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The Impact of Amino Acid Variability on Alloreactivity Defines a Functional Distance Predictive of Permissive HLA-DPB1 Mismatches in Hematopoietic Stem Cell Transplantation

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A major challenge in unrelated hematopoietic stem cell transplantation (HSCT) is the prediction of permissive HLA mismatches, ie, those associated with lower clinical risks compared to their nonpermissive counterparts. For HLA-DPB1, a clinically prognostic model has been shown to be matching for T cell epitope (TCE) groups assigned by cross reactivity of T cells alloreactive to HLA-DPB1*09:01; however, the molecular basis of this observation is not fully understood. Here, we have mutated amino acids (aa) in 10 positions of HLA-DPB1*09:01 to other naturally occurring variants, expressed them by lentiviral vectors in B cell lines, and quantitatively measured allorecognition by 17 $CD4^+$ T cell effectors from 6 unrelated individuals. A significant impact on the median alloresponse was observed for peptide contact positions 9, 11, 35, 55, 69, 76, and 84, but not for positions 8, 56, and 57 pointing away from the groove. A score for the "functional distance" (FD) from HLA-DPB1*09:01 was defined as the sum of the median impact of polymorphic aa in a given HLA-DPB1 allele on T cell alloreactivity. Established TCE group assignment of 23 alleles correlated with FD scores of $\leq$0.5, 0.6 to 1.9 and $\geq$2 for TCE groups 1, 2, and 3, respectively. Based on this, prediction of TCE group assignment will be possible for any given HLA-DPB1 allele, including currently 367 alleles encoding distinct proteins for which T cell cross reactivity patterns are unknown. Experimental confirmation of the in silico TCE group classification was successfully performed for 7 of 7 of these alleles. Our findings have practical implications for the applicability of TCE group matching in unrelated HSCT and provide new insights into the molecular mechanisms underlying this model. The innovative concept of FD opens new potential avenues for risk prediction in unrelated HSCT.

INTRODUCTION

It is generally accepted that high-resolution matching for 8/8 HLA-A, B, C, and DRB1 alleles between unrelated donors (UD) and recipients significantly improves the clinical outcome of hematopoietic stem cell transplantation (HSCT), at least in patients with early stage onco-hematologic disorders [1-6] or with nonmalignant disorders [7]. However, everyday transplantation practice has to face the frequent presence of 1 or more mismatches in the majority of transplantations. This is due to the difficulties of finding an 8/8-matched UD for 30% to 60% of patients, depending to their ethnic origin [8,9], as well as the presence of HLA-DPB1 mismatches in over 80% of unrelated HSCT resulting from the weak linkage disequilibrium between HLA-DPB1 and the other class II loci [10]. However, evidence for the existence of certain permissive mismatch combinations, ie, mismatches associated with lower clinical risks compared with their nonpermissive counterparts, is accumulating.

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Attempts for identifying permissive mismatches include the statistical analysis of the clinical risks associated with certain HLA mismatches involving defined amino acid (aa) positions [11,12]. These data point to the presence of qualitative differences between individual polymorphisms in the HLA molecule, possibly reflecting the relative impact of aa variability on alloreactive T cells mediating the clinical effects of histocompatibility barriers. In line with this notion, our group developed and clinically validated a functional algorithm for nonpermissive HLA-DPB1 T cell epitope (TCE) mismatches [13-16]. This model is based on the observation of reproducible cross reactivity patterns displayed by T cells alloreactive to nominal HLA-DPB1*09:01, towards 23 different HLA-DPB1 alleles studied to date. This led to the classification of HLA-DPB1 alleles into at least 3 different TCE groups based on their ability to be recognized by all (TCE group 1; DPB1*09:01, 10:01, 17:01), some (TCE group 2; DPB1*03:01, 14:01, 45:01, 86:01, 104:01), or none (TCE group 3; DPB1*01:01, 02:01, 02:02; 04:01, 04:02, 05:01, 06:01, 11:01, 13:01, 15:01, 16:01, 19:01, 20:01, 23:01, 46:01) of 7 different T cell clones studied [16,17]. UD-recipient HLA-DPB1 mismatches were classified as permissive or nonpermissive, according to whether they involved alleles from the same or from different TCE groups, respectively. Nonpermissive HLA-DPB1 TCE group mismatches were shown to be associated with significantly higher clinical risks after well-matched unrelated HSCT compared with permissive mismatches. This was demonstrated retrospectively in many, though not all, multicenter retrospective studies [13,14,16,18-21], as well as in 2 recent independent investigations involving over 5000 patients each [15,22]. Based on this, an online Web tool for the identification of nonpermissive HLA-DPB1 TCE group mismatches in UD searches has been developed (www.ebi.ac.uk/ipd/imgt/hla/dpb.html) [23]. A practical constraint to the clinical applicability of this Web tool is the limited number of HLA-DPB1 alleles that have been functionally assigned to TCE groups, ie, 23 of 390 (5.9%) alleles encoding distinct proteins known to date (http://www.ebi.ac.uk/ipd/imgt/hla/).

Here, we have sought to investigate the correlation between TCE groups and aa sequence variability at individual positions of HLA-DPB1 to determine the relationship between functional and structural characteristics in a clinically relevant model of permissive mismatches in unrelated HSCT.

MATERIALS AND METHODS

Cells, Cell Lines, and HLA Typing

Peripheral blood mononuclear cells (PBMC) were obtained from healthy blood or stem cell donors after written informed consent approved by the San Raffaele Ethic Committee. B lymphoblastoid cell lines (BLCL), either locally established or purchased from the European Collection of Animal Cell cultures, are listed along with their respective HLA types in Table S1. High-resolution molecular HLA-A, B, C, DRB1, DQB1, and DPB1 typing of locally obtained PBMC or BLCL was performed by standard techniques under the quality guidelines from the European Federation for Immunogenetics (http://www.efiweb.eu).

Monoclonal Antibodies and Flow Cytometry

Monoclonal antibodies (mAbs) included anti–low-affinity nerve growth factor receptor (ΔLNGFR)-Allophycocyanin (clone ME20.4) (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4-PE-Cyanin7 (clone RPA-T4), anti-CD137-Allophycocyanin (clone 4B4-1) (Becton Dickinson, Franklin Lakes, NJ), anti-pan HLA-DP (clone B7/21) (Cancer Research Technology Limited, London, UK), anti-HLA-DP-DEAV$_{[84-87]}$ (clone TL-3B6), anti-HLA-DR (clone L243) (Biolegend, San Diego, CA), and anti-human IgG-FITC (Jackson ImmunoResearch, Newmarket, UK). T cell receptor (TCR) variable beta chain region (Vβ) typing was performed using the IOTest Beta Mark TCR-Vβ Repertoire Kit (Beckman Coulter, Indianapolis, IN).

Flow cytometry data acquisition was performed on a FACS Canto II (Becton Dickinson) using FACSDiva software (Becton Dickinson). Subsequent data analysis was performed using FCS Express 4.0 software (De Novo Software, Los Angeles, CA).

*Site-directed Mutagenesis of HLA-DPB1*09:01 and Expression in Read-out Cell Lines*

Site-directed mutagenesis (SDM) of wild type (WT) HLA-DPB1*09:01, PCR-amplified from cDNA extracted from local PBMC, was performed using the QuikChange II kit (Agilent Technologies, Santa Clara, CA), according to manufacturer's instructions, and confirmed by Sanger sequencing.

WT and mutant HLA-DP expression by BLCL was driven by a bidirectional lentiviral expression platform, as previously described [24,25], inducing coordinate expression of transgenic HLA-DPB1 and the ΔLNGFR reporter. After magnetic bead purification for reporter gene expression, cell surface expression levels of transgenic ΔLNGFR or HLA-DPB1 were quantitatively assessed by flow cytometric determination of Molecules of Equivalent Soluble Fluorochrome [26] using SPHERO Rainbow Calibration Particles (Spherotech, Lake Forest, IL) [25].

*Isolation and Characterization of CD4$^+$ T Cells Alloreactive to HLA-DPB1*09:01*

PBMC from responder-stimulator pairs matched for 9 to 10/10 of the HLA-A, B, C, DRB1, and DQB1 alleles but mismatched for HLA-DPB1, were used for the set-up of 1-way mixed lymphocyte reactions (MLR) as previously described [25]. PBMC were from a patient before transplantation or at the time of HSCT allograft rejection [27] or from 5 different HLA-typed healthy subjects. For complete HLA typing of the PBMC, see typing of the respective BLCL in Table S1. After 2 rounds of stimulation, T cells were either cloned by limiting dilution or left uncloned and maintained by repeated restimulations, as described [25]. Allospecificity of T cell clones was established by IFN-γ ELISpot assays according to standard protocols [28,29] and

Table 1
Site-Directed Mutants of HLA-DPB1*09:01

HvR	Mutant Name	Naturally Occurring HLA-DPB1*	Aa Position									
			8	9	11	35	55	56	57	69	76	84
-	WT	09:01	V	H	L	F	D	E	D	E	V	D
A	V8L	-	L	-	-	-	-	-	-	-	-	-
	H9Y	88:01	-	Y	-	-	-	-	-	-	-	-
	H9F	-	-	F	-	-	-	-	-	-	-	-
	L11G	-	-	-	G	-	-	-	-	-	-	-
B	F35Y	-	-	-	-	Y	-	-	-	-	-	-
	F35L	-	-	-	-	L	-	-	-	-	-	-
C	D55A	-	-	-	-	-	A	-	-	-	-	-
	E56A	-	-	-	-	-	-	A	-	-	-	-
	D57E	10:01	-	-	-	-	-	-	E	-	-	-
D	E69K	35:01	-	-	-	-	-	-	-	K	-	-
E	V76M	17:01	-	-	-	-	-	-	-	-	M	-
F	D84G	-	-	-	-	-	-	-	-	-	-	G

HvR indicates hypervariable regions.
The naturally occurring counterparts listed for individual mutants have identical aa sequences in the peptide antigen-binding groove.

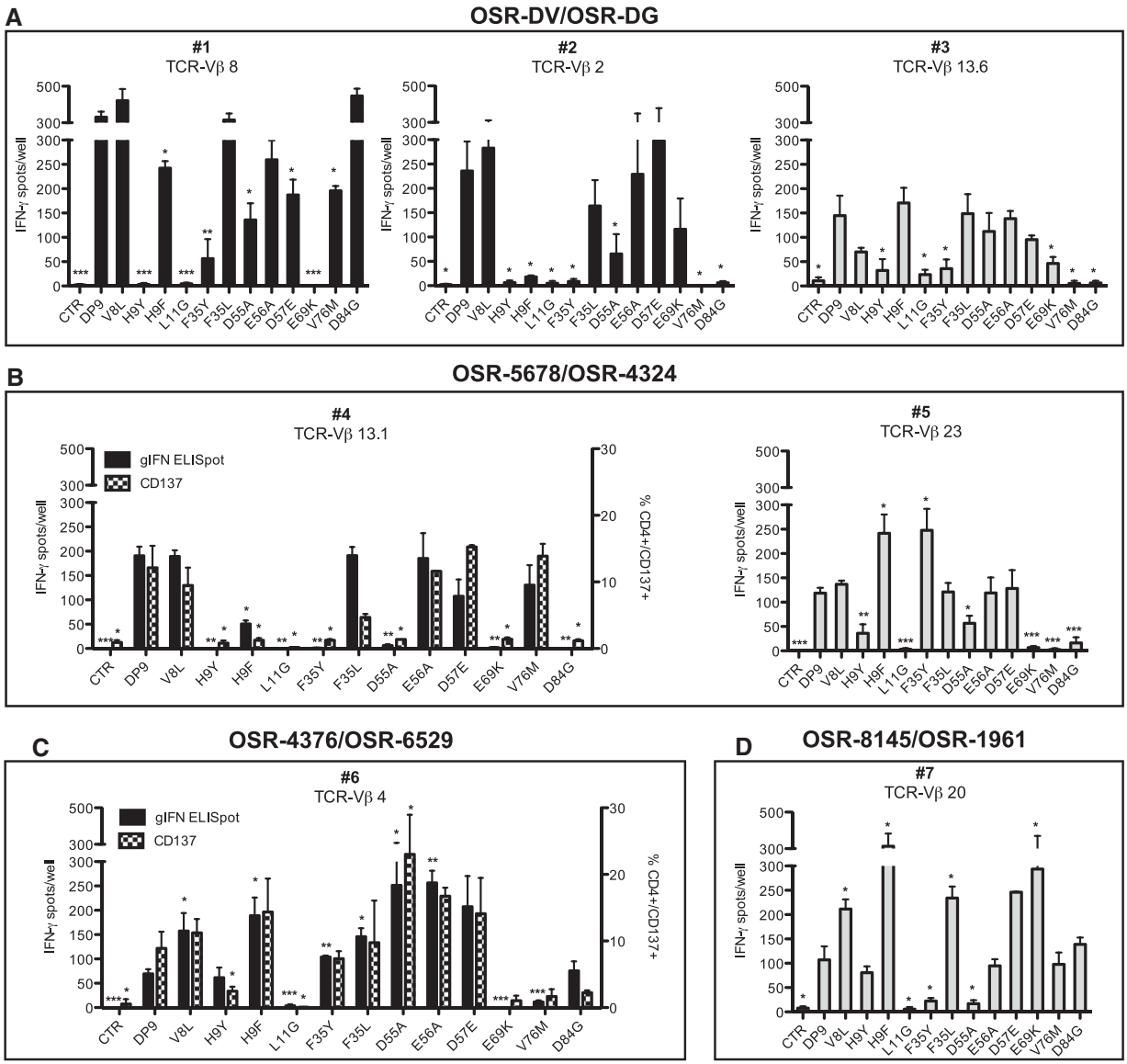

Figure 1. Intra- and interindividual variability of sensitivity to single aa substitutions for allorecognition of HLA-DPB1*09:01 by $CD4^+$ T cell clones. Allorecognition of MGAR (black and hatched bars) or VAVY (grey bars) BLCL after lentiviral transduction with WT (DP9) or mutant HLA-DPB1*09:01 (Table 1), or with irrelevant control HLA-DPB1 (CTR), by clonal alloreactive $CD4^+$ T cells (Table S2). CTR was HLA-DPB1*01:01 for MGAR and HLA-DPB1*04:01 for VAVY. Shown is for all clones the number of spots counted in IFN-γ ELISpot assays in response to transduced BLCL (filled bars). For clones 4 and 6, the percentage of T cells expressing CD137 in response to transduced BLCL is additionally shown on the right axis (hatched bars). (A) T cell clones from PBMC obtained from a patient at the time of HSCT allograft rejection (clones 1 and 2), or before transplantation (clone 3). (B-D) T cell clones from PBMC obtained from 3 unrelated healthy responder-stimulator pairs. Results are expressed as mean ± SD of 3 to 5 (IFN-γ ELISpot assays) and 2 (CD137 assays) independent experiments. Statistical comparisons between alloresponses to each mutant versus WT DPB1*09:01 were performed by the unpaired 2-tailed *t*-Test (*$P < .05$, **$P < .01$, ***$P < .001$).

confirmed by incubation with HLA locus-specific mAbs at a final concentration of 1 μg/mL. Alloreactive TCR-Vβ subfamilies in uncloned T cell cultures were analyzed by flow-cytometric CD137 upregulation assays as described [25,30,31], using appropriately labeled anti-CD4 and anti-CD137 mAb in combination with mAb for different TCR-Vβ subfamilies. A list of alloreactive T cells used, along with their TCR-Vβ subfamilies and their allospecificity, is included as Table S2.

The relative response (RR) of $CD4^+$ T cells to mutant versus WT HLA-DPB1*09:01 was calculated as $response_{[mutant]} - response_{[negative\ control]} / response_{[WT]} - response_{[negative\ control]}$, where response was the number of IFN-γ dots in ELISpot assays or the percentage of $CD4^+$ $CD137^+$ positive T cells per TCR-Vβ family in CD137 upregulation assays.

Statistical Analyses

Statistical comparisons were performed using the unpaired *t*-test, with $P < .05$ as threshold for significance. All statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA).

RESULTS

*SDM of HLA-DPB1*09:01 and Expression in Read-out BLCL*

HLA-DPB1 TCE groups used to identify clinically permissive mismatches in unrelated HSCT have been defined in our previous studies on the basis of reproducible cross reactivity patterns displayed by T cells nominally alloreactive to HLA-DPB1*09:01 [16,17]. We therefore used HLA-DPB1*09:01 as reference allele for SDM of polymorphic aa in the α2 domain encoded by exon 2. A total of 10 polymorphic aa residues within hypervariable regions A through F, located at position 8, 9, 11, 35, 55, 56, 57, 69, 76, and 84, were swapped into a residue naturally encoded by other HLA-DPB1 alleles, resulting in a total of 12 point mutants of HLA-DPB1*09:01 (Table 1). The 10 positions were selected based on their

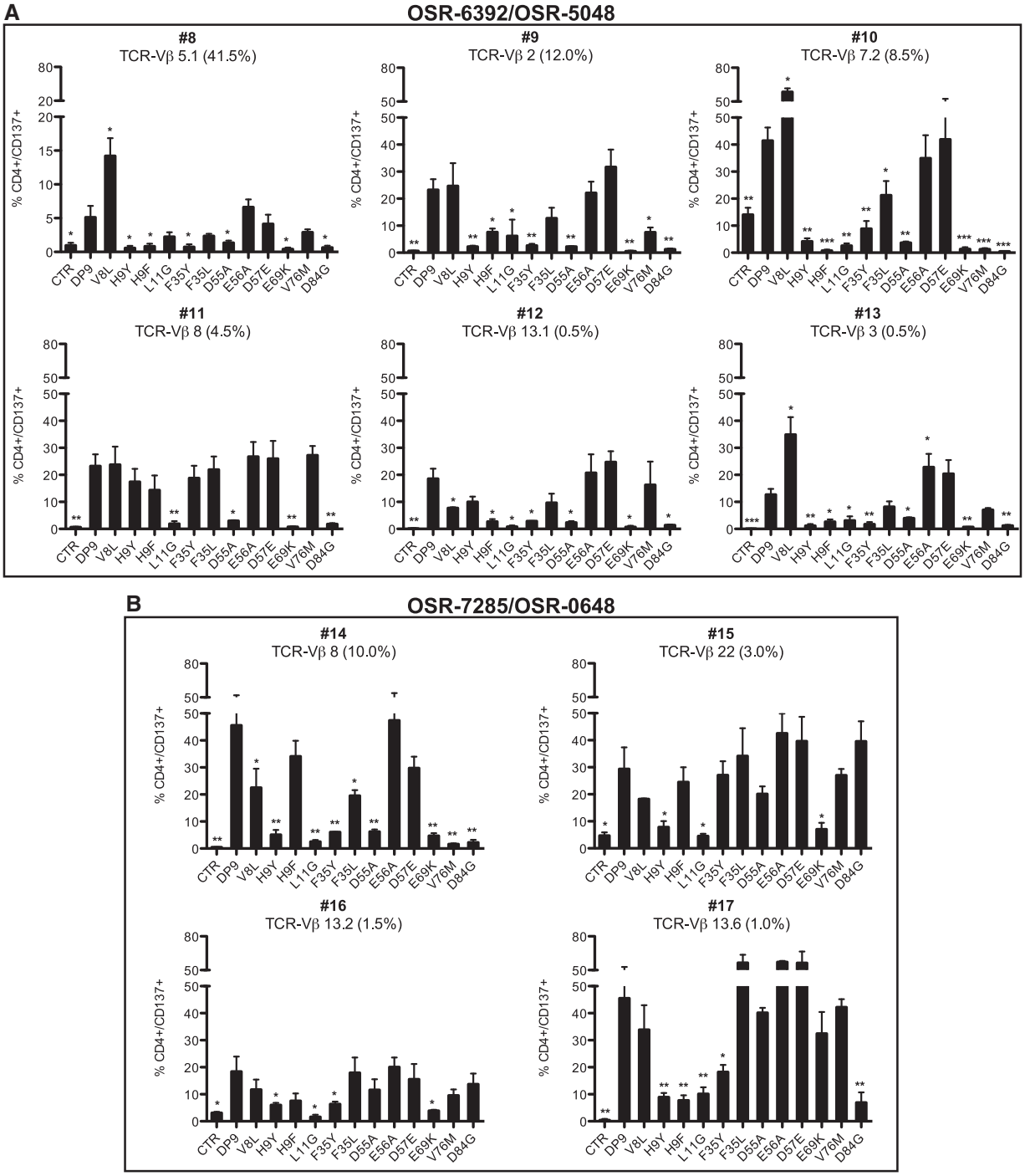

Figure 2. Intra-individual variability of sensitivity to single aa substitutions for allorecognition of HLA-DPB1*09:01 by different TCR-Vβ subpopulations. Shown is the percentage of CD4 + T cells upregulating the activation marker CD137 in response to MGAR BLCL transduced with HLA-DPB1*01:01 (CTR), WT (DP9) or mutant HLA-DPB1*09:01 (Table 1), in 6 and 4 TCR-Vβ subpopulations present in 2 uncloned T cells obtained after 2 rounds of stimulation in MLR between the responder/stimulator pairs OSR-6392/OSR-5048 (A) and OSR-7285/OSR-0648 (B) (Tables S1 and S2). The percentage of the relevant TCR-Vβ subpopulation within the total of $CD4^+$ T cells is shown on top of each graph. CD137 upregulation assays were performed as described in Materials and Methods. Only TCR-Vβ subpopulations present at ≥0.5% and significantly ($P < .05$) upregulating CD137 after stimulation with WT HLA-DPB1*09:01 compared to CTR were analyzed. Results are mean ± SEM of 3 independent experiments. Statistical comparison between alloresponses to each mutant versus WT HLA-DPB1*09:01 were performed by the 2-tailed unpaired *t*-Test (*$P < .05$, **$P < .01$, ***$P < .001$).

predicted role for peptide binding and/or TCR interaction, as suggested by HLA-DP homology modeling (data not shown) and the available literature [32-36].

WT or mutant HLA-DPB1*09:01, or HLA-DPB1*01:01 or 04:01 as negative controls, were lentiviral transduced into the read-out BLCL MGAR or VAVY, homozygous for HLA-DPB1*04:01/DPA1*01:03 and HLA-DPB1*01:01/DPA1*02:01, respectively. Transgene expression levels were assessed by quantitative PCR for transgenic HLA-DPB1 and by cell surface staining for the ΔLNGFR reporter and, in the case of MGAR, for the HLA-DPB1 $DEAV_{[84-87]}$ epitope selectively encoded by transgenic but not endogenous DPB1 for this

BLCL. Compared with WT HLA-DPB1*09:01, these experiments revealed no significant differences in transgene expression levels for any of the mutants except for F35Y and D57E, which showed consistently lower cell surface expression levels compared with WT on MGAR but not on VAVY BLCL (data not shown).

*CD4$^+$ T Cell Effectors Alloreactive to HLA-DPB1*09:01*

A panel of 17 CD4$^+$ T cell effectors alloreactive to HLA-DPB1*09:01 or to 10:01, both from TCE group 1, was obtained from 6 different individuals, including a patient at the time of graft rejection after allogeneic HSCT [27], by 1-way MLR as described in Materials and Methods (Table S2). The 17 effectors included 7 T cell clones obtained by limiting dilution cloning and 10 different HLA-DP allospecific TCR-Vβ subpopulations from 2 CD4$^+$ T cell lines. Allospecificity of the T cell effectors was demonstrated by IFN-γ ELISPOT and/or CD137 upregulation assays against allogeneic stimulator versus autologous responder BLCL, and for T cell clones by mAb inhibition studies on stimulator BLCL (Table S2). According to the HLA-DPα chain preference for allorecognition of WT HLA-DPB1*09:01 displayed by the different T cell effectors (data not shown), MGAR or VAVY BLCL were chosen as read-outs for recognition of mutant HLA-DPB1*09:01 in further studies.

*Role of Individual aa Substitutions for Allorecognition of HLA-DPB1*09:01*

To study the impact of single aa substitutions on allorecognition of HLA-DPB1*09:01, the ability of individual alloreactive T cell effectors to recognize single aa mutants of HLA-DPB1*09:01 was evaluated in comparison to WT. Seven T cell clones were analyzed by IFN-γ ELISPOT and 2 of them in parallel also by CD137 assays with superimposable results (Figure 1). Marked intra- and interindividual variability of sensitivity to different aa substitutions was found for the 7 clones, with none of them displaying identical patterns of sensitivity. Similar results were obtained when the alloresponse to WT or mutant HLA-DPB1*09:01 was tracked for 10 TCR-Vβ subpopulations in oligoclonal T cell lines using the CD137 assay [30] (Figure 2).

On the basis of these data, an overall impact of individual aa substitutions on allorecogntion of HLA-DPB1*09:01 could be assessed as the median RR of all 17 T cells to mutant compared with WT (Figure 3A). The highest functional impact was observed for mutations L11G, E69K, and D84G, each with a median RR of < 0.07, reflecting abrogation of allorecognition by nearly all T cells studied, followed by H9Y and F35Y with a median RR of 0.15 and 0.13, respectively. In contrast, no significant impact was observed for mutations F35L, V8L, E56A, and D57E, with a median RR of 0.95 or >1.0, reflecting similar or even better allorecognition of the mutants compared to WT HLA-DPB1*09:01 by most of the T cells studied (Figure 3A).

The Median Impact of aa Variability on T Cell Alloreactivity Defines a Functional Distance between HLA-DPB1 Alleles

To compare the relative impact of individual aa substitutions on allorecognition, each aa was assigned with a numerical value termed *FD* from the corresponding residue in WT HLA-DPB1*09:01, defined as 1-median RR. According to this definition, all aa present in WT HLA-DPB1*09:01 have a FD_{aa} value of 0, and FD_{aa} values for individual mutations

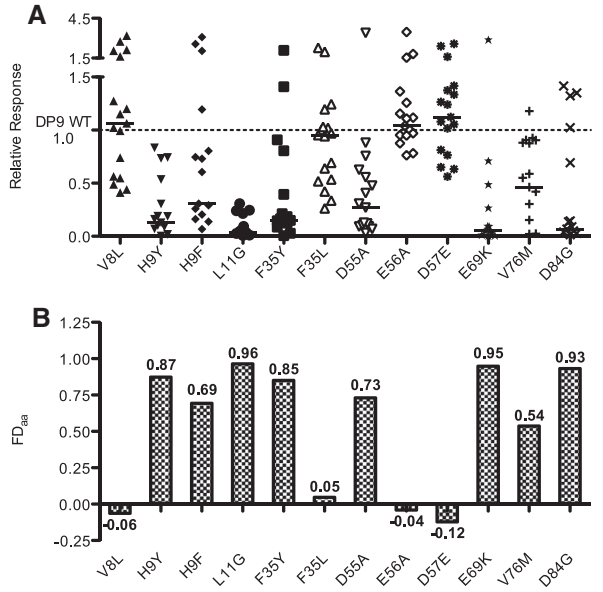

Figure 3. Overall impact of individual aa substitutions on allorecognition of HLA-DPB1*09:01 by an alloreactive CD4$^+$ T cell panel. (A) Relative response (RR) of 17 CD4 + T cells (Table S2) to individual aa substitutions in HLA-DPB1*09:01 (Table 1) compared to WT set as 1.0 (dotted line). Each aa mutation is represented by a different symbol. RR values were calculated according to the formula $Response_{[mutant]}-Response_{[negative\ control]}/Response_{[WT]}-Response_{[negative\ control]}$. Data were derived from those reported in Figure 2 for T cell clones and in Figure 4 for TCR-Vβ subpopulations. (B) FD_{aa} value of each aa mutation, calculated as [1- median RR] from panel A. FD_{aa} values are distributed from lowest (−0.12) to highest (0.96) overall impact on allorecognition of HLA-DPB1*09:01.

		Hypervariable Region (HvR)										
		A			B	C			D	E	F	
TCE	DPB1*	8	9	11	35	55	56	57	69	76	84	FD_{allele}
	09:01	**V**	**H**	**L**	**F**	**D**	**E**	**D**	**E**	**V**	**D**	**0.00**
1	10:01	-	-	-	-	-	-	E	-	-	-	-0.12
	17:01	-	-	-	-	-	-	-	-	M	-	0.54
	03:01/104:01	-	Y	-	-	-	-	-	K	-	-	1.82
2	14:01	-	-	-	-	-	-	-	K	-	-	0.95
	45:01	-	-	-	-	-	-	E	K	-	-	0.83
	86:01	-	-	-	-	-	-	-	-	M	G	1.47
	01:01	-	Y	G	Y	A	A	E	K	-	-	4.20
	02:01	L	F	G	-	-	-	E	-	M	G	2.94
	02:02	L	F	G	L	-	-	E	-	M	G	2.95
	04:01	L	F	G	-	A	A	E	K	M	G	4.58
	04:02	L	F	G	-	-	-	E	K	M	G	3.89
	05:01	L	F	G	L	**E**	A	E	K	M	-	2.97
	06:01	-	Y	-	-	-	-	-	-	M	-	1.41
3	11:01	-	Y	-	Y	A	A	E	**R**	M	-	2.83
	13:01	-	Y	-	Y	A	A	E	-	**I**	-	2.29
	15:01	-	Y	G	Y	A	A	E	**R**	M	**V**	3.79
	16:01	L	F	G	-	-	-	E	-	M	-	2.01
	19:01	L	F	G	-	**E**	A	E	-	**I**	-	1.43
	20:01	-	Y	-	-	-	-	-	K	M	-	2.36
	23:01	L	F	G	-	A	A	E	K	M	G	4.58
	46:01	L	F	G	-	-	-	-	-	M	G	3.06

Figure 4. HvR variability of HLA-DPB1 alleles according to their functional TCE group classification and FD_{allele} scores. Shown are 23 HLA-DPB1 alleles previously classified into TCE groups according to T cell cross reactivity patterns [16,17], along with their aa polymorphism at the 10 positions subjected to SDM in the present study (Table 1). The FD_{allele} score of each of these alleles was calculated as the sum of the FD_{aa} values of each mutation, as reported in Figure 5B. Variants D55E, E69R, V76I, and D84V, naturally occurring in HLA-DPB1*05:01, 19:01, 11:01, 13:0,1 and 15:01 respectively, are indicated in bold because they were not analyzed in this study and were not considered (FD_{aa} value 0).

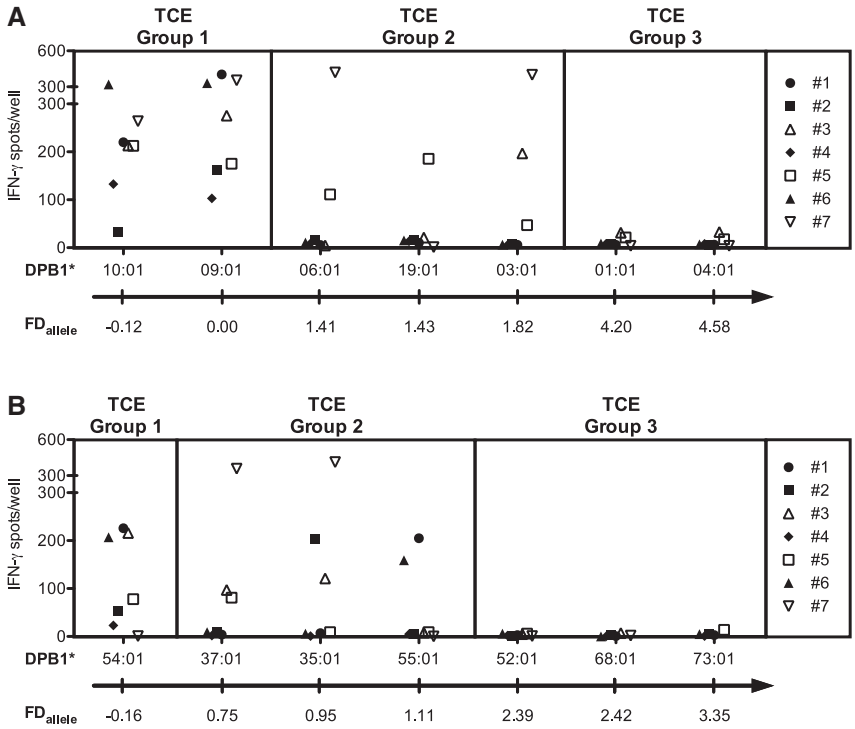

Figure 5. Comparative evaluation of HLA-DPB1 TCE group classification by alloreactive T cell cross reactivity patterns and by FD_{allele} scores. Shown is the number (mean of 3 to 5 independent experiments) of spots counted in IFN-γ ELISpot assays in response to BLCL expressing different endogenous HLA-DPB1 alleles, by 7 T cell clones from this study (Table S2). HLA-DPB1 alleles are shown in the order of rising FD_{allele} scores. Note recognition by most, some or none of the T cell clones for HLA-DPB1 alleles in TCE groups 1, 2, and 3, respectively. (A) Recognition of 7 of 23 HLA-DPB1 alleles previously assigned to TCE group 1, 2, or 3 on the basis of alloreactive T cell cross reactivity patterns [16,17]. (B) Recognition of 7 of 367 HLA-DPB1 alleles newly assigned to TCE groups 1, 2, or 3 on the basis of their FD_{allele} scores.

range from 0 or below to a maximum of 1 with increasing overall negative impact on T cell allorecognition (Figure 3B).

Because all mutations analyzed correspond to naturally occurring variants, individual FD_{aa} values could be used to calculate an overall FD_{allele} score of each HLA-DPB1 allele as the sum of individual FD_{aa} values. In this model, the FD_{allele} score of the reference WT HLA-DPB1*09:01 is 0, as all individual FD_{aa} values are 0. A list of FD_{allele} scores for all 390 HLA-DPB1 alleles encoding different proteins known to date is given in Table S3. The FD_{allele} scores range from -0.16 for HLA-DPB1*54:01 to 5.67 for HLA-DPB1*158:01. It should be noted that aa polymorphisms different in position or type from those studied here were not considered and assigned with an FD_{aa} value of 0, suggesting that some of the assigned FD_{allele} scores might be underestimated.

*The Functional Distance from HLA-DPB1*09:01 is Predictive of HLA-DPB1 TCE Groups*

In our previous studies, 23 HLA-DPB1 alleles were assigned to TCE groups 1, 2, and 3 on the basis of their cross recognition by 5 of 5, 2 of 5, or 0 of 5 T cell clones from a patient before or at the time of HSCT allograft rejection, including clone numbers 1 through 3 from the present study [16,17]. The FD_{allele} scores of these 23 HLA-DPB1 alleles were found to be predictive of their respective TCE group assignment, with TCE groups 1, 2, and 3 falling into FD_{allele} score ranges of $\leq$0.5, 0.6 to 1.9, and $\geq$2, respectively (Figure 4). The only 2 exceptions were HLA-DPB1*06:01 and 19:01, which had been assigned to TCE group 3 because they were recognized by 0 of 5 T cell clones studied [16] but displayed FD_{allele} scores of 1.41 and 1.43, respectively, compatible with TCE group 2. When these 2 alleles were reanalyzed using the 7 alloreactive T cell clones from this study, lack of recognition by clone numbers 1 through 3 was confirmed; however, both alleles were recognized by at least 1 of the other clones, compatible with their assignment to TCE group 2 (Figure 5A).

On the basis of their FD_{allele} scores, TCE group assignment was predicted also for those 367 HLA-DPB1 alleles encoding distinct proteins that so far lacked a functional assignment (Table 2). For 7 of these alleles, the predicted assignment was functionally tested according to the ability of 7 alloreactive T cell clones from this study to recognize BLCL endogenously expressing the relevant alleles (Figure 5B). For all 7 alleles, the predicted TCE group assignment was confirmed, with 6 of 7, 2 to 3 of 7, and 0 of 7 T cell clones recognizing alleles assigned by the relevant FD_{allele} score to TCE group 1 (DPB1*54:01), TCE group 2 (DPB1*37:01, 35:01, 55:01), or TCE group 3 (DPB1*52:01, 68:01, 73:01), respectively. On the basis of these results, TCE group assignment by FD_{allele} score can be performed not only for all existing HLA-DPB1 alleles but also for any potential new allele that will be reported in the future.

DISCUSSION

In this study, we present the innovative concept of FD for the prediction of permissive HLA-DPB1 mismatches in unrelated HSCT. Taking the lead from our clinically validated model for functional matching [13,15-17,21], we show that HLA-DPB1 TCE groups reflect their FD from HLA-DPB1*09:01, defined as the combined median impact of individual aa polymorphism in the hypervariable HLA-DPβ-chain on T cell alloreactivity. This model takes into account both the

Table 2
TCE Group Classification of HLA-DPB1 Alleles based on FD_{allele} Scores

HLA-DPB1*	FD_{allele}	TCE Group
09:01, 10:01, 17:01, 30:01, 54:01, 131:01, 137:01, 156:01, 157:01, 167:01, 168:01, 241:01, 245:01, 251:01, 265:01, 266:01, 288:01, 289:01, 313:01, 314:01, 324:01, 343:01, 361:01, 384:01, 386:01	≤0-0.5	1
03:01, *06:01*, 08:01, **14:01, *19:01***, 21:01, 25:01, 29:01, 35:01, 37:01, 44:01, **45:01**, 55:01, 58:01, 67:01, 69:01, 70:01, 76:01, 78:01, 79:01, **86:01**, 88:01, 91:01, 92:01, 93:01, 98:01, **104:01**, 106:01, 110:01, 111:01, 113:01, 114:01, 119:01, 124:01, 125:01, 132:01, 136:01, 166:01, 197:01, 207:01, 208:01, 221:01, 222:01, 234:01, 242:01, 243:01, 249:01, 264:01, 269:01, 270:01, 287:01, 293:01, 295:01, 311:01, 312:01, 325:01, 329:01, 344:01, 349:01, 351:01, 358:01, 371:01, 383:01, 385:01, 390:01, 391:01, 394:01, 400:01, 404:01, 405:01	0.6-1.9	2
01:01, 02:01, 02:02, 04:01, 04:02, 05:01, 11:01, 13:01, 15:01, 16:01, 18:01, **20:01**, 22:01, **23:01**, 24:01, 26:01, 27:01, 28:01, 31:01, 32:01, 33:01, 34:01, 36:01, 38:01, 39:01, 40:01, 41:01, **46:01**, 47:01, 48:01, 49:01, 50:01, 51:01, 52:01, 53:01, 56:01, 57:01, 59:01, 60:01, 62:01, 63:01, 65:01, 66:01, 68:01, 71:01, 72:01, 73:01, 74:01, 75:01, 77:01, 80:01, 81:01, 82:01, 83:01, 84:01, 85:01, 87:01, 89:01, 90:01, 94:01, 95:01, 96:01, 97:01, 99:01, 100:01, 101:01, 102:01, 103:01, 105:01, 107:01, 108:01, 109:01, 112:01, 115:01, 116:01, 117:01, 118:01, 121:01, 122:01, 123:01, 126:01, 127:01, 128:01, 129:01, 130:01, 133:01, 134:01, 135:01, 138:01, 139:01, 140:01, 141:01, 142:01, 143:01, 144:01, 145:01, 146:01, 147:01, 148:01, 149:01, 150:01, 151:01, 152:01, 153:01, 155:01, 158:01, 160:01, 162:01, 163:01, 164:01, 165:01, 169:01, 170:01, 171:01, 172:01, 173:01, 174:01, 175:01, 176:01, 177:01, 178:01, 179:01, 180:01, 181:01, 182:01, 183:01, 184:01, 185:01, 186:01, 187:01, 188:01, 189:01, 190:01, 191:01, 192:01, 193:01, 194:01, 195:01, 196:01, 198:01, 199:01, 200:01, 201:01, 202:01, 203:01, 204:01, 205:01, 206:01, 209:01, 210:01, 211:01, 212:01, 213:01, 214:01, 215:01, 217:01, 219:01, 220:01, 223:01, 224:01, 225:01, 226:01, 227:01, 228:01, 229:01, 230:01, 231:01, 232:01, 233:01, 235:01, 236:01, 237:01, 238:01, 239:01, 240:01, 244:01, 246:01, 247:01, 248:01, 250:01, 252:01, 253:01, 254:01, 255:01, 256:01, 257:01, 258:01, 259:01, 260:01, 261:01, 262:01, 263:01, 267:01, 268:01, 271:01, 272:01, 273:01, 274:01, 275:01, 276:01, 277:01, 278:01, 279:01, 280:01, 281:01, 282:01, 283:01, 284:01, 285:01, 286:01, 290:01, 291:01, 292:01, 294:01, 296:01, 297:01, 298:01, 299:01, 300:01, 301:01, 302:01, 303:01, 304:01, 305:01, 306:01, 307:01, 308:01, 309:01, 310:01, 315:01, 316:01, 317:01, 318:01, 319:01, 320:01, 321:01, 322:01, 323:01, 326:01, 327:01, 330:01, 331:01, 332:01, 333:01, 334:01, 335:01, 336:01, 337:01, 338:01, 339:01, 340:01, 341:01, 342:01, 345:01, 346:01, 347:01, 348:01, 350:01, 352:01, 353:01, 354:01, 355:01, 356:01, 359:01, 360:01, 362:01, 363:01, 364:01, 365:01, 366:01, 367:01, 368:01, 369:01, 370:01, 372:01, 373:01, 374:01, 375:01, 376:01, 377:01, 378:01, 379:01, 380:01, 381:01, 387:01, 388:01, 389:01, 392:01, 393:01, 395:01, 396:01, 397:01, 398:01, 399:01, 402:01	>2.0	3

Listed are all 390 HLA-DPB1 alleles encoding different proteins known to date (HLA Nomenclature Report, Release 3.18.0, 2014-10-10). Twenty-three HLA-DPB1 alleles that had previously been classified into TCE groups by T cell cross reactivity patterns [16,17] are indicated in bold. Of these, 21 and 2 alleles with concordant and discordant TCE group classification by both methods are indicated in regular type or in italics, respectively.

heterogeneity of the T cell alloresponse to HLA-DPB1, demonstrated previously by others [37] and confirmed in this study, and qualitative differences between individual aa substitutions in the antigen-binding groove. Interestingly, aa residues with a significant impact on the median alloresponse were those predicted to be able to make contact with bound peptide (positions 9, 11, 35, 55, 69, 76, and 84), whereas no such impact was observed for residues pointing away from the groove (positions 8, 56, and 57). This is in line with recent data indicating that HLA-C*03:03/03:04 is a permissive mismatch combination, possibly due to a limited impact of the single aa difference at position 91 of the HLA class I heavy chain encoded by the 2 variants, on peptide binding and alloreactivity [38].

There are several peculiar characteristics to the FD model that deserve attention. First, we investigated the functional impact of 1 aa at a time, calculating the FD_{allele} scores as the sum of individual impacts. The possibility that certain aa polymorphisms may have antagonizing rather than additive effects when present in combination cannot be ruled out. Second, some of the less frequent polymorphisms in HLA-DPB1, with regards to both position and type, were not studied and their impact was neglected in our FD_{allele} score calculations, raising the possibility that these scores might be underestimated for some of the alleles. Third, FD_{allele} scores were determined here in reference to HLA-DPB1*09:01, the nominal alloantigen used to establish T cell cross reactivity patterns. Further studies using other reference DPB1 alleles, such as HLA-DPB1*03:01 or 04:01, frequent alleles from TCE groups 2 and 3, respectively, as well as HLA-DPB1*02:01, which we proposed to represent a yet distinct fourth TCE group [13], are necessary to determine if the classification established here is linear or complex. Based on these considerations, the FD_{allele} score boundaries for the TCE groups should not be regarded as rigid. However, our results are compatible with an emerging picture that HLA-DPB1 TCE groups, and in turn their respective FD_{allele} scores, reflect structural differences that are correlated with the strength of the alloreactive T cell response. In fact, we and others have shown that the median magnitude of the MLR response is higher against mismatched HLA-DPB1 alleles from different TCE groups, compared with the MLR response against mismatched HLA-DPB1 alleles from the same TCE group [39-41]. Although TCE groups 1 and 2 are characterized by relatively narrow FD_{allele} score ranges of 0.0 to 0.5 and 0.6 to 1.9, respectively, TCE group 3 included a broader range of FD_{allele} scores from 2.0 to 5.5, raising the possibility of further functional heterogeneity of HLA-DPB1 alleles from this group. Interestingly, HLA-DPB1*02:01 and 02:02 have been proposed to represent a distinct fourth TCE group that maintained clinical relevance when analyzed in algorithms of permissive mismatches [13,15]. These 2 alleles have FD_{allele} scores of 2.94 and 2.95, respectively, which might delineate an intermediate FD_{allele} score boundary within TCE group 3. Accordingly, TCE group 4 might include not only HLA-DPB1*02:01 and 02:02, as previously suggested, but also other alleles with FD_{allele} scores ranging, for instance, between 2.0 to 3.0, including, for instance HLA-DPB1*05:01, 11:01, 13:01, 16:01, and 20:01. Further experimental and clinical studies are needed to verify this point.

The ability to predict TCE group assignment of any HLA-DPB1 allele based on the relevant FD_{allele} score is of practical relevance for UD searches, for which a free online Web tool is available for use in clinical practice as well as for research purposes [23]. Indeed, in a recent study of 80

UD-recipient pairs from a Dutch transplantation center, the TCE model could not be applied in 2 pairs (2.5%) because of the presence of an HLA-DPB1 allele with unknown classification [42]. It is possible that the frequency of HLA-DPB1 alleles with unknown classification might be even higher in patients of non-Caucasian origin. In silico prediction of TCE assignment for all known and future HLA-DPB1 alleles on the basis of the data from the present study will allow us to constantly update the Web tool, thereby eliminating the problem of nonclassified rare HLA-DPB1 alleles once and for all. In view also of the rapidly growing number of reported HLA-DPB1 alleles, which rose by 224 alleles since January 2014, only (IMGT/HLA database release 3.16.0 and 3.18.0), this possibility is particularly appealing.

The FD model represents a step forward from historical [43] and more recent [12] observations correlating clinical risks after unrelated HSCT with the matching status for defined aa residues in HLA class I by providing a weighted aa scoring system based on functional evidence. It is intriguing to speculate that the aa residues found to be involved in permissive HLA-A, B, C mismatches [12] might reflect the FD_{allele} scores of the relevant alleles. In more general terms, it can be envisaged that the experimental approach pioneered here of validating established TCE groups in HLA-DPB1 by their FD could also be undertaken inversely, ie, FD_{allele} scores could be used to identify TCE groups that could subsequently be evaluated for risk prediction in unrelated HSCT. This would be useful for HLA loci other than DPB1, where T cell cross reactivity patterns that were the basis of the original TCE grouping are likely to be less well defined due to more complex patterns of aa polymorphism. Ultimately, this might represent an innovative experimental platform for the identification of permissive mismatches in unrelated HSCT.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.10.017.

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