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Canine DLA-79 gene An improved typing method, identification of new alleles and its role in graft rejection and graft-vs.-host disease

Gopalakrishnan M. Venkataraman¹, Dan Geraghty¹, Jenifer Fox¹, Scott S. Graves^{1,2}, Eustacia Zellmer¹, Barry E. Storer¹, Beverly J. Torok-Storb¹, and Rainer Storb^{1,2}

¹Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, 98109

²Department of Medicine, University of Washington, Seattle Washington 98195

Abstract

Developing a preclinical canine model that predicts outcomes for hematopoietic cell transplantation (HCT) in humans requires a model that mimics the degree of matching between human donor and recipient major histocompatibility complex (MHC) genes. The polymorphic class I and class II genes in mammals are typically located in a single chromosome as part of the MHC complex. However, a divergent class I gene in dogs, designated dog leukocyte antigen-79 (DLA-79), is located on chromosome 18 while other MHC genes are on chromosome 12. This gene is not taken into account while DLA matching for transplantation. Though divergent, this gene shares significant similarity in sequence and exon-intron architecture with other class I genes, and is transcribed. Little is known about the polymorphisms of DLA-79 and their potential role in transplantation. This study was aimed at exploring the reason for high rate of rejection seen in DLA matched dogs given reduced intensity conditioning, in particular, the possibility that DLA-79 allele mismatches may be the cause. We found that about 82 percent of 407 dogs typed were homozygous for a single, reference allele. Due to the high prevalence of a single allele, 87 of the 108 dogs (~80 %) transplanted were matched for DLA-79 with their donor. In conclusion, we have developed an efficient method to type alleles of a divergent MHC gene in dogs and identified two new alleles. We did not find any statistical correlation between DLA-79 allele disparity and graft rejection or GVHD, among our transplant dogs.

Keywords

dog; DLA; MHC; MHC class I; graft rejection; GVHD; GVHD model

Correspondence and reprint requests: Rainer Storb, Clinical Research Division, Fred Hutchinson Cancer Research Center, P.O. Box 19024; D1-100, Fairview Ave, N., Seattle, WA 98109, rstorb@fhcrc.org.

Conflicts of Interest (None)

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Authors' contributions:

Gopalakrishnan M Venkataraman: performed the experiments, analyzed data and co-authored the manuscript.

Dan Geraghty: Developed software tools and edited the manuscript.

Jenifer Fox: Assisted in typing of archival DNA samples.

Scott Graves: Supervised dog leukocyte typing core and co-authored the manuscript.

Eustacia Zellmer: Edited the manuscript and reviewed typing data.

Barry Storer: Provided statistical analysis.

Beverly Torok-Storb: Provided support and edited the manuscript.

Rainer Storb: Designed the study and edited the manuscript.

Introduction

The polymorphic class I and class II major histocompatibility complex (MHC) antigens in mammals are coded for by genes typically on a single chromosome. In dogs, the genes for the MHC, termed dog leukocyte antigen (DLA), are located principally on chromosome 12. Three functional genes, DLA-88, DLA-12 and DLA-64 constitute canine class I genes while the class II genes include the polymorphic genes DLA-DRB1, DQA1 and DQB1. In addition, and unique among mammals, dogs have an unlinked divergent class I gene, DLA-79 borne on chromosome 18, which shows limited polymorphism (1,2).

DLA-79 has several unique features compared to other class I genes. The mRNA is broadly expressed in most tissues like other class I genes, but unlike other class I genes it is highly expressed in skeletal muscle (3). The protein structure differs from other classical class I proteins by the addition of one amino acid in the peptide binding groove of the $\alpha 1$ binding domain resulting in an altered peptide binding structure which suggests the molecule may bind to a distinct set of peptides (3). Compared to the highly polymorphic DLA-88, DLA-79 has limited polymorphism with only four alleles previously reported (1).

For over 40 years the dog has served as a clinically relevant model for allogeneic hematopoietic cell transplantation (HCT) in humans. In this preclinical model, histocompatibility matching or mismatching of donor/recipient pairs for HCT has traditionally focused solely on the most polymorphic DLA genes located on chromosome 12 while a possible role of DLA-79 alleles in transplantation outcome has remained unknown. DLA-79 gene is not taken into account while DLA matching for transplantation. Because DLA-79 is unlinked to other class I and class II MHC genes, it is important to determine whether it plays a role in graft rejection or induction of GVHD. In the present study, we improved the method for typing DLA-79 alleles, identified two new alleles and tested the hypothesis that mismatches in the alleles of DLA-79 gene of 136 donor and HCT recipients could result in either graft rejection or graft-versus-host disease (GVHD) in dogs otherwise identical for class I and class II alleles.

Materials and methods

Experimental animals

Randombred litters of beagles and mini-mongrel crossbreeds including Basenji, Golden Retriever, hounds and harriers, were raised at the Fred Hutchinson Cancer Research Center, Seattle, WA, or purchased commercially. HCT were performed mostly using beagles, beagle/mini-mongrels and beagle mixtures of other breeds. In addition, 147 dogs that were not part of these donor/recipient pairs were typed with the aim of identifying new alleles. All transplantation experiments were approved by Institutional Animal Care and Use Committee of the FHCRC. Standard care for dogs was provided as described previously (4,5). Unless otherwise noted, the donor/recipient pairs were siblings and DLA-identical based on matching DLA class I and II-associated VNTR micro-satellite markers (6), which was further confirmed by sequence based DRB1 allele typing (7) and, in some experiments additionally confirmed by DLA-88 typing (8). In all cases, the dogs were matched for transplantation (and called DLA-identical by virtue of the close linkage of these DLA genes) only with reference to DLA genes located on chromosome 12. None of the dogs were typed for DLA-79 alleles prior to transplantation.

Source of DNA

Archived samples of genomic DNA were obtained from dogs given nonmyeloablative 200 cGy total body irradiation (9,10) or 200 or 450 cGy total lymph node irradiation (5) and marrow grafts from DLA-identical littermates. In these as well as in 100 and 50 cGy TBI

dose studies, the general aim was to develop methods to compensate for the reduced immune-suppressive effects of reduced TBI (5,9–14). For the current study, DNA samples were obtained from diverse experimental investigations including induction of recipient tolerance towards hematopoietic cells before transplantation (4,5,11,15), intensifying immunosuppression after transplantation (16), and other methods of enhancing hematopoietic cell engraftment (4,14,15). In GVHD studies, DNA samples were collected from dogs that received 920 cGy TBI and marrow from DLA-identical littermates with standard care and no postgrafting immunosuppression. In these dogs, GVHD developed within 14–44 days in about 50% of the dogs.

DLA-79 typing

Genomic DNA from dogs was isolated from whole blood using either Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) or QIAamp DNA blood mini kit (Qiagen, Valencia, CA). The primers used for gene-specific amplification and sequencing are shown in Table 1. Additional information on the sequence alignment of all 4 class I loci and locus specificity of the primers is shown in the supplementary Figure 1. Amplification reactions (typically 30 μ l) contained about 100 ng of genomic DNA and 200 nM each of the primers and 200 μ M each of dNTPs. The products were amplified using the PCR primers shown in Table 1 and 2 $\times$ Immomix or MyTaq HSRed mix (both from Bioline, Tauton, MA). The reactions were run in a Biorad C1000 Cycloer (Bio-Rad, Hercules, CA). The amplification conditions were 96°C for 2 min; 30 cycles of 96°C for 30 s, 65°C for 15 s, 72°C for 60 s; and 68°C for 5 min. About 2 μ l of the PCR reaction was used for gel analysis, and purified products were directly sequenced with a set of 3 to 5 primers, including the amplification primers. The combinations of alleles were resolved using an in-house custom built software (developed by one of the co-authors, D.G.). Alternatively, and especially when new allele possibilities are presented, a high fidelity Velocity DNA polymerase (0.5 μ l) was used for PCR amplification with the 5 $\times$ GC buffer provided by the manufacturer (Bioline). About 2 μ l of the reaction was used for gel analysis or cloning. The amplified DNA fragments were A-tailed with Taq DNA polymerase at 72°C for 5 min and immediately cloned in a TOPO TA cloning vector according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). DNA from at least 10 and up to 20 clones were sequenced with the same primers described to obtain the identity of both the alleles unambiguously. The intron exon boundaries were identified by aligning with previously described DLA-79 genomic sequence (1), and the derived amino acid sequence of the new alleles were aligned with that of closely related known alleles and the reference allele DLA-79*001:01 using ClustalW2 (17) [<http://www.ebi.ac.uk/clustalw2/>]. A web based program at Phylogeny.fr was used in the 'One Click' mode which is a suite of programs recognized for their accuracy and speed (Muscle for multiple alignment, optional Gblocks for alignment curation, PhyML for phylogeny and TreeDyn for tree drawing (18)). The tree was rooted by including HLA-A*02 as an outgroup.

New allele discovery and designation

All new alleles were verified by cloning and sequencing multiple independent PCR products, derived from at least two dogs. The allele identification method easily met the criteria set forth by the International Society of Animal Genetics (ISAG) DLA nomenclature committee (19), which assigned the allele designations. Only alleles encountered in multiple animals and repeatedly confirmed by independent DNA isolations, PCR, and sequencing reactions were considered for new allele designations. New alleles encountered only once in the breeders were confirmed by typing multiple pups. All allele sequences were submitted to Dr. Lorna Kennedy, ISAG for verification and designation.

Statistical analysis

Statistical analyses were done using Fisher 2-tailed test.

Results

Development of an improved method for typing DLA-79 alleles

The DLA-79 typing strategy involved amplifying polymorphic exons 2 and 3, as well as exon 1 and the two intervening introns, as a single fragment, from genomic DNA. The amplification primers used were specifically designed for this purpose after aligning all four class I genes in this context (Table 1 and supplementary figure 1). The primers were found to specifically amplify the DLA-79 gene. Further, new DLA-79-specific internal primers that provide extensive overlapping reads were designed and used for sequencing. Further improvements in the methods include use of direct sequencing of PCR product and software-aided allele calling of duplex reads, and employment of very high fidelity DNA polymerases and TOPO cloning and sequencing for unambiguous allele calling in cases where new allele specificities were a possibility. The choice of PCR enzymes and buffers, PCR conditions, product purification, and cloning methods used were after extensive testing of various available choices. Use of direct sequencing of PCR products, as well as use of a high fidelity polymerase for amplification, when cloning was employed eliminated incorrect base calls. The typing of 407 dogs revealed four corrections to the originally published sequence, irrespective of the allelic differences, all within intron 2. All of these involved oligo G-runs (Fig 1A.). In addition, additional polymorphic non-coding nucleotides were found within introns 1 and 2 (Fig 1A and Supplementary Fig. 2.).

Identification of new DLA-79 alleles

Four DLA-79 alleles were found by typing a total of 407 dogs from our colony; 240 dogs were from low-dose TBI HCT studies, 30 dogs were from GVHD studies, 43 dogs were breeders, and the remaining 94 were other dogs not part of the transplantation studies. *All* four of the DLA-79 alleles reported earlier, that did not have ISAG designations until this study, have been named 79*001:01, 79*001:02, 79*002:01 and 79*002:02. Two of the four DLA-79 alleles from this study were new, named 79*001:03 and 79*003:01 (Figure 1B). Only one new allele, namely 79*001:03, was found among the HCT donor/recipient pairs. Both of the new alleles, 79*001:03 and 79*003:01, were found in breeders from our colony. The most common 79*001:01 allele was found to be homozygous in over 80 percent of the dogs (Table 2.). One dog was homozygous for the 79*001:02 allele. All the remaining HCT dogs were heterozygous, either 79*001:01, 001:02 or 79*001:01, 001:03. The 79*001:02 allele, differs from the reference allele 79*001:01 by two amino acid substitutions at positions 33 (TTC to TTG; F to L) and 37 (AAC to GAC; N to D). The first new allele, 79*001:03, differed from the reference allele at just one non-synonymous amino acid position, 9 (AAC to GAC; N to D). All the three noted changes were previously unreported polymorphic positions and are found in the alpha 1 domain coded by exon 2, outside the hyper-variable regions, HVR I–III. The second new allele, 79*003:01, differed from the reference allele at multiple non-synonymous codons, namely, 66 (AAC to GAC; N to D), 73 (GTC to TTC; V to F), 76 (AGG to GGG; R to M), 98 (ACT to ATT; T to I), 100 (ACA to GCA; T to A), 115 (CAG to CTG; Q to L), and 152 (ACT to AGT; T to S). In this allele, all seven polymorphic positions fall within the HVR I, II and III, as 3, 3 and 1, respectively. The amino acid sequences of these two new DLA-79 alleles are shown aligned with the previously reported alleles in Figure 1B. A phylogenetic tree, derived from amino acid sequences, showing all the currently known DLA-79 alleles and their relationship to the reference allele for the other class I genes, was derived using a web based program at Phylogeny.fr in the ‘One Click’ mode (see *Materials and methods* for details) and is shown in Figure 2. The divergence of DLA-79 gene can be clearly seen.

Marrow graft rejection and DLA-79 matching

Analysis of DLA-79 allele identity of donor/recipient pairs from 24 different DLA-identical HCT experiments spanning over 14 years, was undertaken to retrospectively investigate whether DLA-79 played a role in graft rejection. DNA from dogs that underwent HCT under standard conditions of 200 cGy TBI and postgrafting immunosuppression with CSP + MMF or CSP + rapamycin (10,13), that led to successful engraftment in 9 out of 10 dogs, was typed for the DLA-79 alleles. All 10 pairs were matched *for alleles of the* DLA-79 gene and were of one genotype, 79*001:01,*001:01, homozygous for the most common and reference allele (Table 3). Statistical analysis by Fisher 2-tailed analysis comparing rejection in DLA-79 matched dogs (63/87; ~72%) vs. mismatched dogs (10/11; 91%), excluding the 200 cGy TBI conditions, gave a p value of 0.28 for rejection due to DLA-79 mismatch. When mismatch in the GVH direction only was included as part of the matched group (71/97; ~73% rejection) and compared with the mismatch in the HVG direction (10/11; ~91% rejection), the p value for rejection due to DLA-79 mismatch was similar, at 0.2850.

GVHD and DLA-79 matching

We next investigated whether DLA-79 mismatch played a role in development of GVHD. Among the 18 donor/recipient pairs typed for DLA-79 in which the recipients were given DLA-identical bone marrow after 920 cGy TBI, followed by postgrafting immunosuppression with CSP+MMF, nine recipients developed acute GVHD. Among these 13 matched pairs, GVHD was seen in five recipients; all of 79*001:01, 001:01 genotype. GVHD was also observed in one of two pairs mismatched for DLA-79 in the HVG direction.—In the remaining three donor/recipient pairs, mismatch was in the direction of GVHD (3 donors: 79*001:01, 001:01, 3 recipients 79*001:01, 001:03). In each case, the recipient developed GVHD (Table 4). The 79*001:03 allele differs from the reference allele 79*001:01 in just one amino acid (N9D) that resides outside of all three HVRs. Thus, only 6 out of 15 matched dogs, including the two that were mismatched in the HVG direction, developed GVHD, and all 3 out of 3 dogs mismatched in the GVH direction developed GVHD. However, statistical analysis of these data failed to indicate a significant relationship between DLA-79 mismatch and GVHD (p= 0.1).

Discussion

We have developed an efficient method to type alleles of the DLA-79 gene and have identified two new alleles. Our study has validated the adequacy of our method of DLA typing of only the most polymorphic genes (DLA-88 and DRB1) for histocompatibility matching of siblings. Among dogs receiving reduced intensity conditioning, 80–90% of the dogs were matched for DLA-79 and in these dogs rejection rate was comparable to that of DLA-79 mismatched dogs. In DLA-79 matched dogs, the rejection was likely due to insufficient conditioning. Experiments involving large mismatches, for example, allele *003:01, which has seven amino acid substitutions versus the reference allele, with all changes mapping to HVR, may result in clear demonstration of a role for DLA-79 allele mismatch in transplantation. Further elucidation of the potential role for DLA-79 in graft rejection and GVHD will require specific breeding between prescreened dogs to obtain DLA identical sibling pairs that differ in specific DLA-79 alleles, in light of the low level of polymorphisms.

It is likely that the DLA-79 gene originated before the divergence of other class I genes by gene duplication events as suggested by its sequence divergence. The small number of polymorphic positions in DLA-79, as well as low allele count, when compared to the highly polymorphic classical class I gene DLA-88, suggests that it is not as subject to divergent allele advantage, and may not be involved in immune response to foreign antigens. Unlinked

MHC genes are known in chicken and zebra fish (20,21). A second DQA gene is known in horse, which appears syntenic with rest of the MHC (22). A polymorphic HLA-DPA like locus has been shown to be unlinked and likely non-syntenic in rats (23). A non-syntenic class I gene has not been described in other mammals. A DLA-79 related gene has been found in giant panda (24) and more recently in brown bear (25) and pinniped marine carnivores (26). According to the latter study, this divergent gene likely originated subsequent to the divergence of cat-like carnivores from dog-like carnivores, but before the separation of dog, giant panda and seals. This gene shows limited polymorphism in each of these species, is related to DLA-79, and is transcribed. Additionally, this analysis of a limited number of harbor seals and gray seals indicated variable gene content with a minimum of 5 or 6 class I genes and a maximum in the range of 11 to 15 genes in individuals, a range and variation greater than that seen in other mammals. The other class I genes in these species, except for the DLA-79 related gene, resemble classical class I genes and belong to a single diversity lineage and do not have a counterpart in terrestrial carnivores. Whether the non-syntenic divergent class I gene is unique to canids or is present in other mammals is presently unknown.

The localization of different MHC genes on different chromosomes enhances MHC plasticity and can be an evolutionary advantage. While the function of DLA-79 protein is not known and studies by Burnett and Geraghty (3) suggest that DLA-79 protein is expressed on the surface of the cells, we are not yet able to demonstrate an antibody response in dogs sensitized to lymphocytes that are matched to DLA-88 and DRB1 but are DLA-79 mismatched. Canine DLA-79-specific antibody reagents are currently not available to study tissue distribution and function in normal and disease states. Efforts are underway in our lab to generate a monoclonal antibody against DLA-79 to further understand the function of this protein.

In conclusion, we have developed an efficient method to type a divergent MHC gene in dogs and identified two new alleles. We did not find any statistical correlation between DLA-79 allele disparity and graft rejection or GVHD, among our transplant dogs (p values 0.28 and 0.1, respectively). The transplantation experiments we analyzed were limited to a small set of breeds, and included only two mismatched DLA-79 alleles that are closely related to the most common allele. Defining the role of DLA-79 allele mismatch in graft rejection or GVHD was hampered by a high prevalence of one particular allele for this gene. To explore this role, targeted studies are needed using specifically bred donor/recipient pairs with greater DLA-79 allele disparity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CSP	cyclosporine
DLA	dog leukocyte antigen
FHCRC	Fred Hutchinson Cancer Research Center
G-CSF	granulocyte colony stimulating factor
GVH	graft-versus-host
GVHD	graft-versus-host disease
Gy	gray
HCT	hematopoietic cell transplantation
HLA	human leukocyte antigen
HVG	host -versus-graft
HVR	Hyper-variable region
MHC	Major Histocompatibility Complex
MMF	mycophenolate mofetil
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
TBI	total body irradiation
VNTR	variable number tandem repeat

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Figure 2. Phylogenetic tree of DLA-79 alleles

A rooted phylogenetic tree derived from the amino acid sequences of exons 2 and 3 of all known DLA-79 alleles and their relationship to the DLA-88, DLA-12 and DLA-64 reference alleles, with HLA-A*002:01 as an out group. A web based program at Phylogeny.fr was used in the 'One Click' mode (see *Materials and methods* for details). Use of advanced options with 100 replicates in a bootstrap analysis gave the same result. Use of exon 2 and 3 nucleotide sequences instead of amino acid sequences from the same data set resulted in a similar tree (Supplementary Fig. 3).

Table 1

Primers Used for DLA-79 Typing

Primer	Sequence	Method
DLA-79F	5'–CAGGGCTGACAGTGTCTCCTCTGC–3'	PCR, sequencing
DLA-79R	5'–CACTGAGTCCACCCTCCCTTCC–3'	PCR, sequencing
DLA-79I1F	5'–CCCTGGCCCCCTTCCCCAC–3'	sequencing
DLA-79I2F	5'–GGGTCCAGGTCGCCATCCCCGTC–3'	sequencing
DLA-79I2R	5'–CCCAGTCGCGTCCCCAGGRGTC–3'	sequencing

Table 2

Frequency of DLA-79 alleles among all dogs typed.

DLA-79 alleles	HCT	partial	GVHD	breeders	other	Total	%#
79*001:01, 001:01	195	13	22	35	70	335	82.3
*001:01, 001:02	16	1	4	6	20	47	11.5
*001:01, 001:03	14	0	4	0	4	22	5.4
001:01, 003:01	0	0	0	1	2	1	0.3
001:03, 003:01	0	0	0	1	1	1	0.3
*001:02, 001:02	1	0	0	0	0	1	0.3

The table lists the number of animals analyzed in different categories: partial: either donor or recipient not typed for DLA-79; other: animals that were not part of the previous groups, from other experiments.

* New allele 79*003:01 was found in two unrelated dogs. This allele was confirmed in multiple pups, which were not included in the calculation of allele frequencies.

The frequency table is with reference to our experimental dogs included for this study only and includes many related dogs. This is not meant to represent allele frequencies in any other population. Almost all pure bred dogs typed in this study were beagles. The two new alleles typed were found only in mini-mongrel mixed breeds. All other alleles observed were found in purebred beagles as well as in mini-mongrel hybrids.

Table 3

Relationship between HCT Graft Rejection and DLA-79 matching

									mismatch direction			
									DLA-79 matched		GVH	
									eng.	rej.	eng.	rej.
Group 200 cGy	TBI/TLI* cGy	donor PBMC	Posttransplant Immunosuppression	References #	n	eng.	rej.					
Combined Group	200	no	CSP + MMF or CSP + rapamycin	(9,10)	10	9	1		0	0	0	0
	450*	no	CSP + MMF	(5)	5	3	2		0	0	0	0
	200*	no	CSP + MMF	(5)	4	0	2		0	1	0	1
	100	+/-	various	(4,9,12-14,16)	57	17	33		0	3	1	3
	50	+/-	various	(15)	42	4	26		2	4	0	6
					108	24	63		2	8	1	10
							87			10		11

eng.: engrafted, rej.: rejected, CSP: cyclosporine, GVH: mismatch in graft-vs.-host direction, i.e., graft is heterozygous and host homozygous with reference to the DLA-79 allele, HVG: mismatch in host-vs.-graft direction, MMF: mycophenolate mofetil;

see here for a detailed account of transplantation conditions and experimental design. Statistical analysis by Fisher 2-tailed analysis comparing rejection in DLA-79 matched dogs (63/87; ~72%) vs. mismatched dogs (10/11; 91%), excluding the 200 cGy TBI conditions, gave a p value of 0.28 for rejection due to DLA-79 mismatch. When mismatch in the GVH direction only was included as part of the matched group (71/97; ~73% rejection) and compared with the mismatch in the HVG direction (10/11; ~91% rejection), the p value for rejection due to DLA-79 mismatch was similar, at 0.2850.

Table 4

Relationship between HCT GVHD and DLA-79 matching

Recipient	Alleles	Donor	Alleles	GVHD
Matched at DLA-79				
G621	*001:01, 001:01	G626	*001:01, 001:01	no
G849	*001:01, 001:01	G853	*001:01, 001:01	no
G975	*001:01, 001:01	G974	*001:01, 001:01	no
H088	*001:01, 001:01	H090	*001:01, 001:01	no
H090	*001:01, 001:01	H088	*001:01, 001:01	no
H042	*001:01, 001:01	H041	*001:01, 001:01	no
H041	*001:01, 001:01	H042	*001:01, 001:01	+
G688	*001:01, 001:01	G687	*001:01, 001:01	+
G693	*001:01, 001:01	G466	*001:01, 001:01	+
G761	*001:01, 001:01	G762	*001:01, 001:01	+
G797	*001:01, 001:01	G800	*001:01, 001:01	+
G452	*001:01, 001:03	G458	*001:01, 001:03	no
G672	*001:01, 001:02	G669	*001:01, 001:02	no
Mis-matched at DLA-79				
<i>- donor target (direction of rejection)</i>				
G738	*001:01, 001:01	G737	*001:01, 001:02	no
G997	*001:01, 001:01	G998	*001:01, 001:02	+
<i>- recipient target (direction of GVHD)</i>				
G458	*001:01, 001:03	G539	*001:01, 001:01	+
G627	*001:01, 001:03	G626	*001:01, 001:01	+
G852	*001:01, 001:03	G853	*001:01, 001:01	+