

Presensitization to HY antigens in female donors prior to transplant is not associated with male recipient post-transplant HY antibody development nor with clinical outcomes

It has been shown that chronic graft-*versus*-host disease (cGVHD) incidence is significantly increased in male recipients receiving allogeneic hematopoietic cell transplant from female donors (F→M HCT).^{1,2} B cell immunity plays a critical role in cGVHD development,^{3,4} and we have so far investigated clinically relevant immune responses against minor antigens encoded by the Y-chromosome (HY antigens) following F→M HCT.⁵⁻⁷ Recently, we have demonstrated that in F→M HCT, antibody responses against HY antigens (HY-Ab) are correlated with cGVHD and that the cumulative number of HY-Ab three months post-HCT could significantly predict cGVHD development and non-relapse mortality.⁸

In this study, we investigated whether HY-Ab detected in female donors prior to donation can predict cGVHD or other clinical outcomes following F→M HCT. We hypothesized that HY seropositive female grafts would lead to antigen-specific HY-Ab production in male recipients along with a higher incidence of cGVHD. We first measured HY-Ab in 289 female donors and tested their correlation with clinical outcomes of male recipients in collaboration with the National Marrow Donor Program (NMDP)/Center for International Blood and Marrow Transplant Registry (CIBMTR). Thereafter, we studied adoptive HY-Ab development in our Stanford F→M HCT cohort (n=90).⁸

Briefly, we measured IgG against five HY antigens using our protein microarray technology (*Online Supplementary Document*)⁹ in pre-donation sera collected from 289 female donors of 8/8 HLA-matched HCT with myeloablative conditioning, facilitated by the NMDP between 1990 and 2002. Almost all patients in the NMDP cohort (93%) received a bone marrow graft from an HLA-matched unrelated donor (*Online Supplementary Table S1*). Furthermore, we separately studied pre-donation plasma from 90 adult female donors and their corresponding male recipients who underwent HCT at Stanford University Hospital between 2005 and 2012. Almost all of the Stanford patients (97%) received a

peripheral blood graft, for which an HLA-matched related donor was selected in 76%, and reduced-intensity conditioning was used for 57% (*Online Supplementary Table S1*). The Stanford data confirmed female donor HY-Ab prevalence and assessed the association between the presence of HY-Ab in female donors and post-HCT HY-Ab development in male recipients. This study was approved by The Institutional Review Board of Stanford University and the NMDP. All patients gave written informed consent for the cryopreservation and analyses of blood samples in accordance with the Declaration of Helsinki.

In the 289 NMDP female unrelated donor cohort, half of the female donors (49%) were found to have at least one of the five HY-Ab (Figure 1A), and antibody was detected most frequently against UTY and DBY which are the immune-dominant HY antigens following F→M HCT.^{5,8} Of the 289 patients, 75 patients (26%) had one HY-Ab, 39 patients (13%) had two HY-Ab, and 29 patients (10%) had three or more HY-Ab. A similar distribution of HY-Ab was observed in the 90 Stanford female donor cohort (mainly from related sisters, Figure 1A) and the prevalence of individual HY-Ab did not significantly differ from the NMDP cohort.

Next, we assessed the association of donor HY-Ab with donor parity and donor age (range: 18-60) in the NMDP cohort. Given that women who give birth to multiple boys are reported to have anti-HY immunity,^{10,11} we expected multiparous female donors would have higher levels of HY seropositivity. However, detection of any HY-Ab in female donors was not associated with donor parity (*Online Supplementary Table S2*). One possible explanation for the absence of association is that the information on the gender of births had not been collected. Further information on birth gender might reveal a positive association, although privacy concerns should be considered. Alternatively, HY immunity may result from other male interactions besides childbirth.^{12,13} While there was also no difference in age between female donors testing seropositive and seronegative for any HY-Ab, the donors who had three or more HY-Ab detected (high HY score) were significantly older ($P=0.02$, *Online Supplementary Table S2*). We believe the increased HY score in older female donors may have resulted from cumulative male exposures and not isolated birth events. Analysis of the data from the Stanford cohort similarly

Table 1. Association between individual donor HY seropositivities and their development post-HCT in the Stanford cohort.

		3 months post-HCT (N=90)			Over 1 year post-HCT (N=66)			Anytime post-HCT (N=90)		
		Pt (-)	Pt (+)	P	Pt (-)	Pt (+)	P	Pt (-)	Pt (+)	P
DBY	Donor (-)	55 (79%)	15 (75%)	0.76	30 (68%)	19 (86%)	0.14	37 (84%)	33 (72%)	0.21
	Donor (+)	15 (21%)	5 (25%)		14 (32%)	3 (14%)		7 (16%)	13 (28%)	
UTY	Donor (-)	34 (63%)	24 (67%)	0.82	28 (72%)	14 (52%)	0.12	31 (72%)	27 (57%)	0.19
	Donor (+)	20 (37%)	12 (33%)		11 (28%)	13 (48%)		12 (28%)	20 (43%)	
RPS4Y	Donor (-)	65 (89%)	17 (100%)	0.34	35 (85%)	24 (96%)	0.24	43 (90%)	39 (93%)	0.72
	Donor (+)	8 (11%)	0 (0%)		6 (15%)	1 (4%)		5 (10%)	3 (7%)	
ZFY	Donor (-)	69 (95%)	17 (100%)	>0.99	43 (93%)	20 (100%)	0.55	52 (93%)	34 (100%)	0.29
	Donor (+)	4 (5%)	0 (0%)		3 (7%)	0 (0%)		4 (7%)	0 (0%)	
EIF1AY	Donor (-)	66 (99%)	23 (100%)	>0.99	48 (98%)	17 (100%)	>0.99	56 (98%)	33 (100%)	>0.99
	Donor (+)	1 (1%)	0 (0%)		1 (2%)	0 (0%)		1 (2%)	0 (0%)	

HCT: allogeneic hematopoietic cell transplantation; Pt: patient. We selected the time point of three months as a representative for an early time point because our previous study⁸ demonstrated the prediction potential of three months HY-Ab for cGVHD incidences, and one year for a late time point to analyze HY-Ab persistence if it was transferred.

showed no association between donor age and individual HY-Ab seropositivity in female donors. Parity information was not available in the Stanford cohort.

As our primary research endpoint, cGVHD incidence was assessed in F→M HCT recipients in the NMDP cohort. Overall cGVHD incidence was 46% (95%CI: 40-52%). Analysis was first carried out for individual HY-Ab detection in female donors prior to transplant. There was no statistical difference in cGVHD incidence in male recipients according to the detection of individual HY-Ab in female donors (Figure 2A). Analysis was then expanded to include detection of multiple HY-Abs (as reported by the HY score) in female donors, and no significant association with cGVHD incidence in male recipients was observed in either univariate or multivariate analyses (Figure 2B and *Online Supplementary Table S3*). Furthermore, we did not find any association between donor HY score and other recipient clinical outcomes (*Online Supplementary Figure S1* and *Online Supplementary Table S3*). This absence of association between HY score in female donors and cGVHD development in corresponding male recipients was similarly observed both in the analysis of the Stanford cohort and when the two cohorts were combined for analysis.

This absence of female donor HY-Ab association with male recipient clinical outcomes raised the question of whether specific humoral immunity against each HY antigen could be truly transferred from female donors to male recipients. To address this directly, HY-Ab was tested in the 90 Stanford female donors and their male recip-

ients. Figure 1B shows DBY-IgG development as representation of all five HY antigens. For each donor-recipient pair, one of the four following patterns was observed: 1) HY humoral immunity was possibly transferred, 2) never transferred, 3) developed from seronegative donors, and 4) HY-Ab was detected in neither the donor nor recipient in the pair (Figure 1B). Of note, antibody detected in female donors against each specific HY antigen was not associated with post-HCT development in their corresponding male recipients (Table 1). Rather, individual HY-Abs post-HCT seem to develop frequently from donors who were seronegative for the specific HY antigen, and the positive predictive value for HY-Ab development three months post-HCT was 25% in DBY-IgG, 38% in UTY-IgG, and 0% in the other three antigens (Table 1). Thus, these results provide little evidence that adoptive humoral HY immunity transfers from female donors to male recipients.

Consistent with our HY-Ab findings, a prior CIBMTR analysis showed that the greatest increase in cGVHD risk in F→M HCT was due to sex mismatch disparity itself rather than donor parity (41% for multiparous female donors vs. 39% for nulliparous female donors vs. 30% for male donors).² This lack of difference in cGVHD incidence between those with nulliparous and parous female donors can be supported by the absence of HY immunity transfer from female donors to male recipients in our current study. Together, this clinical study and our donor HY-Ab analysis suggests alloimmunity results primarily from de novo HY antigen stimulation following F→M

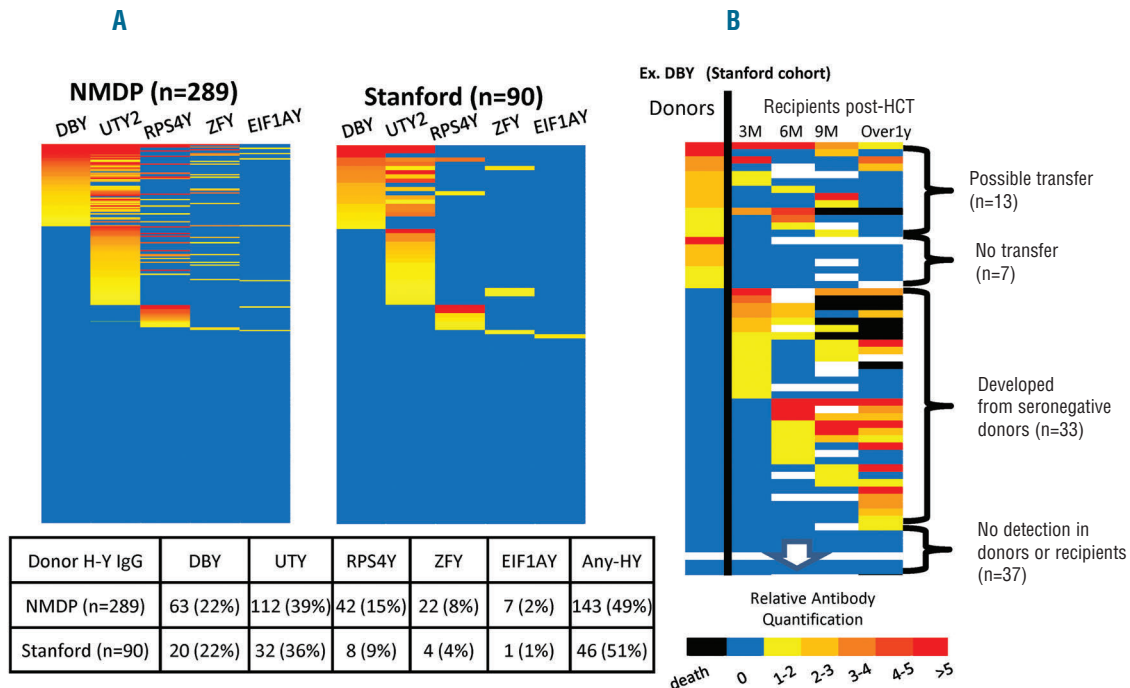


Figure 1. (A) Prevalence of female donor HY antibodies in the NMDP (n=289, left) and the Stanford cohorts (n=90, right). The various heat colors indicate relative antibody quantification as a factor of each HY seropositive threshold. UTY2 are shown as representative of the three UTY1-3 fragments. No statistical difference was observed between the cohorts. (B) Specific HY antibody in female donors and its temporal changes in corresponding recipients post-transplant. DBY-IgG is shown as representative. From left to right, the columns represent donors and recipients at 3, 6, and 9 months, and beyond 1 year post-transplant. The various heat colors indicate relative antibody quantification as a factor of each HY seropositive threshold. Black denotes death. For each donor-recipient pair, one of the following four patterns was observed: 1) HY humoral immunity was possibly transferred, 2) never transferred, 3) developed from seronegative donors, and 4) HY-Ab was detected in neither donors nor recipients.

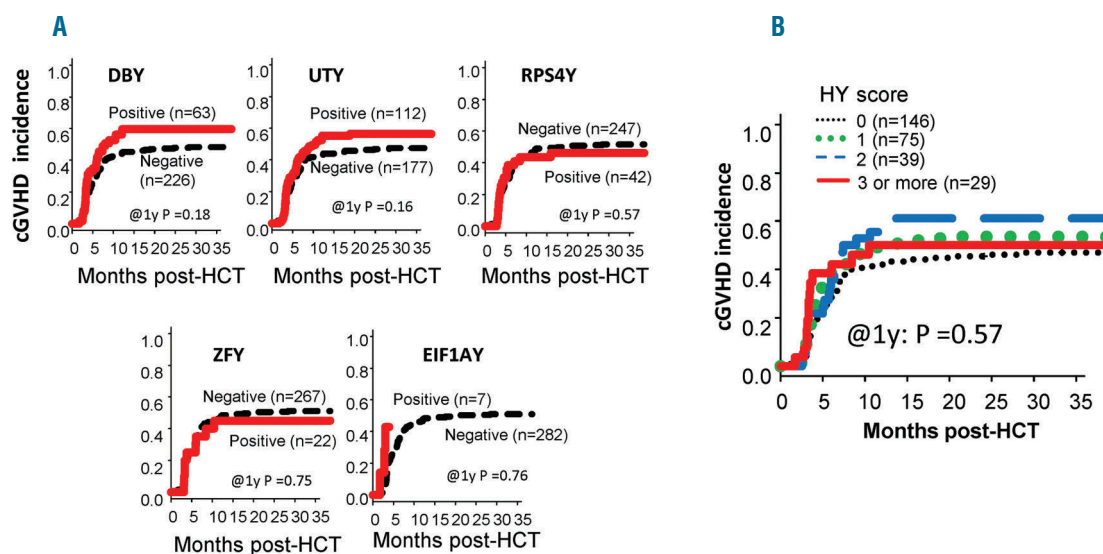


Figure 2. Chronic GVHD incidence (A) according to the detection of individual HY antibodies in female donors prior to transplant and (B) according to the detection of multiple HY antibodies in female donors (donor HY score) prior to transplant. Neither the detection of individual donor HY-Abs prior to transplant nor increased donor HY score prior to transplant was associated with chronic GVHD development in male recipients. (Figures are for the NMDP cohort).

HCT, and that female donor HY-Ab testing does not improve donor selection. The *de novo* development of humoral HY immunity post-F→M HCT predicts cGVHD development, while female donor HY-Ab status does not. Furthermore, the identification and monitoring of allogeneic HY-Ab development post-HCT may help assess the biological effects of novel therapeutic agents in order to optimize when and how to use these B cell targeted drugs.

The absence of adoptive humoral HY immune transfer might raise a concern for the efficacy of donor vaccination strategies to augment humoral graft-versus-leukemia benefits. Similar to HY immunity, presensitized donor anti-tumor humoral immunity may not be effectively transferred to recipients. Human clinical trials in multiple myeloma have included donor immunization followed by a recipient booster vaccination. In a recent trial, an increase of anti-idiotype IgG was limited to only 3 out of 10 recipients following a post-HCT booster vaccination, even though it could be induced and detected in most similarly vaccinated donors.¹⁴ Post-HCT vaccination/sensitization may be critical for the development of effective allo- and anti-tumor humoral immunity.

Our study may have some limitations. First, the current study was retrospective, but the two different cohorts independently produced similar results, providing strong evidence for the absence of an association of donor HY seropositivity with clinical outcomes. Second, the sample size of this study might have been too small to detect a significant impact of donor HY seropositivity. If a sample size larger than the almost 300 patients in this study is needed to detect an association, the impact for female donor HY seropositivity is unlikely to be clinically significant or influential for donor selection criteria. Third, measuring HY-Abs is an indirect tool to assess the transfer of HY reactive B cells. Perhaps the direct clone identification of HY reactive B cells via high throughput sequence analysis would reveal adoptive B cell transfer from donors to recipients post-HCT.¹⁵ The absence of HY-Ab transfer from female donors to male recipients

was assessed only in the Stanford cohort, where donors predominately provided G-CSF mobilized grafts (97%). It would be warranted to confirm the HY-Ab transfer in the case of bone marrow transplantation.

In summary, half of the female donors were shown to be HY seropositive using our novel microarray system. However, there was little evidence to suggest that female donor HY serostatus prior to transplant was associated with post-HCT HY-Ab development or clinical outcomes following F→M HCT. Most humoral HY immunity may develop post F→M HCT from naïve B cells being stimulated by HY antigen following sex mismatched transplantation.

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