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ORIGINAL ARTICLE

Evaluation of alloreactive T cells based on the degree of MHC incompatibility using flow cytometric mixed lymphocyte reaction assay in dogs

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Abstract

It has become anticipated that regenerative medicine will extend into the field of veterinary medicine as new treatments for various disorders. Although the use of allogeneic stem cells for tissue regeneration is more attractive than that of autologous cells in emergencies, the therapeutic potential of allogeneic transplantation is often limited by allo-immune responses inducing graft rejection. Therefore, a methodology for quantifying and monitoring alloreactive T cells is necessary for evaluating allo-immune responses. The mixed lymphocyte reaction (MLR) is widely used to evaluate T cell alloreactivity. In human, flow cytometric MLR with carboxyfluorescein diacetate succinimidyl ester has been established and used as a more useful assay than conventional MLR with radioisotope labeling. However, the available information about alloreactivity based on the differences of dog major histocompatibility complex (MHC) (dog leukocyte antigen, DLA) is quite limited in dog. In this paper, we describe our established flow cytometric MLR method that can quantify the T cell alloreactivity while distinguishing cell phenotypes in dog, and T cell alloreactivity among DLA-type matched pairs was significantly lower than DLA-mismatched pairs, suggesting that our developed flow cytometric MLR method is useful for quantifying T cell alloreactivity. In addition, we demonstrated the advantage of DLA homozygous cells as a donor (stimulator) for allogeneic transplantation. We also elucidated that the frequency of alloreactive T cell precursors was almost the same as that of mouse and human (1–10%). To our knowledge, this is the first report to focus on the degree of allo-immune responses in dog based on the differences of DLA polymorphisms.

Keywords Dog · Major histocompatibility complex · Dog leukocyte antigen · Mixed lymphocyte reaction with flow cytometer · Quantification of alloreactive T cells in dogs

Introduction

Recent developments of veterinary medicine with regard to both internal and surgical medicine have contributed to increasing longevity of the domestic dog (*Canis lupus familiaris*); these dogs are now developing refractory age-related disorders, most of which have human homologs (Kaeberlein et al. 2016), such as arthritis (Black et al. 2007), atopic dermatitis (Hall et al. 2010), diabetes (Adin and Gilor 2017), spinal cord injury (Jung et al. 2009), and disc degeneration (Bach et al. 2014). Recently, it has been anticipated that regenerative medicine with mesenchymal stem cell (MSC) transplantation will be extended to veterinary medicine in the form of new treatments for these various refractory and age-related diseases. Regenerative medicine using autologous MSC is disadvantageous compared with using allogeneic MSCs for clinical use because it is time-consuming, laborious,

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and costly to prepare the MSCs for each individual. Therefore, allogeneic transplantation is expected to expand clinically to meet the emergency requirements.

Major histocompatibility complex (MHC) molecules, which are glycoproteins expressed on the cell surface and induce acquired (adaptive) immunity by presenting peptides to T cells, play a central role in controlling the survival or rejection of grafted tissues and cells. The cellular interaction through the MHC-peptide complex contributes to tissue histocompatibility due to cellular discrimination of "self" and "non-self," providing essential information for matching donor and recipient in transplantation medicine (Ali et al. 2013). MHC genes that encode the MHC molecules were characterized as being among the most polymorphic genes (Shiina et al. 2009). For instance, in human MHC (human leukocyte antigen, HLA) genes, 17,191 HLA class I and 6716 class II alleles have been published to date in the IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/, Release 3.37). Of them, protein variants of HLA class I and class II genes are 11,441 and 4491, respectively. The selection of an appropriate donor with an MHC type that matches that of the recipient was often limited by this tremendous allelic and haplotypic diversity. Hence, to advance allogeneic transplantation, there is a need for detailed information about the polymorphisms and haplotypes of MHC genes.

For the last three decades, the genomic organization and polymorphisms of dog MHC (dog leukocyte antigen, DLA) have been elucidated. Three DLA class I loci (*DLA-88*, *DLA-12/88L*, and *DLA-64*) and four class II loci (*DLA-DRA*, *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) are located on chromosome 12, and one divergent DLA class I gene, *DLA-79*, is on chromosome 18 (Burnett et al. 1997; Burnett and Geraghty 1995; Debenham et al. 2005; Miyamae et al. 2018; Sarmiento et al. 1992; Sarmiento et al. 1993; Sarmiento and Storb 1990). From DLA polymorphism analysis, at least 175 DLA class I alleles (133 *DLA-88*, 20 *DLA-12*, 10 *DLA-88L*, 6 *DLA-64*, and 6 *DLA-79* alleles) have been published to date in the IPD/MHC (https://www.ebi.ac.uk/ipd/mhc/) and NCBI databases (https://www.ncbi.nlm.nih.gov/) (Graumann et al. 1998; Miyamae et al. 2018; Ross et al. 2012; Venkataraman et al. 2013; Venkataraman et al. 2017). Of them, a total of 157 protein variants (131 *DLA-88*, 16 *DLA-12*, 10 *DLA-88L*, 4 *DLA-64*, and 6 *DLA-79* proteins) have been identified. Previous polymorphisms analysis suggested that *DLA-64* and *DLA-79* are putative non-classical DLA class I genes (Miyamae et al. 2018; Venkataraman et al. 2013). Polymorphism analysis of DLA class II genes was revealed and at least 207 DLA class II alleles (121 *DLA-DRB1*, 60 *DLA-DQB1*, and 26 *DLA-DQA1* alleles) and 201 protein variants (119 *DLA-DRB1*, 59 *DLA-DQB1*, and 23 *DLA-DQA1* proteins) were published to date in the IPD-MHC database (Kennedy et al. 2007). In addition to the information on those DLA polymorphisms, our previous study also revealed that dogs had a high MHC homozygous rate and several DLA homozygous haplotypes were detected across different breeds, which may facilitate the selection of DLA-matched donors for transplantation across the breeds (Miyamae et al. 2018).

Although polymorphism information on DLA genes has accumulated, there has been little study of alloreactivity to difference of DLA types among dogs. The mixed lymphocyte reaction (MLR) is a widely used assay for evaluating the alloreactivity in both experimental and clinical transplantations. Instead of the traditional tritiated thymidine ([3H]-thymidine) MLR assay, flow cytometric analysis of alloreactive T cell proliferation in MLR by sequential halving of carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescent intensity in accordance with cell divisions, referred to as CFSE MLR assay, has been developed in humans and mice (Lyons 2000; Suchin et al. 2001). CFSE MLR has an advantage over [3H]-thymidine MLR because CFSE MLR assay allows tracking of the history of cell division and analyzing the phenotype and intracellular cytokines of proliferating cells by multi-parameter flow cytometry (Tanaka et al. 2004). Indeed, previous reports showed that CFSE MLR assay was useful for predicting graft rejection or determining the course of subsequent immunosuppression treatment due to enabling the monitoring of T cell alloreactivity in detail both pre- and post-transplantation (Kreijveld et al. 2008; Tanaka et al. 2005). Therefore, MLR assay combined with flow cytometry contributes to the development of biomedical research with allo-transplantation.

In the present study, we developed a flow cytometric MLR assay for dogs using CytoTell, which, like CFSE, is one of the most powerful indicators of cell proliferation (Adachi et al. 2018). Then, we evaluated the T cell alloreactivity using our newly developed MLR assay in 13 beagles, of which the DLA alleles of five loci (*DLA-DRB1*, *DLA-DQA1*, *DLA-DQB1*, *DLA-88*, and *DLA-12/88L*) were completely determined. Our present study provides the essential information and techniques for introducing transplant medicine into the field of veterinary medicine.

Materials and methods

Animals and blood samples

We obtained blood samples from 38 related dogs from four beagle families (Oriental Yeast Co. Ltd. Tokyo, Japan) and 13 unrelated beagles (Kitayama Labes, Japan) that were owned by the veterinary radiology laboratory in Nihon University and Terumo Corporation (Japan). The blood collection was conducted in accordance with the guidelines for animal experiments and animal welfare in each institution.

RNA isolation and cDNA synthesis

Total RNA was isolated from peripheral white blood cells of the 13 beagles using TRIzol LS Reagent (Invitrogen/Life Technologies/Thermo Fisher Scientific, USA), in accordance with the manufacturer's protocol. cDNA was synthesized with oligo-dT primer using the RevaTra Ace reverse transcriptase reaction (TOYOBO, Japan), after isolated RNA had been treated with DNase (Invitrogen/Life Technologies/Thermo Fisher Scientific, USA).

Primers for determination of DLA class I and II allele sequences

Primer pairs used for DLA class I (*DLA-88* and *DLA-12/88L*) cDNA sequence determination were as previously published (Miyamae et al. 2018). Three primer pairs for the determination of cDNA sequences of *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1* alleles were newly designed based on *DLA-DRB1* (accession no. M29611) and *DLA-DQA1* (accession no. NM_001011726) cDNA sequences and *DLA-DQB1* (accession no. AH006318) genomic sequences. Primer information for PCR amplification and sequencing is presented in Supplementary Table 1 and exact primer locations are described in Supplementary Fig. 1. The primer pairs for genotyping three DLA class II loci were certificated

RT-PCR amplification

RT-PCR was performed with a 20-μL amplification reaction containing 30 ng of cDNA template, 0.4 units of KOD FX polymerase (TOYOBO, Japan), 1× PCR buffer, 2 mM of each dNTP, and 0.4 mM of each primer. The cycling parameters were as follows: initial denaturation of 94 °C for 2 min, followed by 33 cycles of 94 °C for 10 s, 60 °C for 30 s, and 68 °C for 45 s for DLA class II (*DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*). These RT-PCRs were conducted with the thermal cycler GeneAmp PCR system 9700 (Applied Biosystems/Life Technologies/Thermo Fisher Scientific, USA).

Sanger sequencing and sub-cloning

RT-PCR products were sequenced by an ABI3130 genetic analyzer (Applied Biosystems/Life Technologies/Thermo Fisher Scientific) with the protocol of BigDye terminator (Ver. 1.1) (Applied Biosystems/Life Technologies/Thermo Fisher Scientific, USA). To resolve phase ambiguity and determine putative novel alleles in heterozygous form, PCR products were cloned into the pTA2 cloning vector with the TA cloning kit (TOYOBO, Japan), in accordance with the manufacturer's protocol, transfected to *Escherichia coli* JM109 competent cells, and four clones per dog were sequenced.

Determination of DLA class I and class II allele sequences and haplotype estimation

Allele sequences were determined by comparing them with already published DLA alleles in the NCBI database (https://www.ncbi.nlm.nih.gov/) and the IPD-MHC DLA database (https://www.ebi.ac.uk/ipd/mhc/) with Sequencher ver. 5.0.1 DNA sequence assembly software (Gene Codes, USA). Allele sequences were also assigned with Assign ATF ver. 1.0.2.45 MHC allele assignment software (Conexio, Australia) from direct sequencing data. DLA haplotypes including all five loci were estimated with the PHASE program (Stephens et al. 2001).

Reagents for cell preparation and culture

Ammonium-chloride-potassium (ACK) buffer (pH 7.4) containing 5.95 M NH_4Cl, 0.1 M $KHCO_3$, and 10^{-3} M EDTA-4Na was used for lysing erythrocytes. RPMI1640 (Sigma-Aldrich, UK), including L-glutamine, supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin solution (Fujifilm Wako Pure Chemical Corporation, Japan), 10 mM Hepes buffer (Dojindo, Japan), and 1×10^{-4} M 2-mercaptoethanol solution (Fujifilm Wako Pure Chemical Corporation, Japan), was used for MLR assay culture.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were prepared from the freshly isolated and heparinized whole blood of 13 beagles by density gradient centrifugation with Optiprep (Axis Shield, UK). The density and osmolality of the Optiprep solution were adjusted to 1.077 ± 0.001 g/mL and 242 mOsm in accordance with the manufacturer's application sheet C43 (Bøyum et al. 1991). Whole blood was diluted with an equal volume of Tricine-buffered saline and layered carefully over the Optiprep solution at the ratio of 2:1. Mononuclear cells were collected after centrifugation at 700×*g* for 20 min at 20 °C and washed once with Eagle's minimal essential medium (MEM; Nissui, Japan). After erythrocytes had been lysed with ACK buffer and centrifuged at 350×*g* for 5 min at 4 °C, these cells were resuspended with phosphate-buffered saline (PBS).

One-way mixed lymphocyte reaction assay with [3H] -thymidine

Freshly isolated PBMCs from each beagle were divided for use as a responder or stimulator for MLR assay. PBMCs used as a stimulator were treated with 50 μg/mL mitomycin C (Fujifilm Wako Pure Chemical Corporation, Japan) at 37 °C for 20 min. After incubation, stimulator cells were washed

five times with PBS. PBMCs from both responder and stimulator were adjusted to a concentration of 5×10^6 cells/mL with RPMI1640. A total of 100 μL of responder cell suspension was added to each well in triplicate in a 96-well plate, and these responder PBMCs were stimulated with the same volume of cell suspension of stimulator cells at 37 °C in 5% CO_2. Then, 18.5 kBq of [3H]-thymidine (PerkinElmer, USA) was added to each well for the last 18 h of culture. After the 18 h of incubation, cells were harvested, washed thrice with PBS, and were lysed with Soluene-350 (PerkinElmer, USA). Seven milliliters of scintillation cocktail Hionic-Fluor (PerkinElmer, USA) was added to each cell lysate and the scintillation was stabilized for 30 min. Count per minute (CPM) was obtained by counting the scintillation with Tri-Carb 4810 (PerkinElmer, USA). Stimulation indices (SI) for assessing the allo-specific reactivity of responder T cells were calculated by dividing the [3H]-thymidine uptake of allo-MLR (CPM) by the [3H]-thymidine uptake of self-MLR (CPM).

CytoTell labeling and one-way CytoTell-based MLR

CytoTell UltraGreen (AAT Bioquest, USA), like CFSE, is a fluorescent dye that is useful tool for monitoring cell proliferation by a flow cytometer. Here, a 500× CytoTell stock solution was prepared in accordance with the manufacturer's protocol. PBMCs freshly isolated from each beagle used as a responder were adjusted to a concentration of 1×10^6 cells/mL and 0.2 μL of the 500× CytoTell solution was added to label these cells. After incubation with protection from light at 37 °C in 5% CO_2 for 30 min, these cells were washed once. Both responder PBMCs labeled with CytoTell and stimulator PBMCs treated with mitomycin C were adjusted to a concentration of 5×10^6 cells/mL with RPMI 1640. One hundred microliters of each cell suspension was mixed in triplicate in each well of a 96-well plate and incubated at 37 °C in 5% CO_2 with protection from light. After finishing the culture period, cells in each well were harvested, washed once, and resuspended with PBS at a concentration of 2×10^5 cells/100 μL.

Cell surface marker staining

The proliferation of responder T cells labeled with CytoTell was quantified by analysis with a BD FACSCanto II cytometer (BD Biosciences, USA). The monoclonal antibodies (mAbs) used for identifying T cells and the T cell subsets were mouse anti-canine CD3 (IgG1, CA17.2A12; Bio-Rad, USA), rat anti-canine CD4 (IgG2a, YKIX302.9; Bio-Rad, USA), and rat anti-canine CD8 (IgG1, YCATEE55.9; Bio-Rad, USA). The appropriate dilution of these antibodies was determined by preliminary titration studies. Cells were stained with anti-CD3 mAbs at 4 °C for 40 min and secondary staining was performed using Alexa Fluor 647-conjugated goat anti-mouse IgG H&L (Invitrogen/Life Technologies/Thermo Fisher Scientific, USA) at 4 °C for 30 min. Then, these cells were divided into two equal samples, washed once with PBS, and each set of cells was stained using either anti-CD4 or anti-CD8 mAbs at 4 °C for 40 min. Secondary staining was performed with F(ab′)2-goat anti-rat IgG (H+L) cross-absorbed secondary antibody conjugated with PE (Invitrogen/Life Technologies/Thermo Fisher Scientific, USA) at 4 °C for 30 min. After finishing cell surface staining, these cells were washed with PBS and resuspended in 100 μL of PBS supplemented with propidium iodide for detecting dead cells in FACS analysis and analyzed immediately.

Quantification of proliferation in CytoTell-based MLR assay

For quantification of CytoTell-labeled T cells as a responder reacting to allo-stimulation, the calculation was performed as previously reported (Tanaka et al. 2004; Wells et al. 1997). When T cells labeled with CytoTell are activated and divide into two cells, the CytoTell fluorescent intensity corresponding to one round of division is decreased by half in comparison with that of the non-proliferating cell population. The cell division rounds from 0 to *n* of alloreactive T cells, which were identified by their CytoTell fluorescent dilution profiles, were obtained from the fluorescent histogram of CytoTell in CD3+, CD4+, or CD8+ positive T cells. The number of precursor T cells at *n* division was extrapolated by dividing the number of daughter T cells that had divided *n* times by 2^n. The total number of proliferation events was obtained by subtracting the total number of daughter T cells from the total number of precursor T cells. The proliferation indices (PI) were calculated by dividing the total number of precursors by the proliferation events (Supplementary Fig. 3). The SI was calculated using the PI of allo- and self-MLR. Precursor frequency was calculated by dividing the total number of alloreactive precursor T cells that had divided once or more by the total number of all alloreactive precursor T cells including non-dividing precursors, as previously described (Noorchashm et al. 1999).

Statistical analysis

Pearson's product-moment correlation and the paired *t* test program with two samples assuming equal or unequal variances models were performed with the R statistical program (ver. 3.3.2).

Modeling of 3D structure for MHC molecules

The SWISS-MODEL server (https://swissmodel.expasy.org/) was used as fully automated protein structural modeling for *DLA-DRB1*001:01*, *DLA-DRB1*001:02*, and *HLA-DRB1*01:01:01* (IMGT/HLA accession no. HLA00664).

Results

Evaluation of the DLA class II polymorphism detection method and confirmation of five DLA loci haplotypes using four beagle families

To evaluate our newly designed DLA class II gene-specific primers and PCR conditions for amplifying and sequencing of *DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*, we initially performed genotyping and haplotyping using 38 related beagles from four families that had well-defined familial relationships (Supplementary Fig. 2). Six *DLA-DRB1*, five *DLA-DQA1*, and five *DLA-DQB1* alleles were identified. *DLA-88* and *DLA-12/88L* alleles of these beagles were determined in our previous study (Miyamae et al. 2018, #95). *DLA-64* and *DLA-79* were excluded from the analysis due to their low polymorphism and expression level (Miyamae et al. 2018; Venkataraman et al. 2013). A total of 10 DLA allelic haplotypes was confirmed without any pedigree discrepancies. To our knowledge, this is the first report to determine DLA haplotypes including both two DLA class I and three DLA class II genes.

DLA polymorphism analysis for unrelated 13 beagles using MLR assay

We performed genotyping and haplotyping of the two DLA class I loci (*DLA-88* and *DLA-12/88L*) and three DLA class II loci (*DLA-DRB1*, *DLA-DQA1*, and *DLA-DQB1*) in 13 beagles before the MLR assay. Although no *DLA-88L* allele was detected, 7 *DLA-88*, 3 *DLA-12*, 11 *DLA-DRB1*, 6 *DLA-DQA1*, and 8 *DLA-DQB1* alleles were detected and a total of 11 haplotypes were estimated, compared with the DLA haplotypes confirmed by our pedigree analysis (Table 1). One of the 11 *DLA-DRB1*, *DLA-DRB1*novL* (DDBJ accession number

Table 1 DLA allele and haplotype information in 13 beagles

Dog no.	Haplotype ID	DLA locus				
		DLA-88	*DLA-12*	*DLA-DRB1*	*DLA-DQA1*	*DLA-DQB1*
Dog 1	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
Dog 2	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
Dog 3	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
Dog 4	Hp.1	**502:01*	**1*	**001:02*	**001:01*	**002:01*
	Hp.3	**502:01*	**1*	**008:01*	**004:01*	**003:01*
Dog 5	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
	Hp.4	**502:01*	**1*	**001:01*	**001:01*	**002:01*
Dog 6	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
	Hp.4	**502:01*	**1*	**001:01*	**001:01*	**002:01*
Dog 7	Hp.2	**012:01*	**1*	**015:01*	**009:01*	**001:01*
	Hp.5	**005:01*	**nov2*	**020:01*	**004:01*	**013:01*
Dog 8	Hp.4	**502:01*	**1*	**001:01*	**001:01*	**002:01*
	Hp.6	**501:01*	**1*	**006:01*	**005:011*	**007:01*
Dog 9	Hp.6	**501:01*	**1*	**006:01*	**005:011*	**007:01*
	Hp.7	**045:01*	**1*	**novL*	**004:01*	**013:03-*017:01*
Dog 10	Hp.6	**501:01*	**1*	**006:01*	**005:011*	**007:01*
	Hp.7	**045:01*	**1*	**novL*	**004:01*	**013:03-*017:01*
Dog 11	Hp.6	**501:01*	**1*	**006:01*	**005:011*	**007:01*
	Hp.8	**041:01*	**1*	**013:01*	**003:01*	**005:01*
Dog 12	Hp.6	**501:01*	**1*	**006:01*	**005:011*	**007:01*
	Hp.9	**045:01*	**1*	**101:01*	**003:01*	**005:01*
Dog 13	Hp.10	**508:01*	**nov1-3*	**002:01*	**009:01*	**001:01*
	Hp.11	**508:01*	**nov1-3*	**011:01*	**002:01*	**013:03*

The **013:03-*017:01* in *DLA-DQB1* locus indicates a haplotype relationship that are composed of *DLA-DQB1*013:03* and *DLA-DQB1*017:01*. This haplotype was previously reported in 2007 (Kennedy et al. 2007). Of the allele names of *DLA-12* "**1*," "**nov1-3*" and "**nov2*" are personal allele names that have not been given official names from IPD-MHC database (Burnett et al. 1997; Miyamae et al. 2018)

LC494674), was newly detected in this study. Three *DLA-DQB1* alleles (*DLA-DQB1*007:01*, *DLA-DQB1*013:03*, and *DLA-DQB1*017:01*) were identified in two dogs, Dog 9 and Dog 10, and of them *DLA-DQB1*013:03* and *DLA-DQB1*017:01* showed a haplotype relationship as reported (Kennedy et al. 2007). Of these 13 beagles, only Dog 1 was homozygous regarding the DLA haplotype.

Development of CytoTell MLR assay

To determine the duration of MLR culture, we initially performed MLR with both CytoTell and [3H]-thymidine MLR assays. We selected Dog 7 and Dog 11 for this initial MLR because this pair has highly disparate DLA alleles (nine DLA allele mismatches). We performed four independent MLR with both assays using Dog 7 or Dog 11 as a responder and calculated SI at four different co-culture periods (4, 5, 6, and 7 days). The SI of the CytoTell MLR assays in both cases using Dog 7 and Dog 11 as a responder kept increasing in the incubation period and peaked at day 7 (Fig. 1a, c). On the other hand, the SI of the [3H]-thymidine MLR assay was almost at the same level as with the CytoTell MLR assay; however, SI elevated faster than CytoTell MLR and peaked at day 6 (Fig. 1b, d). We considered that the different kinetics of SI values between the two assays might be caused by a difference in quantifying cell activation upon allo-stimulation. Namely, in the CytoTell MLR assay, cell activation can be detected only after the cells labeled with CytoTell react to the allo-stimulation and divide into two cells. In contrast, in the [3H]-thymidine MLR assay, cell activation can be detected once the cells reacting to the allo-stimulation incorporate the radioactive thymidine into the DNA for duplication. This fundamental difference between the two MLR assays was likely to be reflected in the different peaks and kinetics of their SI values. However, it was notable that all of SI at day 7 except [3H]-thymidine assay using Dog 7 (Fig. 1a, c, d) were significantly higher than those at day 4 ($p < 0.05$, Bonferroni-corrected). Furthermore, the SI of Dog 7 at day 7 with [3H]-thymidine MLR was also higher than that of day 4, although not significantly (Fig. 1b). Therefore, we used a 7-day culture as the duration for MLR in the subsequent experiments.

Then, based on the result of DLA typing, we divided the 13 beagles into groups of DLA full match (3 pairs), 1 to 4 DLA allele mismatches (8 pairs), and 5 to 10 DLA allele mismatches (10 pairs). Both CytoTell and [3H]-thymidine MLR assays were performed once or twice using each pair and the quantification of T cell proliferation was compared between the two assays. The combinations of dogs for performing MLR assays are shown in Supplementary Table 2A. SI values obtained from both methods were low in DLA-matched dog pairs, whereas they were high in DLA-mismatched pairs. These trends of SI in both methods represented a highly

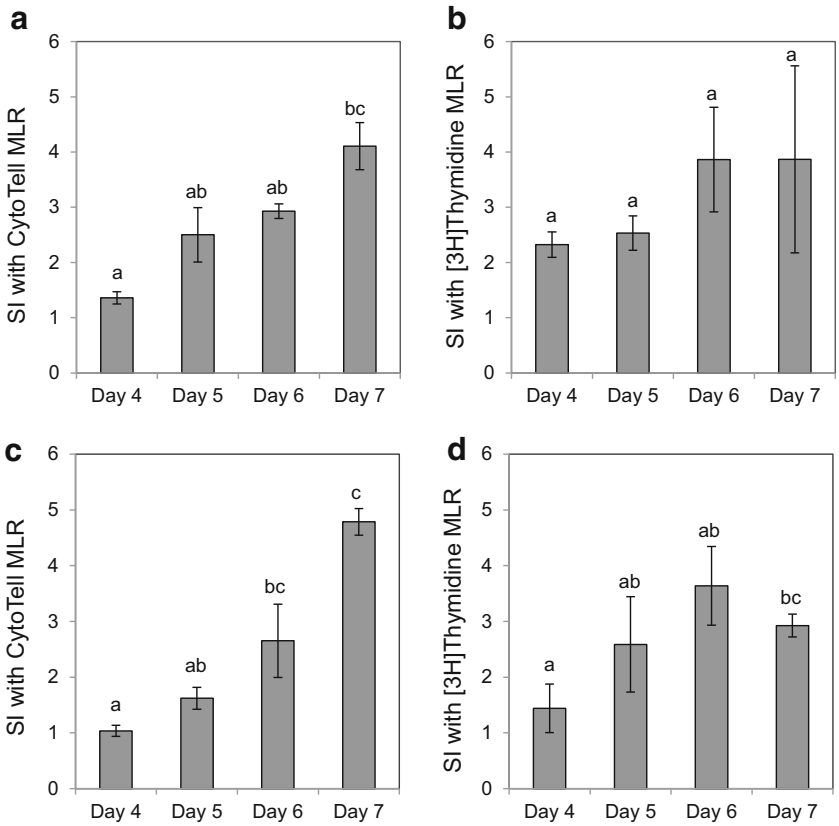


Fig. 1 Kinetics for means of SI quantified with both CytoTell and [3H]-thymidine MLR assay. Means of SI from day 4 to 7 calculated by MLR assay using both Dog 7 (**a**, **b**) and Dog 11 (**c**, **d**) are shown. Error bar represents standard deviation. Different lowercase letters on top of each bar indicate a statistically significant difference of SI among different co-culture periods ($p < 0.05$) (Bars with different letters are significantly different, but bars with same letters are not: e.g., if the same lowercase letter such as "a" or "ab" appears above two bars, then there is no significant differences between those two bars in each graph.). The SI values among four different co-culture periods were compared in each other. Only the SI values within each panel were compared; the SI values between panels were not compared. The *p* values were corrected for multiple comparisons with the Bonferroni procedure

positive correlation (correlation coefficient: $r = 0.709$, $p < 0.001$), suggesting that the newly developed CytoTell MLR assay had almost the same ability to quantify T cell proliferation as the conventional [3H]-thymidine MLR (Fig. 2).

Quantification of T cell alloreactivity with CytoTell MLR assay

To further confirm the relevance between the level of SI and the degree of DLA matching, we performed CytoTell MLR assay with additional pairs and compared the SI among three groups. The pairs and total numbers of MLR tests are listed in Supplementary Table 2A. Although some pairs even in the group with 5 to 10 allele mismatches indicated an SI as low as that in the DLA full match group, in most cases, the intensity of responder T cell alloreactivity and the degree of DLA allele mismatching were well correlated. Importantly, the T cell alloreactivity in the DLA full match group (SI = 1.22 ± 0.60) was significantly lower than that in both groups with 1 to 4 DLA allele mismatches (SI = 2.44 ± 1.35) and 5 to 10 DLA allele mismatches (SI = 3.61 ± 1.73) (Fig. 3). In the dog pairs in which SI was higher than 3 (14 pairs, $n = 27$), the precursor frequency of the responder dogs that reacted to stimulator dog cells was 3.32 ± 2.10%. This suggested that there were about 1 to 5% of alloreactive T cells in dogs.

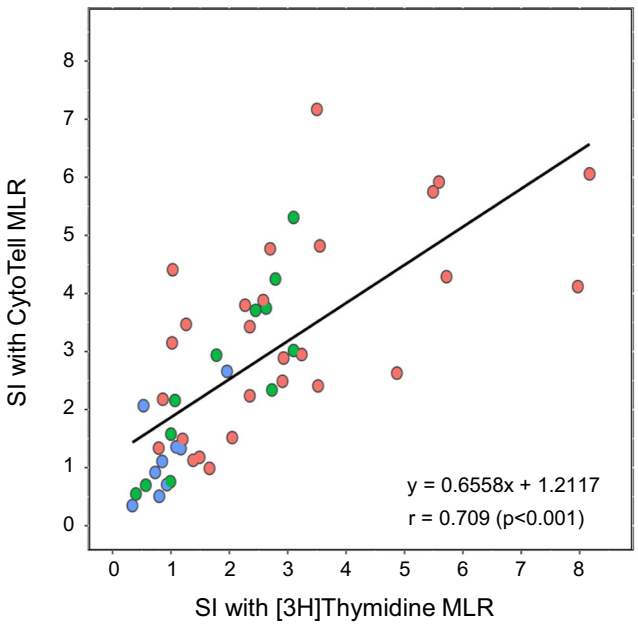


Fig. 2 Comparisons of SI obtained from both CytoTell MLR and [3H]-thymidine MLR assay. Blue, green, and red circles represent the SI from DLA full match, 1 to 4 DLA allele mismatch, and 5 to 10 DLA allele mismatch pairs, respectively. Detailed information about the combinations of dogs is presented in Supplementary Table 2A. The solid line represents the regression line. Regression equation and correlation coefficient by Pearson's product-moment correlation (r) are shown

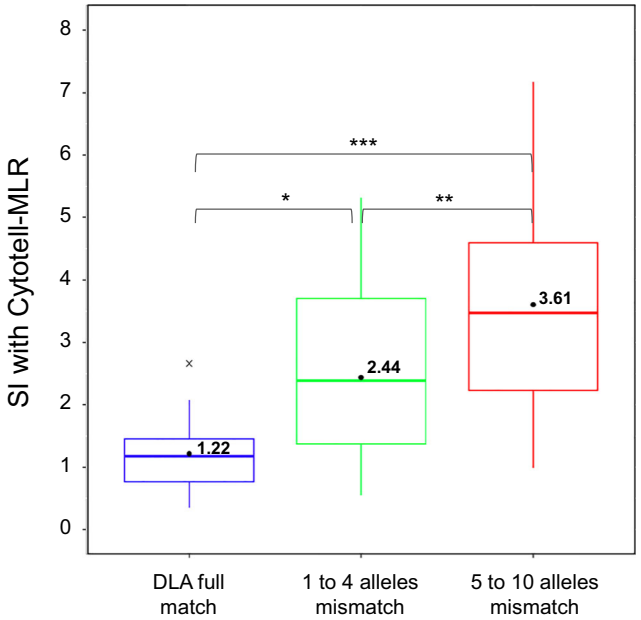


Fig. 3 Comparing the T cell alloreactivity with CytoTell MLR assay among three groups. Blue, green, and red boxplots represent the SI between dog pairs with DLA full match (3 pairs, $n = 14$), 1 to 4 DLA allele mismatch (9 pairs, $n = 18$), and 5 to 10 DLA allele mismatch (11 pairs, $n = 35$), respectively. Detailed information about dog pairs and combinations for MLR are shown in Supplementary Table 2A. "・" and values within each boxplot indicate the means of SI in each group. "×" above the boxplot indicates an outlier. Asterisks denote the significant differences between groups (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, respectively)

Alloreactive CD4 and CD8 T cell proliferation was also analyzed 4 times using two pairs: Dog 7–Dog 9 (8 allele mismatches) and Dog 7–Dog 11 (9 allele mismatches) (Fig. 4 and Supplementary Table 2B). When using Dog 7 cells as a responder, the alloreactivity of CD4 T cells in each pair was significantly higher than that of CD8 T cells in MLR. However, when using Dog 7 PBMCs as a stimulator, although it was not significant, the alloreactivity of CD4 and CD8 T cells varied, suggesting that these results reflected the differences of inherited susceptibility to allo-antigens in the T cell population in each dog.

Assessment of allo-immunogenicity of DLA homozygous dogs with CytoTell MLR assay

Theoretically, using MSCs from a DLA haplotype homozygous individual should facilitate DLA matching between donor and recipient and avoid graft rejection in allogeneic transplantation. To confirm the immunogenicity of DLA homozygous dog, we performed the CytoTell MLR assays between Dog 1 (DLA homozygous) as a stimulator and Dog 2, Dog 3, or Dog 4 as a responder, each of which contains at least one same haplotype as that of Dog 1. In addition to these cells, PBMCs of Dog 7 with 9 DLA allele mismatches to Dog 1 were also used as the stimulator for comparing the T cell

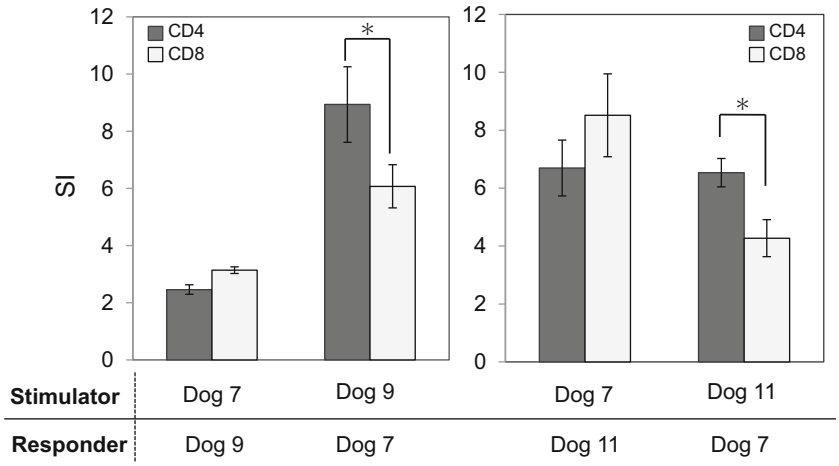
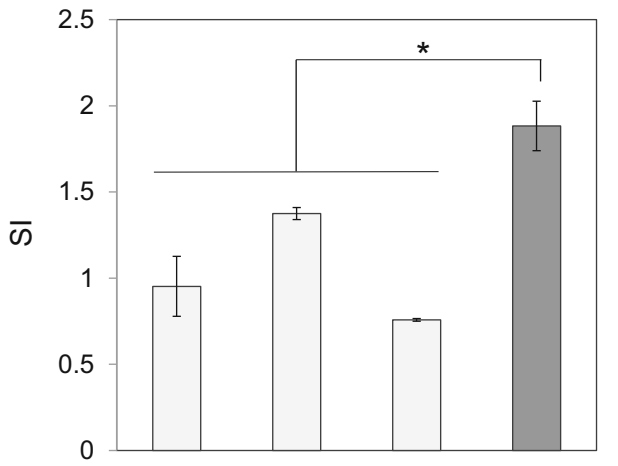


Fig. 4 Quantification of CD4 and CD8 T cell proliferation with CytoTell MLR. Each bar represents mean ± standard deviation of SI for CD4 or CD8 T cells with CytoTell MLR assay in Dog 7–Dog 9 or Dog 7–Dog 11 pairs. Gray and white bars indicate SI calculated in CD4 and CD8 T cell populations after 7 days of MLR culture, respectively. The combination of donor and recipient in each pair for MLR is shown below each graph. Detailed DLA allele and haplotype information is shown in Table 1. An asterisk denotes the significant difference (*$p < 0.05$)

alloreactivity with DLA-matched pairs. CytoTell MLR assays were performed thrice for each pair. These CytoTell MLR assays indicated that T cell allo-responses to Dog 1 of Dog 2 (SI = 0.95 ± 0.17), Dog 3 (SI = 1.36 ± 0.04), or Dog 4 (SI = 0.76 ± 0.01) were significantly lower than that of Dog 7 (SI = 1.88 ± 0.14, $p < 0.05$) (Fig. 5).



Stimulator (Dog 1)	Hp.1 / Hp.1	Hp.1 / Hp.1	Hp.1 / Hp.1	Hp.1 / Hp.1
Responder	Hp.1 / Hp.2 (Dog 2)	Hp.1 / Hp.2 (Dog 3)	Hp.1 / Hp.3 (Dog 4)	Hp.2 / Hp.5 (Dog 7)

Fig. 5 Quantification of allo-immunogenicity of DLA homozygous dog cells with CytoTell MLR. White and gray bars represent the SI of the dogs (Dog 2, Dog 3, and Dog 4) with a haplotype identical to that of the DLA homozygous dog (Dog 1) and a dog with 9 allele mismatches (Dog 7) to the DLA homozygous dog (Dog 1), respectively. CytoTell MLR assays were performed thrice for each pair and the error bar represents standard deviation. The table below the graph shows the combination of dogs for MLR and haplotypes (Hp) in each dog. An asterisk denotes the significant differences ($p < 0.05$)

Discussion

In the present study, we developed and validated the flow cytometric MLR assay in dogs using CytoTell initially by comparing it with conventional [3H]-thymidine MLR assay. Our results demonstrated that the detectability of alloreactive T cell proliferation was almost the same between CytoTell and the conventional [3H]-thymidine MLR assay. Besides, we quantified CD4 and CD8 T cell proliferation by combining the CytoTell MLR assay with cell surface immunostaining. These results suggest that the CytoTell MLR assay is more useful than the conventional [3H]-thymidine MLR assay for both biomedical research and clinical investigation of transplantation because we enable focusing on only alloreactive cells by tracking the proliferation history and exploring their characteristics by detecting cell surface markers and intracellular proteins.

MLR with [3H]-thymidine has often been used for assessing the differences of MHC types among dogs (Raff et al. 1985; Wagner et al. 2002). However, no evaluation of the magnitude of alloreactivity in dogs based on the differences of DLA types determined precisely by a sequence-based typing method has been performed. Our present study indicated that T cell alloreactivity became significantly low in DLA full matched pairs (SI = 1.22 ± 0.60) and higher as the degree of DLA mismatch increased (5–10 allele mismatch group: SI = 3.61 ± 1.73), suggesting that MHC matching between donor and recipient is an essential factor for avoiding graft rejection in dogs as it is in humans.

In the 1-4 alleles mismatched group, a single *DLA-DRB1* allele mismatch between *DLA-DRB1*001:01* and *DLA-DRB1*001:02* was observed in 4 pairs (Dog 2–Dog 5, Dog 2–Dog 6, Dog 3–Dog 5, and Dog 3–Dog 6) (Table 1 and Supplementary Table 2). Interestingly, in comparison among

these four pairs, T cell alloreactivity with Dog 2 and Dog 3 as responders was significantly higher than with Dog 5 and Dog 6 as responders ($p < 0.01$, Supplementary Table 3). A single amino acid disparity at position 47 was the only difference between the both alleles (tyrosine in DLA-DRB1*001:01 and phenylalanine in DLA-DRB1*001:02), and this position was estimated to constitute the peptide-binding sites at pocket 7 by 3D structural analysis compared with HLA-DRB1 (Stern et al. 1994) (Supplementary Fig. 4). This position 47 is thought to induce T cell alloreactivity to donor cells due to the different types of peptides presented, and this consideration may be one of the indicators for predicting allograft rejection in dogs. Since amino acid positions that affect the immune response in the peptide-binding sites and the T cell recognition sites of the DLA molecule are still unknown in dogs, the present study will help to elucidate the differences in immune responses between MHC-type-defined dogs. These trends of disparity of alloreactive T cell populations among allogeneic individuals were observed more obviously in CD4 and CD8 T cell populations and corresponded to those in a previous study using mice; namely, the SI for CD4 and CD8 T cells were significantly different in MLR assays using BALB/c and B6 mice when replacing the stimulator with a responder (Tanaka et al. 2004). Therefore, the strength of alloreactivity when switching recipients and donors is thought to be the differences in each individual's T cell repertoire.

Next, we calculated the precursor frequency for the first time using CytoTell MLR assay by tracing the proliferation history in responder dog T cells that strongly react to stimulators (SI > 3). This result indicated that dogs had $3.32 \pm 2.10\%$ alloreactive T cells. Although the alloreactive T cell precursor frequency can vary among species and depending on the method used for measurement, it has generally been stated that the alloreactive T cell frequency in vivo and in vitro is between 1 and 10% in mice (Suchin et al. 2001; Tanaka et al. 2004), rats (Wilson and Blyth 1968), and humans (Van Oers et al. 1978). Our results indicated that the alloreactive T cell frequency in dogs fitted almost the same range as in other species.

A recent pedigree study indicated that the inbreeding of dogs is steadily advancing due to breed-specific selective breeding for meeting the standards of each breed, resulting in rapid decrease in the genomic diversity within breeds (Jansson and Laikre 2018; Lewis et al. 2015). Loss of genetic diversity by inbreeding affects health and fertility (Leroy et al. 2015; Pedersen et al. 2015). However, low genetic diversity is considered to be one of the advantages for performing allogeneic transplantation because of the facilitation of selecting a donor who is matched to and accepted by the recipient. Indeed, in our previous study, 22 different DLA haplotype homozygotes were identified from only 404 dogs; however, in humans, it is believed that the examination of at least 15,000 individuals is required for detecting 30 different HLA haplotype homozygotes (Miyamae et al. 2018; Nakatsuji et al. 2008). The actual homozygous rate in dogs (24.5%) is about 23 times higher than that of the Japanese population (1.3%). This research clearly suggests that MHC homozygous donors are obtained in dogs by analyzing far fewer individuals than is required for humans. In fact, to resolve the difficulty to perform fully HLA matched transplantation, acceptable disparities of HLA molecules between the recipient and donor have been defined in human hematopoietic transplantation (Heemskerk et al. 2007). This strategy has been used to break through the limitation of HLA matching in transplant medicine. It might be also applied in veterinary region to facilitate choosing an appropriate donor more easily. Further analysis about alloreactivity with DLA incompatible combinations both in vitro and in vivo should help to define acceptable disparities and enlarge the potential pool of grafted stem cell donors. Moreover, in the present study, the CytoTell MLR assay showed that T cell proliferation of DLA heterozygous dogs that had the same haplotype as the DLA homozygous stimulator dog (Dog 1) was inhibited as much as in DLA-identical dog pairs, in accordance with the results of MLR performed in the cynomolgus macaque (Shiba et al. 2016). Thus, we believe that both a high proportion of DLA homozygosity and low allo-immunogenicity of DLA homozygous donor cells could make major contributions to achieving successful allogeneic transplantation using MSCs in veterinary medicine. However, in addition to the cytotoxic T cell-mediated allograft rejection, many reports in humans have shown that donor-specific antibodies (DSAs) against different HLA and non-HLA molecules induce chronic allograft rejection in organ and hematopoietic stem cell transplantation (Ciurea et al. 2011; Zhang et al. 2011). In contrast, since there currently are no publications related to the antibody-mediated allograft rejection in dogs, we need to consider the detection of dog DSAs and evaluation of the impact of allograft rejection in the future.

MSCs are among the most attractive tools for regenerative medicine, including veterinary medicine. Several studies of tissue regeneration in dogs using MSCs demonstrated that allogeneic MSCs with mismatched DLA types had the potential for promoting the engraftment and repair of tissue without an allo-immune response (Arinzeh et al. 2003; Kim et al. 2013; Liu et al. 2013; Silva et al. 2005). Those results are likely to be associated with the immune-modulating effect of MSCs (Aggarwal and Pittenger 2005). Whereas, many reports that the transplantation of allogeneic MSC induces T cells and DSAs to allo-antigens have been published from studies in various species such as the baboon (Beggs et al. 2006), rabbit (Liu et al. 2006), pig (Poncelet et al. 2007), rat (Schu et al. 2012), rhesus macaque (Isakova et al. 2014), and horse (Pezzanite et al. 2015), suggesting that allogeneic MSC from MHC-incompatible individuals could elicit a substantial allo-immune response resulting in graft rejection. Moreover, it has been reported that mouse MSCs expressed MHC class II molecules under proinflammatory conditions and they displayed functions like antigen-presenting cells and this likely indicate induction of an allogeneic immune response in vivo (Eliopoulos

et al. 2005; Stagg et al. 2006). Adipose tissue- and bone marrow-derived canine MSCs were expressed DLA class I but not DLA class II molecules under normal conditions (Screven et al. 2014). The potential for antigen presentation via DLA class II under proinflammatory conditions and the immune-modulating effects, however, has not yet been evaluated properly in canine MSCs. Therefore, to fully understand the potential of allogeneic MSC therapy in dogs, further exploration of the immune responses to allogeneic MSCs is warranted. We emphasize that the CytoTell MLR assay is useful for investigating the allo-immune response and mechanisms about the induction of immune tolerance by MSCs in detail by combining with immunostaining of the cell surface and intracellular proteins.

In summary, herein we developed the CytoTell-based MLR assay with a flow cytometer. Then, using this assay, we evaluated the magnitude of T cell alloreactivity based on the degree of DLA matching, calculated the frequency of alloreactive T cells, and validated the utility of DLA homozygous donor cells in allogeneic transplantation. This information about alloreactivity in dogs could be essential to achieve allogeneic MSC transplantation in veterinary medicine.

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Compliance with ethical standards

Conflicts of interest The authors report no potential conflicts of interest.

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