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The utility of DLA typing for transplantation medicine in canine models

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ABSTRACT. Transplantation medicine is used for the treatment of severe canine diseases, and the dog leukocyte antigen (DLA) is considered to be important in graft rejection. However, the utility of direct sequencing of both DLA classes I and II has not been assessed thoroughly. Eight healthy beagles with identified DLA genes were divided into two sets of four dogs, each including one donor and three recipients for skin transplantation. The following recipients were selected: one dog with a complete match, one with a haploidentical match, and one with a complete mismatch of the DLA gene with the donor. Full-thickness skin segments were obtained from each donor and transplanted to the recipients. A mixed lymphocyte reaction (MLR) assay was performed and analyzed by flow cytometry. Skin grafts of DLA haploidentical and mismatched pairs were grossly rejected within 14 days, whereas in fully matched DLA pairs, survival was as long as 21 days. Histopathological evaluation also showed moderate to severe lymphocytic infiltration and necrosis in DLA mismatched pairs. As seen in the MLR assay, the stimulation index of DLA mismatched pairs was significantly higher than that of fully matched DLA pairs in both sets ($P<0.001$). The allogeneic transplantation results suggested that it is possible to prolong transplant engraftment by completely matching the DLA genotype between the donor and recipient. Additionally, the MLR assay may be used as a simplified *in vitro* method to select donors.

KEY WORDS: cell therapy, dog, major histocompatibility complex, skin graft

The major histocompatibility complex (MHC) is a glycoprotein expressed on the cell surface and involved in self and non-self antigen presentation to the immune system. This antigen presentation is a major immunological barrier that leads to graft rejection and plays a fundamental role in immune responses, such as the elimination of pathogen-infected cells and rejection of transplanted non-self organs and cells [24]. Decreasing numbers of allelic mismatched loci of the MHC gene are related to longer survival and graft acceptance in human allogeneic organ transplantation [13, 21, 27]. Additionally, MHC-mismatched mesenchymal stem cells induce immune responses, such as antibody production, and reduced survival of the transplanted cells [1, 2, 17]. Therefore, in human organ transplantation, matching the MHC gene between the donor and recipient is frequently undertaken.

Allogeneic organ transplantation is an important option for dogs with end-stage organ failure [9], and cell-based transplantation medicine has also gained great interest in this area [7]. It is necessary to match the MHC gene in dogs as graft rejection is the most important factor in allogeneic transplantation. Based on our previous analysis of canine MHC, dog leukocyte antigens (DLAs), and polymorphisms, we established the sequence-based typing method for both DLA class I (*DLA-88* and *DLA-12/88L*) and II (*DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) loci [14, 15]. In addition, we developed the flow cytometric mixed lymphocyte reaction (MLR) for dogs, which is a useful tool for evaluating and characterizing the alloreactive T-cells in transplantation [15]. However, there is limited information currently available regarding DLA matching based on sequence-based typing of DLA class I and II loci for canine transplantation. The aim of this study was to experimentally assess the utility of DLA sequence-based matching between the donor and recipient beagles using skin transplantation (*in vivo*) and an MLR assay (*in vitro*).

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MATERIALS AND METHODS

Dogs

Eight healthy beagles selected based on DLA sequencing were enrolled in this study. Table 1 shows the DLA sequencing and haplotype estimation performed based on previous studies [14, 15]. The dogs were divided into two sets of four, including one donor and three recipients (Set 1 and 2). Based on the estimated haplotypes, the three recipients were selected as follows: one dog with complete match of the DLA gene with the donor, one with haploidentical DLA gene with the donor, and one with complete mismatch of the DLA gene with the donor. Age was matched for both sets and the median was 2.7 years (range; 2.4–3.0 years) and 5.0 years (range; 4.4–5.0), respectively. Each set consisted of males except Set 2, which included one female as a recipient (Dog 6). For blood crossmatching and typing, sample collection was performed from the jugular vein of all dogs into a tube containing EDTA as an anticoagulant. Collected samples were submitted to a commercial laboratory (FUJIFILM VET Systems Co., Ltd., Tokyo, Japan) for both blood cross matching and typing. For each dog, the blood type of dog erythrocyte antigen (DEA) 1.1 and 1.2 was tested using the tube agglutination method. Additionally, a blood cross matching test between the donor and recipient of each group was conducted on EDTA-treated blood using the tube agglutination method. All procedures were conducted in accordance with the Guide for Animal Experimentation and the ethics committee of Nihon University (permission number: AP18BRS008-1).

Skin transplantation

All dogs were anesthetized with general anesthetic, and the transplantation procedures were performed by the same operator. Two full-thickness skin segments were obtained from the lumbar back using an 8 mm-diameter disposable biopsy punch (Kai Industries, Gifu, Japan) from each donor (Fig. 1). Skin segments of the same size were excised from the recipient dogs, and the excised donor skin was grafted by suturing onto the graft bed of the same region of the recipient dog. To eliminate the possibility of adverse effects due to this technique, autologous skin transplantation was performed adjacent to the allogeneic skin graft in the recipient as a control. Bandages were changed every two to three days after the procedure and the grafts were observed at the same time points. On Day 7, one of the two control grafts and one of the two allografts were excised to include the entire skin graft under general anesthesia. The remaining specimens (the second control graft and allograft) were grossly observed until the allograft was rejected and were then biopsied for histopathological evaluation at the same time. Each of the biopsied specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with hematoxylin-eosin, and evaluated under light microscopy. The presence of necrosis and infiltration was assessed on Day 7. Lymphocytic infiltration of the skin tissue observed on the graft on Day 7 was qualitatively measured and scored as follows: minimal, mild, moderate, or severe.

Mixed lymphocyte reaction assay

An MLR assay was performed on the donor and recipient dogs as previously reported [15]. In brief, after freshly isolating peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation from each dog, responder (recipient) PBMCs were stained with CytoTell^{TM}(AAT Bioquest, Sunnyvale, CA, USA), a fluorescent dye similar to 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester. The CytoTell^{TM}-labeled responder T-cells were mixed with stimulator (donor) PBMCs treated with Mitomycin C (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) in a 96-well plate. The responder and stimulator T-cells were adjusted to a concentration of 1×10^6 cells/m*l*. After seven days of incubation, cells in each well were harvested and analyzed with a flow cytometer. The alloreactive T-cell proliferation of the responder (recipient) was quantified by tracing the history of alloreactive

Table 1. Sex, dog leukocyte antigen (DLA) alleles, and haplotype information of the eight beagles

Dog No.	Sex	Haplotype ID	DLA locus				
			DLA-88	*DLA-12*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
Dog 1	Male	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
		Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
Dog 2	Male	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
		Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
Dog 3	Male	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
		Hp.3	**502:01*	**1*	**001:01*	**001:01*	**002:01*
Dog 4	Male	Hp.3	**502:01*	**1*	**001:01*	**001:01*	**002:01*
		Hp.4	**501:01*	**1*	**006:01*	**005:011*	**007:01*
Dog 5	Male	Hp.4	**501:01*	**1*	**006:01*	**005:011*	**007:01*
		Hp.5	**045:01*	**1*	**novL*	**004:01*	**013:03–*017:01*
Dog 6	Female	Hp.4	**501:01*	**1*	**006:01*	**005:011*	**007:01*
		Hp.5	**045:01*	**1*	**novL*	**004:01*	**013:03–*017:01*
Dog 7	Male	Hp.4	**501:01*	**1*	**006:01*	**005:011*	**007:01*
		Hp.6	**041:01*	**1*	**013:01*	**003:01*	**005:01*
Dog 8	Male	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
		Hp.7	**005:01*	**nov2*	**020:01*	**004:01*	**013:01*

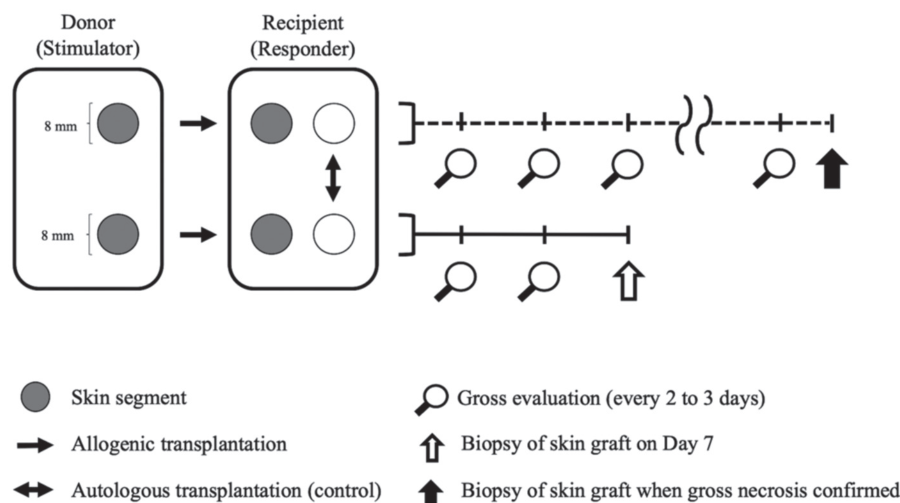


Fig. 1. Experimental design and timeline of skin grafts between donor and recipient dogs. Two full-thickness skin segments were obtained from the donor and transplanted to the recipient. Autologous skin transplantation was performed adjacent to the allogeneic skin graft in the recipient as a control. One of the two grafts was biopsied on Day 7, and the other was biopsied when necrosis was confirmed grossly.

T-cell proliferation in accordance with the halving of the CytoTell™ fluorescence intensity. Cell surface staining to identify T-cell subsets and calculate the stimulation index (SI) was performed as previously reported [15].

Statistical analysis

Paired *t* tests, assuming an equal variances model, were performed and Bonferroni correction was used for multiple comparisons. Additionally, correlations between the SI value and the duration of grossly confirmed engraftment were evaluated by Spearman's correlation coefficients. Statistical analysis was performed with the R statistical program (ver. 3.3.2, R Foundation for Statistical Computing, Vienna, Austria). Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Blood crossmatching and blood typing of eight dogs

Set 1 included one recipient positive for DEA 1.1; however, blood cross matching was negative for both major and minor testing. Set 2 included two positive recipients for DEA 1.1, and minor cross matching was positive in two dogs.

Assessment of allogenic skin transplantation

Table 2 shows the gross duration of skin graft engraftment and histopathological features on Day 7 in each pair. The allogenic skin graft was grossly rejected in the DLA mismatched pairs on Day 11. The grafts in the haploidentical DLA pairs (one allele mismatch pair and four alleles mismatch pair) were rejected on Day 11 and 14, respectively. In the DLA identical pairs, graft engraftments were grossly observed on Day 14 but were eventually rejected on Day 18 for Set 2 and Day 21 for Set 1, respectively (Fig. 2).

On Day 7, moderate to severe lymphocytic infiltration was histopathologically observed in the center and marginal area between the graft and recipient skin, and focal necrosis was observed within the graft tissue of the mismatched pair. Mild to moderate cell infiltration was observed in the center and margin of the graft in the haploidentical pair, with no necrosis in Set 1. In the identical

Table 2. Stimulation index, histopathology of Day 7, and gross duration of graft engraftment results in each pair of beagles

	Donor (Stimulator)	Recipient (Responder)	Grouping by haplotype	Number of mismatched alleles	Stimulation index	Lymphocytic infiltration on Day 7	Features of necrosis on Day 7	Duration of graft engraftment
Set 1	Dog 1	Dog 2	Full-match	0	0.96 ± 0.29	±	-	21 days
	Dog 1	Dog 3	Haploidentical	1	0.99 ± 0.38	+	-	14 days
	Dog 1	Dog 4	Mismatch	5	2.98 ± 0.31[a)]	+++	+	11 days
Set 2	Dog 5	Dog 6	Full-match	0	1.15 ± 0.38	±	-	18 days
	Dog 5	Dog 7	Haploidentical	4	2.57 ± 0.31[a)]	++	+	11 days
	Dog 5	Dog 8	Mismatch	8	6.21 ± 0.47[b)]	++	+	11 days

a ($P<0.01$) and b ($P<0.001$) in the column of Stimulation index denote the significant differences in comparing dog leukocyte antigen (DLA) full-match pairs in each set. Lymphocytic infiltration on Day 7 was scored as minimal (±), mild (+), moderate (++), or severe (+++).

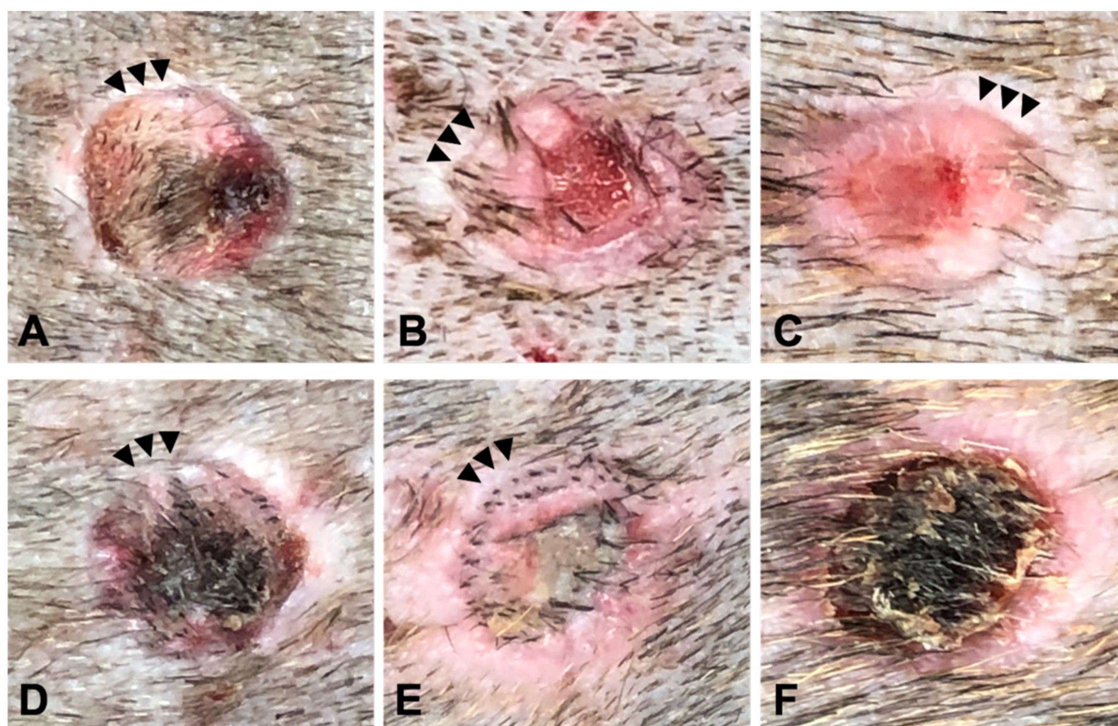


Fig. 2. Gross evaluation features of skin grafts in the recipient of a DLA complete matched pair (Set 2). The center of the skin graft was wounded due to the suturing procedure in both the autologous control (A–C) and allogeneic transplant (D–E). However, the peripheral margin was engrafted on Days 7 (A, D) and 14 (B, E). The autologous control was engrafted until Day 18 (C); however, the allogeneic graft turned a dark, black color and was considered rejected in gross appearance (F). The arrowhead indicates the peripheral margins of the engrafted skin.

DLA pair, the center of the graft was wounded, and with partial epithelial defect, neutrophilic cell infiltration, and necrosis were observed. However, the center included minimal lymphocytic cell infiltration, and the margin of the graft was unclear with minimal cell infiltration observed (Fig. 3). In the histopathology of the rejected specimen, severe lymphocytic infiltration and necrosis were observed for both DLA haploidentical and DLA matched pairs. The mismatched pair showed less lymphocytic infiltration, and the graft was partially replaced by fibroblastic cells and granulation tissue.

The engraftment of control autologous skin grafts was verified both grossly and histopathologically at each time point. On Day 7, gross evaluation indicated that the centers of the control autologous skin grafts were wounded, which was suspected to be caused by the suturing procedure. Histopathological analysis showed partial epithelial defect, neutrophilic cell infiltration, and necrosis in the centers of the grafts, while the margins of the grafts were unclear. These mechanical wounds had improved by the second biopsy.

Quantification of alloreactive T-cells with MLR assay

Based on the DLA sequenced data, Dog 1 and Dog 5 were used as stimulators (donor) in Set 1 and Set 2, respectively. In both sets, the magnitude of the responder (recipient) T-cell alloreactivity and the degree of DLA mismatch between the stimulator (donor) and responder (recipient) were well correlated ($P<0.001$). Additionally, the SIs of DLA haplotype full-mismatched pairs were significantly higher than DLA full-matched pairs in both sets ($P<0.001$). The value of the DLA haploidentical pair SI in Set 2 was also significantly higher than that of the DLA full-matched group ($P<0.01$); however, no significant differences in the DLA haploidentical pair in Set 1 were observed. Further, there was a negative correlation between the SI value and the duration of graft engraftment ($r_s= -0.88$, $P=0.02$). We also quantified the alloreactive CD4- and CD8-positive T-cell proliferation of responder dogs in Set 2 (Fig. 4). The SI value of both CD4- and CD8-positive T-cells in the DLA mismatched group was significantly higher than that in the DLA fully-matched group ($P<0.01$).

DISCUSSION

To our knowledge, this is the first study to perform canine skin allografts in an *in vivo* model while fully determining and matching MHC genes based on molecular typing between donors and recipients. The MHC is the major barrier that contributes to immunological reaction following transplantation, and the success of transplantation relies on the level of compatibility between the donor and recipient for the MHC gene. In humans, organ transplantation based on MHC typing is common and

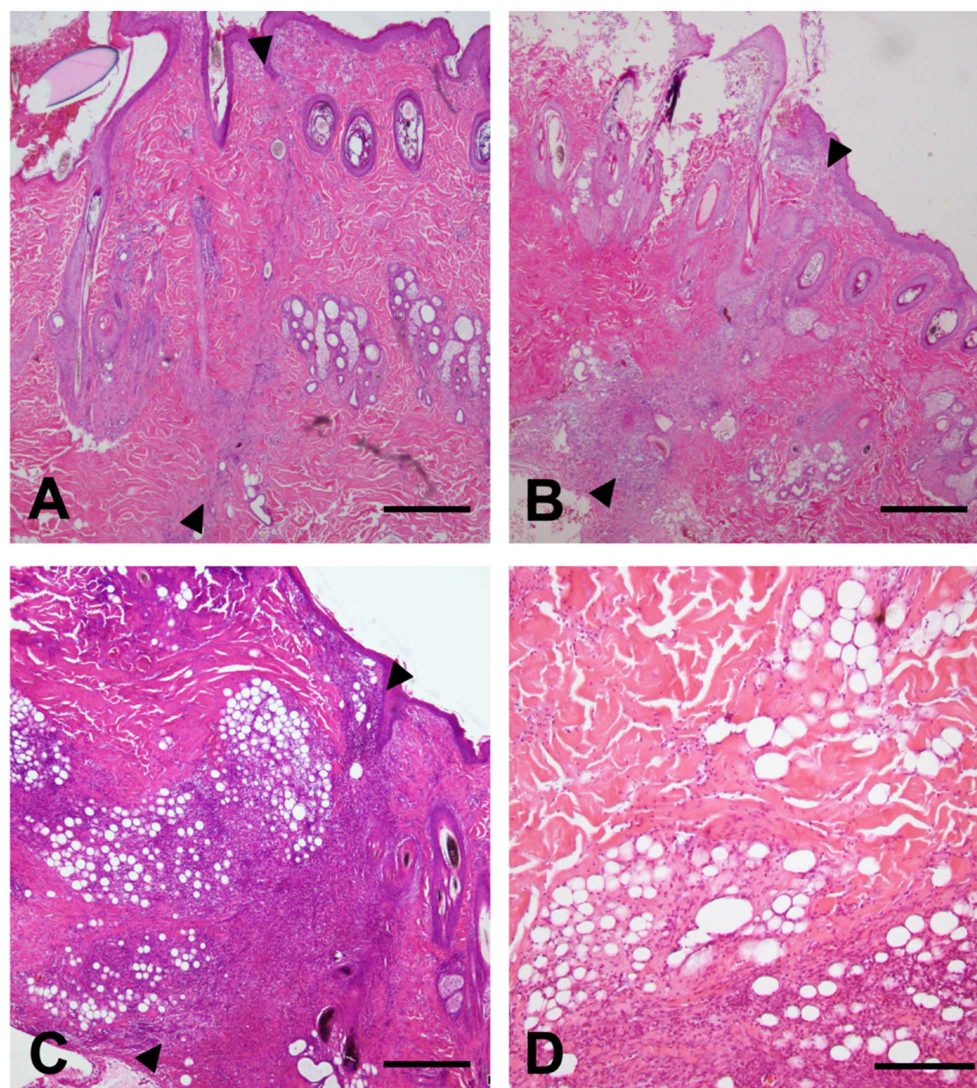
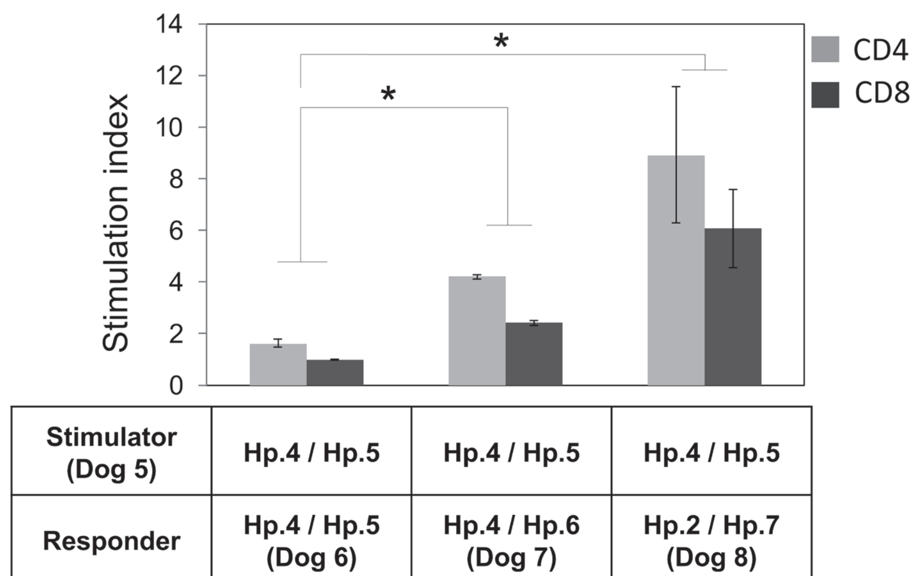


Fig. 3. Hematoxylin and Eosin staining of the allogeneic skin grafts. The center of the figure represents the graft margin (the virtual line between the two arrowheads), and the graft is to the left. The DLA matched graft (A) had less cellular infiltration with unclear margins compared to the haploidentical (B) and mismatched pairs (C). The mismatched pair had the most severe lymphocytic cell infiltration with necrosis of the graft (D). Scale bars=300 μm for A–C and 200 μm for D.

the transplantation of organs with MHC matching reduces acute rejection, prolongs allograft survival, and improves mortality rates [13, 21, 27]. In dogs, intestinal allografts prolonged survival in the DLA matched pair compared to the mismatched pairs [8]. Full-thickness skin grafts are rejected on Days 8 to 12 in complete mismatched murine models [3]. Our skin grafts of DLA haploidentical and mismatched pairs were rejected within 14 days, whereas in the DLA identical pairs, they survived for as long as 21 days. Therefore, our results highlight the importance of DLA matching in prolonging graft survival in dogs.

In the present study, molecular-based typing of the DLA gene was performed to select the donor and recipient. Although several previous studies have attempted to select donors and recipients based on limited DLA loci sequenced or used microsatellite markers located in the DLA genomic region for transplantation [12, 25], there has been no *in vivo* transplantation study involving donors and recipients wherein their DLA types are determined and matched completely based on sequence-based typing. Microsatellite markers used for DLA typing are inadequate in the clinical setting because they have not been characterized in different dogs breeds with distinct DLA alleles. Among different dog breeds, the variation and polymorphisms of DLA genes have been well-characterized by sequence-based typing analysis rather than by using microsatellite markers [11, 14]. Additionally, previous studies that have assessed the rejection of transplanted organs by the degree of DLA agreement are scarce for dogs. In a canine intestinal allograft, the tissue typing was only based on an *in vitro*, serological method and the MLR assay [8]. Although the serological method is a conventional assay for MHC typing, it is likely to be difficult to use in clinical veterinary medicine because stable anti-sera for specific DLA molecules are not often available. Additionally, in humans, the efficiency and accuracy of molecular genotyping is supported by the higher survival rates for unrelated transplantation with matching at the allelic level, compared with



Stimulator (Dog 5)	Hp.4 / Hp.5	Hp.4 / Hp.5	Hp.4 / Hp.5
Responder	Hp.4 / Hp.5 (Dog 6)	Hp.4 / Hp.6 (Dog 7)	Hp.2 / Hp.7 (Dog 8)

Fig. 4. Calculation for alloreactive CD4- and CD8-positive T-cell proliferation among dogs in Set 2. Each bar represents mean ± standard deviation of the stimulation index (SI) for CD4- or CD8-positive T-cells with CytoTell MLR assay among dogs in Set 2 (Dog 5, Dog 6, Dog 7, and Dog 8). The table below the graph shows the combination of dogs for MLR and haplotypes (Hp) in each dog. An asterisk denotes the significant differences (*P*<0.01).

serologically matched pairs [4]. Therefore, the present study provides the utility of sequence-based matching of the DLA gene in canine transplantation medicine.

In human transplantation medicine, although matching of the MHC is advantageous, the tremendous allelic and haplotypic diversity between individuals makes the search for a complete MHC matched donor difficult. Therefore, donors are provided with lifelong administration of immunosuppressants, which may induce side-effects, such as infection and malignancies [28]. In a canine study reporting long-term outcomes post-allogeneic kidney transplants, life-time immunosuppression was required and infectious complications were considered to be the cause of death in 23% of the dogs [10]. To successfully perform long-term management post-transplantation in dogs, withdrawal or reduction of immunosuppressants would be beneficial. In a primate transplantation model of MHC-matched induced pluripotent stem cells, it was reported that MHC matching reduced immune responses by suppressing the infiltration of microglia and lymphocytes into the graft [16]. This study also confirmed that immunosuppressive effects were not sufficient to withdraw immunosuppressive therapy; however, a possibility of reducing immunosuppressant dosage was discussed [16]. In the present study, we demonstrated that the matching of DLA types between the donor and recipient may reduce lymphocytic responses and prolong graft survival. Although it may require appropriate immunosuppression to avoid graft rejection by the minor antigen incompatibility, there may be a possibility of reducing the dose of immunosuppressants.

Our study suggests that matching of the DLA genotype prolongs graft survival in dogs. However, even with a complete DLA-matched donor, the skin graft was eventually rejected. The acceptance of certain organ allografts differs between organs. Skin is recognized as a strongly immunogenic organ and is considered a difficult candidate for allogeneic transplantation [5]. One explanation for the high immunogenicity of the skin is the existence of skin-specific minor alloantigens, which induce weaker immune responses than other alloantigens, such as MHC molecules [5]. A previous study also indicated that cytotoxic T-cells reacting to minor alloantigens of hematopoietic progenitor cells originated in dogs, which had the potential to induce graft rejection [26]. Further, we have included one female recipient in Set 2. In humans, minor histocompatibility encoded on the Y-chromosome is related to reduced graft survival, and the existence of minor antigens related to the Y-chromosome in dogs has also been studied [19, 26]. Additionally, in the present study, blood type minor-mismatched skin transplantation was performed in two dogs. Although the frequency of graft rejection was low compared to that among the blood type incompatible cases, it was reported that rejection can also be caused in blood type minor-mismatched groups in human kidney transplantation [22]. It has also been reported that ABO histo-blood group antigens expressed in vascular endothelial cells in humans are associated with acute rejection after transplantation [6, 20]. Therefore, immune responses to minor antigens may have resulted in delayed rejection in our study.

In the present study, the results of the MLR assay showed that high SI was related to reduced graft survival. The MLR assay mimics the immune response, especially T-cell alloimmune responses related to MHC incompatibility between the donor and recipient in the pre-transplantation state *in vitro* [15]. In human medicine, it has been suggested that the activation of donor-reactive T-cells obtained by MLR assay are closely linked to lymphocytes infiltrating the allografts and, thus, are useful for predicting and monitoring acute rejection during transplantation [23]. In the present study, alloreactive CD4- and CD8-positive T-cell proliferation was observed via the MLR assay in both haploidentical and mismatched groups in Set 2. It is likely that this T-cell alloreactivity

primarily induced the T-cell-mediated acute skin graft rejection 11 days after transplantation. We also detected significant alloreactive T-cell proliferation in the DLA mismatched group in Set 1 (Dog 4). Although we did not quantify the alloreactivity of CD4- and CD8-positive T-cells in Set 1, the graft rejection was observed at the same time after transplantation as in Set 2, suggesting that T-cell-mediated acute graft rejection may have been induced in Dog 4. The present study reveals the discrimination of alloreactive T-cell proliferation in canines in combination with *in vivo* transplantation. Moreover, the study results show that the flow cytometric MLR assay may be used to predict alloreactive T-cell-mediated graft rejection *in vivo* before the administration of any transplant medicine in dogs.

In the present study, when the amino acid sequences of the predicted T-cell recognition site and peptide binding region (PBR) in *DLA-88* alleles were compared among dogs, one residue in the PBR was found to differ between Dog 1 and Dog 4 (p.S21R) (Supplementary Fig. 1A). This disparity may have affected the affinity for peptides presented by the MHC molecule, thus, invoking the activation of the alloreactive CD8-positive T-cells associated with acute rejection. However, as there is only limited information available regarding the structure of DLA molecules and characteristics of the presented peptide, further analysis is required to confirm the exact residues in DLA molecules that influence differential peptide presentation capability.

In recent polymorphism studies involving dogs, DLAs have shown the same allelic polymorphism as in human MHC [11, 14]. The genetic diversity of the DLA gene is lower than that of human MHCs, of which inbreeding is considered a cause [18]. Our previous study revealed that dogs have a remarkably high MHC homozygosity rate and several DLA homozygous haplotypes were detected across many breeds [14, 15]. The small number of polymorphisms, low diversity, and high DLA homozygosity rates are thought to be advantages for allotransplantation in dogs due to facilitation of the matched DLA types between the donor and recipient, resulting in easier allograft acceptance.

In conclusion, we confirmed the utility of sequence-based DLA typing in dogs using *in vivo* (skin transplant) and *in vitro* (flow cytometric MLR) methods, which has not previously been assessed in dogs. DLA matching with sequence-based typing between the donor and recipient in a canine skin transplantation model revealed prolonged allograft survival compared to the mismatched pairs. Additionally, the results of the *in vitro* MLR assay represented the results of *in vivo* skin transplantation well in our study. These results suggest that DLA sequencing may be useful in transplantation medicine and cell-based therapy in veterinary medicine.

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