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Impact of horse anti-thymocyte globulin on graft-*versus*-host disease after human leukocyte antigen-matched related bone marrow transplantation for severe aplastic anemia

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Severe aplastic anemia (SAA) is a life-threatening bone marrow failure disorder curable with allogeneic bone marrow transplantation (BMT). Historically, conditioning with cyclophosphamide (CY) alone was associated with high rejection rates among transfused patients, leading to the addition of horse anti-thymocyte globulin (hATG). Subsequent advances in transfusion practices markedly reduced the risks of alloimmunization and graft rejection, questioning the role of hATG in preventing graft rejection and/or reduce the risk/severity of graft-versus-host disease (GVHD). To address this uncertainty, we retrospectively analyzed 392 patients with SAA who underwent HLA-matched related BMT (HLA-MRD) following CY-based conditioning at our center between 1970 and 2024. After multivariable adjustment for major practice changes and confounding by era, the adjusted odds ratio (aOR, hATG vs. no hATG) of grades II-IV acute GVHD was 1.48 (95% CI, 0.50-4.38) and for grades III-IV acute GVHD, aOR was 2.67 (0.61-11.62). The adjusted hazard ratio (aHR) for chronic GVHD was 0.89 (0.41-1.94). These findings were consistent in sensitivity analyses restricted to recipients of GVHD prophylaxis with cyclosporine plus methotrexate. In the absence of definitive randomized data in this specific transplant setting, these observations suggest that the incremental contribution of hATG to GVHD prevention in HLA-matched related transplantation for SAA may be limited in the context of modern transfusion support and contemporary GVHD prophylaxis.

Severe aplastic anemia (SAA) is a bone marrow failure disorder that can be cured with allogeneic bone marrow transplantation (BMT). Allogeneic transplantation for SAA evolved through a series of iterative laboratory-to-clinical investigations pioneered in Seattle beginning in the early 1970s. Cyclophosphamide-based conditioning, initially

developed in preclinical primate models, enabled durable engraftment in non-transfused patients but was complicated by high rejection rates among previously transfused recipients. Mechanistic studies subsequently identified transfusion-induced alloimmunization and insufficient effective stem-cell dose as key contributors to graft failure. Sequential strategies were therefore introduced to overcome these barriers, including augmentation of marrow grafts with donor buffy-coat cells and later incorporation of anti-thymocyte globulin into conditioning regimens. Concurrent improvements in transfusion practices, particularly leukocyte depletion and irradiation of blood products, progressively reduced rejection risk and helped shape contemporary transplantation paradigms for SAA.

Early clinical application of a cyclophosphamide (CY)-based conditioning regimen (50 mg/kg/day × 4 successive days) for HLA-MRD grafts in SAA achieved durable engraftment in most non-transfused patients but was complicated by graft rejection rates of approximately 35–40% among transfused recipients.[1,2,3,4] Prospective pre-clinical studies in the 1970s predicted this adverse outcome and demonstrated graft rejection was due to host immunization to minor histocompatibility antigen (MHA) disparities expressed on donor hematopoietic cells following exposure to unmodified, leukocyte-rich blood products. In contrast, graft rejection was rare—occurring in approximately 4–8%—among non-transfused patients conditioned with CY-alone.[3,5]

To overcome transfusion-related rejection, marrow grafts were augmented with donor buffy-coat (BC) infusions based on preclinical studies.[3] This reduced the rejection rate

among multiply transfused patients to 8%, but increased chronic graft-versus-host disease (cGVHD), likely due to the addition of donor lymphocytes.[3] Pre-clinical studies pioneered at our institution optimized conditioning regimens via combining horse anti-thymocyte globulin (hATG) with an alkylating agent (i.e., CY) while, at the same time omitting donor BC infusions. Horse ATG was ultimately utilized as the ATG modality based on various pharmaceutical factors and to align with prior studies on HLA-match related donors for SAA pioneered in Seattle. The CY/hATG regimen—our current institutional standard since 1988—maintained high engraftment rates, reduced cGVHD and improved survival.

From the 1970s-1980s onward, transfusion practices and GVHD prophylaxis evolved substantially. Leukocyte removal from blood products via BC-depletion and *in vitro* irradiation markedly reduced transfusion-mediated alloimmunization in preclinical studies and was subsequently translated into the clinic. Moreover, GVHD prevention shifted from prolonged methotrexate monotherapy to combined cyclosporine (CSP) and short-course methotrexate (MTX), optimized GVHD control. Together, these advances largely eliminated the immunologic conditions that originally necessitated hATG for prevention of graft rejection. Consequently, whether hATG continues to have an essential role in the prevention of GVHD, especially compared to rATG, is an increasingly relevant clinical question.

Although hATG is widely incorporated into conditioning regimens based on its immunomodulatory effects and prolonged *in vivo* lymphocyte depletion, it remains

unclear whether GVHD reductions attributed to hATG reflected a true benefit or were confounded by concurrent changes in graft composition, transfusion practices, prophylaxis. This distinction is critical given hATG's side effects, especially infections. Herein, we assess whether hATG affects GVHD outcomes post-HLA-MRD transplantation for SAA in the context of evolving transfusion and prophylaxis practices. This is particularly relevant given the lack of randomized data clearly establishing hATG as a GVHD-preventive agent in this setting, despite evidence from other transplant contexts.

We retrospectively identified 392 patients with SAA who underwent HLA-MRD grafts after CY-based conditioning between 1970 and 2024. All were treated on prospective trials registered on ClinicalTrials.gov. We excluded 11 patients with Fanconi anemia and 2 patients with dyskeratosis congenita. Early on donor-recipient pairs were selected based on serological matching for HLA-A and HLA-B and non-reactivity of patient and donor lymphocytes in mixed leukocyte culture. Since the early 2000s, molecular matching for at least 12 HLA alleles was utilized. We assessed the association between hATG use and GVHD outcomes using multivariable regression models (logistic for aGVHD, Cox for cGVHD). Covariates in adjusted models included use of BC infusions, marrow, age at transplant, GVHD prophylaxis strategy, acute GVHD grader, and year of BMT. Subgroup analyses were performed among patients who received combination GVHD prophylaxis due to the strong observed collinearity between hATG and prophylaxis regimen. The adjusted hazard of overall mortality was estimated and compared between those who did/did not receive hATG using Cox regression.

All patients received intravenous CY at a dose of 50 mg/kg/day for days -5 through -2. 124 patients additionally received hATG intravenously (30 mg/kg/day) administered 10 hours after each of the first three CY doses (days −4 through −2). The marrow graft was infused 36 hours after the last dose of CY. 143 multiply transfused patients additionally received non-irradiated BC cells from their respective marrow donors beginning on day +1 for 4-6 days, all transfused from 1978-1988. One patient each received an HLA-identical peripheral blood stem cell graft and an HLA-identical cord blood graft.

GVHD severity was graded by two non-overlapping independent investigators using Seattle criteria for aGVHD and the NIH consensus criteria for cGVHD.[6,7] One person retrospectively scored all patients pre-NIH consensus criteria prior to 2000. GVHD prevention consisted of a long course of MTX in early patients (n=155). Later, 15 patients received CSP alone, and all subsequent patients received CSP for 180 days combined with a short-course of MTX.[8] This research was conducted under a research protocol approved by the IRB of the Fred Hutchinson Cancer Center (FHCC; FHIRB0010043; RG1001735; Approval Date: 4/26/2018). The study was conducted in accordance with the Declaration of Helsinki and other relevant guidelines and regulations. All patients studied provided written informed consent for participation in a data collection. Patients were followed lifelong by the Long-Term Follow-Up Program under an IRB-approved standardized protocol. Patients returned for a comprehensive evaluation at 1-year post-transplantation. Annual questionnaires were sent to obtain information on late events.

In our cohort, 392 patients received HLA-MRD grafts from 1970-2024. Most patients were White (80%), non-Hispanic (90%), and male (57%) (**Table 1**). Most received marrow grafts alone (58%), with 40% receiving additional BC infusions. One-third (32%) received hATG. GVHD prophylaxis consisted of monotherapy in 53% and CSP/MTX in 47%. Among evaluable patients, grades II–IV aGVHD occurred in 35% of those receiving hATG and 28% of those without hATG (**Table 2**). In contrast, grades III-IV aGVHD were more common among patients who did not receive hATG (18%) compared to those who did receive hATG (10%). cGVHD occurred in 20% of patients (n=124) who received hATG versus 30% of patients (n=268) who did not. Two patients lacked aGVHD data; all patients were evaluated for cGVHD.

After adjustment for age, BC infusion, prophylaxis regimen, aGVHD grader, year of BMT, and hATG exposure, the odds ratio (OR) for grades II-IV aGVHD was OR=1.48 (95% CI 0.5–4.38, p=0.48). The OR for grades III-IV aGVHD was OR=2.67 (95% CI 0.61–11.62), and the hazard ratio (HR) for cGVHD was HR=0.89 (95% CI 0.41–1.94; p=0.76). The HR (hATG vs. non-hATG regimens) for overall mortality for the entire cohort was 0.88 (95% CI 0.38–1.81; p=0.64). Almost all patients who received hATG also received dual-agent prophylaxis, while only 22% of patients who did not receive hATG received such prophylaxis. To address this collinearity, subgroup analyses were repeated among patients receiving CSP+MTX (n=123) (**Table 3**). In this subgroup, the adjusted OR of grades II-IV aGVHD was OR=1.87 (95% CI 0.55–6.33; p=0.31) and the adjusted HR of cGVHD was HR=0.77 (95% CI 0.34–1.77; p=0.54).

This retrospective study refines the historical understanding of hATG in CY–based conditioning. While adding hATG to the original CY regimen clearly contributed to improved engraftment and survival in earlier eras characterized by transfusion-induced alloimmunization, its incremental role in preventing GVHD in the context of modern transfusion support and optimized prophylaxis appears less pronounced. Importantly, transplantation strategies for SAA evolved in parallel with major advances in graft composition and supportive care. The use of donor BC augmentation in earlier decades, introduced to increase effective hematopoietic progenitor dose, also increased donor lymphocyte exposure and may have influenced historical patterns of cGVHD. Improvements in blood-product processing and the transition to calcineurin inhibitor-based prophylaxis further altered the immunologic context in which conditioning regimens were applied. These practice-changing variables, many of which were highly colinear across eras, complicate attribution of GVHD outcomes to any single intervention. Accordingly, these findings should not be interpreted as evidence that anti-thymocyte globulin has no role in transplantation for severe aplastic anemia. Rather, they suggest that the historical benefits attributed to hATG may have been context-dependent and influenced by evolving transfusion practices, graft engineering strategies, and GVHD-prevention approaches.

Apart from preventing graft rejection, hATG was believed to reduce GVHD through lymphocyte depletion and immunomodulation. Evidence supporting a GVHD-preventive effect of ATG has been most consistent in unrelated donor transplantation and

peripheral blood stem-cell grafts.[9-11] Randomized trials in these settings have demonstrated significant reductions in moderate-to-severe chronic GVHD with rabbit ATG, and in some studies improved GVHD-free survival. These observations highlight biologic and pharmacologic differences between ATG preparations, including potency, lymphocyte-depletion kinetics, and duration of immune modulation. The present analysis specifically evaluates equine ATG in the setting of HLA-matched related marrow transplantation for a non-malignant disorder and should not be extrapolated to rabbit ATG, unrelated donor transplantation, peripheral blood grafts, or contemporary GVHD-prevention platforms such as post-transplant cyclophosphamide.[9-12] In contrast, in HLA-MRD BMT for SAA, prospective studies primarily evaluated engraftment and rejection rather than GVHD rates. Thus, the role of hATG in this setting was not established as an independent GVHD-preventive intervention.[3] Similarly, our multivariable analyses, when accounting for confounding variables, did not identify an obvious reduction in GVHD associated with hATG use. These findings suggest that with effective CSP/MTX prophylaxis, the additional immunosuppression provided by hATG may be attenuated. The apparent historical GVHD benefit of hATG may reflect earlier prophylaxis strategies and concurrent discontinuation of BC infusions rather than a durable advantage of hATG.

Our findings align with one meta-analysis which demonstrated that hATG did not impact the overall incidence of GVHD.[13] In contrast, one study demonstrated that ATG use decreased cGVHD among HLA-MRD among patients with hematologic malignancies.[14,15] These differences likely reflect heterogeneity in SAA patient

populations, conditioning regimens, and GVHD prophylaxis over five decades. Together, this highlights the need for disease-specific evaluation of hATG in contemporary transplantation. Importantly, considering several prior retrospective EBMT and CIBMTR studies suggest that rATG significantly reduced cGVHD risk, replacing rabbit ATG (rATG) for hATG may be warranted in this setting.[16-18]

Limitations of this study include confounding by era, limited power to detect modest effects in subgroup analyses, and the retrospective nature of the comparisons. Results may not generalize to rATG preparations, unrelated donor transplantation, PBSC grafts, or emerging GVHD-prevention strategies. Strengths include the size and depth of a large, well-characterized single-institution cohort spanning five decades.

In conclusion, in the modern era of leukocyte-depleted transfusions and combination GVHD prophylaxis, the incremental contribution of horse ATG to GVHD prevention in HLA-MRD for SAA appears limited. These observations underscore the importance of interpreting historical transplant interventions within the biologic and clinical context in which they were developed.

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Table 1. Patients and Treatment Regimens.

Characteristics	**Number (%)**
Number of patients	392
Median (range) age at transplant, years	21.4 (1.6 – 68.5)
Female	169 (43)
Race	
American Indian or Alaskan Native	9 (2)
Asian	16 (4)
African American	14 (4)
Native Hawaiian or Other Pacific Islander	7 (2)
White/Caucasian	314 (80)
Multiple	7 (2)
Unknown	30 (8)
Ethnicity	
Hispanic or Latino	26 (7)
Not Hispanic or Latino	353 (90)
Unknown	13 (3)
Decade of BMT	
1970-1979	155 (40)
1980-1989	117 (30)
1990-1999	46 (12)
2000-2009	31 (8)
2010-2019	32 (8)
2020-2024	11 (3)
Conditioning regimen	
CY	268 (68)
CY/ATG	124 (32)
GVHD prevention	

MTX or CSP	208 (53)
MTX/CSP	183 (47)
None	1 (<1)
Graft	
Marrow	228 (58)
Marrow plus buffy coat	156 (40)
Marrow plus PBSC	3 (1)
PBSC	4 (1)
HLA-identical cord blood	1 (<1)

Table 2. Probability of GVHD in BMT for Severe Aplastic Anemia, all patients.

	Grades II-IV, Acute GVHD	Grades III-IV, Acute GVHD	Chronic GVHD
Population	ATG/No ATG (122/268)	ATG/No ATG (122/268)	ATG/No ATG (124/268)
All patients	35%/28% (2 pts missing)	10%/18% (2 pts missing)	20%/30%

Note: Two patients had missing acute GVHD data; all patients were graded for chronic GVHD if they survived long enough.

Table 3. Sensitivity Analysis Restricted to CSP/MTX Recipients.

Parameter	**HR (95% Confidence Interval)**	**P-value**
Chronic GVHD, adjusted	0.77 [0.34-1.77]	P=0.54
Variable	**OR (95% Confidence Interval**)	
Grades II-IV Acute GVHD, adjusted	1.87 [0.55-6.33]	P=0.31
Grades III-IV Acute GVHD, adjusted	2.83* (0.64-12.49)	P=0.17

*16 events, so only adjusted for age and grader