome compared to patients with lower than cutoff ATG levels, using binomial regression models (multivariate analysis adjusting for confounding factors known to be associated with the outcome). For each outcome, a suitable cutoff was determined from a receiver-operator characteristic (ROC) curve, using the point with maximum sum of sensitivity and specificity. Confounding factors (covariates) considered in the multivariate analyses for aGVHD were recipient age (continuous), donor type (HLA-matched sibling versus other), donor/recipient sex (M/M versus other), recipient CMV serostatus (positive versus negative) and conditioning regimen (with versus without TBI). For cGVHD, we considered the same covariates, plus stem cell source (marrow versus blood stem cells). For PTLD we considered donor/recipient EBV serostatus (+/+ versus other including unknown), aGVHD (grade II-IV) and/or cGVHD (NST) before PTLD

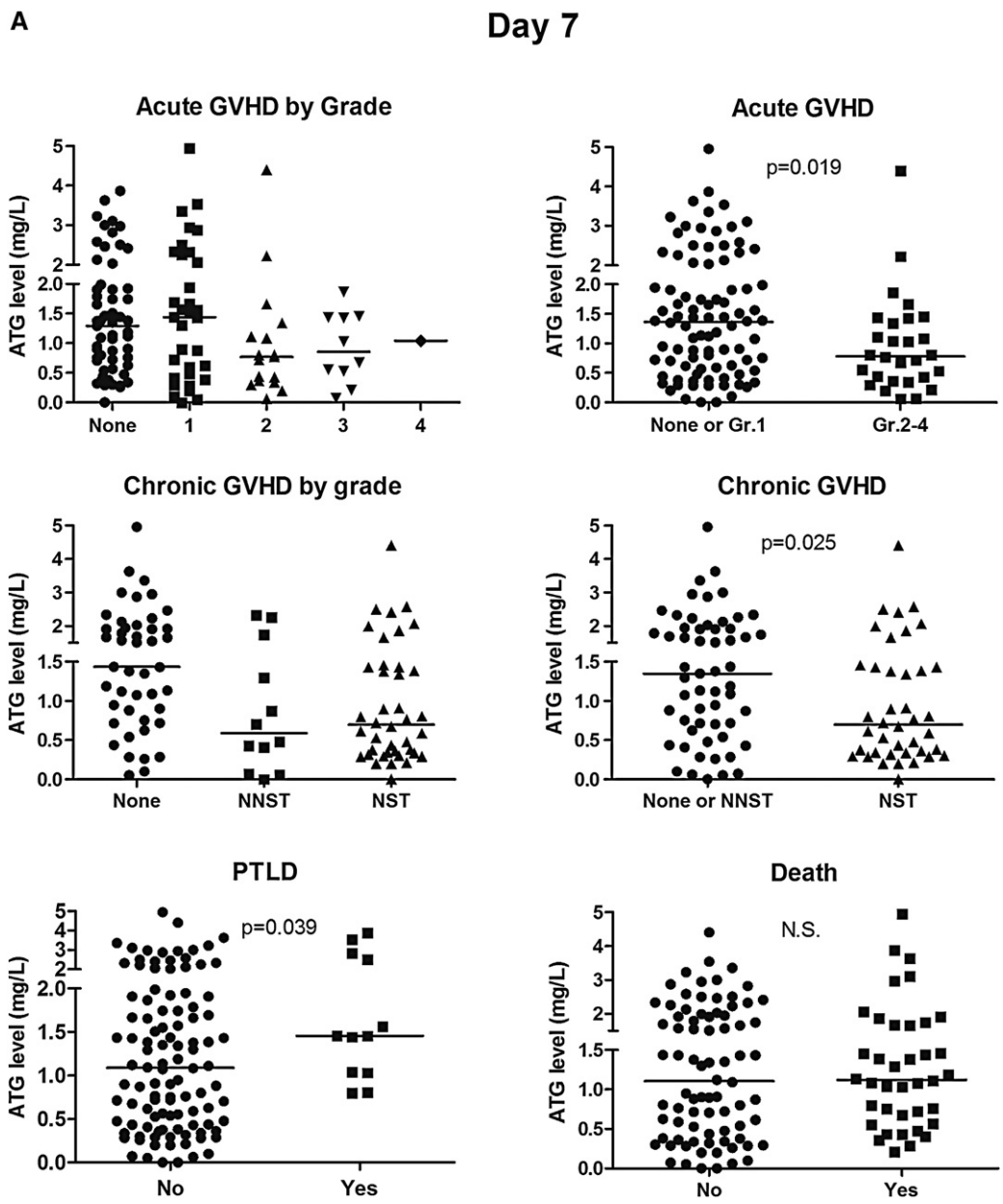

Figure 2. ATG levels in patients with selected clinical outcomes. Horizontal bars indicate median values. NNST indicates "not needing systemic therapy" and NST indicates "needing systemic therapy." *P*-values shown are from the Mann-Whitney-Wilcoxon rank-sum test (univariate analysis). N.S. indicates nonsignificant or no trend toward significance ($P > .15$).

onset (yes versus no), donor type (HLA-matched sibling versus other), and recipient age (continuous) [29,30]. Analysis was performed using STATA software, version 9.2.

RESULTS

Median day 7 ATG levels were 1.109 mg/L (range, undetectable to 4.401 mg/L) and median day 28 levels were 0.053 mg/L (range, undetectable to 0.733 mg/L).

After plotting ATG levels for patients with different grades of aGVHD (Figure 2), it appeared that the medians were similar in patients with grade 0 and I and lower than those in patients with grade II, III, and IV. Therefore, and because grade II-IV aGVHD is clinically significant (treated with systemic immunosuppressive drugs), we primarily compared ATG levels in patients with grade 0 or I versus grade II, III, or IV aGVHD. The latter patients had significantly lower ATG levels ($P = 0.019$ and 0.002 for days 7 and 28, respectively) (Table 2 and Figure 2). We also compared ATG levels in patients with no aGVHD versus any aGVHD; ATG levels appeared higher in the latter group ($P = 0.181$ and .021 for days 7 and 28, respectively) (Table 2). We also compared ATG

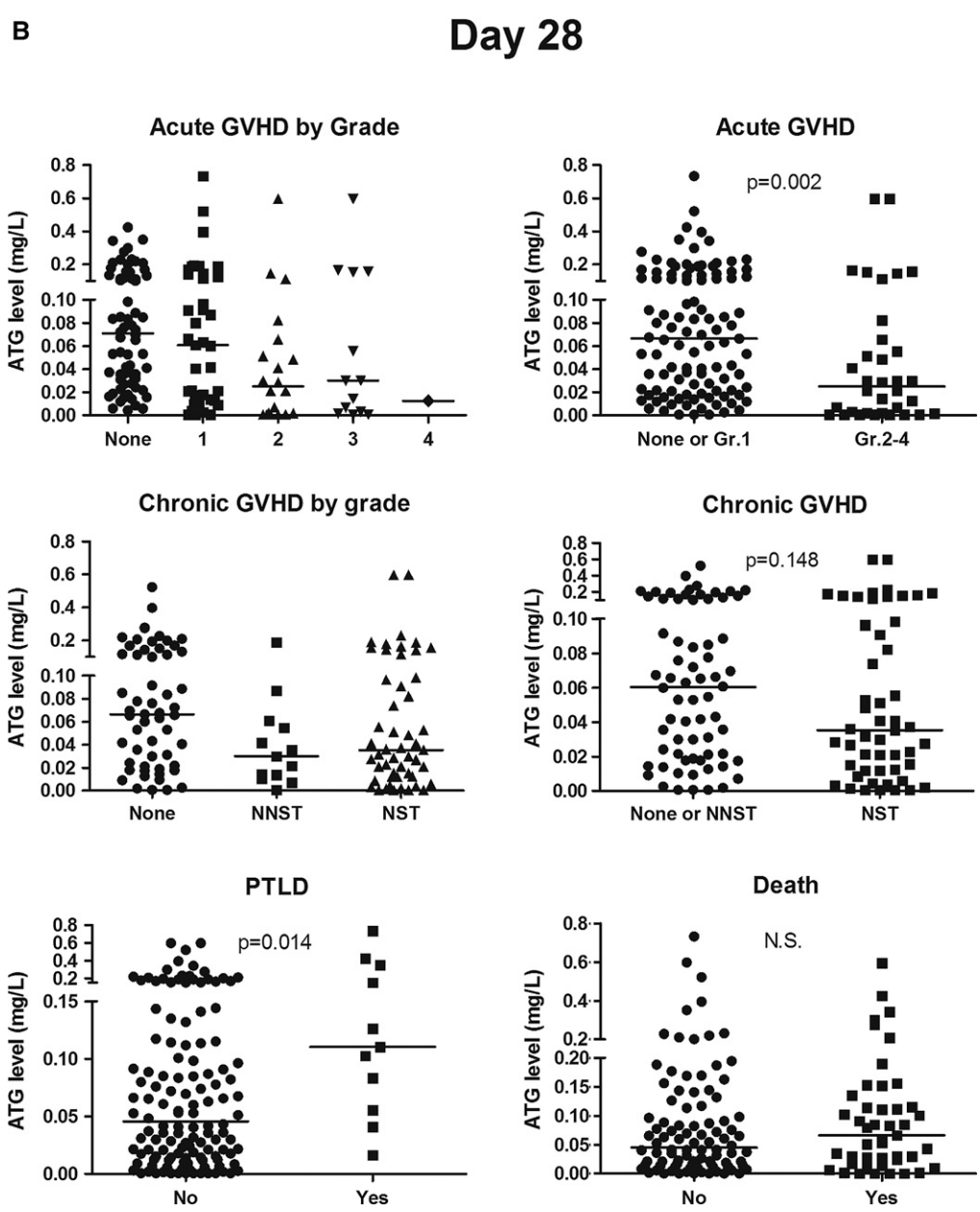

Figure 2. (*continued*).

levels in patients with severe aGVHD (either grade III-IV or associated with death) versus all other patients; the differences were not significant (Table 2).

When plotting ATG levels for patients with no cGVHD, cGVHD NNST, or cGVHD NST (Figure 2), it appeared that the medians were similar in patients with cGVHD NNST and NST, and higher in patients with no cGVHD. This difference was significant ($P = 0.002$ and 0.019 for days 7 and 28, respectively) (Table 2). Nevertheless, our primary comparison was that of ATG levels in patients with no cGVHD or cGVHD NNST versus cGVHD NST, as cGVHD NST is clinically significant. The latter patients tended to have lower ATG levels ($P = 0.025$ and 0.148 for days 7 and 28, respectively) (Table 2 and Figure 2). We also compared ATG levels in patients with cGVHD-associated death versus all other patients; the differences were not significant (Table 2). We also compared ATG levels in patients with any aGVHD or cGVHD-associated death versus all other patients; the differences were also not significant (Table 2).

There was no significant difference in ATG levels between patients who did versus did not develop graft failure (but only 4 patients developed graft failure), relapse, death, nonrelapse death (excluding patients with relapse from analysis), death because of an infection (excluding patients with relapse from analysis), or patients who survived without relapse versus those who died or relapsed (Table 2). Also, there was no significant difference in day 7 ATG levels between

patients who had no versus at least 1 infection between day 7 and 28, day 7 and 56, or day 7 and 365, and no significant difference in day 28 ATG levels between patients who had no versus at least 1 infection between day 28 and 56 or day 28 and 365 (Table 2;only data for the infections between the day of ATG level determination and day 365 are shown). This was also true when the analyses were done separately for microbiologically documented infections, viral infections, bacterial infections, and fungal infections. However, there was a significant difference in both day 7 and day 28 ATG levels in patients who did versus did not develop PTLD; patients who developed PTLD had higher ATG levels ($P = 0.039$ and 0.014 for days 7 and 28, respectively) (Table 2 and Figure 2). The median onset of PTLD was day 57 (range: 38-229). Of the 14 cases of PTLD, 3 were fatal. There was a trend toward higher ATG levels in patients with fatal PTLD than those with nonfatal PTLD (median 1.454 versus 1.100 mg/L for day 7 and 0.153 versus 0.052 mg/L for day 28; not significant for either day 7 or day 28).

The surprising lack of association between ATG levels and non-PTLD infections could be because of the fact that the patients with the lowest ATG levels developed GVHD and subsequently developed infections because of GVHD or its treatment. However, when we excluded patients who developed grade II-IV aGVHD or cGVHD NST from analysis, there was also no significant difference in day 7 ATG levels between patients who had no versus at least 1 infection between day 7 and 28, day 7 and 56, or day 7 and 365 (n = 62), and no significant difference in day 28 ATG levels between patients who had no versus at least 1 infection between day 28 and 56, or day 28 and 365 (n = 74) (data not shown). This was also true when the analyses were done separately for microbiologically documented infections, viral infections, PTLD, bacterial infections, and fungal infections.

We next set out to determine whether patients with higher than cutoff ATG levels had a lower likelihood of developing grade II-IV aGVHD or cGVHD NST or higher likelihood of developing PTLD compared to patients with lower than cutoff ATG levels. The results are shown in Table 2 and Figure 3. ATG levels above 1.454 mg/L on day 7 were associated with 0.35-fold risk of developing grade II-IV aGVHD ($P = 0.030$) and levels above 0.029 mg/L on day 28 were associated with 0.52-fold risk of developing grade II-IV aGVHD ($P = 0.035$). Similarly, ATG levels above 0.803 mg/L on day 7 were associated with 0.52-fold risk of developing cGVHD NST ($P = 0.012$) and levels above 0.052 mg/L on day 28 were associated with 0.60-fold risk of developing cGVHD NST ($P = 0.028$). ATG levels above 1.436 mg/L on day 7 were associated with 5.84-fold risk, and above 0.082 mg/L on day 28 with 6.63-fold risk of developing PTLD ($p = 0.044$ for day 7, $p = 0.015$ for day 28). All patients who developed PTLD had ATG levels above 0.799 mg/L on day 7 and above 0.016 mg/L on day 28; patients with lower levels appeared to be protected.

DISCUSSION

Here, we demonstrated that high levels of ATG on day 7 and 28 predict a low likelihood of developing aGVHD and cGVHD as well as a high likelihood of developing PTLD. The association between ATG levels and aGVHD has been previously noted by Remberger and Sundberg [31]. However, the associations between ATG levels and cGVHD and PTLD are new findings. These were not described by Remberger and Sundberg[31], possibly because of their relatively small sample size (n = 76) or because they measured total rabbit IgG (including both IgG that can bind to lymphocytes as well as IgG that cannot). Consistent with Remberger and Sundberg's results, we also did not observe any association between ATG levels and relapse, death, nonrelapse death, or RFS. Neither Remberger and Sundberg nor we were able to evaluate potential impact of ATG levels on graft failure because of its low occurrence. There appeared to be no impact of high ATG levels on infections in our study; this was not evaluated in the study of Remberger and Sundberg.

The lack of associations between ATG levels and relapse and non-PTLD infections should not be interpreted as "the lack of effect of ATG on relapse or non-PTLD infections." In studies using high dose (10-40 mg/kg thymoglobulin), but not in studies using low dose (4-8 mg/kg thymoglobulin), there was a trend toward higher relapse or non-PTLD infection rates compared to no ATG controls [9,11,21,22,26,32,33]. Therefore, the effects of ATG may be dose-dependent. Low-dose ATG may have anti-GVHD and pro-PTLD effects only, whereas high-dose ATG may have also pro-relapse and pro-viral/bacterial/fungal infection effects. Thus, low-dose ATG might partially protect against GVHD without adversely impacting other outcomes like relapse or infection rates, as long as PTLD incidence could be minimized. Promising anti-PTLD strategies are emerging, for example, preemptive (at the time of high/rising EBV DNAemia) or prompt (early in the course of PTLD) administration of rituximab [34-36] or EBV-specific donor T cells [37-39].

Despite the fact that both aGVHD and cGVHD incidences were lower in patients with higher ATG levels, nonrelapse mortality (NRM) was not affected by ATG levels. This may be because the anti-GVHD effect was in part outweighed by the pro-PTLD effect, or because ATG had no or minimal effect on the most severe (fatal) aGVHD or cGVHD. The latter is supported by the fact that ATG levels were not lower in patients with aGVHD or

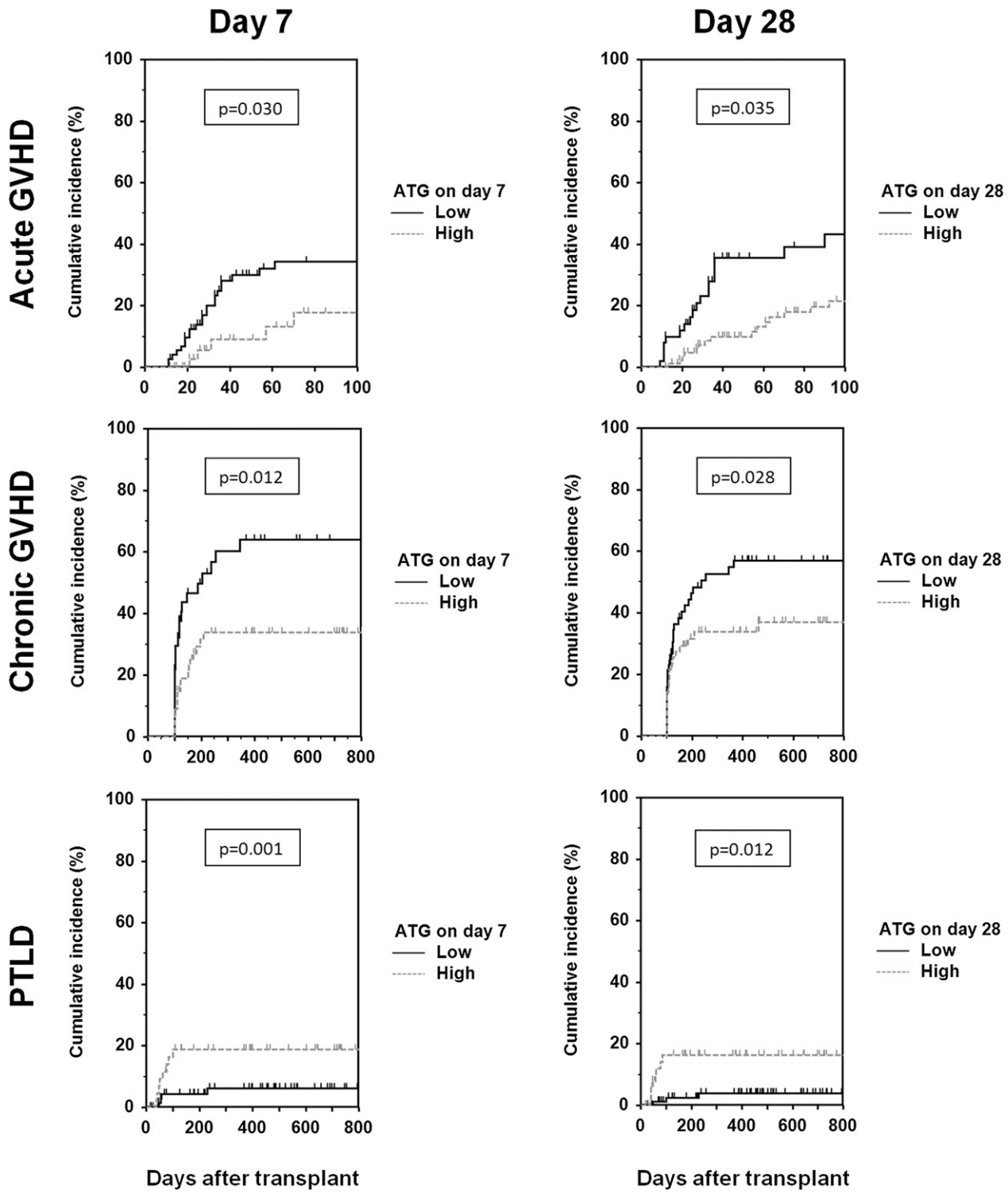

Figure 3. Cumulative incidence of grade II-IV acute GVHD, chronic GVHD NST or PTLD in patients with day 7 or 28 ATG levels above or below the cutoff specified in Table 2. Solid lines represent patients with ATG levels below the cutoff and broken lines patients with ATG levels above the cutoff. *P*-values shown are adjusted for covariates (binomial regression multivariate analysis).

cGVHD-related death compared to other patients (Table 2).

Serum half-life of ATG surmised from our median levels on day 7 and 28 (~5 days) is shorter than that found in previous studies (1-6 weeks) [16,18,20-24]. Most of the previous studies measured total ATG, as opposed to us measuring ATG capable of binding to lymphocytes. It is conceivable that the clearance of antibodies capable of binding to lymphocytes may be faster than the clearance of other antibodies contained in ATG. Somewhat contrary to this hypothesis, Kakhniashvili et al. [22] found that the

Table 2. Association (or lack of association) between ATG levels and clinical outcomes

		ATG levels on day 7 n= 115					ATG levels on day 28 n=137				
		Median ATG levels	P value*	Cut- off ATG level	Adjusted Relative Risk (95% IC)	Adjusted P value**	Median ATG Levels	P value*	Cut-off ATG level	Adjusted Relative Risk (95% IC)	Adjusted P value**
Acute GVHD, any (grade 1-IV)	Yes	1.030	0.181	0.718	0.64 (0.44-0.93)		0.030	**0.008**	0.021	0.61 (0.46-0.82)	**0.001**
	No	1.293					0.071				
Acute GVHD, grade II-IV	Yes	0.781	**0.019**	1.454	0.35 (0.14-0.90)	**0.030**	0.025	**0.002**	0.029	0.52 (0.28-0.95)	**0.035**
	No	1.364					0.066				
Acute GVHD, grade III-IV	Yes	1.030	0.240				0.022	0.152			
	No	1.133					0.055				
Acute GVHD-related death	Yes	1.034	0.835				0.007	0.300			
	No	1.125					0.053				
Chronic GVHD, any (NNST or NST‡)	Yes	0.689	**0.002**	0.871	0.47 (0.31-0.73)	**0.001**	0.034	0.019	0.052	0.58 (0.41-0.85)	**0.004**
	No	1.436					0.066				
Chronic GVHD, NST‡	Yes	0.698	**0.025**	0.803	0.52 (0.32-0.87)	**0.012**	0.035	0.148	0.052	0.60 (0.39-0.95)	**0.028**
	No	1.351					0.060				
Chronic GVHD-related death	Yes	1.865	0.177				0.106	0.477			
	No	1.091					0.053				
Acute or chronic GVHD-related death	Yes	1.455	0.357				0.055	0.893			
	No	1.109					0.053				
Any infection (definite or presumed) §	Yes	0.993	0.885				0.046	0.741			
	No	1.264					0.053				
Definite infection§	Yes	0.927	0.998				0.046	0.768			
	No	1.243					0.053				
Viral infection§	Yes	0.900	0.963				0.040	0.623			
	No	1.119					0.055				
PTLD	Yes	1.456	**0.039**	1.436	5.84 (1.81-18.87)	**0.001**	0.110	**0.014**	0.082	6.63 (1.51-29.08)	**0.012**
	No	1.087					0.046				
Bacterial Infection§	Yes	0.993	0.412				0.051	0.618			
	No	1.264					0.053				
Fungal infection§	Yes	0.633	0.419				0.048	0.586			
	No	1.126					0.053				
Severe infection§,†	Yes	1.056	0.875				0.063	0.697			
	No	1.154					0.053				
Severe definite infection§,†	Yes	0.907	0.932				0.048	0.947			
	No	1.188					0.053				
Relapse***	Yes	0.916	0.428				0.053	0.713			
	No	1.298					0.053				
Relapse or death***	Yes	1.109	0.897				0.055	0.815			
	No	1.209					0.051				
Death	Yes	1.121	0.655				0.066	0.462			
	No	1.105					0.045				
Death due to infection****	Yes	1.271	0.540				0.030	0.923			
	No	1.295					0.055				
Non-relapse death****	Yes	1.337	0.721				0.055	0.800			
	No	1.087					0.053				

*Mann-Whitney-Wilcoxon test (univariate analysis).

**Binomial regression (multivariate analysis).

§Yes indicates at least one infection between the time of ATG level determination (day 7 or 28) and day 365 post-transplant. PTLD is counted among any infections, definite infections, viral infections, severe infections and severe definite infections.

†Severe infection was defined as an infection treated in a hospital. If an infection occurred during hospitalization for another reason, the infection was considered severe only if typically treated in the inpatient setting.

‡NNST indicates "not needing systemic therapy", NST indicates "needing systemic therapy."

***Patients with aplastic anemia (n=3) were excluded from analysis.

****Patients who relapsed were excluded from analysis.

rate of ATG disappearance from serum was similar for antibodies capable of binding to lymphocytes and antibodies capable of binding to granulocytes. However, Kakhniashvili et al. [22] did not compare antibodies capable of binding to lymphocytes versus all other antibodies contained in ATG. In a mouse study of rabbit-antimouse ATG (a model for rabbit-antihuman ATG) it was shown that lymphocyte-specific ATG is more rapidly cleared from serum compared to total ATG [40]. This supports the fact that the relatively short ATG half-life in our study may be because of measuring only the ATG capable of binding to lymphocytes. Another reason for the relatively short half life in our study could be the fact that we administered a relatively low dose of ATG. It has been shown that the higher the total ATG dose, the longer the half-life [20]. Yet another reason for the relatively short half-life in our study could be that we measured the disappearance of ATG between day 7 and 28, that is, the time of a substantial increase of counts of leukocytes (including lymphocytes) presumably adsorbing ATG from serum (median leukocyte count of our patients was 0.1/nL on day 7 and 4.9/nL on day 28, and median lymphocyte count was 0.5/nL on day 28). In support of this hypothesis, there was a significant correlation between absolute lymphocyte count on day 28 and the change of serum level of ATG (capable of binding lymphocytes) from day 7 to day 28 (Spearman rank correlation coefficient $r = 0.302$, $P = .002$).

What could be the reason for the large interpatient variability in serum ATG levels after a uniform dose of ATG (Figure 2)? One reason could be the variable number of leukemic cells adsorbing ATG from serum; this is unlikely, as when we compared the day 7 or day 28 ATG serum level or the change (day 28 minus day 7 level) between patients with acute leukemia in remission versus in relapse/refractory disease, there was no significant difference (data not shown). Another reason could be the variable number of cells infused with the graft leading to a variable amount of ATG transferred from serum to cells at the time of graft infusion. Another reason could be the variable rate of leukocyte recovery (see previous paragraph). Not only a variable amount of ATG may be transferred from the serum to the recovering leukocytes, but also the rate of leukocyte recovery may be a surrogate for the rate of recovery of cells of the "reticuloendothelial system" where antibodies are cleared [41]. In renal transplant recipients, the rate of disappearance from the serum was increased when human-antirabbit antibodies were detected [42]. Probably this is not the case in HCT recipients, as HCT recipients cannot mount antibody responses to neoantigens in the first month posttransplant [43]. ATG may bind to cells not only via Fab but also via Fc. As human Fc receptors are polymorphic (in some individuals binding therapeutic antibodies with high avidity and in others with low avidity [44]), it is conceivable that interindividual variability in Fc receptors may partly explain the interpatient variability in ATG levels.

A limitation of our study is that we determined the levels of rabbit IgG capable of binding to lymphocytes, but not IgG capable of binding to lymphocyte subsets or other immune cell subsets, or IgG exerting a specific function like inducing differentiation of CD4 T cells into regulatory cells or blocking proliferation or chemotaxis of T cells or other immune cells. Further studies could attempt to determine whether the anti-GVHD or pro-PTLD effect of ATG is associated with its ability to bind to a specific immune cell subset or to inhibit a specific immune cell function. If yes, this would give insight into the mechanism of action of ATG and facilitate its further improvement (eg, by depleting the pro-PTLD IgG fraction or enriching for the anti-GVHD IgG fraction).

Our conclusions regarding GVHD are imperfect, as we used historical definitions of aGVHD and cGVHD (before/after day 100). Moreover, instead of the NIH grading of cGVHD [45] we used a treatment-based classification (NNST, roughly corresponding to mild cGVHD, and NST, roughly corresponding to moderate or severe cGVHD per the NIH grading).

Given the highly variable clearance of ATG, could it be beneficial to give an additional dose of ATG to patients with low levels on day 7? In a study randomizing patients, who received 7.5 mg/kg ATG during conditioning, to no ATG versus 2.5-3.75 mg/kg ATG on day 7, there was no survival difference, but the incidences of both aGVHD and cGVHD were reduced, so presumably patient quality of life was improved [46]. Perhaps a survival difference or a greater reduction of the GVHD incidences could have been achieved if ATG was administered on day 7 only to patients with low day 7 ATG levels.

In conclusion, low-dose ATG has anti-GVHD and pro-PTLD effects, but probably no effect on relapse or non-PTLD infections. Research into optimization of transplant outcomes using ATG should include ATG dosing based on its pharmacokinetics and anti-PTLD strategies like preemptive/prompt infusion of rituximab or EBV-specific T cells.

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