A confirmation of chronic graft-*versus*-host disease prediction using allogeneic HY antibodies following sex-mismatched hematopoietic cell transplantation

Jed Paul,[1] Hideki Nakasone,[2] Bita Sahaf,[1] Fang Wu,[1] Kathy Wang,[3] Vincent Ho,[3] Juan Wu,[4] Haesook Kim,[3] Bruce Blazar,[5] Jerome Ritz,[3] Alan Howard,[6] Corey Cutler,[3] and David Miklos[1]

[1]Division of Blood and Marrow Transplantation, Stanford University School of Medicine, Stanford, CA, USA; [2]Division of Hematology, Saitama Medical Center, Jichi Medicial University, Saitama, Japan; [3]Division of Hematological Malignancies, Dana-Farber Cancer Institute, Boston, MA, USA; [4]The Emmes Corporation, Rockville, MD, USA; [5]Division of Pediatrics BMT, Minneapolis, MN, USA and [6]Center for International Blood and Marrow Transplant Research, Minneapolis Campus, Minneapolis, MN, USA

Correspondence: DAVID B. MIKLOS.
dmiklos@stanford.edu
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SUPPLEMENTARY MATERIALS:

A confirmation of chronic graft-versus-host disease prediction using allogeneic HY antibodies following sex-mismatched hematopoietic cell transplantation

Jed Paul[a], Hideki Nakasone[b], Bita Sahaf[a], Fang Wu[a], Kathy Wang[c], Vincent Ho[c], Juan Wu[d], Haesook Kim[c], Bruce Blazar[e], Jerome Ritz[c], Alan Howard[f], Corey Cutler[c], David Miklos[a]

[a]Division of Blood and Marrow Transplantation, Stanford University School of Medicine, 269 West Campus Dr., CCSR #2205, Stanford, CA 94305
[b]Division of Hematology, Saitama Medical Center, Jichi Medicial University, 1-847 Amanuma-cho Omiya-ku, Saitama, 330-8503, Japan
[c]Division of Hematological Malignancies, Dana-Farber Cancer Institute, 44 Binney Street, Mayer 530, Boston, MA 02115
[d]The Emmes Corporation, 401 North Washington Street, Suite 700, Rockville, MD, USA
[e]Division of Pediatrics BMT, MMC 336 Mayo 8366A, 420 Delaware St SE, Minneapolis, MN 55455
[f]Center for International Blood and Marrow Transplant Research, Minneapolis Campus, 500 N 5th Street, Minneapolis, MN 55401-1206

Author emails:
Jed Paul: jedpaul@stanford.edu, Hideki Nakasone: nakasone-tky@umin.ac.jp, Bita Sahaf: bsahaf@stanford.edu, Fang Wu: fangw@stanford.edu, Kathy Wang: kathy_wang@dfci.harvard.edu, Vincent Ho: Vincent_Ho@dfci.harvard.edu, Juan Wu: jwu@emmes.com, Haesook Kim: htkimc@jimmy.harvard.edu, Bruce R Blazar: blaza001@umn.edu, Jerome Ritz: jerome_ritz@dfci.harvard.edu, Alan Howard: ahoward@nmdp.org, Corey Cutler: cscutler@partners.org, David Miklos: dmiklos@stanford.edu

Corresponding Author:
David B. Miklos, MD, PhD
Division of Blood and Marrow Transplantation, Stanford University School of Medicine,
269 West Campus Dr., CCSR #2205, Stanford, CA, 94305
E-mail: dmiklos@stanford.edu
TEL: 650-725-4959, FAX: 650-724-6182

Methods

1.1. Patients and blood samples

We quantified HY antibody levels for 79 BMT CTN subjects (44 from BMT CTN protocol 0201 and 35 from BMT CTN protocol 0402) and 155 DFCI F→M HCT patients who underwent transplant between 2004 and 2014. Samples from the BMT CTN 0402 study were collected as sera, while samples from the DFCI and BMT CTN 0201 studies were collected as EDTA plasma. Samples from both sites were collected at the 3-month and 1-year post-transplant time points. Of the 234 total patients with 3-month samples, 185 also had 1-year samples. To set HY antibody seropositivity thresholds, a control group of 60 paired sera and EDTA plasma samples that were collected from healthy male blood donors was obtained from the NHLBI's BioLINCC repository (REDS II-LAPS study), as healthy males are not expected to have "self" HY antibodies[1]. cGVHD severity for each patient from the BMT CTN studies was obtained from center-reported assessments, all of which were performed in prospective randomized studies by experienced BMT CTN centers. HCT patient and donor characteristics are reported in Table 1. Approval for this study was obtained from the Stanford University Institutional Review Board and informed consent was obtained from all patients and donors.

1.2. Antibody detection

Antibodies against five HY antigens (DBY, UTY2, ZFY, RPS4Y, and EIF1AY) were tested using our HY antibody microarray platform[2,3]. SMCY, another HY antigen, was previously included in our HY panel[3]. However, the protein's large size, needing to be produced in 6 overlapping fragments, makes it a poor antigen assay target. This limitation, coupled with SMCY's lack of reproducible meaningful findings, led us to eliminate the antigen from our panel for future studies. Proteins were printed in quadruplicate spots on microarray slides and incubated with 1:50 dilutions of patient samples. Slides were digitally scanned, and median fluorescence intensity (MFI) for each antigen was determined. In case of printer

error, each set of quadruplicate HY antigen spots was printed twice on each subarray; if the difference in MFI between two duplicate sets of spots was greater than 1000, manual inspection of the scanned microarray images was performed to determine which set of spots to use (Supplemental fig. 2). Antibody quantifications were determined in a blinded manner, with clinical results secured in the BMT CTN data center prior to analysis.

1.3. Statistical Analyses

Serum and EDTA plasma specific seropositivity cutoffs were defined for each antigen as the third quartile added to twice the interquartile range (Q3 + 2IQR) of the MFI for the 60 normal males[2,3]. HY score, or the cumulative number of seropositive HY antibodies for an individual sample, was calculated for each sample to determine the association of multiple HY antibodies and cGVHD development. For example, if a patient had HY antibodies against DBY, UTY2, and ZFY at 3-months, his or her 3-month HY score would be 3. The Q3 + 2IQR cutoff allowed us to make binary distinctions between seropositive and seronegative patients; therefore, the Chi-squared test was used to determine statistical significance for the association of single or multiple HY antibodies and cGVHD development. The paired Wilcoxon test was used to study sample type specific differences in HY antibody quantification between the paired serum and plasma healthy male samples.

HY Score ranged from 0-5 as a measurement of cumulative antigen specific seropositivity. Relating HY score to cGVHD development, covariates were explored with logistic regression. Of the recognized cGVHD risk factors examined, only anti-thymocyte globulin (ATG) usage in the conditioning regimen statistically associated with cGVHD development (Supplemental table 1). However, only a total of 6 patients received ATG, offering minimal adjustment value. We therefore adjusted on a panel of factors consistently shown to be confounders in the HY antibody-cGVHD relationship[3,4]. Odds ratios with 95% confidence intervals were obtained after adjusting for patient age, donor age, disease, donor relation,

cell source, data sources, and acute GVHD (aGVHD) grade. All statistical tests were performed using R v.3.3.2 (The R Foundation, Austria).

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Supplementary Tables

Table S1. Association between 1-year HY score and cGVHD status stratified by aGVHD status.

aGVHD Positive patients	cGVHD positive	cGVHD negative
HY score > 1	33	5
HY score ≤ 1	45	16

Relative Risk: 1.18 p=0.128 (Chi-squared)

aGVHD Negative patients	cGVHD positive	cGVHD negative
HY score > 1	35	7
HY score ≤ 1	28	16

Relative Risk: 1.31 p=0.039 (Chi-squared)

Table S2. Association between patient characteristics and cGVHD development

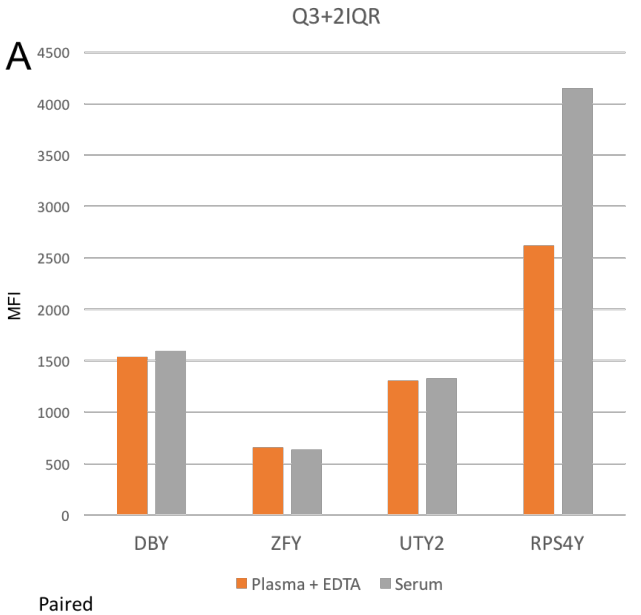
Characteristic	**No cGVHD (n=63)**	**cGVHD (n=171)**	**Fisher p value**
Age			1
< 50	34	94	
>= 50	29	77	
aGVHD			0.377
No	32	75	
Yes	31	96	
Conditioning Regimen			0.631
Busulfan	26	78	
Cytarabine + TBI	31	82	
Others	6	11	
Donor Age			0.614
<50	49	127	
>= 50	14	44	
Donor Relation			0.289
Mismatched	3	18	
MRD	35	80	
MUD	25	73	
Cell Source			0.829
PBSC	54	149	
Bone Marrow	9	22	
Advanced Disease			0.114
ALL	15	30	
AML	27	58	
Non-acute leukemia	21	83	
GVHD Prophylaxis			0.552
Cyclosporine	2	11	
Tacrolimus	59	157	
Others	2	3	
ATG			0.006
No	58	170	
Yes	5	1	

Table S3. Multivariable analyses for the impact of HY antibody seropositivity on other clinical outcomes.

	NRM OR* (95% CI)	P	Relapse OR* (95% CI)	P	OS OR* (95% CI)	P
3 Months						
HY Score 3M As a group variable						
0 to 1	1	ref	1	ref	1	ref
2	1.12 (0.36-3.50)	0.84	1.29 (0.56-3.02)	0.55	1.41 (0.61-3.25)	0.43
3 to 5	0.32 (0.064-1.57)	0.15	1.35 (0.56-3.23)	0.51	0.89 (0.34-2.35)	0.82
DBY alone	0.96 (0.30-3.07)	0.94	1.30 (0.57-3.01)	0.53	1.75 (0.76-4.00)	0.19
UTY2 alone	0.64 (0.25-1.64)	0.35	1.26 (0.66-2.41)	0.49	0.77 (0.39-1.52)	0.45
ZFY alone	0.45 (0.16-1.29)	0.14	1.15 (0.58-2.26)	0.7	0.82 (0.41-1.67)	0.59
RPS4Y alone	0.62 (0.16-2.42)	0.49	0.79 (0.32-1.93)	0.6	0.73 (0.29-1.86)	0.51
EIF1AY alone	0.69 (0.06-8.29)	0.77	1.94 (0.50-7.51)	0.34	2.64 (0.59-11.9)	0.21
1 Year						
HY Score 1Y As a group variable						
0 to 1	1	ref	1	ref	1	ref
2	0.34 (0.08-1.37)	0.12	1.25 (0.50-3.12)	0.63	0.56 (0.21-1.54)	0.26
3 to 5	<0.01 (0-Inf)	0.99	0.39 (0.13-1.20)	0.1	0.11 (0.022-0.55)	0.007
DBY alone	0.15 (0.017-1.26)	0.081	0.30 (0.10-0.88)	0.028	0.09 (0.02-0.42)	0.003
UTY2 alone	0.29 (0.09-0.91)	0.034	1.47 (0.68-3.18)	0.33	0.72 (0.32-1.63)	0.43
ZFY alone	0.60 (0.20-1.80)	0.36	0.45 (0.20-0.998)	0.0495	0.49 (0.21-1.13)	0.094
RPS4Y alone	<0.001 (0-Inf)	0.99	0.41 (0.13-1.32)	0.13	0.13 (0.02-0.70)	0.017
EIF1AY alone	<0.001 (0-Inf)	0.99	1.61 (0.27-9.71)	0.61	0.44 (0.045-4.21)	0.47

*Adjusted for patient age, donor age, disease, donor relation, cell source, data source, and aGVHD grade.
cGVHD indicates chronic graft-versus-host disease; NRM, non-relapse mortality; OS, overall survival; OR, odds ratio; CI, confidence interval.

Supplementary Figures



Paired Wilcoxon	DBY	ZFY	UTY2	RPS4Y
Difference	-56.8	21.8	-24.3	-1530.1
p value	0.6759	0.7105	0.9729	0.5388

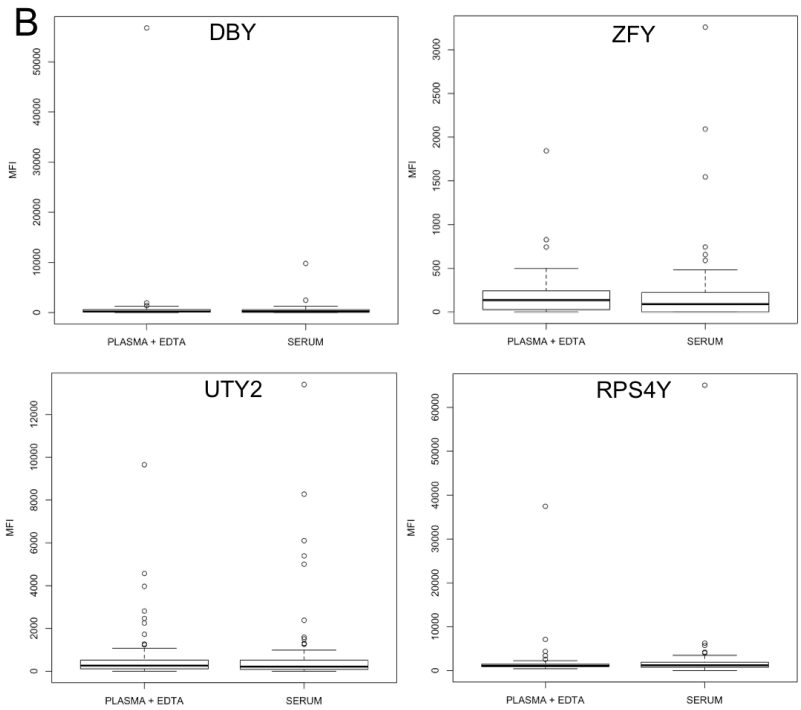
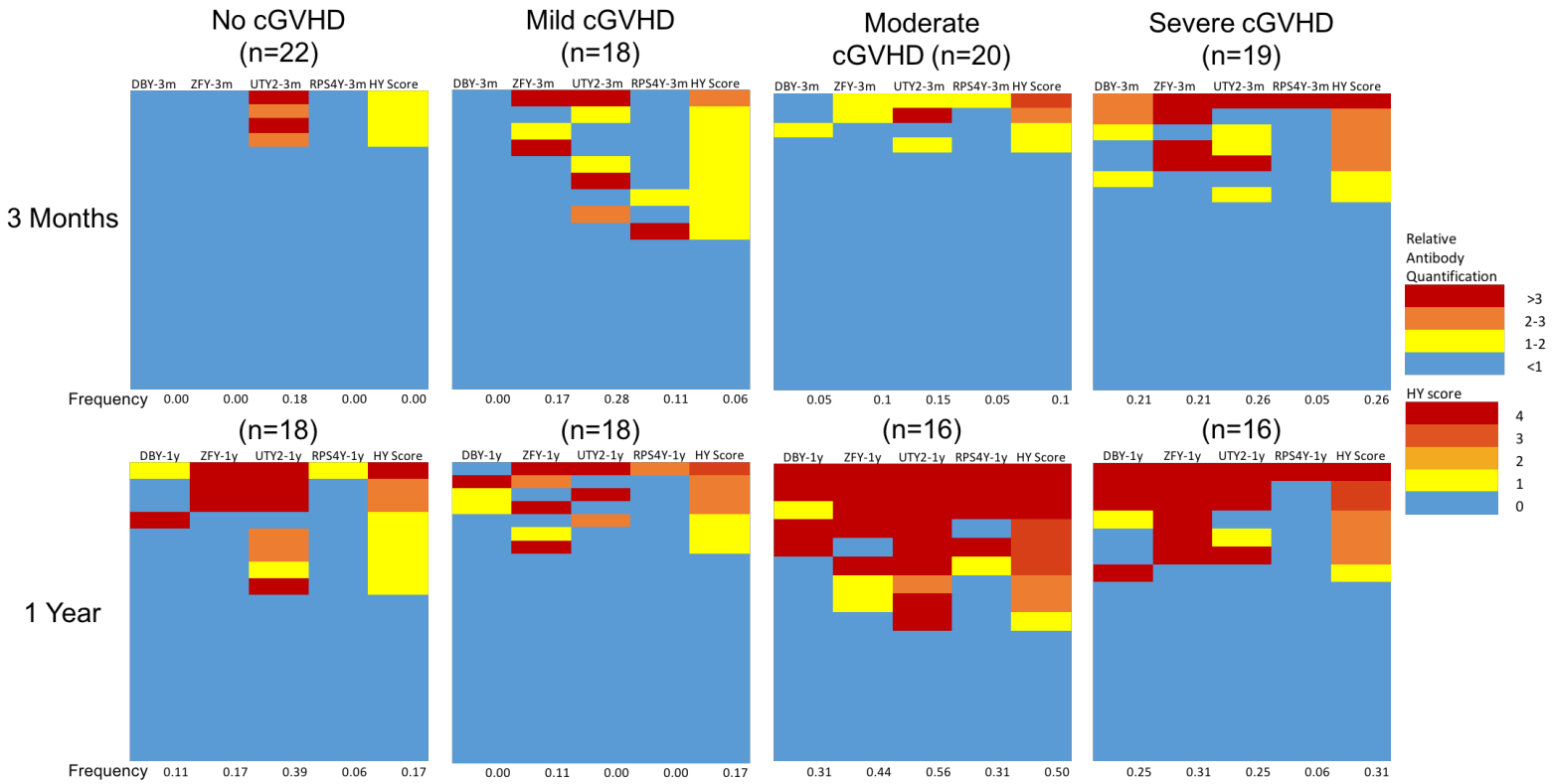


Figure S1. Panel A: MFI-based cutoffs for the four most informative HY antigens determined by measuring 60 paired sera (gray) and EDTA plasma (orange) samples. Panel B: Analysis of 60 paired plasma EDTA plasma versus paired serum samples from the NHLBI. HY antibody measurement did not differ for these two sample types.



P values by Chi-squared:	DBY	ZFY	UTY2	RPS4Y	HY Score > 1 (separated by severities)	HY Score > 1 (no/mild vs moderate/severe)
3-months	0.0212	0.1633	0.7233	0.4673	0.0391	0.0229
1-year	0.4824	0.3852	0.0812	0.0536	0.1612	0.0557

Figure S2. Anti-HY antibody responses in F→M HCT recipients stratified by cGVHD severity at both 3-months and 1-year time points. Intensity of the antibody response is color-coded as a multiple of each HY-seropositivity threshold. HY score represents the cumulative number of seropositive HY antigens. Seropositivity frequencies for each antibody are listed along the bottom border of each heat map. HY score frequency refers to the frequency of an HY score greater than 1. Table with p values by chi-squared for each HY antibody is shown below heat maps.

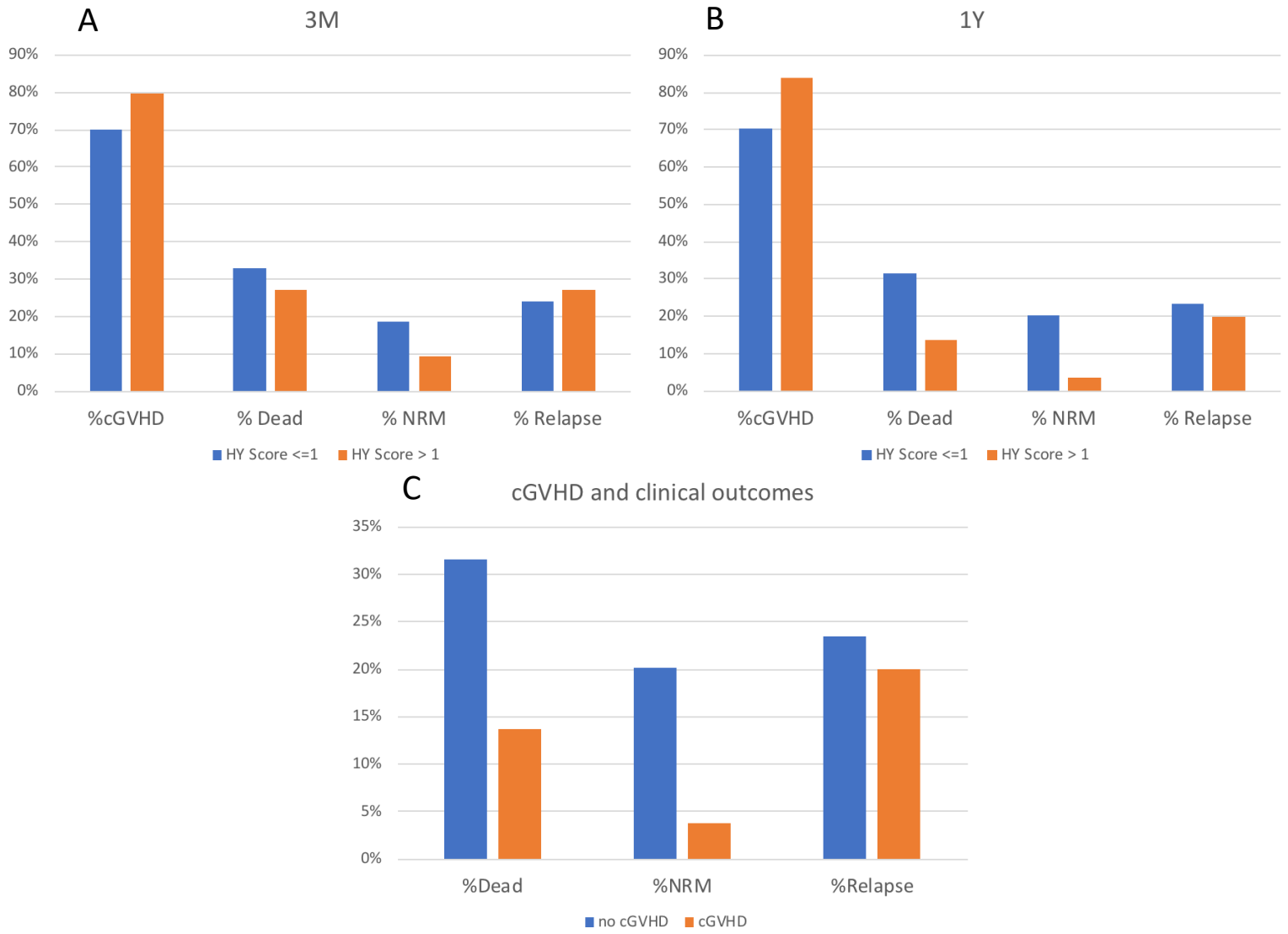
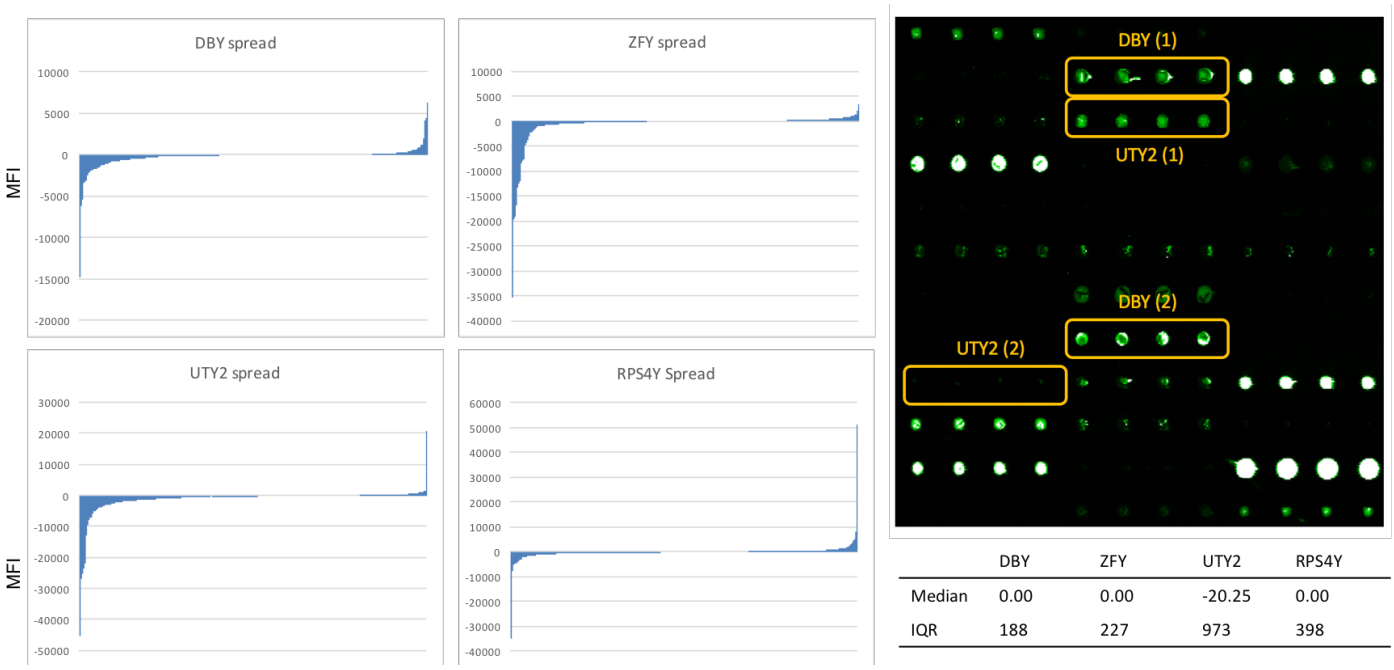


Figure S3. Comparison of rates of developing cGVHD, death, NRM, and relapse between high and low HY scoring patients at 3-months (A) and 1-year (B) post-transplant. In panel C, the frequencies of the same clinical outcomes are graphed against cGVHD status. Note that the frequencies of experiencing death, NRM, and relapse are nearly identical between 1-year HY score and cGVHD status.



	DBY	ZFY	UTY2	RPS4Y
Median	0.00	0.00	-20.25	0.00
IQR	188	227	973	398

Figure S4. Panel A: MFI spread calculated across the four most informative HY antigens, with each bar representing one patient sample. The majority of samples with large spread showed a higher MFI on the first set, suggestive of printing error on the second set. Panel B: A representative subarray image showing a case of high spread but large overall fluorescence between two duplicate sets of spots (DBY) and a case of high spread due to printing error (UTY2).