A phase II multicenter rabbit anti-thymocyte globulin trial in patients with myelodysplastic syndromes identifying a novel model for response prediction

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ABSTRACT

Immune dysregulation is a mechanism contributing to ineffective hematopoiesis in a subset of myelodysplastic syndrome patients. We report the first US multicenter non-randomized, phase II trial examining the efficacy of rabbit(r)-anti-thymocyte globulin using 2.5 mg/kg/day administered daily for 4 doses. The primary end point was hematologic response; secondary end points included duration of response, time to response, time to progression, and tolerance. Nine (33%;95% confidence interval=17%-54%) of the 27 patients treated experienced durable hematologic improvement in an intent-to-treat analysis with a median time to response and median response duration of 75 and 245 days, respectively. While younger age is the most significant factor favoring equine(e)-anti-thymocyte globulin response, treatment outcome on this study was independent of age (P=0.499). A shorter duration between diagnosis and treatment showed a positive trend (P=0.18), but International Prognostic Scoring System score (P=0.150), karyotype (P=0.319), and age-adjusted bone marrow cellularity (P=0.369) were not associated with response classification. Since activated T-lymphocytes are the primary cellular target of anti-thymocyte globulin, a T-cell expression profiling was conducted in a cohort of 38 patients consisting of rabbit and equine-antithymocyte globulin-treated patients. A model containing disease duration, CD8 terminal memory T cells and T-cell proliferation-associated-antigen expression predicted response with the greatest accuracy using a leave-one-out cross validation approach. This profile categorized patients independent of other covariates, including treatment type and age using a leave-one-out-cross-validation approach (75.7%). Therefore, rabbit-anti-thymocyte globulin has hematologic remitting activity in myelodysplastic syndrome and a T-cell activation profile has potential utility classifying those who are more likely to respond (*NCT00466843 clinicaltrials.gov*).

Introduction

Myelodysplastic syndromes (MDS) are diseases of complex and varied biology manifested clinically by ineffective and dysplastic hematopoiesis. In a subset of patients, an immune mechanism has been implicated in disease pathobiology.[1,2] Historically, patients with suspected immune-mediated MDS and aplastic anemia (AA) have been treated with equine(e)anti-thymocyte globulin (e-ATG) with and without cyclosporine-A (CsA).[3-6] Combined treatment with e-ATG and etanercept[7,8] as well as alemtuzumab monotherapy[9] have shown activity in a subset of MDS patients with lower International Prognostic Scoring System (IPSS) risk and specific selection criteria including younger age, shorter duration of red cell transfusion requirements, and HLA-DR15 genotype.[1,10,11] In a series of 129 unselected MDS patients treated at the National Institutes of Health (NIH), responses to e-ATG plus CsA led to durable hematologic improvement (HI) in 30% of patients, with improvement in overall survival and progression-free survival in patients with low or intermediate IPSS risk disease compared to historical outcomes in the International MDS Risk Analysis Workshop (IMRAW) database.[1] Although a similar response rate of 29% was observed in a recent randomized phase III trial, that study failed to show an overall survival benefit or improvement in event-free survival in e-ATG+CsA-treated patients compared to best supportive care (BSC).[12] The latter finding may relate to the cross-over design allowing treatment of patients on BSC and the inadequate statistical power to assess survival advantage. Wide spread acceptance of immunosuppressive therapy (IST) has proven difficult owing to conflicting reports regarding efficacy and safety in unselected MDS patients, unfamiliarity of community hematologist with this modality and lack of cohesive criteria for patient selection.[13]

In addition to e-ATG, rabbit ATG(r-ATG) has also shown activity in patients with AA and in small cohorts of patients with MDS.[14-16] Although e-ATG is associated with superior response rates in AA.[17] Phase II studies in MDS have demonstrated similar rates of response in r-ATG-treated patients. Delineating the underlying pathophysiology in patients

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responsive to IST, development of cohesive selection criteria based on biological features, and optimizing treatment regimens are all key to adoption of this therapy for those MDS patients most likely to benefit. To this end, we performed a multicenter phase II trial to investigate the efficacy and safety of r-ATG in low or intermediate IPSS risk MDS patients, and analyzed pretreatment clinical variables associated with response. Combined data from this study and a second cohort of patients treated with e-ATG was analyzed to develop a cohesive biomarker-based model based on cytometry expression profiles to improve IST selection for MDS patients.

Methods

Patients and eligibility criteria

This was a multicenter phase II clinical study. Details of patient eligibility criteria are available in the *Online Supplementary Appendix*. This trial (BMF RDCRN 5406) received institutional review board approval at all participating sites, and was registered at *clinicaltrial.gov* (*NCT00466843*).

Drug administration

Patients were hospitalized to receive r-ATG (Thymoglobulin®, Genzyme Corp) at a dose of 2.5 mg/kg/day intravenously (IV) for 4 doses (total 10 mg/kg). The daily infusion was administered over at least six hours and slowed as necessary to minimize infusion-related symptoms, as detailed in the Online Supplementary Appendix. All patients were pre-medicated with prednisone (1 mg/kg/day orally) two days prior to the first dose and during the infusion, and then continued on a tapering schedule of prednisone for 14 days after the final r-ATG dose to prevent serum sickness. Antibiotic prophylaxis was administered according to individual institutional practices. Protocol modifications are available in the *Online Supplementary Appendix*.

Study procedures

Response evaluation was conducted at 16 weeks in Int-2 IPSS due to the possible risk of faster disease progression in unresponsive patients and 24 weeks in low/intermediate-1 IPSS risk patients. Patients were hospitalized for treatment and then followed on study weekly for the first month and then monthly until response evaluation. Base-line and on study complete blood counts with differential and chemistry were assessed. Blood product transfusions at baseline and on study were evaluated. Bone marrow aspirate and biopsy was repeated at response evaluation.

Hematologic response criteria and adverse events

The primary end point was best HI for at least eight consecutive weeks according to the International Working Group (IWG) 2000 response criteria.[18] Patients who achieved HI were followed every six months for up to two years and annually thereafter. Disease progression (DP) was defined as outlined in the *Online Supplementary Appendix*. Adverse events were graded according to the Common Toxicity Criteria of the National Cancer Institute (CTCAE version 3.0). Overall response, overall survival (OS), and progression-free survival (PFS) are defined in the *Online Supplementary Appendix*.

Study design of the r-ATG trial and statistical methods

A pre-defined accrual strategy was established for two cohorts based on IPSS classification. Specific information on the accrual goals and early stopping rules is available in the *Online Supplementary Appendix*. Clinical characteristics and adverse event data in patients treated with r-ATG were summarized using descriptive statistics, including mean, median, and range for continuous variables (e.g. age and duration of disease), and frequencies and percentages for categorical variables (e.g. sex and IPSS). Fisher's exact test was used to test any association of drug response and the discrete variables (e.g. IPSS). The Spearman method was used to estimate correlation between continuous variables. Univariate and multivariate analyses were performed using the Cox proportional hazards model. Kaplan Meier estimates for overall survival and log rank test were used for comparison. A detailed description of biomarker statistical analysis is provided in the *Online Supplementary Appendix*.

Table 1. Base-line-clinical characteristics of r-ATG-treated patients.

Characteristics	N. (%)	
Age (yr) (n=27)		
Median 65, mean 63.8, range 26-79		
Duration of disease from diagnosis to treatment initiation---mo. (n=24)		
Median 13, mean 29.8, range 1-89		
	Evaluable for Response (n=21)	Total (n=27)
Age ≤ 61 - yr	7 (33.3)	9 (33.3)
Age > 61 - yr	14 (66.7)	18 (66.7)
Sex		
Male	18 (85.7)	23 (85.2)
Female	3 (14.3)	4 (14.8)
IPSS		
Low	6 (28.6)	8 (29.6)
Int-1	12 (57.1)	15 (55.6)
Int-2	3 (14.3)	4 (14.8)
WHO		
RA	2 (9.5)	2 (7.4)
RCMD	8 (38.1)	8 (29.6)
MDS-U	5 (23.8)	5 (18.5)
RAEB1 and 2	5 (23.8)	5 (18.5)
MDS/MPN	1 (4.7)	1 (3.7)
Missing	0	6 (22.2)
Age-adjusted BM cellularity		
Hypocellular	6 (28.6)	9 (33.3)
Normal cellular	4 (19.0)	4 (14.8)
Hypercellular	11 (52.4)	14 (51.9)
Neutropenia (ANC ≤ 1,000/dL)		
Yes	8 (38.1)	11 (40.7)
No	13 (61.9)	16 (59.3)
Prior therapies		
None	8 (38)	8 (29.6)
At least one	10 (47.6)	14 (51.9)
Missing	3 (14.3)	5 (18.5)
5-azacytidine		
Yes		8 (29.6)
No		14 (51.9)
Missing		5 (18.5)
Lenalidomide/thalidomide		
Yes		9 (33.3)
No		13 (48.1)
Missing		5 (18.5)

IPSS: International Prognostic Scoring System: low, intermediate-1 (Int-1), and intermediate-2 (Int-2); WHO: World Health Organization including refractory anemia (RA), cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blast 1and 2 (RAEB1) and (RAEB2); MDS: unclassified (MDS-U); myeloproliferative neoplasm (MPN).*

Results

Patients' characteristics

Between April 2007 to March 2009, 27 patients were enrolled at three centers including H. Lee Moffitt Cancer Center, Tampa, FL (n=21), Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH (n=3) and UCLA Medical Center, Los Angeles, CA, USA (n=3). Patients' characteristics (n=27) are shown in Table 1 based on an intent-to-treat and for patients evaluable (n=21) for response. A detailed description of individual patients that contributed to the biomarker analysis is provided in *Online Supplementary Table S1*. Of the 27 total patients, the median age was 65 years (range 26 – 79 years) (Table 1) and nine (33%) were under 61 years. Twenty-three (85%) were male and 4 (15%) female. According to the IPSS, 8 (30%), 15 (55%) and 4 (15%) were classified as low, int-1, and int-2 risk categories, respectively.

Morphologic subtypes according to the World Health Organization (WHO) criteria included 2 (7%) with refractory anemia (RA), 8 (30%) patients had Refractory Cytopenia with Multilineage Dysplasia (RCMD), 5 (19%) MDS-unclassified, 5 (19%) had either refractory anemia with excess blasts-1 or -2 (RAEB) and 1 (4%) had a myelodysplastic/myeloproliferative neoplasm (MDS/MPN)-unclassified. WHO classification was unavailable on 6 (22%) patients.

Ten patients (37%) had abnormal cytogenetics by metaphase karotyping including trisomy 8 (n=2), del(20q) (n=4), del(5q) (n=2) unresponsive to lenalidomide, del(11)(q23) (n=1), and complex (n=2). A JAK2V617F mutation was detected by allele-specific PCR in a patient with an otherwise normal karyotype. Age-adjusted bone marrow cellularity was normal in biopsies from 4 (15%), hypercellular in 14 (52%), and hypocellular in 9 (33%) patients (Table 1).

Duration of disease was calculated in 24 patients and ranged from 1 month to 89 months with a median of 13 months (mean 29.8 months) (Table 1). Cytotoxic therapy had been administered in 2 patients for cancers that occurred three years or more prior to enrollment. Fourteen (52%) had failed prior MDS therapies including 8 (30%) who had had no response to 5-azacytidine and 9 (33%) who failed lenalidomide or thalidomide. Eight patients (30%) had had either no prior therapies or growth factors and the data were unavailable on 5 patients (19%).

Adverse events

Most adverse events were classified as grade 1 or 2 in severity (61 out of 70 events, 87%) with the most common being infusion-related fever, rigors, chills and myalgias (Table 2) on the first day of therapy. Infusion reactions with the first dose occurred in 11 patients (43%) requiring interruptions in infusions, and grade 3-4 hypotension and cardiac arrhythmias accompanied the infusion reaction in one patient. One patient developed debilitating motor neuropathy that required prolonged hospitalization related to serum sickness from premature steroid withdrawal. Three deaths occurred on study including one patient with a preexisting line infection and one patient who experienced a complete response at the time of the event. One patient died of pulmonary aspergillosis.

Hematologic response and pre-treatment clinical response co-variates in r-ATG-treated patients

Nine patients (33%, 95%CI: 17%-54%) out of 27 in an intent-to-treat analysis had HI to r-ATG (Table 3). Erythroid response (n=7) included 6 major and one minor response. The median time to achieve a response was 75 days (range 3-114 days) and the median duration of response was 245 days (range 112 to >667 days). Of the 10 patients with moderate-to-severe neutropenia (ANC median 0.275, range 0-0.8), 3 had a sustained increase in neutrophils (30%) for 158-245 days at the date of censor, and one patient continued to maintain the response at the last date of follow up. There were 7 patients with throm-

Table 2. Summary of adverse events on BMF RDCRN 5406.

r-ATG-treated patients	N. of adverse events Grade	Grade	Grade	N. (%)
Type of event	1 or 2	3 or 4	5	(N=70)
Fever/rigors/chills	23	0	0	23 (33)
Hypotension	4	1	0	5 (7.2)
Infection*	3	1	3	7 (8.7)
Myalgia and headaches	6	0	0	6 (8.7)
Pulmonary/upper respiratory	6	0	0	6 (8.7)
Gastrointestinal**	5	0	0	5 (7.2)
Cardiac arrhythmia	3	1	0	4 (5.8)
Neurological***	2	2	0	4 (5.8)
Metabolic/laboratory	3	0	0	3 (4.3)
Renal/genitourinary	3	0	0	3 (4.3)
Neutropenia	1	1	0	2 (2.8)
Hypertension	1	0	0	1 (1.4)
Puritis	1	0	0	1 (1.4)
	61	6	3	70

**Infection included infected central line (n=2) and aspirgillus pneumonia. One patient had prior history of line infection and received a partial course of therapy on Day 1 requiring discontinuation from the study. **Vomiting, diarrhea and nausea. ***Neurological included motor neuropathy, syncope, and fall.*

bocytopenia defined as a platelet count less than 100x10^9/μL and 5 with profound thrombocytopenia (platelets <50x10^9/μL). Of these 13 thrombocytopenic patients, 3 had a sustained improvement in platelets. Eighteen of 27 patients (66.7%) were non-responders and of these 6 (22%) were non-evaluable for response due to either study withdrawal (n=2), death related to infection (n=3), or study discontinuation due to infusion-related SAE (n=1). Twelve of the patients evaluated for response failed to have hematologic improvement at either 16 or 24 weeks among whom 7 had stable disease and 5 had disease progression.

Pre-treatment clinical variables were analyzed for association with hematologic improvement in the 21 patients who were evaluable for response (9 responders and 12 non-responders) (Table 1). Although younger age has emerged as a strong predictor for both survival and hematologic response to immunosuppressive therapy with e-ATG,[1,11] there was no age difference in responders and non-responders (P=1.0) using age as a continuous variable and using an age cut off of 61 years (Table 4). The median time to achieve a response in patients aged 61 years or under was 79 days (range 3-85) and 68 days (range 4-114) in patients over 61 years of age, which did not differ according to age category. Similarly, the duration of response did not differ according to age category (≤ 61 years: median 322, range 274-592; >61 years: median 202, range 112-667). The frequency of HLA-DR15 class II genotype had been previously shown to be higher in both MDS and AA patients19 compared to controls and significantly associated with e-ATG-response in MDS patients.[1,11] Only 4 patients treated with r-ATG had the HLA-DR15 allele (Table 4), but 3 of these patients achieved HI (75%). This difference was not statistically significant as a result of the small sample size. The time to achieve a response and the duration of response were similar in patients with and without the HLA-DR15 allele (*data not shown*). Of the 3 Int-2 category patients, 2 had a sustained improvement in platelets and neutrophil count for a median of 233.5 days (range 222-245 days), respectively, but subsequently relapsed and progressed to AML after a median of 509 (range 373-645) days. Low and Int-1 responsive patients experienced no leukemia progression during the follow-up period. Several additional pre-treatment variables failed to correlate with response in this study (Table 4): ANC, platelet count, hemoglobin, lymphocyte count, bone marrow blast percentage, age-adjusted bone marrow cellularity (hypocellular, normal cellular, and hypercellular), IPSS, and M:E ratio. PNH phenotype, which has shown variable association with e-ATG response,[20,21] was determined in fresh samples from 8 patients (4 responders and 4 non-responders) with no significant association with response.

Prolonged duration of RBC transfusion dependence[11] and a longer interval from diagnosis to treatment[12,14] have been correlated with ATG non-response. In r-ATG-responsive patients treated on this study, a shorter time from diagnosis to the initiation of treatment was observed compared to non-responsive patients (median for responders 8.8 months, range 3.3-45.8 months *vs.* median for non-responders 43.1 months, range 0.9-88.7 months; (P=0.074). This difference was not statistically significant although it may relate to sample size limitations. The presence of bone marrow fibrosis, as defined by the modified European fibrosis scale,[22] was negatively associated with response (0 of 5 responders among patients with fibrosis *vs.* 8 of 14 without fibrosis (57%); P=0.045) as was treatment with more than 2 prior therapies (response among patients receiving <2 prior therapies was 8 of 10 (80%) *vs.* 0 of 8 in patients who received ≥2 prior therapies; P=0.001) (Table 4). Failure to respond or treatment with individual drugs including 5-azacitidine or lenalidomide/thalidomide showed no association with r-ATG response (*data not shown*).

Table 3. Best clinical response to r-ATG.

	N. (total)	% (95%CI)
All responses - intent to treat	9 (27)	33.3 (17-54)
HI-E[#]	7 (18)	38.9
HI-E, major	6	
HI-E, minor	1	
HI-N, major%	3 (10)	30.0
HI-P, major[§]	3 (13)	23.0
No response - intent to treat	18 (27)	66.7 (46-83)
Not evaluated for response (n=6)	6 (27)	22.0
Adverse event	4 (27)	14.8
Death*	3	
SAE	1	
Withdrew	2 (27)	7.4
No response - evaluated at 16 or 24 weeks (n=12)		
Stable disease	7 (27)	25.9
Disease progression	5 (27)	18.5

[#]*Hematologic improvement-erythroid, major or minor (HI-E), total number with anemia; %hematologic improvement-neutrophil per number of patients with neutropenia; defined by an absolute neutrophil count (ANC) < 1000 cell/μL;* [§]*hematologic improvement-platelets per number of patients with thrombocytopenia; defined as platelet counts <100 X 10^9/μL; *death due to infection definitely not related occurred prior to completion of infusion (n=1), and death due to infection possibly related (n=2).*

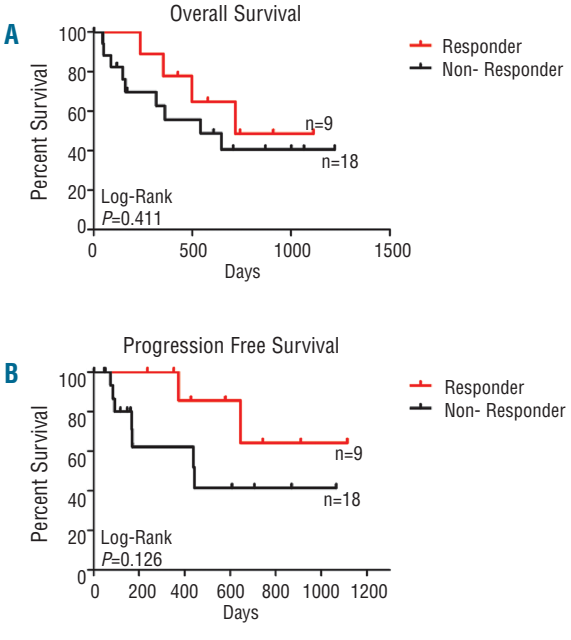

Figure 1. Overall survival (OS) (A) and progression free survival (PFS) (B) after r-ATG therapy. Kaplan-Meier curves are shown for responders (red, n=9) and non-responders (black, n=18) to r-ATG in an intent-to-treat analysis.

Table 4. Pre-treatment clinical variables associated with r-ATG response.

	Response	Min.	Median	Mean (95%CI)	Max.	P**
Age (years)	R (n=9)	54	65	65.2 (60.4, 70.1)	74	
	NR (n=12)	44	66	63.2 (57.7, 68.6)	79	0.499
ANC (k/L)	R (n=9)	0.13	2.3	2.19 (0.8, 3.6)	7.12	
	NR =12)	0	1.70	1.70 (1.0, 2.4)	4.37	0.702
Plts (k/L)	R (n=9)	14	89	106.6 (55.5, 157.6)	228	
	NR (n=12)	16	148.5	199.2 (98.2, 300.3)	551	0.355
Hgb (g/dL)	R (n=9)	7.7	10.2	9.8 (8.9, 10.7)	11.5	
	NR (n=12)	6.6	9.1	9 (8.5, 9.5)	10.5	0.176
Lymphocytes (k/L)	R (n=9)	0.37	1.26	1.30 (0.9, 1.7)	2.47	
	NR (n=12)	0	0.84	0.98 (0.6, 1.4)	2.27	0.303
Blast %	R (n=9)	0.8	2	3.2 (0.9, 5.5)	12	
	NR (n=12)	0	2.4	4.9 (1.5, 8.3)	18	0.774
Duration of disease (months)	R (n=9)	3.8	8.8	14.6 (4.4, 24.9)	46.2	
	NR (n=11)†	1.4	43.1	41.6 (19.2, 64.0)	89.1	0.074
	Variables	**N**	**Responders**	**(%)**		**P*****
Age (years) (n=21)	≤ 61	7	3	(42.8)		
	> 61	14	6	(42.8)		1.00
Age-adjusted BM Cellularity (n=21)	Hypo	6	1	(16.7)		
	Normal	4	2	(50.0)		
	Hyper	11	6	(54.5)		0.369
HLA-DR15 (n=21)	Positive	4	3	(75.0)		
	Negative	17	6	(35.3)		0.272
Karotype (n=20)	Normal	15	8	(53.3)		
	Abnormal	5	1	(20.0)		0.319
IPSS* (n=21)	Low	6	4	(66.7)		
	Int-1	12	3	(25.0)		
	Int-2	3	2	(66.7)		0.150
Number of prior Treatments (n=18)	≤ 2	10	8	(80.0)		
	> 2	8	0	(0.0)		0.001
Fibrosis (n=19)	No	14	8	(57.1)		
	Yes	5	0	(0.0)		0.045

IPSS: International Prognostic Scoring including low, intermediate-1 (Int-1), and intermediate-2 (Int-2); Tampa, FL.BM: bone marrow; ANC: absolute neutrophil count; Plt: platelet count; Hgb: hemoglobin. **Wilcoxon rank-sum test. ***Fisher's exact test. †Data was unavailable for one patient.*

Time-to-event analysis

The median duration of follow up for all patients was 520 days (range 2-1221 days). There were 4 deaths among responders and 9 among non-responders. Median OS in responders was 718 days *versus* 541 days in non-responders (hazard ratio (HR) 0.6133, 95%CI: 0.2036-1.848; *P*=0.411) (Figure 1A). Evolution to AML was observed in 2 responders and 7 non-responders. Median PFS in responders was unreached *versus* 438 days in non-responders (HR 0.2989, 95%CI: 0.0.07-1.127; *P*=0.126) (Figure 1B). Responding patients who progressed were classified as Int-2 by IPSS criteria at the time of treatment indicating that r-ATG is not disease modifying in this group of patients. Of the 5 non-responders who progressed, 4 were classified as Int-1 and one as low risk by IPSS, with progression occurring within four months. Four of the 5 had been heavily pre-treated with multiple therapies including 5-azaditicine, 2 had an abnormal karotype including del5q/del(20) and del(20)/trisomy 8, 4 had trilineage dysplasia and 3 had RAEB by WHO criteria. Bone marrow fibrosis was moderate to severe in 4 of 6 patients with disease progression.

Immunological profile classification in r-ATG and e-ATG-treated patients

Absence of an age association in this study prompted us to examine alternative independent biomarkers associated with HI. To determine if a biomarker profile was associated with response independent of the type of ATG, data were combined with that of 21 patients treated with e-ATG of which 7 patients had a durable hematologic response according to IWG 2000 criteria. Fibrosis and prior treatment data were unavailable on the e-ATG cohort. Comparing the base-line characteristics of e-ATG and r-ATG-treated patients (*Online Supplementary Table S1*), there was no difference in duration of disease or the frequency of HLA-DR15 allele among these 2 cohorts (e-ATG, 9 of 21 (43%) *vs.* r-ATG 4 of 17 (24%); *P*=0.173). However, e-ATG-treated patients were significantly younger in age (mean age 56 and median 60 years old in e-ATG group *vs.* mean 63 and median 65 years old in r-ATG group; *P*=0.017) (*Online Supplementary Table S1*). Immunophenotypic flow cytometry biomarkers studied were reported previously.[23] On the combined cohort, median or less disease duration (combined cohort median

Table 5. Binary logistical regression for univariate analysis of ATG Response.

Variables	Coefficient	P	OR+	95%CI for Exp(B) lower	upper
Age	-0.06	0.06	0.941	0.88	1.00
HLA-DR15+	0.67	0.34	1.94	0.50	7.64
[a]Disease duration (Yes/No ≤ Median)	1.48	0.04	4.40	1.09	17.72
CD4%	-0.04	0.07	0.96	0.92	1.00
CD4 Naïve%	-0.02	0.51	0.98	0.93	1.04
CD8%	0.33	0.14	1.03	0.99	1.08
CD8 Naïve%	0.02	0.69	1.02	0.94	1.11
CD8 TM%	0.06	0.02	1.06	1.00	1.12
total CD4 Ki67%	0.48	0.01	1.61	1.12	2.31
CD4 Naïve Ki67%	0.10	0.16	1.11	0.96	1.27
total CD8 Ki67%	0.41	0.03	1.51	1.05	2.18
CD4/CD8 ratio	-0.57	0.04	0.57	0.33	0.97
Drug type (rATG *vs.* eATG)	0.74	0.28	2.09	0.56	7.85

[a]Median of combined cohort is 23 months. CI: confidence interval; OR: odds ratio; TM: terminal memory; rATG: rabbit anti-thymocyte globulin; eATG: equine anti-thymocyte globulin.

Table 6. Multivariate analysis for models of response.

Variables	Coefficient	P	OR+	95%CI for OR+ lower	upper	Accuracy rate LOOCV**
Multivariable logistical regression five variables						
Age	-0.09	0.18	0.92	0.81	1.04	76%
Drug type (eATG *vs.* rATG)	1.26	0.33	3.52	0.28	44.63	
[a]Disease duration (≤ median)	2.77	0.04	15.89	1.10	230.50	
CD8 TM%	0.09	0.05	1.09	1.00	1.19	
Total CD4 Ki67%	0.71	0.02	2.04	1.14	3.63	
Logistical regression using age and disease duration						
Age	-0.07	0.05	0.93	0.87	1.00	59.9%
Disease duration (≤ median)	1.71	0.03	5.51	1.16	26.13	

*[a]Median of combined cohort is 23 months; CI: confidence interval; +OR: odds Ratio; TM: terminal memory; LOOCV**: leave-one-out cross-validation; TM: terminal memory; rATG: rabbit anti-thymocyte globulin, eATG: equine anti-thymocyte globulin.*

23 months; *P*=0.04, OR 4.40), CD8TM% (*P*=0.02, OR 1.06) as a continuous variable, total CD4 Ki67% (*P*=0.01, OR 1.61) as a continuous variable, total CD8 Ki67% (*P*=0.03, OR 1.51) as a continuous variable, and CD4/CD8 ratio (*P*=0.04, OR 0.57) as a continuous variable were significantly associated with HI using univariate analyses (Table 5). Both age and disease duration (<median of 23 months) were included in a multivariate model with each of the immune parameters significantly associated with response in univariate analyses (*Online Supplementary Table S2*) to identify a novel profile with better accuracy for response classification. Of these immunological variables, CD8TM% (*P*=0.05, OR 1.09) and total CD4 Ki67% (*P*=0.02, OR 2.04) were significant classifiers independent of both age and disease duration. CD4/CD8 ratio (*P*=0.1) and %CD8 Ki67$^+$ T cells (*P*=0.19) were not independent of age and disease duration in multivariable analyses so they were dropped from the final model (*Online Supplementary Table S2*). Using this multi-parameter classification, shorter disease duration (0.04, OR 15.89) and the immune profile (CD8 TM%, *P*=0.05 and CD4 Ki67%, 0.02) added value and were independent of age (*P*=0.18) and drug treatment type (eATG *vs.* rATG; *P*=0.33). Accuracy was tested using a LOOCV approach (Table 6), as described previously.[24-26] Comparing the overall predictive accuracy of the multi-parameter biomarker model to age and disease duration by LOOCV, the final accuracy rate was 75.7% *versus* 59.5%, respectively. These results uniquely identify a signature that independently refines response estimates for ATG patient selection independent of drug treatment type and age (i.e. r-ATG or e-ATG).

Discussion

This prospective multicenter study shows that r-ATG has remitting activity in MDS comparable to that reported historically for e-ATG + cyclosporine.[1] Infusion-related side-effects were manageable by pre-medication with corticosteroids and diphenhydramine before each treatment dose. HI was achieved in 33% of patients including erythroid, neutrophil and platelet responses in patients across all IPSS risk categories. Hematopoietic improvement in some Int-2 risk patients is consistent with previous data[9] suggesting that immune deregulation can impact hematopoietic production in patients beyond the low and Int-1 stage of disease. However, disease progression to AML in the Int-2-responsive patients after initial hematologic improvement indicates that IST is not disease-modifying in this patient population, and that r-ATG should

only be used selectively in lower-risk (low or Int-1) MDS patients. IST is a reasonable treatment alternative for patients with MDS, with response rates that are comparable to other agents such as erythroid stimulating agents, azanucleosides, and lenalidomide in non-del(5q) MDS. The advantages of IST include the need generally for only a single treatment course and the durable duration of response when achieved.

IST was initially applied in patients with hypoplastic MDS based upon presumed overlapping disease pathobiology with aplastic anemia.[3] Both agents are presumed to deplete hematopoietic suppressive effector T-cell populations. In a study of 35 MDS patients randomized to receive e-ATG (15 mg/kg/d for 5 days) or r-ATG (3.75 mg/kg/d for 5 days),[14] there was no difference in the observed response rates between these 2 treated groups. The hematologic improvement rate of 33% in this study is similar to reported rates of response to e-ATG treatment in unselected patients.

Patient selection criteria for e-ATG are currently based on a model from the NIH incorporating age, duration of transfusion dependence, and HLA-DR15. The effect of age has been the most important independent predictor of response to e-ATG with a lower probability of hematologic response in patients over the age of 60 years. When patients were stratified into younger (<61 years) and older (≥61 years) age groups in this study, there was no difference in the response rate (42.8% in both groups; P=1.0). In addition to age, hematologic improvement with e-ATG treatment in prior studies concluded that the HLA-DR15 class II genotype was also an independent covariate for response. Consistent with this finding, 3 of the 4 HLA-DR15 positive patients in this study responded to r-ATG.[11] Including our study, a shorter interval between diagnosis and initiation of therapy has been associated with probability of response in 4 independent studies of patients treated with anti-lymphocyte serotherapy.[11,12,14] Hematologic response rates, however, have shown an inconsistent relationship with bone marrow cellularity, PNH, and WHO classification.[5,21,27,28]

In an effort to define a T-cell profile associated with ATG response independent of age or other covariates, we analyzed biomarkers in a mixed cohort of patients in this study. Several factors were significantly associated with hematologic improvement in univariate analyses. In a multivariate model, shorter disease duration, having a higher CD8TM% and a higher $CD4^+$ T-cell proliferative index ($Ki67^+$) independently discriminated response after adjusting for treatment type (e-ATG *vs.* r-ATG), and age. Longer duration of disease, number of prior therapies, and the presence of bone marrow fibrosis adversely affected response in r-ATG-treated patients. These may all jointly reflect a longer duration of immune-mediated bone marrow injury allowing for selection of clonal autonomy with an immune-independent mechanism of clonal expansion. The precise mechanism underlying T-cell and immune deregulation is unknown, however, we previously reported a primary defect in telomerase function in naïve T cells of MDS patients.[29] Failure to repair telomeres in rapidly expanding cells leads to premature growth arrest, apoptosis, cell exhaustion in stem cells and in lymphocytes, and T-cell repertoire alterations. These changes enhance the risk for autoimmune reactivity.[30-32] Early MDS is characterized by an apoptotic phenotype in the bone marrow with evidence of accelerated telomere shortening in myeloid progenitors and the stem cell compartment.[33] In early MDS, altered T-cell populations may directly suppress hematopoiesis since both $CD8^+$ and $CD4^+$ T cells have the capacity to damage bone marrow by cell-cell mediated interactions, by the Fas/Fas receptor apoptotic pathway, or release of inhibitory cytokines including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) or transforming growth factor-β (TGF-β).[34] The presence of autoreactive or damaging effector T cells are a stimulus for highly suppressive regulatory populations such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs).[35] Recent evidence shows that MDSCs contribute to the dysplastic phenotype[36] and Tregs are a risk factor for disease progression[37,38] in MDS. Repertoire contraction, reduced CD4/CD8 ratio, and high lymphocyte proliferative index were previously reported to be present in e-ATG-responsive patients and to improve after treatment suggesting that ATG may indeed restore the T-cell compartment.[23] In the case of lymphoid ablation with ATG, removal of destructive T cells may reduce the stimulus for suppressor cells, which 'resets' immune homeostasis in the bone marrow. Collectively, this model points to altered T-cell dynamics and a shorter disease duration as indicators of ATG-response in MDS patients.

Although the final model requires prospective validation, this study indicates that T-cell immunoprofiling may be a useful tool to guide MDS patient selection for IST therapy and improve upon age as selection criteria. Furthermore, these results indicate that patients with an immune mechanism should receive T-cell depleting agents early after disease initiation. Benefit from the therapy may be limited after receiving multiple treatments for MDS, development of fibrosis, worsening stem cell depletion or through clonal evolution that are time-dependent processes. Recognition that the dysplastic phenotype, which characterizes MDS, may arise from diverse biological processes, including an immune mechanism, strengthens the need for conducting prospective clinical trials based on biomarker-assigned therapy.

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