The Bovine CD1 Family Contains Group 1 CD1 Proteins, but No Functional CD1d[1]

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The CD1 family of proteins presents lipid Ags to T cells. Human CD1a, CD1b, and CD1c have been shown in humans to present mycobacterial lipid Ags. Cattle, like humans, are a natural host of several mycobacterial pathogens. In this study, we describe the CD1 family of genes in cattle (*Bos taurus*) and provide evidence that *B. taurus* expresses CD1a, CD1e, and multiple CD1b molecules, but no CD1c and CD1d molecules. In mice and humans, CD1d is known to present Ag to NKT cells, a T cell lineage that is characterized by a limited TCR repertoire, capable of rapidly secreting large amounts of IFN-γ and IL-4. In cattle, two CD1D pseudogenes were found and no intact CD1D genes. Consistent with this, we found complete lack of reactivity to a potent, cross-reactive Ag for NKT cells in mice and humans, α-galactosylceramide. Our data suggest the absence of NKT cells in cattle. It remains open whether other cells with the NKT-like phenotype and functions are present in this species. With its functional CD1A and CD1B genes, *B. taurus* is well equipped to present Ags to CD1-restricted T cells other than NKT cells. Cattle can be used as a model to study group 1 CD1-restricted T cell immunity, including its role in the defense against mycobacterial infections that occur naturally in this species. *The Journal of Immunology,* **2006, 176: 4888–4893.**

The CD1 protein family presents a variety of antigenic structures to T cells, including lipids, glycolipids, small aromatic compounds, and lipopeptide Ags. CD1 proteins are structurally related to MHC class I proteins in terms of the overall structure of their three extracellular domains (α1, α2, and α3) and association with β_2-microglobulin, but the CD1 Ag-binding groove has a hydrophobic surface, unlike MHC molecules. The α1 and α2 domains, forming the Ag-binding groove are highly conserved in mammalian species. Based on protein sequence homology, patterns of expression, and functional properties, the five known CD1 isoforms have been divided into two subsets. Group 1 CD1 molecules (CD1a, CD1b, and CD1c) have been shown in humans to present mycobacterial lipid Ags (1–5).T cells that recognize these Ags are activated during the course of acute *Mycobacterium tuberculosis* infections in humans (6, 7).Group 2 CD1 molecules (CD1d) are known to present Ag to NKT cells, a T cell lineage that is characterized by a limited T cell repertoire and an Ag-experienced phenotype. These cells are capable of rapidly secreting large amounts of IFN-γ and IL-4 (8, 9).The invariant α-chain of the TCR of NKT cells and its Ag specificity is highly conserved. Manipulation of the NKT population by stimulation or deletion has profound effects on the course of infectious diseases and autoimmune diseases. CD1e is of intermediate homology and functions intracellularly, assisting Ag loading (10). With the exception of muroid rodents, in which group 1 CD1 genes have been deleted by a major chromosomal rearrangement (11), all mammals studied to date express group 1 CD1 isoforms, but there is considerable variation in the number of CD1 genes and the isoforms that are present.

Mycobacterial Ags that are presented by CD1 include a siderophore-related lipopeptide presented by human CD1a (1), diacylated sulfoglycolipids, lipoarabinomannan, mycolic acid, glucose monomycolates presented by human CD1b (2, 4, 6, 12), and mycobacterial phosphoglycolipids presented by human CD1c (5, 13). Understanding the development of CD1-restricted, T cell-mediated immunity upon infection with mycobacteria may be important for the development of new or improved vaccines. However, there is insufficient knowledge about the protective capacities of CD1-restricted responses against infection, the interplay with other parts of the immune system, and whether CD1-restricted T cells can give rise to immunological memory. These questions are ideally studied in a species that is a natural host of mycobacterial infections, expresses group 1 CD1 isoforms, and can be subjected to vaccination-challenge experiments. *Bos taurus* is the natural host of several pathogenic mycobacteria, among which are *Mycobacterium avium paratuberculosis*, causing Johnes disease, and *Mycobacterium bovis*, causing bovine tuberculosis. Both pathogens pose a zoonotic threat and cause substantial economic losses worldwide. This makes *B. taurus* an important target species for vaccine development, as well as an alternative model to study the role of CD1 in protection against mycobacterial diseases. In this study, we describe the CD1 gene family in *B. taurus* and provide evidence that *B. taurus* expresses CD1a, CD1b, and CD1e molecules, but no CD1c and CD1d molecules. The gene for CD1C is not present in the genome, and both CD1D genes are pseudogenes. Our findings suggest that cattle have the capacity to present mycobacterial Ags, but that CD1d-restricted NKT cells are absent.

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[3] Abbreviation used in this paper: bo, bovine.

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Materials and Methods

Animals and tissue samples

PBMC were isolated from blood by standard Ficoll-Hypaque gradient centrifugation from outbred Holstein cattle and from human blood bank buffy coats. Genomic DNA was prepared from PBMC using the Promega Wizard genomic DNA purification kit. Single-cell suspensions were freshly prepared from bovine thymus, spleen, and liver and used without purification. Freshly isolated bovine PBMC and a cell suspension from bovine thymus were used to isolate RNA with the Qiagen RNAEasy kit, followed by first-strand cDNA synthesis with Multiscribe reverse transcriptase.

Cloning and expression of boCD1

PCR was performed on cDNA from bovine thymus and bovine PBMC using PFU polymerase according to the protocol supplied by the manufacturer. Primers and annealing temperatures were as follows: bovine (bo) CD1A forward, 5′-ATGCTATTTCTGCAACTTCCATTGCTCCTG; boCD1A reverse, 5′-CCATCATCACCTAAGTCTGTTTAAATGTGTC; boCD1B forward, 5′-ATGCTGCTTCTACCATTTCTGTTACTTGG; boCD1B reverse, 5′-GGGCTCACAAGATATTCTGATATGACCA at an annealing temperature of 53°C; boCD1D forward, 5′-GAATGGGGTGCT TGCTGTTTCT; and boCD1D reverse, 5′-TCCAGAg/cAGAc/tAGGTGT GGGa/cAGGAGAGTCAC at an annealing temperature of 55°C. The gap in boCD1B5 was sequenced after cloning of a PCR product that was obtained by nested primer sets: boCD1B5 forward outer set, 5′-TTCAGT GATGAGGAGGTGGCTGAGA; boCD1B5 reverse outer set, 5′-GTGAT GGAGTCAGGAATGCAGCAC; boCD1B5 forward inner set, 5′-GAACTATTCCGAGTCTACTTCATTGGG; and boCD1B5 reverse inner set, 5′-CCAGGGTAAAAGGGAGAGTTACACA, both at an annealing temperature of 67°C. PCR products were isolated from an agarose gel and cloned into PCR4Blunt Topo for sequencing and in pcDNA3.1 for transient expression. 293T cells were transfected using FuGene-6 reagent (Roche) according to the manufacturer's protocol and analyzed 48 h after transfection.

PCR analysis of boCD1D

Forward primers specific for an internal fragment of *boCD1D1* (5′-GT CAGCCCCAGATGCCCCGCCTTTTA) and for *boCD1D2* (5′-CCCCA GATGCCCCGCCTTGGG) were used with the same reverse primer (5′-CAGGCCCAGGACTGGGGCCACTGG) and were shown to be specific at an annealing temperature of 72°C.

mAbs and staining procedures

Anti-bovine CD1 Abs CC20 (IgG2a), CC43 (IgG2b), and CC122 (IgG1) were provided by Dr. C. J. Howard (Institute for Animal Health, Compton, U.K.); 20.27 SBU-T6 (IgG1) was obtained from the European Collection of Cell Cultures; CC14 (IgG1) and isotype-matched control Abs AV29 (IgG2b), AV20 (IgG1), and AV37 (IgG2a) were provided by Dr. J. C. Hope (Institute for Animal Health, Compton, U.K.); and goat anti-mouse PE was obtained from BD Biosciences. Anti-human CD1a (OKT6), CD1b (BCD1b3), CD1c (F10/21A3), CD1d (CD1d42), and IgG1 control (P3) were provided by Dr. D. B. Moody (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). Bovine thymocytes, bovine PBMC, and cells transfected with bovine CD1 were incubated for 30 min with the unlabeled Abs at 20 mg/ml in PBS/BSA, followed by a wash and an incubation with goat anti-mouse PE.

Southern blot

Southern blot was performed with bovine genomic DNA digested with the restriction enzymes *Eco*RI, *Eco*RV, *Pst*I, *Sal*I, and *Stu*I using 0.8% agarose gel electrophoresis and neutral capillary transfer to Nytran membrane (Schleicher & Schuell Microscience). The DIG-High Prime Kit II (Roche Molecular Biochemicals) was used for probe labeling and chemiluminescent detection. A mixture of probes was used consisting of purified PCR products of the α3 domain of boCD1b3 (forward primer, 5′-GTGAAGC CGGAGGCTTGGCTG; reverse primer; 5′-CCAGTACAGGATGAT GTCCTGGTC), CD1a (forward primer, 5′-TACGACCAGAGGCCTG GCTCTC; reverse primer, 5′-CCCAGTAGAGGATGATGTCCTGGC), and CD1e (forward primer, 5′-ACCCCTTTGAGCTCCAGGTAT CATTTG; and reverse primer, 5′-CTTGTTTTTCCAGTTCTGCCTTC CCTG). Hybridization was performed at 42°C and washes at 65°C in 0.5× SSC.

T cell stimulations

For proliferation assays, 2×10^5 PBMC were plated per well in round-bottom 96-well plates. T cell medium was made by supplementing 500 ml of RPMI 1640 medium with 50 ml of FCS (HyClone), penicillin (Invitrogen Life Technologies), streptomycin (Invitrogen Life Technologies), 20 mM HEPES (Invitrogen Life Technologies), and 4 ml of 1 N NaOH solution. Proliferation was measured after culture for 3 days with α-galactosylceramide at concentrations ranging from 1 μg/ml to 1 ng/ml, followed by a 6-h pulse of 1 μCi of [^{3}H]thymidine before harvesting and counting β emissions.

CD1d tetramers and α-galactosylceramide

Mouse and human CD1d proteins were purified from a baculovirus expression system (14) and a eukaryotic expression system (15). Purified CD1d proteins were enzymatically biotinylated using BirA (Avidity) according to manufacturer's instruction. CD1d proteins were loaded with a 10-fold molar excess of α-galactosylceramide or with vehicle only as previously described (16). Tetramers were assembled by mixing CD1d proteins and streptavidin-allophycocyanin (Prozyme) in a 4:1 molar ratio. Cells stained with allophycocyanin-labeled tetramers were double stained with anti-bovine CD3 or with anti-human CD3 and propidium iodide.

Results

The bovine CD1 family consists of nine CD1 genes

In a preliminary assembly of the bovine genome (Btau_2.0), with 6× coverage, (available at ⟨www.ensembl.org⟩), searches were performed with the α1, α2, and α3 domains of all human CD1 isoforms. This revealed the presence of one boCD1A gene, four boCD1B genes, two boCD1D genes, one boCD1E gene, and two incomplete boCD1B gene sequences. These two incomplete sequences were shown to belong to one gene by DNA sequencing of a PCR product generated with nested primer sets in the two independent gene fragments. This PCR product spanned the gap and

Table I. *boCD1 genes*

Gene name	Location in Btau_1.0, version 35[a]	Cloned[b]
boCD1A	SCAFFOLD266049: 15,000–20,000	boCD1a (DQ192541), cDNA
boCD1B1	SCAFFOLD132027: 2,000–7,000	
boCD1B2	SCAFFOLD132027: 10,000–15,000	
boCD1B3	SCAFFOLD1387: 11,000–15,000	boCD1b3 (DQ192542), cDNA
boCD1B4	SCAFFOLD96605: 1,000–5,000	
boCD1B5	SCAFFOLD108448: 0–2,000	
	SCAFFOLD28448: 0–1,000	Gap in genomic DNA (DQ192543)
boCD1D1	SCAFFOLD323092: 0–5,000	boCD1d1 (DQ192544), cDNA
boCD1D2	SCAFFOLD224934: 0–1,800	
boCD1E	SCAFFOLD185078: 2,000–9,000	

[a] The scaffold number and the approximate location of the gene on the scaffold are given. Sequences are available at http://nov2005.archive.ensembl.org/Bos_taurus.

[b] The full-length cloned boCD1 mRNAs as described in this article that are transcripts of the indicated genes are shown with their GenBank accession numbers.

included adjacent sequences (GenBank DQ192543). An overview of the bovine CD1 genes and accession numbers is given in Table I. Alignment of bovine sequences with known CD1 $\alpha 1$ domains of other species was generated with ClustalW software (⟨www.ebi.ac.uk/clustalW⟩), as well as a neighbor-joining tree with branch length showing that the sequences segregate by isoform and not by species (Fig. 1). Comparable results were obtained with CD1 $\alpha 2$ domains (data not shown). Standard blast searches for bovine mRNA or expressed sequence tag with the $\alpha 1$ and $\alpha 2$ domains of all human and bovine CD1 isoforms suggested the transcription of bovine *CD1B1*, *CD1B3*, *CD1D1*, *CD1D2*, and *CD1E* genes.

Cloning and expression of boCD1a and boCD1b3

PCR with boCD1A primers on cDNA from bovine thymus, but not from bovine PBMC resulted in a 900-bp band that was cloned and sequenced and appeared to be a properly spliced *boCD1A* transcript that is predicted to give a full-length CD1a protein (GenBank DQ192541).

PCR with boCD1B primers on cDNA obtained from thymus and PBMC resulted in two bands of ~900 and 1000 bp. Cloning and sequencing of these products revealed that the 1000-bp band was a full-length, in-frame *boCD1B* transcript of the bovine genomic sequence that we had already named *boCD1B3* (GenBank DQ192542), and the 900-bp band was a transcript with a partially deleted $\alpha 2$ domain.

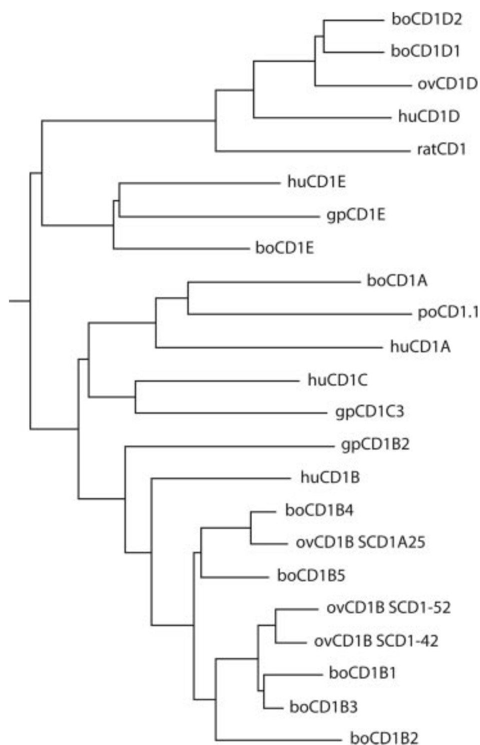

FIGURE 1. Relationship of genomic boCD1 sequences and known mammalian CD1 isoforms. A neighbor-joining dendrogram was created based on alignment of $\alpha 1$ domains of genomic bovine and known mammalian CD1 sequences. The branch length is proportional to the number of substitutions per bp. The sequences included in this figure and their GenBank accession numbers are: human (hu) CD1A BC031645; huCD1B NM_001764; huCD1C NM_001765; huCD1D NM_001766; huCD1E NM_030893; porcine (po) CD1.1 AF059492; guinea pig (gp) CD1B2 AF145484; gpCD1C3 AF145489; gpCD1E AF145490; rat CD1 D26439; ovine (ov) CD1B (SCD1B-42) Z36891; ovCD1B (SCD1B-52) NM_001009425; ovCD1B (SCD1A25) Z36890; ovCD1D (SCD1D) AJ006722; and the $\alpha 1$ domains of the bovine genomic sequences from Table I.

Flow cytometric analysis of bovine thymocytes, bovine PBMC, and 293T cells transfected with full-length boCD1B3 and boCD1A transcript was performed with a panel of Abs against human and boCD1 (Fig. 2 and data not shown). The Abs SBU-T6 (17), CC14 (18), CC20 (19, 20), and CC122 (19, 21) are known to recognize bovine and sheep CD1, but their isoform specificity has not yet been elucidated. Because SBU-T6 was shown to immunoprecipitate sheep CD1e, and at least one other protein (18, 22), it is regarded as a pan-CD1-specific Ab. CC20 and BCD1b3 are known to recognize human CD1b. Our data, summarized in Table II, show that SBU-T6 recognizes thymocytes, peripheral B cells, and 293T cells transfected with boCD1b3 and boCD1a, which is consistent with a pan-CD1 recognition pattern. The Abs BCD1b3, CC14, CC20, and CC122 recognize boCD1b3 transfectant cells and bovine thymocytes, presumably because they express boCD1b, but not boCD1a transfectant cells. The Ab CC43 does not recognize boCD1b3 or boCD1a, but it does recognize bovine peripheral B cells and thymocytes.

Cloning and sequencing of a boCD1d pseudogene

PCR with boCD1D primers on cDNA obtained from PBMC resulted in a single band of ~1200 bp. Lowering the annealing temperature led to the appearance of an extra band of ~900 bp. Cloning and sequencing of these products revealed that the 900-bp band was not related to CD1. The 1200-bp band represented a CD1D-like transcript, which we named *boCD1D1* (GenBank DQ192544). The transcript was homologous to human and sheep CD1D cDNA sequences, except for the presence of an extra stretch of DNA homologous to the intron between the leader and the $\alpha 1$ domain. This putative intron lacked a splice site consensus sequence at its 5′ end. An internal, in-frame stop codon lies in this intron. The other introns were properly spliced out. The two genomic boCD1D sequences showed that the lack of splice site consensus was not a PCR artifact. In addition, we noticed that the genomic sequences contained a mutated start codon. The presence of a nonmutated start codon in the sequence we cloned was probably caused by the forward PCR primer, which contained this start codon. Alignment of human genomic DNA encoding CD1d, the two bovine genomic sequences, which we now call transcribed CD1D pseudogenes, and the cloned *boCD1D1* pseudogene cDNA is shown in Fig. 3.

Blast searches in bovine mRNA sequences resulted in multiple sequences with the mutated stop codon and the unspliced intron between the leader and $\alpha 1$ domains (e.g., GenBank BE486972), and none with that intron properly spliced out.

Because the two boCD1D pseudogenes were highly homologous and could have been alleles, we designed primer sets that were specific for these two genes. PCR on bovine genomic DNA

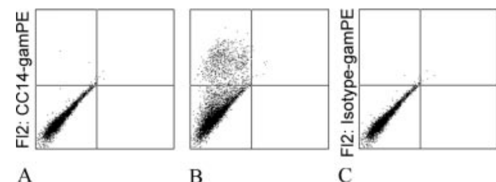

FIGURE 2. Flow cytometric analysis of Abs recognizing boCD1. Expression vector pcDNA3.1 with a cDNA insert encoding boCD1b3 or no insert was used to transiently transfect 293T cells. Cells transfected with a vector with no insert (*A*) or with the vector construct encoding boCD1b3 (*B* and *C*) were stained with mAb CC14 (*A* and *B*) and with an isotype control (*C*), followed by goat anti-mouse PE, before flow cytometric analysis. Results with transfected cells expressing boCD1a, bovine thymocytes, and bovine PBMC, and stainings with other Abs are not shown, but are summarized in Table II.

Table II. *Staining patterns of anti-CD1 Abs*

mAb	boCD1b3 Transfectant	boCD1a Transfectant	Bovine Thymocytes	Bovine B Cells	Published Recognition Pattern	Refs.
SBU-T6	+	+	+	+	Bo/Sh thymocytes, ShCD1e, Bo/Sh B cells	17, 18, 22, 30, 31
CC14	+	−	+	−	Bo/Sh thymocytes, ShCD1B-42	18, 30, 31
CC20	+	−	+	−	Bo/Sh thymocytes, HuCD1b	19, 20, 30, 31
CC43	−	−	+	+	Bo/Sh thymocytes, different from CC20 Ag; Bo B cells, not in *Bos indicus*	21, 32
CC122	+	−	+	−	Intestinal LP cells, Bo/Sh thymocytes	19, 21
OKT6	−	−	−	−	HuCD1a	
BCD1b3	+	−	+	−	HuCD1b	
F10/21A3.1	−	−	−	−	HuCD1c	
CD1d42	−	−	−	−	HuCD1d	

Sh, sheep; Hu, human; LP, lamina propria.

showed that both genes were present in all 12 individual outbred animals studied, arguing against the two genes being alleles of a single locus. Because both boCD1D genes found in the bovine genome were pseudogenes, we decided to investigate the possibility that cattle has no intact CD1D genes.

Southern blotting

Because the bovine genomic sequences containing the CD1 genes were at the time of this writing not assembled into complete chromosomes, we were concerned that basing our search for CD1 genes on the genomic data only may lead to the wrong number of CD1 genes. We thus set out to determine the number of CD1 genes in cattle by Southern blotting. Because CD1 $\alpha3$ domains are usually highly conserved within a species and sufficiently different from $\alpha3$ domains of other MHC-like molecules, we used a mixture of probes encoding the $\alpha3$ domains of boCD1a, boCD1b3, and boCD1e. Fig. 4 shows the presence of nine bands after digestion of genomic DNA with the *Stu*I restriction enzyme and hybridizing with a mixture of the $\alpha3$ domain probes. Digestion with other restriction enzymes led to seven or eight bands. Detection of a lower number of bands than the actual number of genes in a Southern blot is very common because very close sequence homology is likely to generate identical bands, and this may have happened in our case. In addition, when a restriction enzyme does not cut between two genes, these genes will give rise to one band. In summary, our Southern blot and genomic analyses are consistent with each other, and the Southern blot does not suggest a different number of CD1 genes than the number we found by searching genomic data.

Predicted amino acid sequences of boCD1 proteins

Alignment of partial and full-length bovine mRNA sequences and bovine genomic sequences allowed us to predict the amino acid sequences of the expressed *boCD1A*, *boCD1B1*, *boCD1B3*, and *boCD1E* genes (data not shown). In addition to the *boCD1D* pseudogenes that were discussed above, we noted that *boCD1B2* and *boCD1B4* are pseudogenes because of a mutated start codon and/or point mutations that would make the sequences out of frame. We have no evidence for the expression of the boCD1B5 gene. The extracellular domains of boCD1b1 and boCD1b3 proteins are 90% identical. Their predicted cytoplasmic tails are RFMGSHRVGHD and RRWSYQNIL respectively. After translation and transport from the endoplasmic reticulum to the cell surface, CD1 molecules recycle to specific cellular compartments

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BoCD1D1 cDNA      GA-ATGGGG-TGCTTGCTGTTTCTGTTCTTCTGGGGGCTTCCGCAGATTTGGCGGAGTTTTGAGAATGAGTGGAACCGGGCT--------
BoCD1D1 genomic   GAAGTGGGG-TGCTTGCTGTTTCTGTTCTTCTGGGGGCTTCCGCAGATTTGGCGGAGTTTTGAGAATGAGTGGAACCGGGCT--------
BoCD1D2 genomic   GAAGTGGGGGTGCTGGCTGTTTCTGTTCTTCTGGGGGCTTCCCCAGATTTGGGGGAGTTTGGGGGATGAGTGGAACCGGGGATT------
HuCD1d            GATATGGGG-TGCCTGCTGTTTCTGCTGCTCTGGGCGCTCCTCCAGGCTTGGGGAAGCGCTGAAGgtgggtggaacgagggcgcttgagt
      leader peptide  M  G   C  L  L  F  L  L  L  W  A  L  L  Q  A  W  G  S  A  E

BoCD1D1 cDNA      ----------GAGGGCGGAGAGAGGGAGCCAAGCAAGGAAAGGGGCCGACGTTCGCGGCGGGCGGGTGGAGAGTGATGCACCTCGAAAAG
BoCD1D1 genomic   ----------GAGGGCGGAGAGAGGGAGCCAAGCAAGGAAAGGGGCCGACGTTCGCGGCGGGCGGGTGGAGAGTGATGCACCTCGAAAAG
BoCD1D2 genomic   ---CCCGGAAGAGGTCGGCGAGAGGGAGCCGAGCAGGGAAAGGGGAGGACGCTCGCGGCGGGCGGGCGCAGACTGATGCACCTCGGAAAG
HuCD1d            gcactcgcgggagggcggagagagggagctgggtagggacggggagggcaacgcctgatggg--------gactggtgagacccgggacg

BoCD1D1 cDNA      CCCGGAAGTTGATCCACCAGCCCGAGAACCGGCTACTCCCTGCCTTCAGCTGTT-GATCCCTGGAGACGGAGCTCAATTCAGAGCGAGCA
BoCD1D1 genomic   CCCGGAAGTTGATCCACCAGCCCGAGAACCGGCTACTCCCTGCCTTCAGCTGCTTGATCCCTGGAGACGGAGCTCAATTCAGAGCGAGCA
BoCD1D2 genomic   CCGGGAGCTTGATCCAGCAGCTCGAGAACCTGCTACTCCCTGCCTCCAGCTGCTTGGCCCCTGGAGACCGAGCTCGATTCAGAGCGAGCA
HuCD1d            cactg-gcgcgatc---taggtagaaaactcgctgctccctggctccgg-------------ggagaggcagcgcggcacagagttcgct

BoCD1D1 cDNA      GACATCACCCTTTCTTCCCAAACCTAACTGTTTCTTTCTTTCTTTTTTCCTTCCTTCTCCCTTCTGTGCTCTCCCTCCCGTTTCCCCGGC
BoCD1D1 genomic   GACATCACCCTTTCTTCCCAAACCTAACTGTTTCTTTCTTTCTTTTTTCCTTCCTTCTCCCTTCTGTGCTCTCCCTCCCGTTTCCCCGGC
BoCD1D2 genomic   GACATCACCCTTTCTTCCCAAACCTAACTGTTTCTTTCTTTCTTTTTTTTTTCCTTTTCCCTTCTGTGCTCTCCCTCCCGTTTCCCCGGC
HuCD1d            ggcatcagcc--gcctcctgaagctcatctcctcttg-tttctttcttccttctctttatgctggctgctctcccggccacttgctacac

BoCD1D1 cDNA      TCCTCCGATCTGTATTCTCTCC-AGCCCCGCAAACGCCTTTCTCCTTCCAGGGCCTCCAGATCTCCTCGTTCGCCAACCGCAGCTGGACA
BoCD1D1 genomic   TCCTCCGATCTGTATTCTCTCC-AGCCCCGCAAACGCCTTTCTCCTTCCAGGGCCTCCAGATCTCCTCGTTCGCCAACCGCAGCTGGACA
BoCD1D2 genomic   TCCTCGGATCTGTATTCTCTCC-AGACCCGCAAACGCCTTTCTCCTTCCACGGCCTCCAGATCTCCTCCTTGGCCAACAGCAGCTGGACA
HuCD1d            gcctccaatcttcattctctcccagTCCCGCAAAGGCTTTTCCCCCTCCGCTGCCTCCAGATCTCGTCCTTCGCCAATAGCAGCTGGACG
                                       α1 domain  V  P  Q  R  L  F  P  LR  C  L  Q  I  S  S  F  A  N  S  S  W  T
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FIGURE 3. Alignment of human (Hu) and bovine (Bo) CD1D sequences. The two boCD1D genes, the cloned cDNA of *boCD1D1*, and human CD1D are shown from the leader peptide until the first part of the $\alpha1$ domain. The start codon is mutated in the genomic boCD1D sequences and there is no consensus signal for the expected 5′ splice site. Of note, all other introns are spliced out properly from the cDNA of *boCD1D1* (data not shown). Sequences that are present in cDNA are given in upper case, and sequences that are spliced out in cDNA are in lower case letters. The mutated start codons in the genomic sequences are underlined. The translation of human CD1D is given.

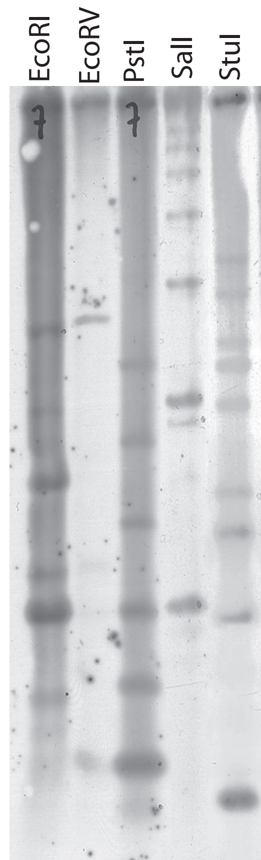

FIGURE 4. Southern blot and detection of CD1-containing fragments. A mixture of DIG-labeled probes of the α3 domains of boCD1b3, boCD1a, and boCD1e was hybridized to a Southern blot of bovine genomic DNA digested with the indicated restriction enzymes.

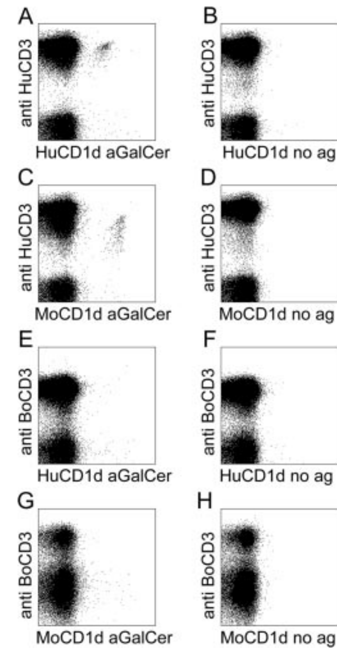

FIGURE 5. Bovine T cells do not recognize CD1d tetramers loaded with α-galactosylceramide (αGalCer). Human (*A–D*) and bovine (*E* and *F*) PBMC and bovine thymocytes (*G* and *H*) were stained with human (Hu) CD1d tetramers (*A* and *B* and *E* and *F*) or mouse CD1d tetramers (*C* and *D* and *G* and *H*) that were fluorescently labeled with allophycocyanin and loaded with α-galactosylceramide (*A, C, E,* and *G*) or with vehicle only (*B, D, F,* and *H*). Human cells were also stained with anti-human CD3-PE, and bovine cells with anti-bovine CD3-PE. Lymphocytes were gated based on low forward and side scatter, and dead cells were excluded based on high propidium iodide staining. Per panel 300,000 events are plotted. Mo, mouse.

(23). With the exception of CD1a, known sorting motifs are present in the cytoplasmic tails of human and mouse CD1 molecules. The cytoplasmic tail of boCD1b1 does not show homology to the tails of other known CD1 proteins, but it shows homology to the cytoplasmic tail of ovine B7.1 (PSMGSHRVGHD, GenBank accession number AY445823). The cytoplasmic tail of boCD1b3 shows high sequence homology to the human CD1b tail, including a putative tyrosine-based sorting motif. The human CD1b tail has no di-leucine-sorting motif, but boCD1b3 has a putative one.

Recognition of α-galactosylceramide

CD1d-restricted NKT cells with invariant TCR are known to be stimulated by α-galactosylceramide. An evolutionarily conserved TCR mediates recognition of α-galactosylceramide in humans and mice. The CD1d/α-galactosylceramide complex is recognized cross-species (24). We investigated whether cattle show reactivity to α-galactosylceramide, to support our genetic data for the absence of an intact CD1D gene, and to investigate the possibility that in cattle another Ag-presenting molecule is able to present α-galactosylceramide to NKT cells. Human and mouse CD1d tetramers loaded with α-galactosylceramide were used to stain bovine thymocytes (Fig. 5, *G* and *H*), PBMC (Fig. 5, *E* and *F*), liver cells (data not shown), and spleen cells (data not shown), but no staining above the background of unloaded tetramers was observed, whereas this was the case for human PBMC that were used as a positive control (Fig. 5, *A–D*). Human and bovine PBMC were stimulated with α-galactosylceramide and tested for proliferation. The proliferative capacity of all cells was confirmed with a polyclonal T cell activator, but even after multiple rounds of stimulation, reactivity against α-galactosylceramide could only be shown in human cells (data not shown).

Discussion

The CD1 family of genes in cattle lacks intact CD1D genes, while group 1 CD1 proteins were expressed. Two transcribed boCD1D pseudogenes, which we named *boCD1D1* and *boCD1D2*, were identified in cattle. Both genes contain a mutated start codon, and an unspliceable intron, consistent with the genomic data. The two CD1D genes are not alleles because both genes were present in 12 of 12 animals studied. Southern blotting confirmed the total number of CD1 genes found in the bovine genome, which is consistent with the absence of an additional, possibly intact bovine CD1D gene. This lack of an intact CD1D gene may be comparable to what has been found in the guinea pig, for which multiple CD1 genes have been identified but no clear homologue of CD1D (25). Lack of recognition of α-galactosylceramide by bovine T cells suggests that there is no functional substitute for CD1d that is able to stimulate NKT cells in cattle. It remains to be determined whether other cells with the NKT-like phenotype and functions compensate for the lack of functional CD1d and NKT cells in this species. The absence of functional CD1d in *Cavia porcellus* and *B. taurus* suggests that the NKT/CD1d system is not universally present in mammals.

The closest related species to cattle in which CD1 genes have been described is sheep (*Ovis aries*). In this species, one CD1D transcript has been described that appears to be functional, although its expression at the protein level has not been confirmed.

Common ancestors of sheep and cattle are thought to have diverged around 22 million years ago (26). Assuming that the inactivated boCD1D genes have arisen from a functional gene in a common ancestor, the inactivation as found in modern cattle must have taken place relatively recently. The highly conserved features of CD1d and NKT cells in humans and mice suggest an important function in the immune system that is otherwise fulfilled in cattle or for some reason not needed. Cattle and sheep share some salient immunological features like the high percentage of $\gamma\delta$ T cells. Ruminant $\gamma\delta$ T cells express members of the WC1 family of scavenger receptors (27) and have an extended hinge region in the constant part of the γ-chain (28), with additional cysteine residues (29). The function of ruminant $\gamma\delta$ T cells and the stimuli they react to are largely unknown, but do not seem to relate to the presence (in sheep) or absence (in cattle) of functional CD1d and NKT cells.

Bos taurus is well equipped to present Ags to T cells with its functional CD1A and multiple CD1B gene products. Most known pathogen-related Ags that are presented by group 1 CD1 are mycobacterial Ags. Cattle is a natural host of several mycobacterial pathogens. Infected cattle may in the future be used to study group 1 CD1-restricted T cell immunity against mycobacterial infections, which can lead to improved vaccines for cattle and a potentially useful model for human mycobacterial diseases and vaccine development.

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Disclosures

The authors have no financial conflict of interest.

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