SUPPLEMENTARY APPENDIX

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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Supplemental Table 1. Detailed patient characteristics of r-ATG and e-ATG-treated patients.

Treatment Type	Local.ID	Best.Response Intent-to-treat	*R.Status	Sex	Age at TX	#IPSS	Karyotype	DR15	**Samples	Dur (mon)
(r)ATG	MCC-06-001	HI-E major	R	M	61	Low	N	Pos	Yes	6.5
(r)ATG	MCC-06-003	SD	NR	M	69	Int-1	N	Neg	Yes	89.1
(r)ATG	MCC-06-004	SD	NR	M	67	Int-2	N	Pos	Yes	12
(r)ATG	MCC-06-006	TLR	R	F	71	Int-2	N	Neg	Yes	8.5
(r)ATG	MCC-06-007	SD	NR	M	70	Int-1	AB	Neg	Yes	80.4
(r)ATG	MCC-06-010	HI-P	R	M	64	Int-1	N	Pos	Yes	46.2
(r)ATG	MCC-06-012	DP	NR	M	68	Int-1	AB	Neg	Yes	1.4
(r)ATG	MCC-06-015	DP	NR	M	79	Int-1	AB	Neg	Yes	73.1
(r)ATG	MCC-06-017	HI-P, HI-N	R	M	65	Int-2	N	Neg	Yes	15.3
(r)ATG	MCC-06-018	HI-E, minor	R	M	72	Low	N	Neg	Yes	8.8
(r)ATG	MCC-06-020	DP	NR	M	69	Low	N	Neg	Yes	43.6
(r)ATG	MCC-06-021	SD	NR	M	61	Low	N	Neg	Yes	43.1
(r)ATG	MCC-06-024	HI-E major	R	M	74	Int-1	N	Neg	Yes	11.3
(r)ATG	MCC-06-026	DP	NR	F	53	Int-1	N	Neg	Yes	4.2
(r)ATG	MCC-06-027	HI-E, major	R	M	54	Low	AB	Neg	Yes	3.7
(r)ATG	CCF-06-001	HI-N, major	R	M	71	Int-1	N	Pos	Yes	6.9
(r)ATG	CCF-06-002	HI-E, major	R	M	55	Low	N	Neg	Yes	24.3
Summary (r)ATG=rabbit anti-thymocyte globulin-treated subset for biomarker study (n=17)			**9=R 8=NR**	**2=F 15=M**	**mean=66 median=68**	**6=low 8=Int1 3=Int2**	**4=AB 13=N**	**4=Pos 13=Neg**	**17=Yes 0=No**	**mean=28 median=12**
(e)ATG	eATG1	**N/A**	R	ND	65	ND	AB	Pos	Yes	3.4
(e)ATG	eATG2	**N/A**	NR	ND	66	ND	AB	Neg	Yes	30.5
(e)ATG	eATG3	**N/A**	R	ND	46	ND	N	Pos	Yes	7.9
(e)ATG	eATG4	**N/A**	R	ND	55	ND	N	Neg	Yes	194.3
(e)ATG	eATG5	**N/A**	R	ND	41	ND	ND	Pos	Yes	12.1
(e)ATG	eATG6	**N/A**	R	ND	19	ND	N	Neg	Yes	12.5
(e)ATG	eATG7	**N/A**	R	ND	41	ND	N	Neg	Yes	39.3
(e)ATG	eATG8	**N/A**	R	ND	37	ND	AB	Pos	Yes	25.4
(e)ATG	eATG9	**N/A**	NR	ND	66	ND	AB	Neg	Yes	98.1
(e)ATG	eATG10	**N/A**	NR	ND	56	ND	N	Neg	Yes	50.6
(e)ATG	eATG11	**N/A**	NR	ND	58	ND	AB	Neg	Yes	5.6
(e)ATG	eATG12	**N/A**	NR	ND	57	ND	N	Pos	Yes	7.6
(e)ATG	eATG13	**N/A**	NR	ND	36	ND	AB	Neg	Yes	61.1
(e)ATG	eATG14	**N/A**	NR	ND	69	ND	AB	Neg	Yes	3.3
(e)ATG	eATG15	**N/A**	NR	ND	67	ND	AB	Pos	Yes	22.7
(e)ATG	eATG16	**N/A**	NR	ND	72	ND	AB	Pos	Yes	14.5
(e)ATG	eATG17	**N/A**	NR	ND	70	ND	N	Neg	Yes	23.9
(e)ATG	eATG18	**N/A**	NR	ND	65	ND	AB	Neg	Yes	25.6
(e)ATG	eATG19	**N/A**	NR	ND	70	ND	AB	Pos	Yes	88.8
(e)ATG	eATG20	**N/A**	NR	ND	60	ND	N	Pos	Yes	86.5
(e)ATG	eATG21	**N/A**	NR	ND	66	ND	AB	Pos	Yes	ND
Summary (e)ATG=analysis of samples from patients treated with equine anti-thymocyte globulin at the NIH (n=21)			**7=R 14=NR**	**no data**	**mean 56*** median 60**	**no data**	**12=AB 8=N 1=ND**	**10=Pos 11=Neg**	**21=Yes 0=No**	**mean=41 median=25 1=ND**

*Response status analyzed for clinical co-variate R=responder, NR=non-responder

#International Prognostic Scoring System (IPSS): Int-1, Intermediate-1, Int-2, Intermediate-2

**Samples: Biological material available for analysis from this subset of patients prior to treatment initiation

Patients without sample collection included those that withdrew due to failure to complete the study (n=6), loss during shipping (n=1), no sample drawn prior to treatment (n=2), sample lost (n=1)

***p<0.05

N/A, not applicable because samples were obtained retrospectively.

ND, no data available

WD, withdrew due to adverse event, death on study, or patient request

F/M= female/male

A/N/ND=abnormal/normal/no data

Pos/Neg=positive/negative

Sample size: r-ATG, n=17 with samples, n=27 total intent-to-treat,n=21 with response information

Sample size: e-ATG, n=21 retrospective samples

Supplemental Table 2. Binary Logistic Regression for Immune Predictors of ATG Response independent of age and disease duration using significant variables from univariate analysis.

Variables	β	*p*	OR[+]	95% CI for OR	
				lower	upper
Age	-0.11	***0.02***	0.90	0.82	0.98
Disease Duration (Yes/No < Median)	1.58	***0.06***	4.84	0.93	25.27
Drug Type (rATG versus eATG)	1.79	***0.06***	5.98	0.87	40.37
Age	-0.06	*0.08*	0.94	0.88	1.01
Disease Duration (Yes/No < Median)	1.67	***0.05***	5.30	0.99	28.27
CD8 TM%	0.06	*0.06*	1.06	1.00	1.13
Age	-0.07	*0.16*	0.93	0.85	1.03
Disease Duration (Yes/No < Median)	2.80	***0.02***	16.45	1.58	171.36
Total CD4 Ki67%	0.62	***0.01***	1.86	1.14	3.05
Age	-0.05	*0.22*	0.95	0.88	1.03
Disease Duration (Yes/No < Median)	1.66	***0.05***	5.23	1.04	26.41
Total CD8 Ki67%	0.30	*0.19*	1.34	0.86	2.10
Age	-0.07	*0.06*	0.93	0.86	1.00
Disease Duration (Yes/No < Median)	1.56	*0.06*	4.78	0.93	24.57
CD4/CD8	-0.49	*0.10*	0.61	0.34	1.10

CI=confidence interval, OR=odds ratio; rATG=rabbit anti-thymocyte globulin, eATG=equine anti-thymocyte globulin.

Supplemental Methods

Data Collection. Data collected included baseline demographics, disease baseline characteristics, MDS classification, prior MDS treatment, HLA-DR15 , IPSS risk was calculated incorporating the percentage of myeloblasts in bone marrow, karyotype, and the number of cytopenias, was used for pre-treatment risk stratification(18). In addition, the bone marrow aspirate and biopsy were assessed for cellularity and fibrosis which was assessed in core biopsies stained for reticulin and graded by the modified European consensus scale as mild/none (score 0/1), moderate (score 2) or severe (score 3)(19). All patients had bone marrow samples reviewed and diagnosis confirmed at the participating institutions. Peripheral blood samples for biomarker analysis were collected at least four weeks prior to initiation of therapy.

Detailed eligibility criteria. Patients were ≥18 years of age with a pathologic diagnosis of MDS and low, intermediate-1 (Int-1), or Int-2 risk disease by IPSS were eligible (18). All patients provided written informed consent. Patients had either symptomatic anemia with an untransfused hemoglobin <9 g/dL, anemia requiring RBC transfusion, platelets <50 x 1,000/μL, or neutropenia (defined by an absolute neutrophil count (ANC) <1.0 x 1,000/μL). Exclusion criteria included therapy-related MDS, history of cancer within <3 years, prior immunotherapy for malignant or autoimmune diseases, prior anti-lymphocyte serotherapy, chronic myelomonocytic leukemia (CMML) with leukocyte count ≥12 x 1,000/μL), and high risk MDS based on IPSS.

Reduction in infusion rate for adverse events. The infusion rate was decreased to 50% for fever, chills, hypotension, dysrhythmia or itching with a maximum infusion length of 24 hours.

Detailed pre-defined accrual strategy and additional stopping rules. The Simon two-stage design was used to calculate sample size based on 80% power and 5% type I

error based on two groups having an IPSS score of low/intermediate-1 (Int-1) or Int-2 at baseline. An underlying objective response rate of 25% was deemed sufficient to warrant further investigation in future studies, whereas a 5% true response rate would have indicated a relative lack of efficacy. With the assumptions, 13 participants were initially accrued in stage 1 and extended to 25 evaluable patients in total. At least 1 response was required in the 1st stage for continuation to stage 2. Adjusting for an expected 5% loss to follow-up, 14 subjects were accrued to stage 1 and 13 subjects were to be accrued to stage 2. Responses were observed in each cohort (Int-1 and Int-2) allowing for the expansion phase, but the Int-2 cohort was closed due to slow accrual. An additional early stopping rule was included for excess toxicity if 3 or more out of the first 15 (20%) experienced a SAEs with documented infection deemed treatment-related and or deaths.

Protocol modifications. The protocol was modified to include a chest X-ray at the screening visit to exclude occult pulmonary infection. Subjects with evidence of infection/infiltrate suspicious for active infection were ineligible until radiographic documentation of resolution.

Overall response, overall survival (OS), and progression free survival (PFS). The duration of overall response was determined from the time that hematologic response criteria were met until the first date that recurrent or progressive disease was objectively documented. Overall survival (OS) and progression free survival (PFS) were evaluated from the time of trial registration until either death or leukemia transformation, respectively. Patients were censored at the time of study withdrawal or event, where appropriate and analyses were performed using an intent-to-treat basis.

Sample collection and biomarker analysis. Pre-treatment peripheral blood samples on r-ATG-treated patients were collected on 17 of the 21 patients, as

described in detail in **supplemental Table 1**. Cryopreserved cells were stored in liquid nitrogen and analyzed at the end of the trial using 7-color flow cytometry, as described previously(20). Frozen samples from 21 patients treated with e-ATG at the National Institutes of Health (NIH) or Cleveland Clinic were similarly analyzed. A description of the T-cell flow cytometry profiling methods is provided in supplemental material. Information about the e-ATG-treated patients is provided in **Supplemental Table 1**.

Briefly, CD4 and CD8 T-cell immune profiles were detected after surface staining with anti-CD3-phycoerythrin (PE) Cy7, anti-CD45RA-FITC, anti-CD62L-APC and either anti-CD4− or CD8-APC Cy7 (BD Biosciences, San Jose, CA USA). The percentage of CD4+ and CD8+ cells in the CD3 gate was used to calculate the CD4/CD8 ratio. Naive and memory CD4 and CD8 T-cell populations were defined by CD45RA and CD62L expression as follows: naïve CD45RA+/CD62L+, central memory CD45RA-/CD62L+, effector memory CD45RA-/CD62L-, and terminal effector memory CD45RA+/CD62L-.[7,8] A viability stain, 4′,6-diamindigo-Z-phenylinodole (DAPI) was used and results were analyzed on a LSRII Benchtop analyzer (BD Bioscience). Ki67 staining was performed on permeabilized cells using BD perm kit and the percentage of proliferating cells examined on naïve and memory as well as total CD3+/CD4+ and CD3+/CD8+ T-cells(20). Exemplary primary dot plot data have been published previously. High resolution PNH testing was performed on freshly isolated blood samples by flow cytometry using liquid fluorescently labeled inactive toxin aerolysin (FLAER), as previously described, to quantify glycophosphatidylinositol (GPI)-anchor proteins(33). A GPI-AP deficient (ie, PNH) phenotype was defined as FLAER-negative granulocytes ≥ 0.003% and FLAER-RBCs ≥ 0.005%(34).

Definition of Disease Progression. Disease progression was defined by a ≥ 50% increase in myeloblasts, depending on baseline myeloblast percentage. For patients with <5% myeloblasts: an increase to ≥10% myeloblasts, or patients with 5% to 10%

myeloblasts: ≥50% increase to >10% myeloblasts, and for patients with 10% to 20% myeloblasts: ≥50% increase to >20% myeloblasts. AML transformation was defined as ≥ 20% myeloblasts in the bone marrow or peripheral blood.

Detailed description of the statistical methods for biomarker analysis. T-cell profiling was examined using continuous data on a subset of patients with peripheral blood samples collected prior to treatment initiation. For biomarker studies, 17 r-ATG-treated patients contributed to this analysis. Data was also used from 21 patients treated with e-ATG at the National Heart, Lung, and Blood Institute (NHLBI). Response in this cohort has been published previously(1). The accuracy rate of the final multivariable model was determined by the "leave-one-out" cross validation (LOOCV) method. This approach used one patient as the validation data and the remaining patients as the training data each time and repeated the process from the 1st patient to the last patient. At each process, the training data were used to build a multivariate model which was then used to predict response status of the patient from the validation data, as reported previously(22-24). All tests were two-sided, with a *p-value* <0.05 determining significance.