Tandem repeats modify the structure of the canine *CD1D* gene

F. A. Looringh van Beeck*, P. A. J. Leegwater†, T. Herrmann‡, F. Broere*, V. P. M. G. Rutten*§, T. Willemse*† and I. Van Rhijn*

*Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands.
†Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3584 CM, Utrecht, The Netherlands.
‡Institute for Virology and Immunobiology, Julius Maximilians University Wurzburg, Versbacher Straβe 7, 97078, Wu rzburg, Germany.
§Department of Tropical Veterinary Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Private Bag X04, Pretoria, 0110, South Africa.

Summary

Among the CD1 proteins that present lipid antigens to T cells, CD1d is the only one that stimulates a population of T cells with an invariant T-cell receptor known as NKT cells. Sequencing of a 722 nucleotide gap in the dog (*Canis lupus familiaris*) genome revealed that the canine *CD1D* gene lacks a sequence homologous to exon 2 of human *CD1D*, coding for the start codon and signal peptide. Also, the canine *CD1D* gene contains three different short tandem repeats that disrupt the expected gene structure. Because canine *CD1D* cDNA lacks sequences homologous to human exon 2 and 3, the functionality of canine CD1d protein may be affected, and this could have consequences for the development and activation of canine NKT cells.

Keywords

CD1, dog, microsatellite, NKT cells, simple sequence repeat

The CD1 family is a group of non-polymorphic glycoproteins that present lipid antigens to T cells (Porcelli & Modlin 1999; Brigl & Brenner 2004; Barral & Brenner 2007). Like MHC class I proteins, CD1 proteins are heterodimers of $\beta 2$ microglobulin and a heavy chain consisting of three extracellular α domains, a transmembrane region and a cytoplasmic tail, each encoded by a separate exon, preceded by an exon that encodes the start codon and signal peptide, and sometimes an additional exon that consists of 5′ UTR only.

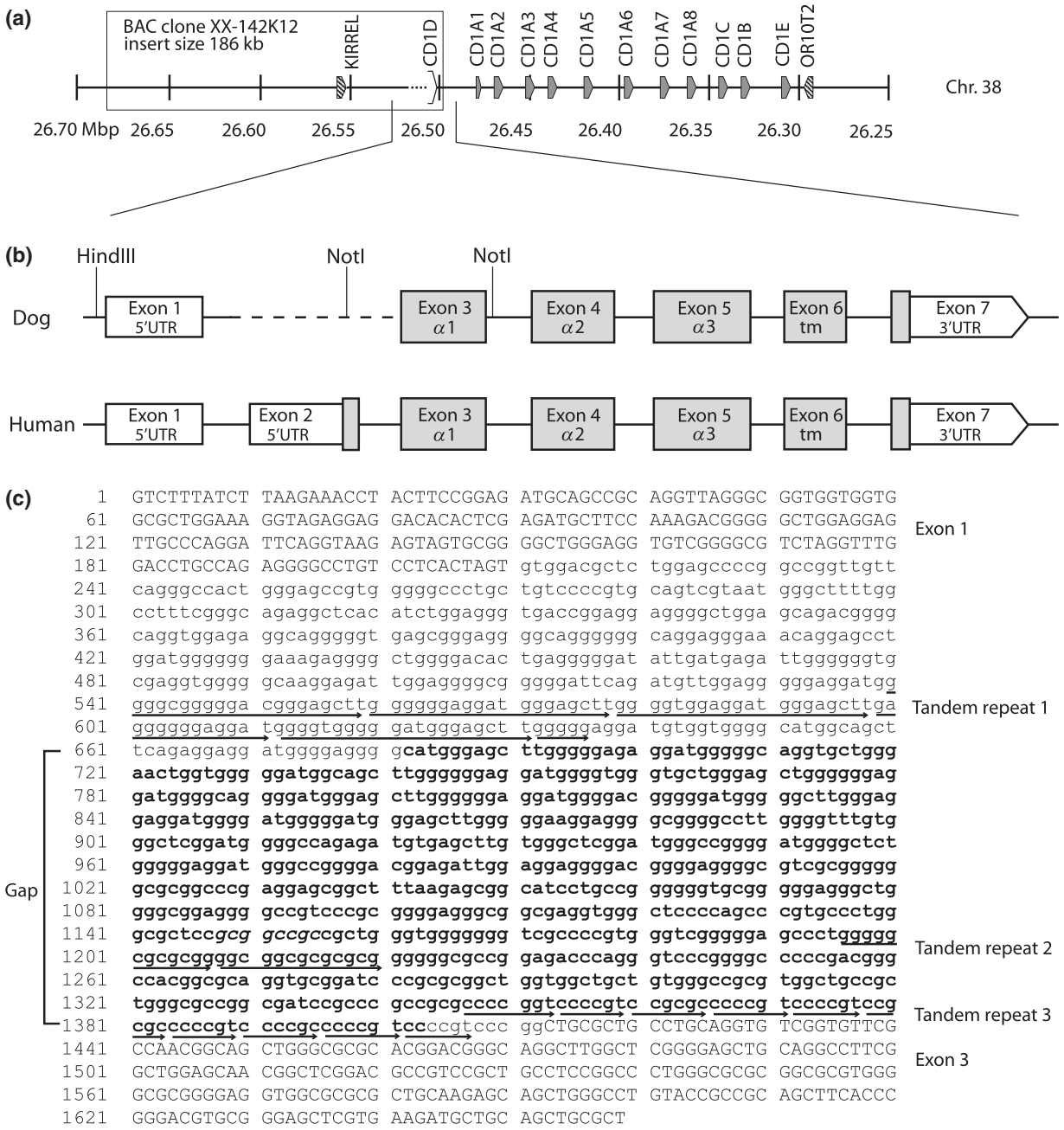
Large variation exists in the number of *CD1A*, *CD1B*, and *CD1C* genes between mammalian species, whereas one or two *CD1D* genes are present in all mammalian species studied to date (Dascher *et al.* 1999; Hayes & Knight 2001; Eguchi-Ogawa *et al.* 2007; Looringh van Beeck *et al.* 2008, 2009). CD1d is crucial for the selection (Bendelac *et al.* 1995) and activation (Kawano *et al.* 1997) of a subset of T cells known as natural killer T (NKT) cells. The canine CD1 locus is located on chromosome 38 and contains one *CD1D* gene, eight *CD1A* genes, one *CD1C*, one *CD1B* and one *CD1E* gene (Fig. 1a) (Looringh van Beeck *et al.* 2008). In the canine genome (CanFam 3.1) and in the sequence of BAC clone XX-14K12 AC183576.27, the canine *CD1D* gene is incomplete due to an internal gap of unknown length. Upstream from this gap we detected a sequence with 62% nt identity to exon 1 of human *CD1D* and downstream from the gap a sequence with 68% identity to human exon 3 (ENST00000368171; www.ensembl.org) (Fig. 1b). No canine homolog of the human *CD1D* exon 2 was found. Putative full-length canine exons were found for *CD1D* exon 4, 5, 6 and 7, with identities of respectively 76%, 84%, 58% and 56% to the corresponding human exons.

To be able to fill the gap in the genomic DNA sequence between exon 1 and exon 3 of canine *CD1D*, we performed a two-step digestion on BAC clone XX-14K12 DNA using restriction endonuclease HpaI followed by HindIII and NotI (Fig. S1). The two restriction fragments containing the gap were subsequently cloned and sequenced. The complete DNA sequence of the gap was obtained and consists of 722 nt (GenBank GU930707) (Fig. 1c). No canine homolog of the human *CD1D* exon 2 was found in this DNA sequence.

Using Tandem Repeat Finder, three different types of tandem repeats were identified in the sequence. Tandem repeats occur more frequently in the canine genome compared to other mammalian genomes as a result of an impaired DNA replication and repair mechanism in dogs

Address for correspondence

I. Van Rhijn, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands.
E-mail: i.vanrhijn@uu.nl




```
   1  GTCTTTATCT TAAGAAACCT ACTTCCGGAG ATGCAGCCGC AGGTTAGGGC GGTGGTGGTG
  61  GCGCTGGAAA GGTAGAGGAG GACACACTCG AGATGCTTCC AAAGACGGGG GCTGGAGGAG
 121  TTGCCCAGGA TTCAGGTAAG AGTAGTGCGG GGCTGGGAGG TGTCGGGGCG TCTAGGTTTG
 181  GACCTGCCAG AGGGGCCTGT CCTCACTAGT gtggacgctc tggagccccg gccggttgtt
 241  cagggccact gggagccgtg ggggccctgc tgtccccgtg cagtcgtaat gggcttttgg
 301  cctttcgggc agaggctcac atctggaggg tgaccggagg aggggctgga gcagacgggg
 361  caggtggaga ggcagggggt gagcgggagg ggcaggggag caggagggaa acaggagcct
 421  ggatgggggg gaaagagggg ctggggacac tgaggggggat attgatgaga ttgggggtg
 481  cgaggtgggg gcaaggagat tggaggggcg ggggattcag atgttggagg gggaggatgg
 541  gggcgggggg cgggagcttg gggggaggat gggagcttgg ggtggaggat gggagcttga
 601  gggggggagga tggggtgggg gatgggagct tgggggagga tgtggtgggg catggcagct
 661  tcagaggagg atggggaggg gcatgggagc ttgggggaga ggatgggggc aggtgctggg
 721  aactggtggg ggatggcagc ttgggggggag gatgggggtgg gtgctgggag ctgggggggag
 781  gatggggcag gggatgggag cttgggggga ggatggggac gggggatggg ggcttgggag
 841  gaggatgggg atgggggatg ggagcttggg ggaaggaggg gcgggggcctt ggggtttgtg
 901  ggctcggatg gggccagaga tgtgagcttg tgggctcgga tgggccgggg atggggctct
 961  gggggaggat gggccgggga cggagattgg aggaggggac ggggaggggc gtcgcggggg
1021  gcgcggcccg aggagcggct ttaagagcgg catcctgccg gggggtgcgg gggagggctg
1081  gggcggaggg gccgtcccgc ggggagggcg gcgaggtggg ctccccagcc cgtgccctgg
1141  gcgctcc*gcg gccgc*cgctg ggtgggggggg tcgccccgtg ggtcgggggа gccctgggggg
1201  cgcgcggggc ggcgcgcgcg ggggggcgccg gagacccagg gtcccggggc ccccgacggg
1261  ccacggcgca ggtgcggatc ccgcgcggct ggtggctgct gtgggccgcg tggctgccgc
1321  tgggcgccgg cgatccgccc gccgcgcccc ggtccccgtc cgcgcccccg tccccgtccg
1381  cgccccgtc cccgccccg tccccgtccc ggCTGCGCTG CCTGCAGGTG TCGGTGTTCG
1441  CCAACGGCAG CTGGGCGCGC ACGGACGGGC AGGCTTGGCT CGGGGAGCTG CAGGCCTTCG
1501  GCTGGAGCAA CGGCTCGGAC GCCGTCCGCT GCCTCCGGCC CTGGGCGCGC GGCGCGTGGG
1561  GCGCGGGGAG GTGGCGCGCG CTGCAAGAGC AGCTGGGCCT GTACCGCCGC AGCTTCACCC
1621  GGGACGTGCG GGAGCTCGTG AAGATGCTGC AGCTGCGCT
```

Figure 1 Canine CD1 locus and *CD1D* gene. (a) Map of the canine CD1 locus containing eight *CD1A* genes, one *CD1C* gene, one *CD1B* gene, one *CD1E* gene and one *CD1D* gene as previously published (Looringh van Beeck *et al.* 2008). The gap in the genomic sequence is indicated by a dashed line. BAC clone XX-142K12 that covers the gap and the *CD1D* gene is indicated by a box. (b) Schematic representation of the alignment of canine and human *CD1D* showing exons (boxes) and introns (line) and the gap (dashed line). Restriction sites for HindIII and NotI in the canine *CD1D* gene are shown. (c) Partial genomic sequence of canine *CD1D*, including: gap sequence (bold, lower case), canine homologs of human exon1 and 3 sequence (upper case), three types of tandem repeats (arrows) and the NotI-site within the gap (italic).

(Laidlaw *et al.* 2007). The repeat fragment size of the first tandem repeat (TR1) is 20 nt and was found five times. The second tandem repeat (TR2) has a fragment size of 12 nt and was repeated twice. The region containing TR1 and TR2 is characterized by a high guanine content (59%), which may explain why this part had not been sequenced before. The motif TR3 consists of a hexanucleotide repeat of the consensus DNA sequence CCCCGT. This tandem repeat was present 10 times and, based on alignment with human *CD1D*, is located at the intron–exon boundary of canine *CD1D* homolog of human *CD1D* exon 3. To determine the presence and the copy number of the tandem repeats in dog breeds other than the Boxer from which BAC clone XX-142K12 was derived, PCR was performed on genomic DNA

of three Beagles, three Labrador Retrievers and a wolf. Using a primer set located outside TR3 (primer set 1, Appendix S1), PCR products were generated and sequenced (Fig. S2). We found a $(CCCCGT)_6$ sequence in wolf genomic DNA (GenBank GU930708). In the PCR products of the three Beagles, we only detected the $(CCCCGT)_8$ sequence (GenBank GU930709). In one Labrador Retriever we found both $(CCCCGT)_8$ and $(CCCCGT)_{10}$, whereas in the other two Labrador retrievers only $(CCCCGT)_{10}$ was found (GenBank GU930710). These findings indicate that TR3 is a variable number tandem repeat with at least three different alleles. This suggests that TR3 is still an active site with tandem duplication events before and after the divergence of domestic dogs from the gray wolf between 15 000 and 100 000 years ago (Lindblad-Toh *et al.* 2005).

Using primer set 5 (Appendix S1), we observed differential transcription of *CD1D* in cDNA from various Beagle tissues (Fig. 2a). The highest transcription levels were found in thymus, lymph node, spleen, and PBMC, showing that tissue distribution of transcription of *CD1D* in dogs is comparable to other species and not hampered by the presence of the tandem repeats. Sequencing of *CD1D* PCR products generated from Beagle thymus-derived cDNA, generated with primer set 6, located outside the full-length coding sequence of human CD1d, revealed that canine *CD1D* transcript contained exon 1 and exon 4–7 (Genbank JX139112). Longer PCR products were not obtained using this primer set. Figure 2b shows the structure of the human (NM_001766.3; coding sequence 1008 bp) and canine *CD1D* cDNA sequences (Genbank JX139112; coding sequence 726 bp). Consistent with the absence of an exon 2 homolog in canine genomic DNA, the canine transcript lacks a sequence similar to human exon 2 encoding the signal sequence of CD1d. However, an alternative start codon in the CD1d reading frame was present in exon 1. No signal peptide could be identified. Exon 3, encoding the $\alpha 1$ domain, was also absent in the cDNA. The deletion of nucleotides flanking a repeat is common, and it is likely that TR3 is responsible for the deletion of the first part of canine *CD1D* exon 3 and its acceptor splice site, explaining the absence of exon 3 in the cDNA. The introns between exon 4–7 were spliced out as expected, and no additional splice variants were found. Because a homolog of exon 3, encoding the $\alpha 1$ domain, was absent, it is not expected that translation of this transcript will lead to a functional CD1d protein. Even though only Beagle CD1d cDNA was analyzed and we cannot formally rule out that other breeds have normal CD1d transcripts, the Beagle CD1d cDNA is consistent with the Boxer *CD1D* genomic sequence, and we have confirmed the presence of TR3 in Labrador and wolf. Therefore we expect that other dog breeds show the same aberrant CD1d transcript. Together, our findings suggest that, despite gene transcription, expression of the canine CD1d protein is possibly absent or altered *in vivo*. Consistent with this, a panel of anti CD1d antibodies, raised against different species, did not stain canine thymocytes (Fig. S3). Our findings raise the question of how the recently described canine NKT cells (Yasuda *et al.* 2009) can be positively selected and activated in the absence of CD1d protein. One possibility is that one of the many canine *CD1A*, *CD1B*, or *CD1C* genes might encode a restriction element for these cells.

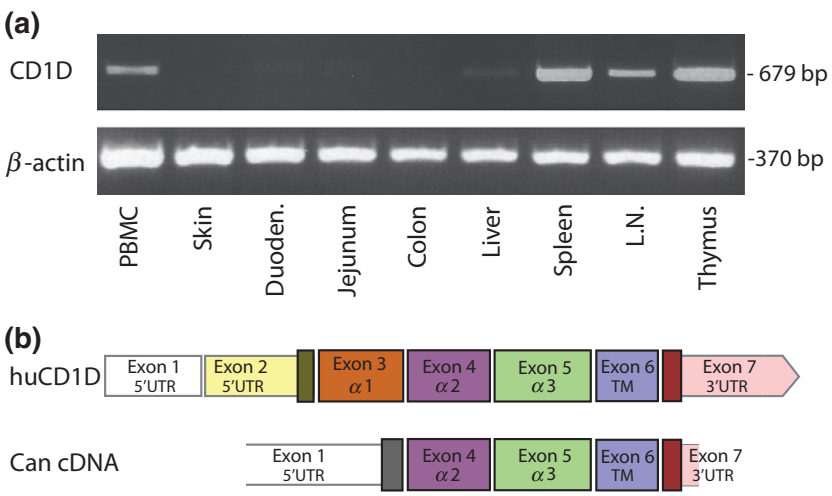


Figure 2 Canine *CD1D* transcription. (a) Transcription of canine *CD1D* in different dog tissues. Primer set 5 was used for the detection of *CD1D* transcription in different dog tissues. To compare the quantity of input of cDNA, a PCR for β-actin was performed. L.N.: Lymph node. (b) Structure of human and canine *CD1D* transcripts, with the untranslated parts represented by narrower boxes in lighter shades, and the coding sequence represented by broader boxes in darker shades. The canine transcript was obtained using primer set 6 and lacks a sequence homologous to human *CD1D* exon 2 and 3. TM: Transmembrane domain. Primer sequences are provided in Appendix S1.

Acknowledgements

IVR was supported by an NWO Meervoud subsidy; Netherlands. FALVB was supported by Royal Canin, France.

References

Barral D.C. & Brenner M.B. (2007) CD1 antigen presentation: how it works. *Nature Reviews Immunology* **7**, 929–41.

Bendelac A., Lantz O., Quimby M.E., Yewdell J.W., Bennink J.R. & Brutkiewicz R.R. (1995) CD1 recognition by mouse NK1 + T lymphocytes. *Science* **268**, 863–5.

Brigl M. & Brenner M.B. (2004) CD1: antigen presentation and T cell function. *Annual Review of Immunology* **22**, 817–90.

Dascher C.C., Hiromatsu K., Naylor J.W. *et al.* (1999) Conservation of a CD1 multigene family in the guinea pig. *Journal of Immunology* **163**, 5478–88.

Eguchi-Ogawa T., Morozumi T., Tanaka M., Shinkai H., Okumura N., Suzuki K., Awata T. & Uenishi H. (2007) Analysis of the genomic structure of the porcine CD1 gene cluster. *Genomics* **89**, 248–61.

Hayes S.M. & Knight K.L. (2001) Group 1 CD1 genes in rabbit. *Journal of Immunology* **166**, 403–10.

Kawano T., Cui J., Koezuka Y. *et al.* (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* **278**, 1626–9.

Laidlaw J., Gelfand Y., Ng K.W., Garner H.R., Ranganathan R., Benson G. & Fondon J.W. 3rd (2007) Elevated basal slippage mutation rates among the Canidae. *Journal of Heredity* **98**, 452–60.

Lindblad-Toh K., Wade C.M., Mikkelsen T.S. *et al.* (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* **438**, 803–19.

Looringh van Beeck F.A., Zajonc D.M., Moore P.F., Schlotter Y.M., Broere F., Rutten V.P., Willemse T. & Van Rhijn I. (2008) Two canine CD1a proteins are differentially expressed in skin. *Immunogenetics* **60**, 315–24.

Looringh van Beeck F.A., Reinink P., Hermsen R. *et al.* (2009) Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants. *Molecular Immunology* **46**, 1424–31.

Porcelli S.A. & Modlin R.L. (1999) The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annual Review of Immunology* **17**, 297–329.

Yasuda N., Masuda K., Tsukui T., Teng A. & Ishii Y. (2009) Identification of canine natural CD3-positive T cells expressing an invariant T-cell receptor alpha chain. *Veterinary Immunology and Immunopathology* **132**, 224–31.

Supplementary Materials and Methods

In silico sequence analysis

A partial canine *CD1D* gene was detected by BLAST searches in the canine genome assembly CanFam3.1 (Broad Institute) using Ensembl (www.ensembl.org), trace files on the NCBI site (www.ncbi.nlm.nih.gov), and the CHORI-82 Canine Boxer BAC library (bacpac.chori.org). Alignments were performed using ClustalW (http://www.genome.jp/tools/clustalw/). To determine the presence of tandem repeats we analyzed the full genomic length of the canine *CD1D* gene with Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) (Benson 1999) using default settings. The presence of splice sites in this sequence was predicted using NetGene2 server (www.cbs.dtu.dk/services/NetGene2), signal peptide analysis was performed with SignalP (www.cbs.dtu.dk/services/SignalP) (Bendtsen *et al.* 2004).

BAC Clone DNA sequencing

BAC clone XX-142K12 from the CHORI-82 Canine BAC library was obtained from BACPAC Resources Center (bacpac.chori.org). The CHORI-82 library formed the basis for the canine genome and consists of genomic DNA of a Boxer cloned into the pTARBAC2.1 vector (Osoegawa *et al.* 1998). Rapid alkaline lysis miniprep method (Qiagen) was used followed, by precipitating BAC clone DNA with ice cold isopropanol. BAC DNA was digested using endonuclease *Hpa*I, succeeded by excision and elution of the band containing the gap in the *CD1D* gene with subsequent digestion with *Hind*III and *Not*I (Stratagene) (Fig. S1). The two fragments containing the gap were ligated into pcDNA3.1+ vector (Invitrogen) and sequenced, using a method for GC rich sequences, by BaseClear (Leiden, Netherlands).

Genomic DNA and cDNA sequencing

Blood from three Beagles, three Labrador retrievers and a wolf (*Canis lupus*) was collected according to the regulations of the Animal Ethics Committee of the University of Utrecht, Netherlands (protocol number 2008.II.11.106). Genomic DNA was isolated from EDTA blood from all seven animals, using the salt extraction method (Miller *et al.* 1988). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized Beagle blood by standard Ficoll-Hypaque gradient

centrifugation. From one Beagle also skin, intestinal tissue, liver, spleen, lymph node and thymus were collected. RNA was isolated from tissues using the RNeasy kit (Qiagen) followed by cDNA synthesis using the iScript kit (Bio-Rad). All genomic DNA and cDNA was frozen at -20˚C until use. To determine the genomic conservation of the CCCCGT-repeat across dog breeds, we performed a hot-start touch down polymerase chain reaction (PCR) using primer set 1 with genomic DNA as template. PCR was performed with *Pfu* Turbo polymerase (Stratagene) and 4% dimethyl sulfoxide under the following conditions: 7 min at 98˚C, followed by 35 cycles of 15 s at 95˚C, 15 s at annealing temperature, 30 s at 72˚C, followed by a final elongation of 5 min at 72˚C. After each of the first 20 cycles, the annealing temperature was decreased with 0.5˚C, starting at 69˚C. PCR products were ligated in a pCR4Blunt-TOPO vector (Invitrogen) and vector DNA was sequenced, using a method for GC rich sequences, by BaseClear (Leiden, Netherlands). The expression of canine *CD1D* was determined in various tissues using PCR conditions as described above with an annealing step at 65˚C for 60 s with primer set 5. To check the quantity of cDNA in each PCR, we performed *β*-actin-specific PCR in parallel.

Staining canine thymocytes with CD1d mAbs

Canine thymocytes were stained with biotinylated anti-rat CD1d (IgG2a) antibodies WTH-1, WTH-2, WTH-3, the latter has also been designated as 58 (Monzon-Casanova *et al.* 2010), and anti-human CD1d (IgG1) antibody CD1d42. Raji cells transfected with rat *CD1D* cDNA and human PBMC were used as positive control, and stainings without primary antibody as negative control. WTH-1 and WTH-2 have been shown to cross-react with murine CD1d, but have never been tested for reactivity with canine CD1. To confirm CD1 expression on canine thymocytes, we used anti-canine CD1a8 (IgG1) antibody CA13.9H11, which was kindly provided by Professor P.F. Moore, University of California, Davis, CA, USA.

The cells were stained with primary antibody for 30 min at 4˚C followed by incubation with streptavidin-R-phycoerythrin or Goat Anti Mouse Ig -R-phycoerythrin (BD Biosciences) for 30 min at 4˚C. Fluorescence was measured using a FACScalibur flow cytometer (Becton Dickinson).

Primers

Primer sequences	Name	Location	Product size (bp)	Template
F 5'-GCGCAGGTGCGGATCCCG	Set 1	Exon 3	158-182	Genomic
R 5'-GCCGTTGGCGAACACCGACA		Exon 3		DNA
F 5'-GATCCTGAGTTTCCAAGGGTCTCAC	Set 5	Exon 4	679	cDNA
R 5'-GCAGCCTTTCTTCACCCTCAGC		Exon 7		
F 5'-GAGGAAACTCTACAAAGAGG	Set 6	Exon 1	1048	cDNA
R 5'-GCAGCCTTTCTTCACCCTCAGC		Exon 7		
F 5'-GTCGTCGACAACGGCTCCG	β-actin		370	cDNA
R 5'-GGCTGGGGTGTTGAAGGTCTC				

References (Supplementary Materials and Methods only)

Bendtsen J.D., Nielsen H., von Heijne G. & Brunak S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**, 783-95.

Benson G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res* **27**, 573-80.

Miller S.A., Dykes D.D. & Polesky H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**, 1215.

Monzon-Casanova E., Steiniger B., Schweigle S., Clemen H., Zdzieblo D., Starick L., Muller I., Wang C.R., Rhost S., Cardell S., Pyz E. & Herrmann T. (2010) CD1d expression in paneth cells and rat exocrine pancreas revealed by novel monoclonal antibodies which differentially affect NKT cell activation. *PLoS One* **5**.

Osoegawa K., Woon P.Y., Zhao B., Frengen E., Tateno M., Catanese J.J. & de Jong P.J. (1998) An improved approach for construction of bacterial artificial chromosome libraries. *Genomics* **52**, 1-8.

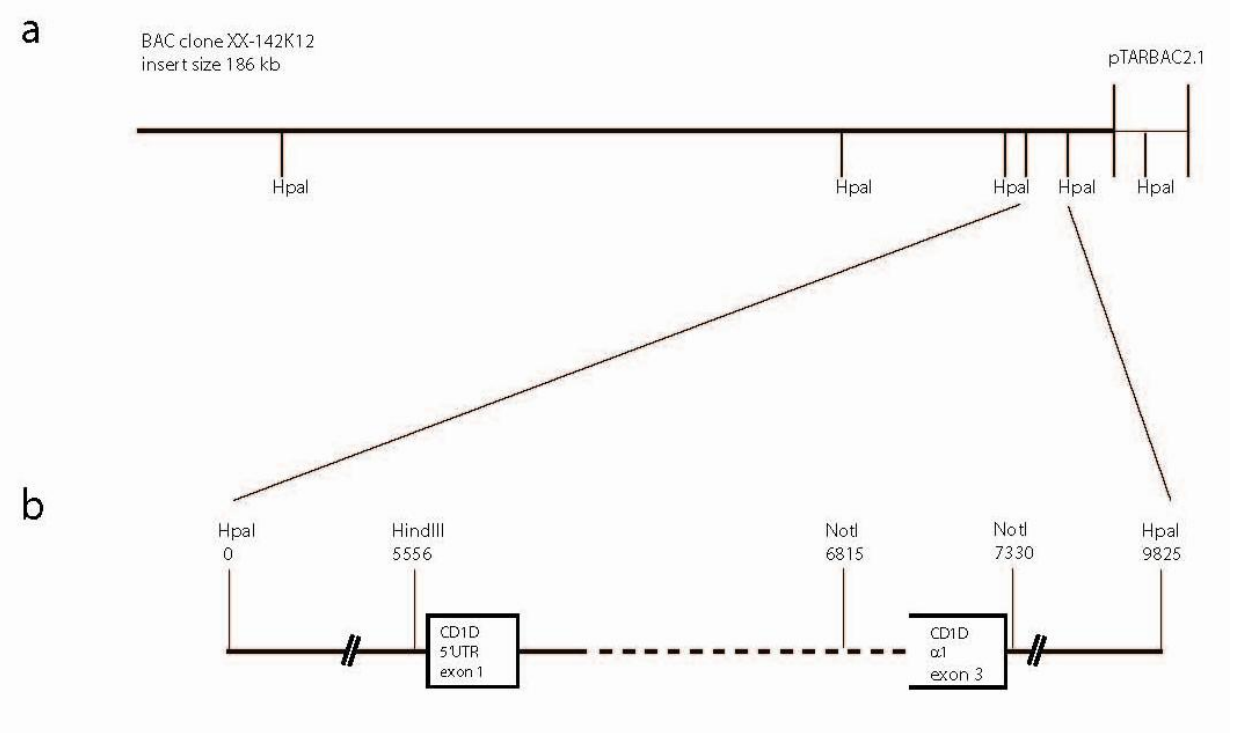


Figure S1 Endonuclease restriction map of BAC clone XX-142K12.
Map showing HpaI restriction sites of BAC clone XX-142K12 which as an insert size of 186kb in vector pTARBAC2.1 (a). Map showing HpaI-fragment of 9825 nt, numbered after retrieving the full length sequence of the gap, containing the gap which was subsequently digested with HindIII and NotI leading to two fragments which were both cloned into pcDNA3.1+ vector (b).

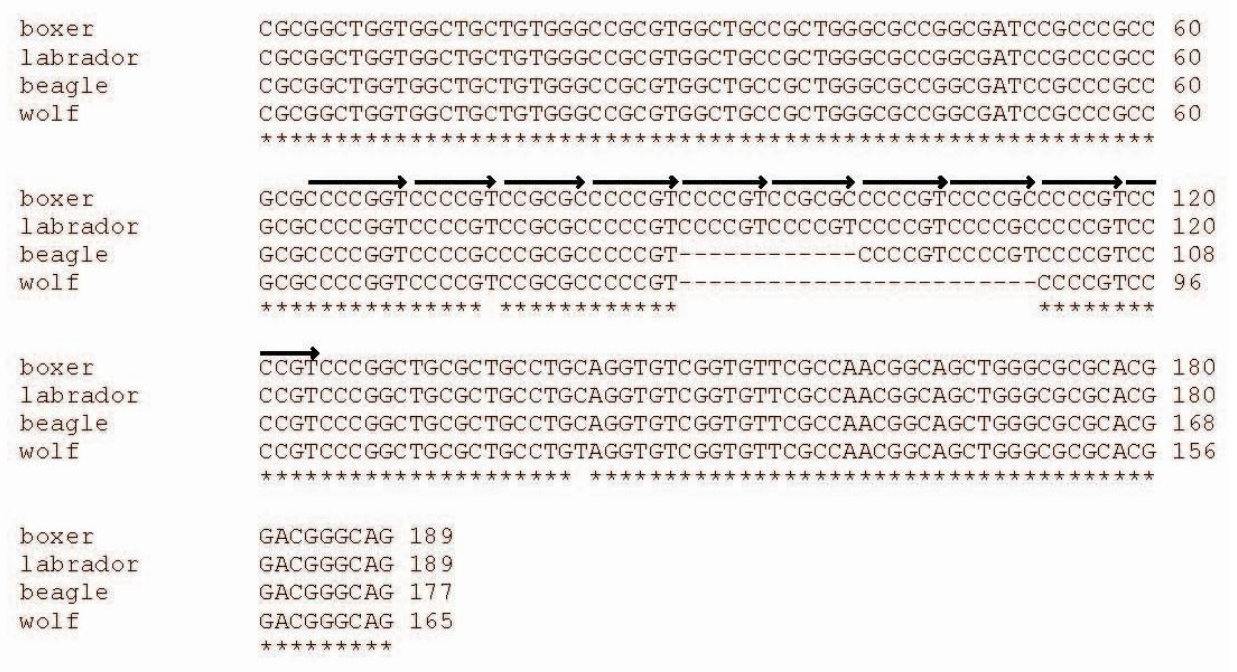


Figure S2 Alleles of tandem repeat 3

Alignment of the 3'part of the gap in the genomic sequence of the CD1D gene of one Boxer, one Beagle, one Labrador retriever and one wolf containing the hexanucleotide CCCCGT tandem repeat TR3. The repeat was shown to be a variable number tandem repeat with 6 – 10 repeat units. Arrows indicate the location of the CCCCGT repeat in the canine sequence. Identical nt are indicated by an asterisk.

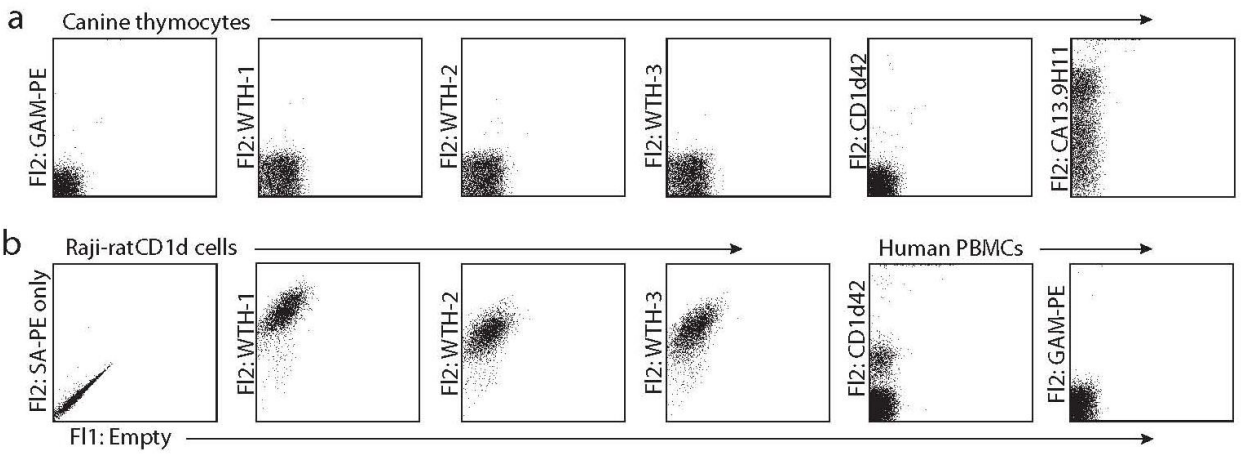


Figure S3 Flow cytometric analysis of expression of CD1d on canine thymocytes.
a. Negative staining of canine thymocytes was observed with biotinylated anti-rat CD1d antibodies (WTH-1, WTH-2 or WTH-3), anti-human CD1d (CD1d42). Anti-canine CD1a8 (CA13.9H11) was used as a positive control for CD1 expression by thymocytes. All primary antibody incubations were followed by incubation with SA-PE or GAM-PE. As a negative control we omitted the primary antibody, and incubated cells only with secondary antibody. b. Staining of ratCD1d-transfected Raji cells and human PBMC was used as a positive control for respectively WTH-1,-2, -3 and CD1d42 (b).