

# Association of a common dog leucocyte antigen class II haplotype with canine primary immune-mediated haemolytic anaemia

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## Key words

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## Abstract

Immune-mediated haemolytic anaemia (IMHA) is the commonest immune-mediated disease of the dog, representing a major health concern to this species. The aim of this investigation was to determine whether genetic susceptibility to IMHA is associated with genes of the canine major histocompatibility complex (MHC; dog leucocyte antigen system, DLA). Samples were collected from 108 dogs with primary idiopathic, Coombs' positive IMHA. This diseased population was subdivided on the basis of Coombs' test results into two groups: 1) dogs with dominant warm-reactive immunoglobulin (Ig) G haemagglutinins and (2) dogs with an additional or dominant cold-reactive IgM haemagglutinin. The DLA class II alleles and haplotypes of the diseased population were characterised, and these data were compared with those derived from a breed-matched control cohort and a much larger group of DLA-typed dogs. Two haplotypes were increased in the patient group: DLA-DRB1\*00601/DQA1\*005011/DQB1\*00701 (in the group with warm-reactive IgG haemagglutinins only) and DLA-DRB1\*015/DQA1\*00601/DQB1\*00301 (in both groups, but more so in the group with cold-reactive IgM haemagglutinins). One haplotype, DLA-DRB1\*001/DQA1\*00101/DQB1\*00201, was decreased in the total patient group, but this decrease was limited to the warm-reactive IgG haemagglutinins group, and it was actually increased in the cold-reactive IgM haemagglutinins group. A second haplotype, DLA-DRB1\*015/DQA1\*00601/DQB1\*02301, was also decreased in the total patient group, and this decrease was found in both subgroups. In addition, all haplotypes carrying DLA-DRB1\*001 were significantly increased in the cold-reactive IgM haemagglutinins group. When the overall patient group was divided on the basis of individual breeds with more than six animals represented, each of the haplotypes could be shown to be implicated in one of the breeds. Thus, it was apparent that different breeds had different MHC associations with canine IMHA, which is similar to the observation that different human ethnic groups can have different HLA associations with the same immune-mediated disease.

## Introduction

Immune-mediated haemolytic anaemia (IMHA) is a common clinical entity in the dog. Intra- or extravascular haemolysis is mediated by immunoglobulin (Ig) G and/or IgM antibodies that bind to the surface of circulating

erythrocytes and activate the classical pathway of the complement system. These immunoreactants may be detected on the surface of red cells of dog by the Coombs' test (direct antiglobulin test), which is used to confirm the clinical diagnosis. The major clinical distinction in such

dogs is between primary and secondary IMHA. A wide range of underlying diseases and trigger factors have now been described for dogs with secondary IMHA – including previous administration of drugs or vaccines (1, 2), neoplasia (particularly lymphoma, myeloproliferative disease and haemangiosarcoma) (3) and infection (e.g. with *Babesia spp.*, *Leishmania major* or *Ehrlichia canis*) (4). In such cases, management of the underlying disease is of major importance. By contrast, in dogs with primary IMHA, detailed clinical and laboratory examination fails to reveal an underlying causation, and the disease is generally considered to be idiopathic and autoimmune in nature (autoimmune haemolytic anaemia, AIHA). In most western countries (including the UK), the majority of affected dogs at this time have primary IMHA.

Primary IMHA generally presents as a profound regenerative anaemia of acute or chronic onset (5). The disease most commonly develops in middle-aged dogs without any apparent gender bias. Breed predispositions for primary IMHA have been reported with the condition being more common in the English Cocker Spaniel, Old English Sheepdog, Poodle and English Springer Spaniel (6, 7). Familial clustering of IMHA is also sometimes observed in pedigrees, and the disease can coexist with other autoimmune conditions, particularly immune-mediated thrombocytopenia (8, 9).

A seasonal incidence of canine primary IMHA has been described (10, 11), and clinical episodes of the disease can be precipitated by stress factors such as oestrous or whelping (7). There has been limited investigation of the immunopathogenesis of canine primary IMHA. The target erythrocyte membrane autoantigens have been defined (10), and peripheral blood lymphocytes from affected dogs are known to make proliferative responses to recombinant peptides derived from the sequence of these molecules (12). This latter observation suggests a role for antigen presenting cells and class II molecules of the major histocompatibility complex (MHC) in the activation of autoreactive T lymphocytes in these dogs. For this reason, the canine MHC genes are considered to represent candidate susceptibility genes in dogs with primary IMHA.

The canine MHC plays a central role in the control of the immune response in dogs. The canine MHC, as in most species, consists of three regions of tightly linked genes (class I, II and III), the first two of which are involved in regulation and the presentation of self and non-self antigens to the immune system. In dogs, the MHC is referred to as the dog leucocyte antigen (DLA) system and molecular characterisation has only recently begun. The DLA system is known to contain class I and II genes although the precise number of genes on different haplotypes has not yet been determined. DLA class II genes appear to be highly polymorphic, but the full extent of this polymorphism has again not been determined.

We have previously investigated DLA-DRB1, DQA1 and DQB1 polymorphism in the dog and established molecular-

based methods suitable for routine DLA genotyping (13–16). There are currently 90 DLA-DRB1, 22 DLA-DQA1 and 54 DLA-DQB1 alleles officially recognised by the International DLA Nomenclature Committee under the auspices of the International Society for Animal Genetics, which is presently co-ordinated by LJK (17, 18). It is likely that additional DLA-DQ and DR polymorphisms will be identified, especially as more breeds are examined. We have identified extensive interbreed but minimal intrabreed DLA variation (16, 18, 19). Strong linkage disequilibrium exists between DLA class II loci with many examples of preferential allelic association. Some 'established' or conserved DLA haplotypes may be characteristic of a particular breed (19, 20). Thus, the pattern of distribution of DLA types in different dog breeds is analogous to the differences seen between different human ethnic groups and populations.

Domestic dogs have been actively line-bred for the past few hundred years to achieve extreme phenotypic variation between different pedigrees, but minimal genetic variation still remains between breeds. Such breeding programmes have inadvertently resulted in differences in breed susceptibility to certain immune-mediated diseases, including IMHA (21, 22), diabetes (23), hypothyroidism (24) and Addison's disease (25). With the completion of the Dog Genome Assembly and initiation of ongoing breed genetic diversity projects, comparative genomic studies in diseases, including IMHA, could provide important information into such complex genetic disorders.

MHC genes are known to be the major contributory factors to the development of autoimmune conditions in humans. Extensive studies have documented the association of particular HLA alleles with specific human autoimmune diseases such as type I diabetes (26–29) and multiple sclerosis (30, 31). It therefore comes as no surprise that a number of canine autoimmune conditions are associated with certain DLA class II alleles and haplotypes, including polyarthritis (32), hypothyroidism (33, 34) and diabetes (35).

Human patients with AIHA have been reported to have increased frequencies of the HLA class I alleles, B8, (36) and B27 (37), and also reduced frequency of HLA-DQ6 (38, 39). There are no other reports of studies on HLA class II for human AIHA, so these class I associations may reflect linkage with other MHC genes. Human AIHA can be subdivided according to whether the antibodies are 'cold' or 'warm' in their *in vitro* reactivity, i.e. whether they cause agglutination at +4°C or at +37°C (40). The autoantibodies in the cold disease are usually of the IgM class, whereas in the warm disease they are IgG. Patients with AIHA mediated by either type of autoantibody show some HLA associations (41). The same spectrum of warm- and cold-reactive antibodies is documented in canine IMHA (10).

The aim of this study was to determine whether the development of canine primary IMHA shows any association with particular DLA class II alleles or haplotypes.

## Materials and methods

### Animals

Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood samples from dogs with IMHA were submitted for a diagnostic Coombs' test to the Clinical Immunology Laboratory at the School of Clinical Veterinary Science, University of Bristol. A full Coombs' test was performed in a microtitre system using serial dilutions of four antisera – a polyvalent canine Coombs' reagent, anti-dog IgG(Fc), anti-dog IgM(Fc) and anti-dog complement C3. These reagents were all preabsorbed with pooled normal canine erythrocytes, and the Coombs' test was performed in duplicate at both 4°C and 37°C. The inclusion criteria for dogs in this study were 1) the presence of anaemia with haematocrit below the normal reference range (37–55%), 2) a positive Coombs' test and 3) absence of underlying disease as shown by brief historical notes provided by the attending clinician for each case. The residual blood from these diagnostic procedures was stored frozen at –20°C for subsequent analysis of DLA genes.

Samples from a total of 108 dogs that met these criteria were collected over a period of 3 years (May 2003–May 2006). These samples comprise 62 females (31 entire and 31 neutered) and 43 males (28 entire and 15 neutered). Gender was not recorded for three dogs. More than 50% of the dogs were one of five breeds. These included 17 English Cocker Spaniels (16.5%), 16 Springer Spaniels (14.7%), 15 Collies (13.8%), six Dobermans (5.5%) and six Labradors (5.5%). The remaining 48 dogs were from 33 other breeds, but either as singletons or as pairs of animals. The dogs with IMHA within this study were all, to our knowledge, individual pet animals owned by different human families. The School of Clinical Veterinary Science at the University of Bristol receives IMHA samples from all over the UK and also from Jersey, which makes it less likely that the samples will be closely related. It was not possible to definitively investigate the relatedness of this population as pedigree certificates were not available for review. The owners of the majority of pet dogs will generally not have pedigree certification unless they intend to show or breed from the animal. An analysis of the date of birth of dogs of specific breed within the IMHA population has confirmed that at least (the majority of) these animals could not have been siblings.

The population of 108 dogs was further subdivided into two groups on the basis of the Coombs' test results. In the first group ( $n = 69$ ), the Coombs' test showed the presence of a dominant warm-reactive IgG antibody associated with the erythrocytes of dog. In the second group ( $n = 27$ ), there was an additional or dominant cold-reactive IgM haemagglutinin. There is anecdotal clinical evidence that the former pattern of reactivity is associated with milder, chronic-onset IMHA, whereas dogs with cold agglutinins often have more severe, acute-onset disease (3).

DNA samples from control dogs without IMHA ( $n = 750$ ) were obtained from residual blood samples taken for diagnostic purposes at the Small Animal Hospital, University of Liverpool. A subset of 178 breed-matched controls was also used in the analysis.

### MHC genotyping for DLA-DRB1, DQA1 and DQB1

DNA was extracted from all samples using a standard phenol–chloroform method. DNA concentration was measured using a spectromax spectrophotometer, and samples were normalised to 20 ng/μl. All the dogs were characterised for three DLA class II loci using sequence-based typing (13, 42).

All polymerase chain reaction (PCR) were performed with 25 ng DNA in a 25 μl reaction containing 1 × PCR buffer, as supplied by Qiagen (with no extra magnesium), Q solution (Qiagen, Crawley, UK), final concentrations of 0.1 μM for each primer, and 200 μM each deoxyribonucleotide triphosphate, with 2 units of Taq polymerase (Qiagen HotStarTaq). A negative control containing no DNA template was included in each run of amplifications to identify any contamination.

Forward and reverse primers used for DLA-DRB1, DQA1 and DQB1 were DRBF: GAT CCC CCC GTC CCC ACA G and DRBR3: CGC CCG CTG CGC TCA, (33), DQAIin1: TAA GGT TCT TTT CTC CCT CT and DQAIin2: GGA CAG ATT CAG TGA AGA GA (43), and DQB1B: CTC ACT GGC CCG GCT GTC TC and DQBR2: CAC CTC GCC GCT GCA ACG TG (42, 43), respectively. All the primers are intronic and locus specific. The product sizes were 303 bp for DLA-DRB1, 345 bp for DLA-DQA1 and 300 bp for DLA-DQB1.

A standard Touchdown PCR protocol was used for all amplifications, which consisted of an initial 15 min at 95°C, 14 touchdown cycles of 95°C for 30 s, followed by 1 min annealing, starting at 62°C (DRB1), 54°C (DQA1) and 73°C (DQB1) and reducing by 0.5°C each cycle, and 72°C for 1 min. Then 20 cycles of 95°C for 30 s, 55°C (DRB1), 47°C (DQA1) and 66°C (DQB1) for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

To check for the presence of a product, 5 μl PCR product was run on a 2% agarose gel. Prior to sequencing, all samples were purified as follows: 2 units of shrimp alkaline phosphatase (Amersham, Little Chalfont, UK), and 10 units of Exo1 (New England Biolabs, Hitchin, UK) were added to 5 μl of PCR product. The mixture was incubated for 1 h at 37°C, then for 15 min at 80°C. Cycle sequencing was performed using Big Dye Terminator V3 (Applied Biosystems, Warrington, UK), and samples were sequenced on an Applied Biosystems 3100 Genetic Analyzer. Sequencing data were analysed using MatchTools and MatchTools Navigator (Applied Biosystems).

### Haplotype assignment

Three-locus, DLA-DRB1/DQA1/DQB1, haplotypes were identified by following a sequential analytical process. First,

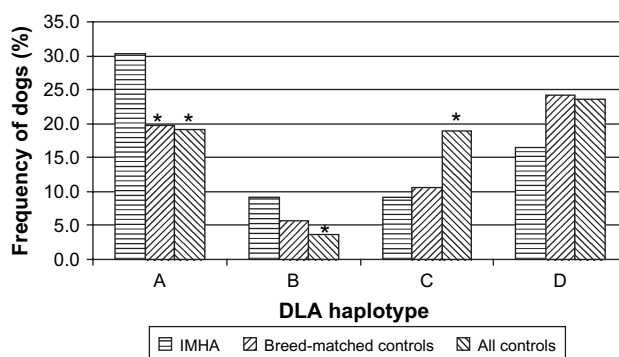
all dogs that were homozygous at all three MHC loci were selected, and from these, several different DLA-DRB1/DQA1/DQB1 haplotype combinations were identified. Dogs that were homozygous at only two loci were then selected. From these dogs, many of the previous haplotypes were confirmed and also several additional haplotypes were identified. The remaining dogs were examined using the haplotype data already identified, and haplotypes were assigned to each of these dogs. From these dogs, further possible haplotypes were identified.

Allele and haplotype frequencies in each group were compared by  $\chi^2$  statistics, and odds ratios with 95% confidence limits were calculated. This analysis involves multiple comparisons, and therefore, the results have to be interpreted with caution as some of these results may be false positives. However, when the same result is found in different data sets, the chances of a result being a false positive are reduced. Ideally, these results should be tested in a second independent data set.

## Results

Three-locus DLA haplotypes were assigned to each case and control dog. The percentage of case and control dogs carrying each haplotype was considered. When the 108 dogs with primary IMHA were compared with the 750 controls, two haplotypes were significantly increased in the diseased dogs: DLA-DRB1\*00601/DQA1\*005011/DQB1\*00701 (30.3% vs 19.1%, OR = 1.8, CI = 1.2–3.0,  $P < 0.01$ ) and DLA-DRB1\*015/DQA1\*00601/DQB1\*00301 (9.2% vs 3.7%, OR = 2.6, CI = 1.1–5.8,  $P < 0.02$ ), while two other haplotypes were reduced: DLA-DRB1\*001/DQA1\*00101/DQB1\*00201 (9.2% vs 18.9%, OR = 0.4, CI = 0.2–0.9,  $P < 0.02$ ) and DLA-DRB1\*015/DQA1\*00601/DQB1\*02301 (16.5% vs 23.6%, not significant). Similar results were shown when comparing the diseased dogs with the subset of matched controls, although only the increase in DLA-DRB1\*00601/DQA1\*005011/DQB1\*00701 haplotype reached significance (Figure 1). The difference between DQB1\*00301 and DQB1\*02301 is one amino acid.

A second analysis was performed on the basis of subgroup defined by Coombs' test reactivity. There was no specific breed association with the nature of this reactivity. The DLA allele and haplotype frequencies in these two subgroups were compared to those of the breed-matched control group ( $n = 178$ ) (Figure 2). This analysis suggests that each of the associations found in the overall IMHA population can be ascribed to either of the subgroups defined by serological reactivity in the Coombs' test. The increase in DLA-DRB1\*00601/DQA1\*005011/DQB1\*00701 was only seen in those cases with a dominant warm-reactive IgG antibody. The increase in DLA-DRB1\*015/DQA1\*00601/DQB1\*00301 was still seen in both subgroups but was more markedly



\* indicates control groups that are significantly different from the cases.

DLA haplotypes:

A, DRB1\*00601/DQA1\*005011/DQB1\*0701;

B, DRB1\*015/DQA1\*00601/DQB1\*00301;

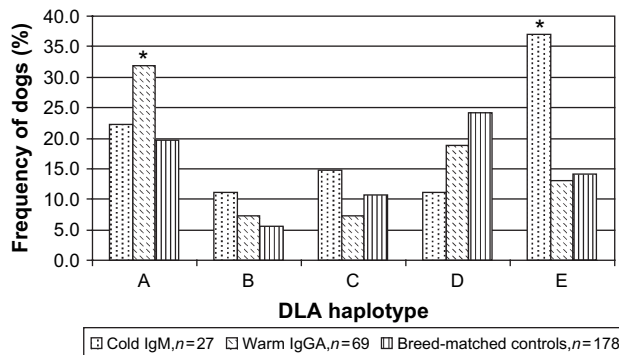
C, DRB1\*001/DQA1\*00101/DQB1\*00201 and

D, DRB1\*015/DQA1\*00601/DQB1\*02301.

**Figure 1** Susceptible and protective haplotypes for immune-mediated haemolytic anaemia.

seen in the subgroup of dogs with a cold-reactive IgM haemagglutinin.

For one of the haplotypes that was decreased in the total patient group, DLA-DRB1\*001/DQA1\*00101/DQB1\*00201, the decrease was limited to the warm-reactive IgG haemagglutinins group, and it was actually increased in the cold-reactive IgM haemagglutinins group. The second haplotype that was decreased in the total patient group, DLA-DRB1\*015/DQA1\*00601/DQB1\*02301, was decreased in both subgroups. Additionally, all haplotypes carrying DLA-DRB1\*001 were greatly increased in the subgroup of dogs with cold-reactive IgM antibodies. Only two of these differences reached significance (Figure 2), which is due to the small numbers in these groups. It was not possible to further analyse these groups by breed as the numbers were too small.



\* indicates control groups that are significantly different from the cases.

Haplotypes A–D are the same as for Figure 1. E, all DRB1\*001 haplotypes.

**Figure 2** Division of immune-mediated haemolytic anaemia on the basis of Coombs' test results.

A third analysis was performed on the basis of breed – selecting the five breeds in the overall diseased dog population with larger numbers represented. This analysis was more complicated, as each breed showed different effects. Dobermans showed no clear differences between cases ( $n = 6$ ) and controls ( $n = 23$ ). Collies showed a decrease in DLA-DRB1\*002/DQA1\*00901/DQB1\*00101 (4/15 cases vs 15/39 controls, 26.7% vs 38.5%), which was not different in the overall group (Figure 3). However, none of the six Labradors with IMHA had DLA-DRB1\*015/DQA1\*00601/DQB1\*02301, whereas 30/69 (43.5%) of controls had this haplotype. Similarly, 34.8% of controls but no dogs with IMHA had DLA-DRB1\*001/DQA1\*00101/DQB1\*00201 (Figure 4).

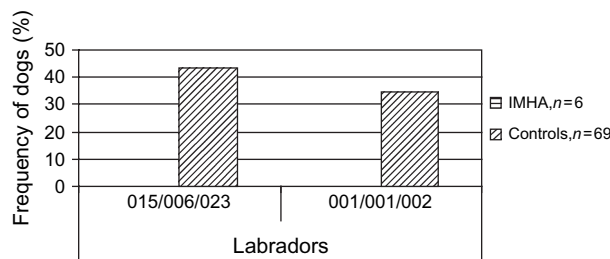
Sixteen of 17 Cocker Spaniels with IMHA had DRB1\*00601/DQA1\*005011/DQB1\*00701, and only one had the related haplotype carrying DQB1\*02001 instead of DQB1\*00701. However, although 26/28 Cocker spaniel controls had DRB1\*00601, 20 (71.4%) had the haplotype with DQB1\*00701 and 12 (42.9%) had the haplotype with DQB1\*02001 (Figure 5).

English Springer Spaniels with IMHA showed an increase in DLA-DRB1\*015/DQA1\*00601/DQB1\*00301, which was present in 5/16 (31.3%) cases compared with 3/15 (20.0%) controls. These dogs also showed an increase in another haplotype DRB1\*00101/DQA1\*00201/DQB1\*01303, which was present in 6/16 (37.5%) cases compared with 2/15 (13.3%) controls (Figure 6).

**Discussion**

The results of this study suggest that an underlying DLA association exists with primary IMHA in dogs and that this may represent an important immunological risk factor in the aetiology of this condition. A genetic component to IMHA has long been suspected, given the predilection of this disease to occur in particular breeds and reports of familial occurrence.

Two potential DLA risk haplotypes: DLA-DRB1\*00601/DQA1\*005011/DQB1\*00701 and DLA-DRB1\*01501/DQA1\*00601/DQB1\*00301, and one protective haplotype: DLA-DRB1\*00101/DQA1\*00101/DQB1\*00201 were identified, although only the association with DLA-DRB1\*00601/

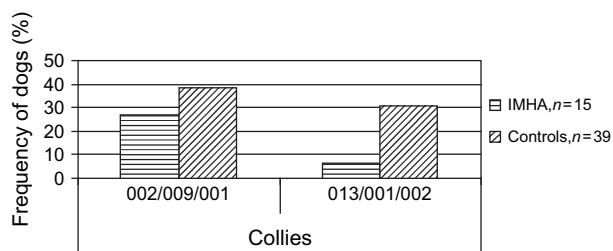


**Figure 4** Dog leucocyte antigen haplotypes in Labradors with immune-mediated haemolytic anaemia.

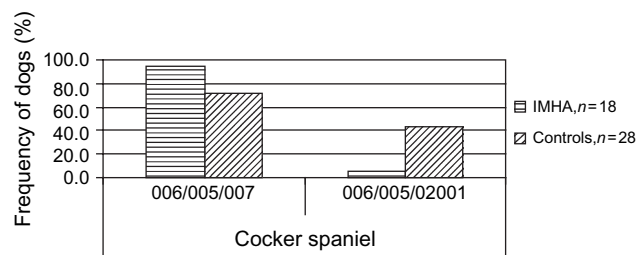
DQA1\*005011/DQB1\*00701 was maintained at the level of significance when the comparison was restricted to breed-matched controls. Further heterogeneity in DLA haplotype association was observed when analysis was restricted to particular breeds including Dobermans, Collies, Labradors, Cocker Spaniels and English Springer Spaniels. Our data provide, for the first time, evidence that that some breeds may have unique MHC associations with IMHA and some may even lack a strong MHC association. However, given the small numbers present in some breeds, results should be interpreted with caution until larger sample sizes can be examined.

IMHA, like other immune-mediated conditions, is likely to have a complex aetiology, whereby multiple genetic and environmental components interact to trigger and drive the continued disease progression. The genetic basis for canine disease is likely related to the fact that most dog breeds have undergone extensive selection and inbreeding where major phenotypic traits have largely been fixed, along with particular susceptibility genes that have been inadvertently carried along and enriched (44). This also appears to be the case in some human populations where different HLA and other gene associations can be seen with the same disease (45). Such disease-associated genetic heterogeneity is also likely to occur in dog breeds.

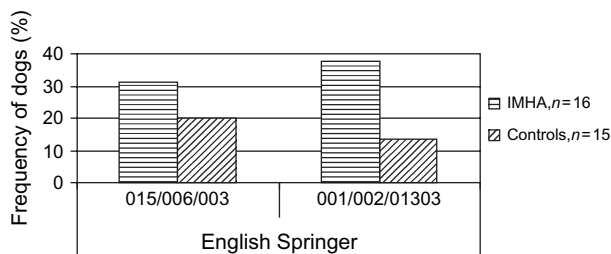
The explanation for the observation that a number of different DLA haplotypes appear to be associated with IMHA could be that the ‘true’ disease gene mutation has been fixed within different DLA haplotypes and is now in strong linkage disequilibrium. As in the human MHC, the



**Figure 3** Dog leucocyte antigen haplotypes in Collies with immune-mediated haemolytic anaemia.



**Figure 5** Dog leucocyte antigen haplotypes in Cocker Spaniels with immune-mediated haemolytic anaemia.



**Figure 6** Dog leucocyte antigen haplotypes in English Springer Spaniels with immune-mediated haemolytic anaemia.

DLA region is likely to contain hundreds of immunologically relevant genes, and this explanation remains a distinct possibility. A further explanation is that only certain combinations of DLA-DRB1 and DQ alleles are optimal for maintaining good immune regulation and for the balance between adequate immuneresponsiveness and immune surveillance without increased risk of developing autoimmunity. Some combinations enriched within certain breeds may encode susceptibility to IMHA.

In humans, there is some evidence that HLA-DQ molecules are particularly important in regulating antibody responses, including the production of autoantibodies. This is likely to only relate to the production of IgG antibodies, and the production of IgM immunoglobulin appears to be less strongly MHC regulated. As such, it is possible that DLA associations observed with IMHA are underestimates of the true associations as a minority of dogs only develop IgM autoantibodies and not IgG. Further stratification of the dogs in this study by serological response showed clearer and stronger DLA associations.

It is clear that only very subtle differences in amino acid sequence within MHC molecules can relate to major differences in risk to particular diseases. For example, the basis for HLA-encoded susceptibility to type I diabetes in humans is considered to relate to the presence of an amino acid other than aspartic acid at position 57 of the HLA-DQ beta-chain (DQB non-Asp57) (26) and also with the presence of arginine at position 52 of the HLA-DQ alpha-chain (DQA Arg52) (29). In a recent study, we also observed that susceptibility of dogs to Leishmaniasis was associated with a DLA-DRB1 allele that only differed from another non-associated DRB1 allele by one amino acid (46). A similar situation also possibly exists in IMHA.

Advances in the therapeutic management of immune-mediated diseases in dogs and humans are likely to require specific knowledge of the interaction between the self-derived peptides and the class II MHC molecules that present these autoantigens to autoreactive T cells. Indeed, many strategies have now been devised to block such molecular interactions or subvert them towards the generation of immunoregulatory, rather than immunopathogenic, T-cell responses (47). The dog provides a unique

large-animal model for the development of such modalities as this species spontaneously develops disease homologous to humans, while sharing the same range of environmental trigger factors. The knowledge of class II molecules associated with canine IMHA is one part of the information required to design and test such immunotherapeutic strategies in a canine disease model. Preliminary work has already reported peptide epitope mapping on canine autoreactive T cells using a panel of overlapping 15mer peptides derived from the sequence of one of the dominant autoantigens for the canine disease (erythrocyte membrane glycoprotein) (12). The molecular modelling of such peptide-MHC interactions forms the basis for advances in this area.

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