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Bovine T cell receptor gamma variable and constant genes: combinatorial usage by circulating γδ T cells

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Abstract Studies here describe expression and sequence of several new bovine T cell receptor gamma (TRG) genes to yield a total of 11 TRG variable (TRGV) genes (in eight subgroups) and six TRG constant (TRGC) genes. Publicly available genomic sequences were annotated to show their placement. Homologous TRG genes in cattle and sheep were assigned, using four accepted criteria. New genes described here include the bovine TRGC6, TRGV2, and TRGV4, homologues of ovine TRGC4, TRGV2, and TRGV4, respectively. The bovine Vγ7 and BTGV1 clones (previously TRGV4 and TRGV2, respectively) were reassigned to new subgroups TRGV7 and TRGV8, respectively, with approval by the IMGT Nomenclature Committee. Three TRGV subgroups (TRGV5, TRGV6, and TRGV8) were further designated as TRGV5-1 and TRGV5-2, TRGV6-1 and TRGV6-2, and TRGV8-1 and TRGV8-2 because each subgroup is comprised of two mapped genes. The complete sequence of bovine TRGC5 is also reported, for which a limited number of nucleotides was previously available, and shown to be most closely related to ovine TRGC5. Analysis of circulating γδ T cells revealed that rearrangement of TRGV genes with TRGC genes is largely dictated by their proximity within one of the six genomic V-J-C cassettes, with all TRG genes expressed by bovine peripheral blood γδ T cells. Cattle are useful models for γδ T cell biology because they have γδ T cells that respond to isopentenylpyrophosphate (IPP) antigens, while mice do not, and some bovine TRGV genes cluster closely with human genes.

Keywords γδ T cells · γδ T cell receptor genes · TRGC · TRGV · Ruminant

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank database under the accession numbers DQ179591, DQ179592, DQ179593, and DQ179594.

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Introduction

Ruminants are considered among the "γδ T cell high" species, which includes not only the ruminant species of sheep and cattle but also another mammalian species within artiodactyls, i.e., swine (Hein and Mackay 1991), as well as nonmammalian species such as chickens (Cooper et al. 1991). "γδ high" refers to the level of γδ T cells in circulation rather than in epithelial-rich tissues, because even in "γδ low" species such as mice, the number of γδ T cells can comprise up to 50% of lymphocytes in skin, intestine, and the reproductive tract. Moreover, at least in the γδ T cell high ruminant species, the large proportion of γδ T cells decreases to 5% of peripheral blood mononuclear cells (PBMC) by adulthood (Rogers et al. 2005). This is similar to humans, in whom the proportion of γδ T cells in the blood decreases between adolescence and adulthood (Parker et al. 1990).

While γδ T cells share many functions with αβ T cells, particularly in their role as inducers of inflammation through their ability to produce interferon gamma (IFN-γ), they are distinct in the manner by which their T cell receptor interacts with antigen and the types of antigens that stimulate them. For example, γδ T cells do not see antigenic peptides in the context of self major histocompatibility complex (MHC) molecules, but rather, they can respond to autologous molecules on cells and react with nonpeptidic molecules, such as isopentenylpyrophosphate (IPP), derived from mycobacteria (for review, see Hayday 2000). γδ T cells also differ from αβ T cells in that expression of particular T cell receptor gamma (TRG) and T cell receptor delta (TRD) genes may be developmentally controlled and the γδ T cell receptor genes expressed direct

tissue localization. That is, the murine TRGV1–TRGC4/TRAV15/DV6 subgroup (Bosc and Lefranc 2003) T cell receptor genes are expressed by cells early in ontogeny and reside as intraepithelial lymphocytes, while the TRGV5–TRGC1/TRDV1-expressing cells localize in the intestine (Ota et al. 1992). Similarly, human TRGV1 subgroup (Lefranc et al. 1986a)/TRDV1-expressing T cells that react with the autologous MHC-like protein, MICA, localize in the intestine (Groh et al. 1998). While many different γ and δ T cell receptor genes (Lefranc and Lefranc 2001) are expressed by cells in human cord blood, shortly after birth there are generally only two major subpopulations in blood (Morita et al. 1994): specifically the TRGV9/TRDV2 population, which responds to IPP and expands with age, and the TRGV1 population that decreases with age.

While γδ T cells from humans and mice have been extensively studied, antigen responsiveness by γδ T cells from cattle, a γδ high species, has also been given serious attention. Bovine γδ T cells were shown to respond to components of mycobacteria, including IPP, when cells are from mycobacteria-infected cattle (Welsh et al. 2002), to autologous antigens on monocytes (Sathiyaseelan et al. 2002a) and protozoan-transformed lymphocytes (Daubenberger et al. 1999), to antigens of *Leptospira* after in vivo priming (Naiman et al. 2001), and to peptides from *Anaplasma marginale* (Lahmers et al. 2005). Moreover, like human and murine γδ T cells, bovine γδ T cells can produce IFN-γ after stimulation (Naiman et al. 2001; Rogers et al. 2005). It is important to expand our capacity to study the biology of γδ T cells in other mammalian species, since mice, one of the main models for humans, do not respond to mycobacterial IPP antigen. However, a full understanding of immune responses by bovine γδ T cells requires an understanding of the T cell receptors they express. While quite extensive study of the ovine γδ T cell receptor genes has been carried out, the understanding of bovine γδ diversity is less complete. This became apparent in our studies of the γδ T cell response to *Leptospira* bacterial antigens (Blumerman et al., submitted for publication), which is by a discrete population of cells expressing the γδ T cell lineage-specific molecule WC1.1 (Naiman et al. 2001; Rogers et al. 2005). While we were able to clone and sequence the δ-chain from cDNA obtained from responsive cells, we were unable to identify corresponding γ-chain cDNA. Thus, it became clear that the coverage of the bovine genes was incomplete rather than the alternative hypothesis proposed previously (Su et al. 1999) that the bovine TRG locus is less complex than that of sheep.

Here, we identified new bovine TRG variable (TRGV) region and TRG constant (TRGC) region genes and determined their location on the TRG loci relative to other TRGV and TRGC genes. Analysis of circulating γδ T cells revealed that they use all eleven TRGV genes and six different TRGC genes. Moreover, the association of TRGC genes with particular TRGV genes in cDNA is largely dictated by their proximity to one another on the chromosome.

Materials and methods

Isolation of cells

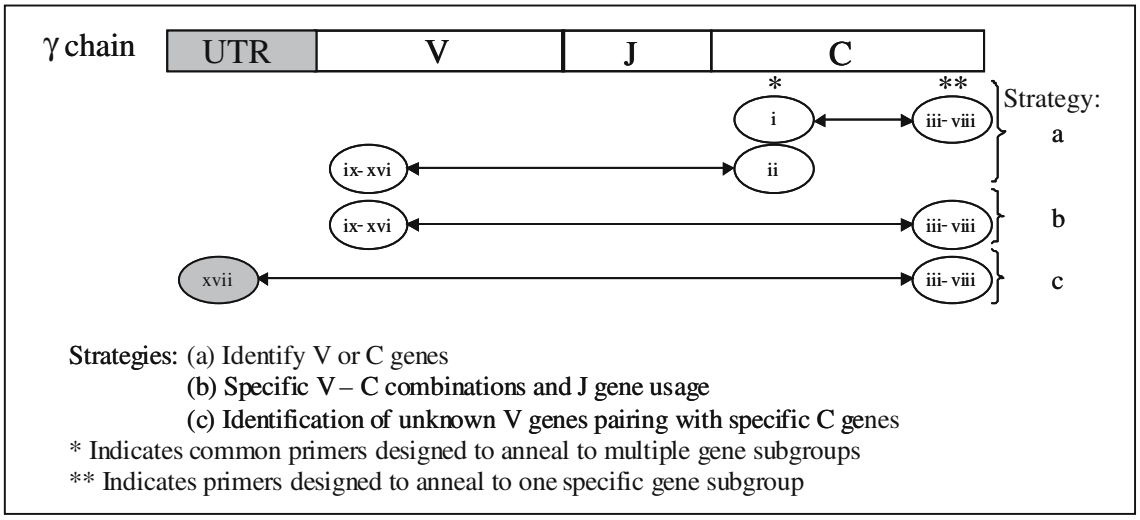
Blood was collected into heparin from cattle aged 14 to 20 months by venipuncture of the jugular vein. PBMC were isolated from blood via density gradient centrifugation over Ficoll-Hypaque (Ficoll-Paque, LKB-Pharmacia Biotechnology, Piscataway, NJ), using standard techniques, and immediately resuspended in Trizol (Invitrogen, Carlsbad, CA) for isolation of ex vivo RNA.

RNA isolation and reverse transcription-polymerase chain reaction

RNA was isolated from cells using Trizol according to the manufacturer's instructions (Invitrogen). Total RNA was used for cDNA synthesis, employing a commercial Reverse Transcription (RT) kit and random primers (Promega, Madison, WI). Polymerase chain reaction (PCR) was carried out using primer pairs described in Fig. 1 based on publicly available sequences from the National Center for Biotechnology Information GenBank website (http://www.ncbi.nih.gov/Genbank/), the ImMunoGeneTics information system (IMGT; http://imgt.cines.fr; Lefranc et al. 2005), or referenced publications as indicated. PCR reagents (Promega) were used at a final concentration of 2 mM $MgCl_2$ and cycling conditions were 30 s at 95°C, 1 min at 55°C, and 45 s at 72°C for 35 cycles. PCR products were analyzed in 2% Tris–acetate–EDTA agarose gels and were then cloned into the pCR2.1 vector (Invitrogen). Between two and ten cDNA clones from each PCR reaction (a total of more than 100 clones) were sequenced commercially using the T7 forward and M13 reverse primers (GeneWiz, North Brunswick, NJ), for verification of product identity and for subsequent sequence analysis.

Sequence analyses

Analysis of nucleotide and deduced amino acid sequences including alignment of sequences, percent identity determination, and construction of a coding tree (cladogram) was performed using BioEdit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalW version 1.83 using the default parameters (Chenna et al. 2003). Unless otherwise indicated, TRGV sequences were truncated after the leader sequences (L-REGION) at amino acid position 1 and at the beginning of the CDR3 region, as defined according to the IMGT unique numbering (http://imgt.cines.fr; Lefranc et al. 2003).



Primer Number	Bovine Gene Subgroup	Primer Design and Orientation	Primer Sequence (5'-3')
i	Common bovine TRGC	bovine TRGC(1-4) forward	CAGGGAAATACCATGAAGAC
ii	Common bovine TRGC	bovineTRGC(1-4) reverse	AGCCAGCTGAACTTCATGTA
iii	TRGC1	bovineTRGC1 reverse	CCTGTAAGTTTGCTTTCATCGGTC
iv	TRGC2	bovineTRGC2 reverse	GTATCTGTAATGCTTTCATCAGTC
v	TRGC3	bovineTRGC3 reverse	CCAGGCTAGATTCTGAGCAG
vi	TRGC4	bovineTRGC4 reverse	GTGACTTCACTTTCATCTTTCAG
vii	TRGC5	ovineTRGC5 reverse	GATGGAGAAGTAGATCATGC
viii	TRGC6	ovineTRGC4 reverse	GTAGATGGCACTCTTGAGG
ix	TRGV1	bovineTRGV1 forward	ATGTTGTGGGCCCTAGTGCTGCCTT
x	TRGV2	ovineTRGV2S1&2 forward	ATGATGCGGGTCCCAGC
xi	TRGV3	bovineTRGV3 forward	ATGTCACCATTGGAAGCATTCACA
xii	TRGV4	ovineTRGV4 forward	CGTTGTGCACTGGTATCAAG
xiii	TRGV5	bovineTRGV5 forward	ATGCGGGCCCCAGCACTGCTTCTG
xiv	TRGV6	bovineTRGV6 forward	ATGGGCTCTTCTCTCGGGGCA
xv	TRGV7	bovine TRGV7 forward (previously TRGV4)	ATGGCATTCCTGGAAGCGGTCCTC
xvi	TRGV8	bovineTRGV8 forward (previously TRGV2)	ACAAGTTGTCAGTCATGAGGGCTA
xvii	TRGV 5' untranslated region	UTRG forward	CTGTCACATCTGCTGAGAA

Fig. 1 RT-PCR strategies used to identify TRGV and TRGC genes expressed by bovine cells and the primer sequences employed in each strategy as indicated by *lower case Roman numerals* (*i–xvii*). Primers were designed based on available sequences for TRG genes for cattle and sheep and were specific for sequences that were conserved among known TRGC genes (those in the column marked with *one asterisk*), specific for a transcript within a single gene subgroup (those in the column marked with *two asterisks*), or for sequence in the untranslated region (UTR)

TRGC sequences were truncated at the first codon of the *C* region and at the first stop codon. We employed four accepted criteria to assign homologies to the ovine TRG genes including: (1) at least 60% deduced amino acid identity, (2) proximity on a coding tree, (3) positional information within the chromosomal locus, and (4) distinct features (Clark et al. 1995; Glusman et al. 2001; Lefranc and Lefranc 2001).

Genomic sequences employed included those submitted to GenBank by Conrad and coworkers (http://www.ncbi.nih.gov/Genbank/; accession nos. AY644517 for TRG1@ and AY644518 for TRG2@). cDNA sequences used were found at the IMGT Web site (http://imgt.cines.fr/textes/IMGTrepertoire/LocusGenes/) and the GenBank Web site. Accession numbers of the TRGV and TRGC sequences used for primer design and sequence analysis are in parentheses following the gene name:

cattle (Bos taurus), TRGV1S1 (D16119), TRGV1S2 (D16123), TRGV1S3 (D16131), TRGV3S1 (U73186), TRGV3S2 (U73187), TRGV5S1 (D13653), TRGV5S2 (D16121), TRGV5S3 (D16122), TRGV5S4 (D16125), TRGV5S5 (D16128), TRGV5S6 (D16129), TRGV5S7 (D16130), TRGV5S8 (D13649), TRGV5S9 (D13652), TRGV5S10 (D16132), TRGV5S11 (D16126), TRGV5S12 (D16127),TRGV5S13 (D13651), TRGV5S14 (D13654), TRGV5S15 (D16120), TRGV5S16 (D16133), TRGV5S17 (D13650), TRGV5S18 (D16117), TRGV5S19 (D16118), TRGV5S20 (D16124), TRGV6S3 (Vγ6; AY560834), TRGV7S1 (Vγ7, previously called TRGV4; U73188), TRGV8S3 (BTGV1, previously called TRGV2; D13648), TRGC1 (*01: D90409, *02: D90410, *03: D90413), TRGC2 (*01: D90411, *03: D90415, *03: D90417, *04: D90416), TRGC3 (*01: D90414), TRGC4 (*01: X63680), TRGC5 (AY735449), TRGC6 (AY735450), TRG1@ (AY644517), TRG2@ (AY644518);

sheep (Ovis aries), TRGV1S1 (Z12998), TRGV2S1 (Z12999), TRGV2S2 (Z13000), TRGV2S4 (Z13002), TRGV3S1 (Z13003), TRGV4S1 (Z13004), TRGV5S1 (Z13005), TRGV5S2 (Z13006), TRGV6S1 (Z13007), TRGV8S1 (previously called TRGV2S3; Z13001), TRGC1 (*01: Z12964), TRGC2 (*01: Z12965), TRGC3 (*01: Z12966), TRGC4 (*01: Z12967), TRGC5 (*01:

Z13986), TRGC6 (*01: AF312559, AF312560, AF312561), TRG2@ (AY362775).

Accession numbers of the previously unidentified or incompletely sequenced bovine TRGV and TRGC sequences are as follows: TRGV2S2 (DQ179591), TRGV4S2 (DQ179592), TRGC5 (DQ179593), TRGC6 (DQ179594). Accession numbers of the newly designated subgroup genes, for those subgroups containing two mapped genes, are as follows: TRGV5-1 (DQ179595), TRGV5-2 (DQ179596), TRGV6-1 (DQ179597), TRGV6-2 (DQ179598), TRGV8-1 (DQ179599), TRGV8-2 (DQ179600). These sequences were derived from cloned RT-PCR products.

Results

Determining TRG genes expressed in bovine PBMC

While five TRGC region genes have been previously identified for cattle and six for sheep, with one ovine gene being nonfunctional [i.e., TRGC6 (ovCγ6); Miccoli et al. 2003], on the basis of preliminary analysis of antigen-expanded bovine γδ T cells in our lab, it became apparent that the previously described bovine gene sequences did not represent exhaustive coverage. While others have suggested that the duplication of TRG genes in cattle is more limited than in sheep (Su et al. 1999), resulting in the disparity in number, we postulated the analysis of the bovine TRG genes was incomplete and that homologues for all ovine TRG genes existed in cattle.

To address this, primers were designed for the constant and variable genes of the TRG chain based on publicly available bovine and ovine gene sequences using the strategies outlined in Fig. 1. Sequences from both species were used because of the known high degree of homology between genes for which both ovine and bovine sequences exist. The TRGC gene sequences were aligned to identify a common gene primer (Fig. 1, primers i and ii) that annealed to known bovine and ovine TRGC1 through TRGC5 sequences. Primers for the individual TRGC genes were

a

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TRGV2    ATGATGCGGGTCCCAGCGCTGCTTCTGGTTTTCCTGGCTCCTGTCACGCAGGTGTCTTCCAACACAGAAGGGGAGAAGATGTCAATCACT 90
TRGV2    AAGGCAACTGGGTCTGGGTCGTCTGTTGAAATCATTTGTGATCTTATTACCCAAACCCTCAAATATATCCACTGGTACAAGTACCAGGAG 180
TRGV2    GGCACAGCTCCTCGACGCCTTCTTTACTACGACATCTCCTACTCAAAGGTTGTGTTGGAATCAGGAGTCAGTGAAGGGAAGTACAAAGTT 270
TRGV2    TATAAAGAGAAGAGCTATACTTTTGCAATCTTAAACCTGCAAGAAAGTGATTCTGGAATGTACTACTGT 339

TRGV4    TCTACCCGAAGACCAGACAGAACAGTGCACATAAGTTGCAAGCTGTCAGGGGTTCCCCTGGAGAATGCCATTATGCACTGGTATCAAGAG 90
TRGV4    AAAGAAGGGGAGCCCCTGAAACGAATCCTTTATGGCTCAGCTAACAGTTATAAGCTGGACAAACCTAATTCCCGCTTGGAGATAGATAAC 180
TRGV4    AAAAAGAATGGAATCTTTTACCTGATAATCAATAATGTTGTCAAGTCCGATGAAGCCACCTACTACTGT 249
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b

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boTRGV2   MMRVPALLLVFLAPVTQVSSNTEGEKMSITKATGSGSSVEIICDLITQTLKYIHWYKYQEGTAPRRLLYYDISYSKVVLESGVSEGKYKV 90
ovTRGV2S1 ................M.....M.R........AVP.AF...........V.......H..................I.....I....... 90
boTRGV2   YKEKSYTFAILNLQESDSGMYYC 113
ovTRGV2S1 ..........S............ 113

boTRGV4   STRRPDRTVHISCKLSGVPLENAIMHWYQEKEGEPLKRILYGSANSYKLDKPNSRLEIDNKKNGIFYLIINNVVKSDEATYYC 83
ovTRGV4S1 ........................VV..................................M.......................... 83
```

c

	FR1-IMGT (1-26)	CDR1-IMGT (27-38)	FR2-IMGT (39-55)	CDR2-IMGT (56-65)
	10 20 *	30	40 50	60
	····|····|····| * ····|····|·	···|····|···	·|····|····|····|	····|····|
TRGV2	SSNTEGEKMSITKATGSGSSVEIICDLI	TQTLKY.....	IHWYKYQEGTAPRRLLY	YDISYSKV..
TRGV4	----------STRRP..DRTVHISCKLS	GVPLENAI....	MHWYQEKEGEPLKRILY	GSANSY....

	FR3-IMGT (66-104)			
	70	80	90	100
	····|····|····|····|····|····|····|····			
TRGV2	VLESGVSEGKYKVYK...EKSYTFAILNLQESDSGMYYC			
TRGV4	KLDKPNSRLEIDNKK...NGIFYLIINNVVKSDEATYYC			

Fig. 2 Nucleotide sequences of previously unidentified expressed bovine TRGV genes. Nucleotide and amino acid identities are indicated by a *dot* (.) and sequence that is not available is indicated by a *dash* (–). Gene names of the subgroup sequence used in the analyses are as indicated; however, no gene name is given if only one sequence exists in a particular subgroup at this time. See "Materials and methods" for GenBank accession numbers. **a** TRGV2 (primer designed using ovine TRGV2S1 and TRGV2S2 sequences) and TRGV4 (primer designed using ovine TRGV4 sequence) nucleotide sequences. **b** Comparison of the deduced amino acid sequences of the previously unidentified bovine TRGV genes (*bo*) and publicly available ovine (*ov*) gene sequences. **c** The framework regions (FR-IMGT) and complementarity determining regions (CDR-IMGT) according to the IMGT unique numbering for V-DOMAIN (Lefranc et al. 2003) are indicated for the newly identified bovine TRGV2 and TRGV4 genes. Amino acids 15A and 15B are indicated by an *asterisk* (*). **c** Dots (.) indicate gaps according to the IMGT unique numbering

also designed (primers iii–viii). Primer pairs were designed to uniquely amplify each of the known ruminant TRGV region gene subgroups (primers ix–xvi). The common TRGC primer was paired either with the gene-specific TRGC primers to verify expression of specific TRGC genes or with TRGV primers to identify expressed TRGV genes (Fig. 1, strategy a). Possible expressed TRGV–TRGJ–TRGC gene combinations were determined by pairing primers for all individual TRGC genes with primers for all TRGV genes (Fig. 1, strategy b). Finally, a primer specific for ovine sequences found in the 5′ untranslated region (UTR) of TRGV genes (primer xvii; Hein and Dudler 1993; Ishiguro et al. 1993) was also used to identify TRGV genes that had been previously unidentified for either cattle or sheep by pairing the UTR primer with the unique TRGC primers (Fig. 1, strategy c). A total of more than 100 cDNA clones from PCR products were sequenced to confirm insert identity and specificity of the primer pairs used and to obtain sequence of expressed genes that had not been previously described.

Bovine TRGV genes

The analysis conducted here identified expression of two new TRGV genes by bovine cells including TRGV2 and TRGV4, whose nucleotide sequences are reported (Fig. 2a), along with their deduced amino acid sequences, which are compared to the ovine genes assumed at initiation of these studies to be their homologues (Fig. 2b). The structure of the expressed gene product for the TRGV genes including complementarity determining region (CDR-IMGT) and framework (FR-IMGT) sequences was modeled on previously available information at IMGT, based on the IMGT numbering for V-DOMAIN (Lefranc et al. 2003; Fig. 2c).

Bovine TRGV gene sequences obtained in this study and those previously published were compared with publicly available ovine sequences to determine homology based on the degree of identity of nucleotide and inferred amino acid sequences. The homologous bovine and ovine TRGV gene subgroups and the new subgroup names approved by the IMGT Nomenclature Committee are summarized in Table 1 and Fig. 3. The newly identified bovine gene TRGV4 reported in this paper was 98% identical at the nucleotide level with the ovine TRGV4S1, indicating that these genes are homologous. Comparison of the newly identified bovine TRGV2 sequence reported in this study revealed the greatest identity, 94%, with ovine TRGV2S1 and TRGV2S2 (Fig. 4). The bovine TRGV2 and TRGV4 gene sequences reported here (Fig. 2) are currently the unique representatives of the bovine TRGV2 and TRGV4 subgroups. The bovine Vγ7 (Hein and Dudler 1997), previously called TRGV4, and BTGV1 (Ishiguro et al.

Table 1 Summary of homology of the bovine and ovine TRGV genes based on cladograms and percentage identity of nucleotide and deduced amino acid sequence

Homologous genes				Previous or alternative bovine gene names	
IMGT subgroups		Percent nucleotide identity	Percent inferred amino acid identity	Gene names	Reference[b]
Bovine[a]	Ovine				
TRGV1	TRGV1	92	83	Vγ1	Ishiguro et al. 1993
TRGV2	TRGV2S1 TRGV2S2	94	87	–	–
TRGV3	TRGV3	90	85	Vγ3	Hein and Dudler, 1997
TRGV4	TRGV4	98	96	–	–
TRGV5-1 TRGV5-2	TRGV5	95	88	V2 /V6 Vγ5	GenBank/Conrad, Ishiguro et al. 1993
TRGV6-1 TRGV6-2	TRGV6	93	88	Vγ6	Vaccarelli 2004[c]
TRGV7	none	–	–	Vγ7, previously TRGV4	Hein and Dudler, 1997 IMGT
TRGV8-1 TRGV8-2	TRGV8	92	88	V3.1/V3.2, BTGV1, previously TRGV2S3	GenBank/Conrad, Ishiguro et al. 1993, IMGT

[a]Bolded designations indicate bovine genes previously unidentified. A designation containing a dash, such as TRGV8-1 and TRGV8-2, indicates that these are two separate genes. Underlined IMGT gene names are those that are both mapped (or unambiguously identified) genes and for which the nomenclature is definitive

[b]Designations are referenced to GenBank (Conrad et al. submission, accession numbers AY644517 for TRG1@ and AY644518 for TRG2@) and IMGT http:// www.imgt.cines.fr)

[c]G. Vaccarelli, M.C. Miccoli, C. Lanave, E.P. Cribiu and S. Ciccarese. Comparative analysis of human and bovidae T cell receptor variable and joining gamma genes (unpublished); Direct submission Feb 27, 2004, Dipartimento Di anatomia Patologica E Di Genetics, University of Bari, via amendola 165/A, Bari, BA 70126, Italy

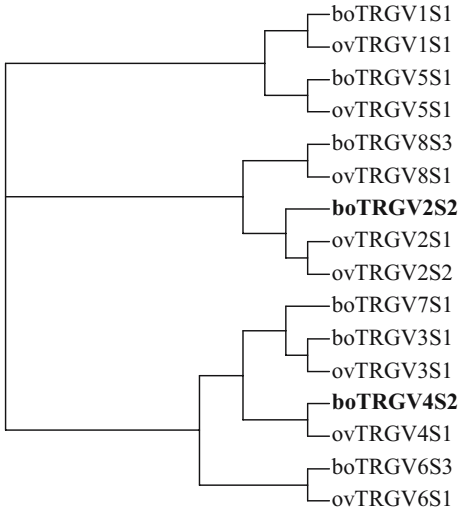

Fig. 3 Cladogram constructed to determine relationship between TRGV bovine (*bo*) (new sequences reported in this study are *bolded*) and ovine (*ov*) amino acid sequences of TRGV genes. Bovine and ovine gene subgroup (s) sequences used are as indicated (see "Materials and methods" for accession numbers). Full-length sequences for ovine and bovine TRGV4 are not available; thus, analyses were performed by truncating at amino acid position 10 and at the beginning of the CDR3 region

1993), previously called TRGV2, sequences were assigned the new subgroup names TRGV7 and TRGV8, respectively. This was because the expressed bovine TRGV8 sequence and the predicted transcripts from the two bovine genomic sequences, TRGV8-1 and TRGV8-2 (identified in the 5′ end of the gamma locus TRG1@; Conrad et al., GenBank), were most similar to the ovine TRGV8S1 gene (previously TRGV2S3) (Fig. 4). The former Vγ7 shared little identity to any other bovine or ovine TRGV sequence. Ruminant TRGV2 and TRGV8 subgroups can be distinguished from one another by a different CDR1-IMGT length (six and three amino acids, respectively).

Comparisons among the bovine TRGV gene subgroups indicated that the levels of identity ranged from 34 to 75% for the nucleotide sequences and from 17 to 66% for the inferred amino acid sequences (Table 2), which were much less than the proposed homologous genes between ruminant species (see Table 1). The levels of identity for individual sequences that fall within bovine TRGV gene subgroups were also determined. The TRGV5 subgroup had 20 publicly available cDNA sequences, which were >93% identical for all comparisons (data not shown). For three cDNA sequences in the TRGV1 subgroup and two in the TRGV3 subgroup, the identities were ≥90% (data not shown). These slight sequence variations within TRGV gene subgroups likely represent polymorphisms among breeds or individuals (IMGT Repertoire, http://imgt.cines.fr). Overall, the level of similarity among bovine TRGV subgroups was not greater than that between homologous ovine and bovine TRGV genes that fall within the same subgroups.

Bovine TRGC genes

Expression of one new TRGC gene, TRGC6, was identified in this study, along with the complete sequence for bovine TRGC5 (Fig. 5). At the conclusion of these studies, additional bovine TRGC5 and TRGC6 sequences became available (Lahmers et al. 2005) and were retrospectively included in sequence analyses reported here. Sequence analyses, comparing sequences obtained here and those previously published for bovine TRGC genes with ovine sequences, reflect the gene homology between these closely related ruminant species, but it should be noted that the numeric gene designations are not necessarily analogous between the two ruminant species (Table 3). For example, the new TRGC6 gene, whose nucleotide sequence is reported here (Fig. 5) and by Lahmers et al. (2005), has the highest degree of identity (92%) with the ovine TRGC4 gene. Relationships between bovine and ovine TRGC genes were confirmed by cladogram analyses (Fig. 6).

The identity among bovine TRGC genes (Table 4) ranged from 55 to 90% for the nucleotide sequence and from 41 to 88% for the amino acid sequence among bovine TRGC genes. Sequence analysis showed that the TRGC1 and TRGC2 are the most highly related, although TRGC1 has an insertion of ten amino acids when compared with TRGC2. Thus, except for those two genes, the nucleotide identity among bovine TRGC genes was generally less than that between the homologous bovine and ovine genes, which ranged from 88 to 96% at the nucleotide level. The exception was the ovine TRGC6, which had the lowest percentage of identity with its closest bovine genes TRGC1 and TRGC2 being 72 and 79%, respectively. Upon evaluation of individual sequences within TRGC gene subgroups, we found that the percent identity among three available TRGC1 gene sequences and among four available TRGC2 gene sequences was 98–99% (data not shown). Overall, the level of similarity among bovine TRGC subgroups was not greater than that between homologous ovine and bovine TRGC genes that fall within the same subgroups.

TRGV and TRGC gene combinations found in PBMC

RT-PCR was conducted on RNA from ex vivo PBMC from two animals of differing breeds to ascertain which TRGV–TRGJ–TRGC gene combinations were used by bovine γδ T cells. To do this, primers for all possible V–C combinations were employed in a checkerboard comparison. The results showed eleven V–C combinations occurred, as detected by RT-PCR (Fig. 7a) with a schematic representation of the successful recombinations found in this study (Fig. 7b). Sequencing of the products was conducted to confirm the identity of the gene combinations amplified by RT-PCR. Combinations that did not yield a product are not shown.

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              Exon 1 →                          ← 
boTRGV8-1 ATGCCGTGGGTCCCAGCACTGTTTCTGGTTTTCCTGGCTCCTGGTAAGTGTGTTGCCTGGATGCTCTGGGGATTTTTCTGTACTTTTTGG 90
boTRGV8-2 ....T...............C....................A............................................... 90
ovTRGV8S1 ....T...............CC.G~~~A..............~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 39

ovTRGV2S1 ...AT.C..........G...C....................~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 42
ovTRGV2S2 ...AT.C..........G...C....................~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 42
boTRGV2   ...AT.C..........G...C....................~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ 42

                                                                                      → Exon 2
boTRGV8-1 CAATGGTGAGGGGGAGGGTCATACTAAGAAATGGTGTTTCCTCTTTCTCATGATTTTTTGTTGCTTTCCCCATTGCAGTCACACAGGTAT 180
boTRGV8-2 .......................................................................................... 180
ovTRGV8S1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~............. 52

ovTRGV2S1 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~....TG.....G. 55
ovTRGV2S2 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~....TG.....G. 55
boTRGV2   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~.....G.....G. 55

boTRGV8-1 CTTCCAACATGGAAGAGGACAAGTTGTCAGTCATGAGGGCT~~~~~~ACTGGGTCATCTATTGTAATCGATTGTGATCTTACAGGAAGC~ 263
boTRGV8-2 ........................................G~~~~~~............G.............................~ 263
ovTRGV8S1 ............G....................C......G~~~~~~............G.......................CA..A.~ 135

ovTRGV2S1 ..............AG...G...A.....A.T.CT.A...AGCTGTGC.....G...T.G...A....AT...........TCACCCAAA 145
ovTRGV2S2 ...............G...G...A.....A.T.CT.A...AGCTGTGC.....G...T.G...A....AT...........TCACCCAAA 145
boTRGV2   .........CA....G...G...A.....A...CT.A...AACTGGGT.......G...G...A....AT...........TTACCCAAA 145

boTRGV8-1 ~~~~~~~~TATATCCACTGGTACAAATTCCAGGAGGGAGCTGTACCCCGACGCCTCCTCTACTACGATGTCTCCAACTCAAAGGCTGTGT 345
boTRGV8-2 ~~~~~~~~................................................................A.T.........T..A.. 345
ovTRGV8S1 ~~~~~~~~..............................A....G...........T................A.T.........T...A. 217

ovTRGV2S1 CCGTCAAA.................GCA.........CA.A.CT...........T..T........CA.....T........AT..... 235
ovTRGV2S2 CCGCCAAA.................G.A.........CA.A.CT...........T..T........CA.....T.........T..... 235
boTRGV2   CCCTCAAA.................G.A.........CA.A.CT..T........T..T........CA.....T.........T..... 235

boTRGV8-1 TGGACTCAGGAATCAGTGAAGGGAAATACCATGCTTACAAAGGCACAGGGAAGAGCTACACGTTTGTAATCTCCAACTTGCAAGAAAGTG 435
boTRGV8-2 ....T...............A.......T.G..T...........A.~~~~~~~~~~~~~~~A..T........................ 420
ovTRGV8S1 ...............................C.T..............................C........A................ 307

ovTRGV2S1 ....A....................G...A.A.T...T....AG~~~~~~........T..T....C......A...C............ 319
ovTRGV2S2 ....A....................G.....A.T...T....AG~~~~~~........T..T....C......A...C............ 319
boTRGV2   ....A......G.............G...A.A.T...T....AG~~~~~~........T..T....C.....TA...C............ 319

boTRGV8-1 ATTCTGGCACCTACTACTGT 455
boTRGV8-2 .................... 440
ovTRGV8S1 ..........G......... 327

ovTRGV2S1 .......A.TG......... 339
ovTRGV2S2 .......A.TG......... 339
boTRGV2   .......A.TG......... 339
```

Fig. 4 Comparison of the two coding regions of the genomic bovine (*bo*) TRGV8-1 and TRGV8-2 on TRG1@ (refer to Fig. 8 for base pair locations of these coding regions) with expressed sequence of ovine (*ov*) TRGV2S1, TRGV2S2 and TRGV8S1 and the newly identified bovine TRGV2. Nucleotide identities are indicated by a *dot* (.) and sequence gaps are indicated by a *tilde* (~)

Table 2 Percent identity of nucleotide and predicted amino acid sequences among bovine TRGV gene subgroups

Gene subgroups	Gene names	Gene subgroups (gene names)							
		TRGV1 (*TRGV1S1*)	TRGV2 (*TRGV2S2*)	TRGV3 (*TRGV3S1*)	TRGV4[a] (*TRGV4S2*)	TRGV5 (*TRGV5S1*)	TRGV6 (*TRGV6S3*)	TRGV7 (*TRGV7S1*)	TRGV8 (*TRGV8S3*)
TRGV1	*TRGV1S1*	*	*70*	*37*	*34*	*77*	*46*	*39*	*69*
TRGV2	*TRGV2S2*	56	*	*41*	*38*	*68*	*45*	*39*	75
TRGV3	*TRGV3S1*	18	19	*	*47*	*38*	*37*	*58*	*38*
TRGV4[a]	*TRGV4S2*	20	24	22	*	*36*	*34*	*49*	*37*
TRGV5	*TRGV5S1*	66	57	17	23	*	*42*	*37*	*68*
TRGV6	*TRGV6S3*	27	29	23	19	27	*	*38*	*45*
TRGV7	*TRGV7S1*	22	21	44	25	18	25	*	*37*
TRGV8	*TRGV8S3*	52	65	19	25	56	31	21	*

Numbers in italics above the asterisks (forming a diagonal line) indicate percentage nucleotide identities, while numbers below the diagonal indicate percentage amino acid identities

[a]Full-length sequence for bovine TRGV4 is not available, and thus, analyses were performed by truncating at amino acid position 10 and at the beginning of the CDR3 region

a

```
TRGC5    GATAGAAGGCTTGATGGAGACTTATTTCCCAAGCCCACTATATTTTTTCCTTCAGTTGAGGAAGTAAAACTCCACGGGGCTGGAACACAT 90
TRGC5    CTTTGCCTTCTTCAGAATTTTTTCCCTGATGCTATTAAGATACAATGGAAAGAAAAGAATGTCAATACAATTCTGGAATCTTATCAGGGA 180
TRGC5    AATATCATCAAGACTAATGACACATACATGAAATTCAGCTGGCTAACCTTGACTAAAAAGGCAATGGATAAAGAACATGTATGTATCGTC 270
TRGC5    AAACACGAGAATAACAAAGGAGGACGTGATCAACAGATTCTTTTTTCTCCAGTTAAAAAAGAGGTCGCTACACATGCCTGCATGAAAAAA 360
TRGC5    GAAAGTGATACCCTGCAGCTGCAGTTTGCGAACACCTCTGCCTATTATACCTACCTCCTCCTCCTCAAGAGCATGATCTACTTCTCC 450
TRGC5    ATCATCGCCTTCTGTGTGTTTTGGAGAACAGGCATCTTCAGCGATGGGAAGATTTTC 507

TRGC6    GATAAAAAACTTCCTGCAGATACCTCCCCAAAACCCACAATTTTTCTTCCTTCAATTAACGAAGTCAACCATCAACAGGCTGCAACATAT 90
TRGC6    CTTTGTCTTCTGGAAAAATTTTTCCCTGATGTTATTAAGGTTTCTTGGAAAGAAAAGAATGACAACAGAGTTCTGCCATCCCAGCAGGGA 180
TRGC6    AATACCATGAAGACCAATGACACATACATGACGTTCAGCTGGCTGACCGTGACAGAAAACTCCATGAAGGAAGAGCACATGTGTATCGTC 270
TRGC6    AAACATGAGAAAAATACAAGAGGAAAAGATCAAGAGATTCTTTTTCCTGCAGTGAATGAAGTTTTCACCCCAGTTGTCACTACTACTGAG 360
TRGC6    CCTCCAAATGATTGTTTGAAAGATGGAAGTGAAGTCACTGATACTGATCTTACAAAAGCTTGTGCGAGAGATGAAAGTGAATTCGCTAAT 450
TRGC6    TCTACAAAAGCATGTCTGAAAGATGAAAACAATACCGTGCAGCTGCAGTTCACGTACAACTCTGCTTACTACACCTACCTCCTCCTCCTC 540
TRGC6    CTCAAGAGTACCATCTAC 558
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b

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boTRGC5  DRRLDGDLFPKPTIFFPSVEEVKLHGAGTHLCLLQNFFPDAIKIQWKEKNVNTILESYQGNIIKTNDTYMKFSWLTLTKKAMDKEHVCIV 90
ovTRGC5  .....................R.S.................V......G......H.....................G..... 90
boTRGC5  KHENNKGGRDQQILFSPVKKEVATHACMKKESDTLQLQFANTSAYYTYLLLLLKSMIYFSIIAFCVFWRTGIFSDGKIF 169
ovTRGC5  ..........E......N..........G.........S.....................I....N.... 169

boTRGC6  DKKLPADTSPKPTIFLPSINEVNHQQAATYLCLLEKFFPDVIKVSWKEKNDNRVLPSQQGNTMKTNDTYMTFSWLTVTENSMKEEHMCIV 90
ovTRGC4  ..N..T.II.................T........N.............GK.............N...K........D...K..... 90
boTRGC6  KHEKNTRGKDQEILFPAVNEVFTPVVTTEPPNDCLKDGSEVTDTDLTKACARDESEFANSTKACLKDENNTVQLQFTYNSAYYTYLLLL 180
ovTRGC4  RL...AG.............S...A.....D...Q.E.......F..V.S.G...VTD.............E..LA.....  180
boTRGC6  LKSTIY 186
ovTRGC4  ...A.. 186
```

Fig. 5 Nucleotide sequences of expressed bovine TRGC genes previously unidentified or incompletely sequenced. Nucleotide and amino acid identities are indicated by a *dot* (.). See "Materials and methods" for GenBank accession numbers. **a** TRGC5 [primer designed using ovine TRGC5 sequence; partial sequence of TRGC5 was previously reported (Hein and Dudler 1997)] and TRGC6 (primer designed using ovine TRGC4 sequence) nucleotide sequence. **b** Comparison of the deduced amino acid sequences of the previously unidentified or incompletely sequenced bovine TRGC genes (*bo*) and publicly available ovine (*ov*) gene sequences. *Underlined* sequence corresponds to the connecting regions according to the IMGT unique numbering for C-DOMAIN (Lefranc et al. 2003)

Genomic mapping

The information from the V–C combinatorial study was useful to empirically model a chromosome map of V–C ensembles. These were confirmed and/or modified when bovine genome sequence data became available by Conrad and coworkers (GenBank accession nos. AY644517 and AY644518). In addition, the annotation of the ovine chromosome 4 indicated that the ovine TRG genes are coded for on two distinct and distant regions of this chromosome designated as locus TRG1@ and locus TRG2@, separated by a wide distance on the chromosome (Miccoli et al. 2003). We further annotated the bovine genomic sequences and constructed two loci coding for the bovine TRG genes, as shown in Fig. 8, based on this. The genomic annotation assured us that our coverage of the expressed γ gene usage was largely complete. The mapping on the chromosome also indicated that our TRGV–TRGC gene combinations deduced from RT-PCR were physically probable.

The results indicated that while the ovine TRGC6 gene is a pseudogene, the most closely related bovine genes, TRGC1 and TRGC2, are not. The data indicated that this area was likely to have duplicated on the bovine locus, giving rise to those two highly similar genes (see Table 4). Analysis of the connecting region suggested that ovine TRGC6 is most closely related to bovine TRGC2 on the basis that in the hinge region bovine TRGC2 has two repeats of the TTEPP or TTKPP motifs and ovine TRGC6 has one repeat, while bovine TRGC1 has four repeats and ovine TRGC2 has three repeats.

The TRGV5, TRGV6, and TRGV8 subgroups have been further divided because each subgroup is comprised of two mapped genes (in this study and Conrad et al.). We found mRNA sequences corresponding to each of the mapped genes TRGV5-1 and TRGV5-2, TRGV6-1 and TRGV6-2, and TRGV8-1 and TRGV8-2, and those sequences were submitted to GenBank by us (see "Materials and methods" for accession numbers). Analysis of TRG2@ revealed that the two TRGV6 subgroup genes (TRGV6-1 and TRGV6-2) are upstream of TRGC2 and TRGC6, respectively, and thus in different V-J-C cassettes (Fig. 8). RT-PCR analysis confirmed that TRGV6-1 and TRGV6-2 associated with both TRGC2 and TRGC6, respectively (Fig. 7). Thus, it is likely that the combinations identified by RT-PCR were derived from the two different V-J-C cassettes. A similar situation was found for TRGV5-1 and TRGV5-2. Therefore, when the existence of two genes in these three subgroups are taken into account, the only V–C combination that was between two V-J-C cassettes is that of TRGV8–TRGC4.

Table 3 Summary of homology of the bovine and ovine TRGC genes based on cladograms and percentage identity of nucleotide and deduced amino acid sequence

Homologous genes				Previous or alternative bovine gene names	
IMGT subgroups		Percent nucleotide identity	Percent inferred amino acid identity	Gene names	Reference[b]
Bovine[a]	Ovine				
TRGC1	TRGC6	72	70	Cγ1, C6	Takeuchi et al. 1992, GenBank/ Conrad
TRGC2	TRGC2	89	86	Cγ2, C2	Takeuchi et al. 1992, GenBank/ Conrad
TRGC3	TRGC3	94	87	Cγ3, C3	Takeuchi et al. 1992, GenBank/ Conrad
TRGC4	TRGC1	96	95	Cγ4, C1	Ishiguro et al. 1993, GenBank/ Conrad
TRGC5	TRGC5	95	92	Cγ5	Hein and Dudler 1997
TRGC6	TRGC4	92	85	C4, Cγ6	GenBank/ Conrad Lahmers et al. 2005

[a]Bolded designations indicate bovine genes previously unidentified or incompletely sequenced but for which complete sequence is provided here. Underlined IMGT gene names are mapped (or unambigously identified) genes for which the nomenclature is definitive

[b]Designations are referenced to GenBank (Conrad et al. submission, accession numbers AY644517 for TRG1@ and AY644518 for TRG2@) and IMGT http://www.imgt.cines.fr)

Areas of uncertainty are the placement of the bovine TRGC5-containing cassette, which was found to be associated with TRGV3, TRGV4, and TRGV7 subgroup genes, as well as the placement of TRGV2. For the first case, the RT-PCR data of combinatorial usage of various TRGV and TRGC genes supported the placement of the TRGV3, TRGV4, and TRGV7 genes shown within the TRGC5-containing cassette (Fig. 8). The mapping data and further analysis of the chromosome upstream and downstream of the identified cassettes indicated that the TRGC5-containing cassette was not found in the bovine locus named TRG2@. However, it was unclear whether this cassette is at the 5′ or 3′ end of bovine locus TRG1@ because no genomic sequence is available for cattle or sheep for this region. The placement of TRGV2 is also uncertain, but is likely to be next to the related TRGV8 subgroup genes previously identified and for which genomic sequence on the chromosome exists. Moreover, although no genomic sequence was available to confirm the TRGV2 placement because of truncation of the bovine genomic sequencing at this point, RT-PCR data of combinatorial usage (Fig. 7) supported the placement of TRGV2 within the TRGC3-containing cassette.

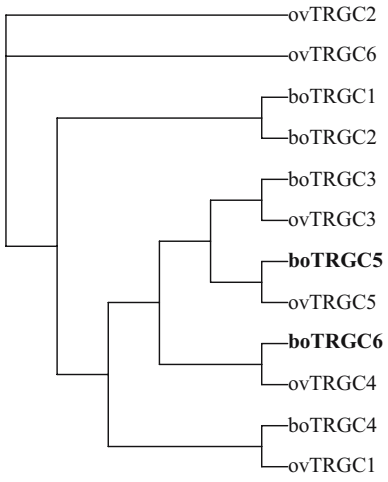

Fig. 6 Cladogram constructed to determine relationship between TRGC bovine (*bo*) (new sequences reported here are *bolded*) and ovine (*ov*) amino acid sequences of TRGC genes. Bovine and ovine gene sequences used are all TRGC*01 (see "Materials and methods" for accession numbers)

Discussion

The studies reported here have provided additional description and understanding of the bovine TRG genes and their homology with those of sheep, another ruminant species. Genomic mapping and sequencing has helped resolve the homology between bovine and ovine TRGC genes and a table and figure are provided here as a guide using the accepted nomenclature as prescribed by the WHO Nomenclature Committee (see Tables 1 and 3 and Fig. 8). The genomic organization of bovine TRGC genes was found to be generally more similar to that of mice than to that of humans, with extensive duplication of V-J-C cassettes over two loci. While the arrangement in the TRG2@ locus was definitive with three V-J-C cassettes, we predict that within the TRG1@ locus, there will also be three V-J-C cassettes. Although we have not confirmed placement of the TRGC5-containing cassette within this locus, the placement of the ovine homologue TRGC5 is within the ovine TRG1@ locus, as mapped by Miccoli et al. (2003). While Miccoli and coworkers predicted that the ovine TRG1@ would contain only one TRGV gene, i.e., Vγ1.1 (referenced in Miccoli et al. 2003 as "in preparation"), the annotation here of the bovine genome sequence, as well as the determination of the expressed V–C gene combinations, suggests many more are likely to be found in the ovine locus given the overall high level of conservation between sheep and cattle TRG genomic sequence reported here and in previous studies.

It has been suggested that within the ovine TRG2@ locus, the two V-J-C cassettes containing the two TRGV5 subgroup genes represent a recent duplication because their leader sequences are 95% similar with only one to two

Table 4 Percent identity of nucleotide and predicted amino acid sequences among bovine TRGC gene subgroups

Gene subgroups[a]	Gene subgroups[a]					
	TRGC1	TRGC2	TRGC3	TRGC4	TRGC5	TRGC6
TRGC1	*	*90*	*69*	*76*	*55*	72
TRGC2	88	*	*75*	*81*	*60*	*78*
TRGC3	56	58	*	*76*	*66*	*76*
TRGC4	64	69	63	*	*62*	*81*
TRGC5	41	44	52	49	*	*62*
TRGC6	60	65	62	66	49	*

Numbers in italics above the asterisks forming a diagonal line indicate percentage nucleotide identities, while numbers below the diagonal indicate percentage amino acid identities

[a]Bovine gene sequences used are all TRGC*01 (see "Materials and methods" for accession numbers)

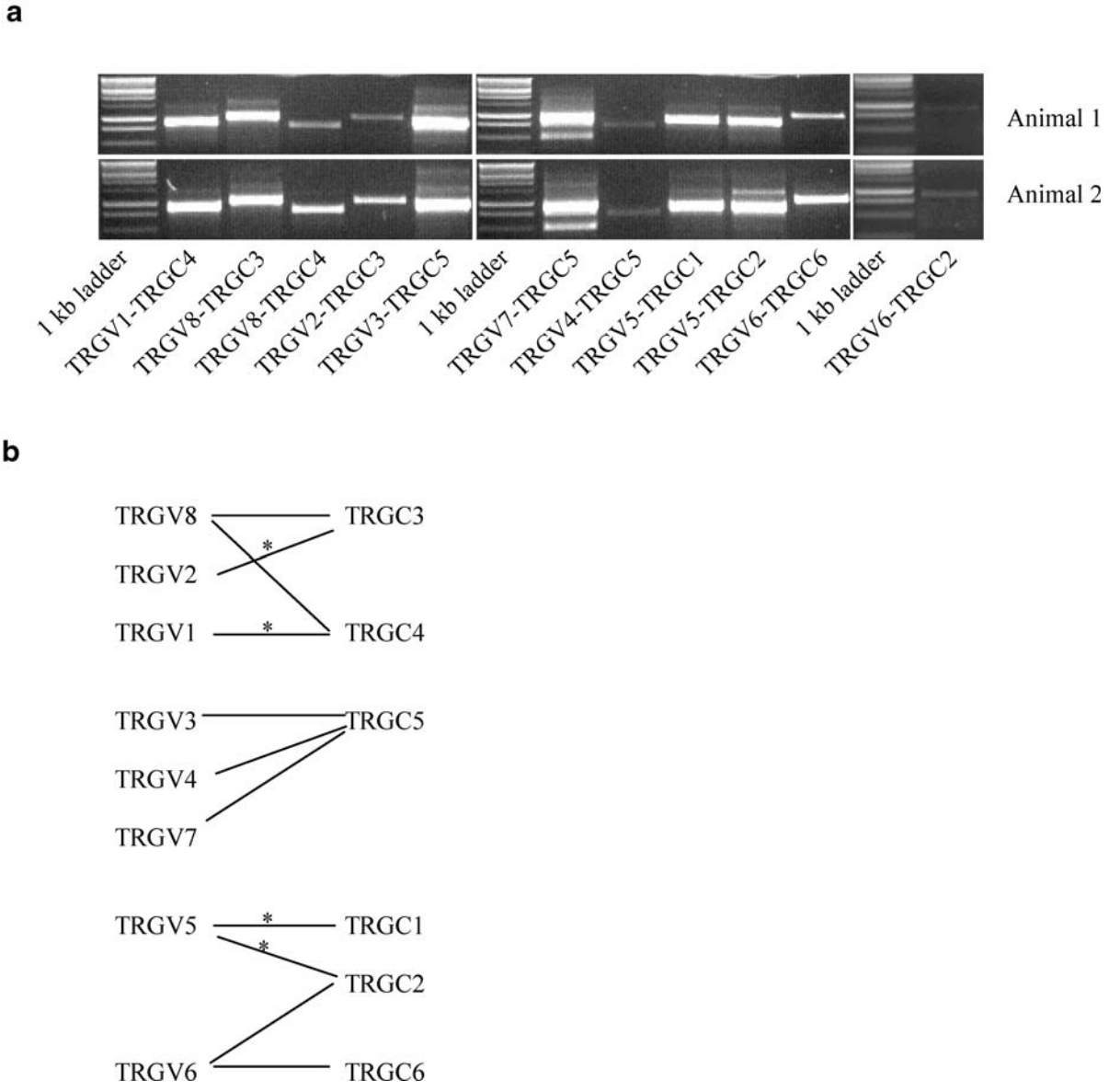

Fig. 7 **a** RT-PCR analysis using RNA derived from two cattle of differing breeds to determine the combinations of bovine TRGC genes associated with TRGV genes expressed by T cells in bovine peripheral blood. **b** Schematic representation of the combinations used by bovine peripheral blood γδ T cells (*Asterisk* confirms previously identified combinations by sequencing through junctional region of multiple clones; Ishiguro et al. 1993)

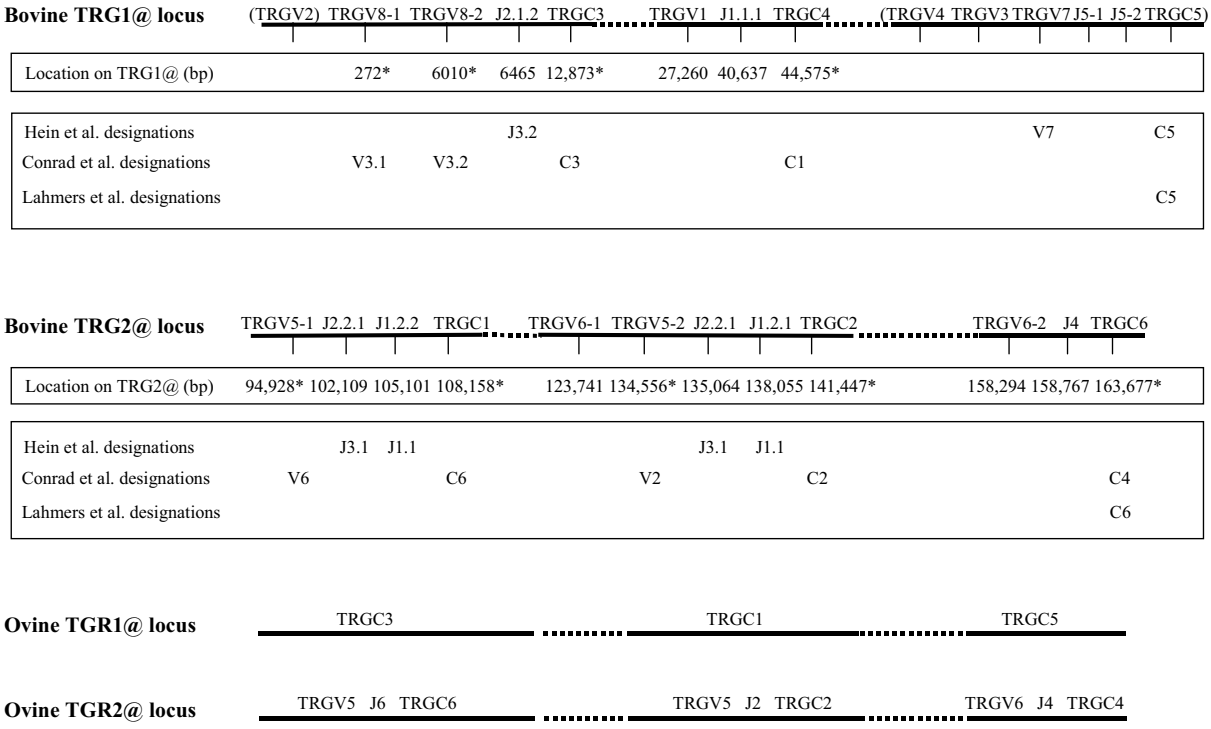

Fig. 8 Annotated map of the TCR gene loci TRG1@ and TRG2@ based on genomic sequence for bovine available as part of the Bovine Genome Sequencing project (http://www.ncb.nml.nih.gov/genome/guide/cow/) and at GenBank (Conrad et al., submission accession nos. AY644517 for TRG1@ and AY644518 for TRG2@) and a comparison with the ovine TRG1@ and TRG2@ loci organization as per Miccoli et al. 2001. Base pair locations of variable (*V*), junction (*J*), and constant (*C*) genes are shown beneath each locus, although the exact *J* region boundaries are somewhat ambiguous. TRGJ5-1 and TRGJ5-2 were designated here based on homologous ovine genes and the TRGC5 gene with which they associate. These previously unidentified genes have the following deduced amino acid sequences, respectively: STWIKVFGEGTKLVVIPP and YVKIFGDGTKLVVT. TRGV and TRGC locations previously idntified by Conrad et al. are designated with an *asterisk* (*). *Parentheses* indicate a proposed placement on the TRG locus, while all other placements are confirmed. Multiple names have been given for some genes in primary publications (Hein and Dudler 1997; Lahmers et al. 2005). The IMGT standardized gene names (http://imgt.cines.fr) have been approved by the IMGT Nomenclature Committee (other names are referred to as clone names as designated by submitter.)

amino acid differences (Miccoli et al. 2003; Takeuchi et al. 1992). Annotation of the bovine TRG2@ reported in this study and by Conrad et al., based on genomic sequences they submitted to GenBank (GenBank Accession no. AY644518), indicate that TRGC gene duplication occurred before these ruminant species separated 18 million years ago (see Su et al. 1999). That is, for both cattle and sheep there seem to be homology unit duplications because the TRGC1 and TRGC2 V-J-C cassettes both contain TRGV5 subgroup genes and were found contiguous on the chromosome at the TRG2@ locus. While the duplicated ovine TRGC6 is considered to be nonfunctional, bovine TRGC1 and TRGC2 are both functional. A precedent for the maintenance of a functional gene in a closely related species is found in comparisons of chimpanzees and humans, where TRGV10, the single member of its subgroup, is functional in the chimpanzee and the gorilla but not in humans (Zhang et al. 1996). In the TRB locus, four functional TRBV genes have been maintained in chimpanzees but not in humans (Meyer-Olson et al. 2003).

In cattle, TRGC1 and TRGC2 are distinguished by differences in the connecting region, with TRGC1 having four and TRGC2 having only two TTEPP/TTKPP motifs (Takeuchi et al. 1992; IMGT Protein display, http://imgt.cines.fr). Bovine TRGC1 is the homologue of ovine TRGC2 based on the distinctive features of the connecting region; however, using chromosome localization, bovine TRGC1 is comparable to ovine TRGC6, while bovine TRGC2 is comparable to ovine TRGC2. Moreover, the duplicated bovine TRGC1 and TRGC2 genes were both found to be most similar to ovine TRGC2 and least similar to ovine TRGC6, based on percentage identity and cladogram analysis. On the basis of these findings, we assigned bovine TRGC1 as the ovine TRGC6 homologue and bovine TRGC2 as the ovine TRGC2 homologue (Table 3). There is precedence for the TRGC gene duplication in humans, where the two existing TRGC genes are very similar and differ only in the connecting region, although they are encoded by a different number of exons: one for TRGC1 and two or three for TRGC2 (Buresi et al. 1989; Ghanem et al. 1991; Lefranc et al. 1986b; Lefranc and Lefranc 2001). Thus, the human TRGC2 gene encodes gamma chains that are longer and only noncovalently linked to the delta chain because the cysteine is replaced by a tryptophan (Lefranc and Rabbitts 1989; IMGT Protein display, http://imgt.cines.fr). In mice, the connecting regions of the TRGC gene products are also of different lengths, with TRGC2 being shorter by five amino

acids than TRGC1, and TRGC4 having a duplicated exon encoding 18 amino acids (IMGT Protein display, http://imgt.cines.fr).

Our V–C combinations agreed with the few previously reported by Ishiguro et al. (1993), and both their group and our group found differences among individual animals in relative gene expression (C. Herzig and C. Baldwin, unpublished data). This could account for the discrepancy of our results with an earlier study that suggested ovine TRGC5-expressing cells were restricted to skin (Hein and Dudler 1997). Hein and Dudler (1993) also suggested that ovine TRGC3, TRGC1, and TRGC5 were expressed only by adult γδ T cells. Since these genes and their bovine homologues (TRGC3, TRGC4, and TRGC5, respectively) were shown to map to a single TRG gene cluster, i.e., TRG1@, it is plausible that one locus could remain inaccessible until adulthood. Moreover, the ovine TRGC2 and TRGC4 genes were suggested to be transcribed only in fetuses. Since their bovine homologues (TRGC2 and TRGC6) are found in the second cluster proposed here, TRG2@, it is equally plausible that this other locus becomes inaccessible with age, as occurs for murine TRGV1–TRGC4 genes. However, since we found all six V–C genomic ensembles expressed by cells from peripheral blood of adult cattle, the cells would have to be maintained into adulthood.

While the TRGV and TRGC genes that we found to be expressed may not be exhaustive, it is highly likely that we detected most, if not all, TRGV and TRGC expressed genes. We sequenced more than 100 clones derived from PCR reactions, using common and specific primers, and every TRGV and TRGC sequence obtained corresponded to genes described here. That is, there were no anomalous TRGV or TRGC genes. Also, analysis of the available gamma loci genomic sequence (GenBank Accession nos. AY644517 and AY644518) did not reveal any further TRGV or TRGC genes in addition to the ones described in this study by sequencing RT-PCR products (though the TRG1@ locus appears to be truncated at both ends). Nor were additional highly homologous TRGV or TRGC genes identified when the bovine genome, available through the Bovine Genome Sequencing Project (http://www.ncbi.nlm.nih.gov/projects/genome/guide/cow/), was examined for TRG genes described here by blast searching (Herzig, unpublished data). Thus, though our methods of detecting TRG expressed genes may not be completely exhaustive, it is thorough enough to ensure that genes with significant homology have been described here or previously.

It is generally theorized that since T cell receptors are never secreted, the C portion of the molecule is irrelevant to its function. The high number of ruminant TRGC genes contrasts with the TRDC and TRAC genes coding for the delta and alpha chains of the T cell receptor, where in ruminants, like other mammals, there is only one TRDC and one TRAC gene in the genome. It also contrasts with the TRGC genes in other mammalian species because there are only two highly homologous TRGC genes in human and four, though one is a pseudogene, in mice. Nevertheless, there is precedence for C-gene duplication in ruminants, because ruminants have three TRBC genes (Conrad et al. 2002) compared to two in humans (Lefranc and Lefranc 2001) and mice (Glusman et al. 2001). It has been suggested that the γδ high species have more diversity of γδ T cell receptor genes expressed within a tissue than occurs in mice and humans, and this clearly extends to the TRGC genes as well. While expression of TRGC genes has been shown to correlate with tissue localization of the expressing cell, with murine TRGV1–TRGC4-expressing T cells developing in the thymus and migrating to the skin while TRGV5–TRGC1-expressing cells develop extrathymically and migrate to the intestine (Ota et al. 1992), it is unclear what role the *C* region plays in this, if any.

Different *C* region amino acid sequences could theoretically affect γδ T cell function through their interaction with CD3. For example, two charged lysines are needed in the tail of the T cell receptor to interact with CD3 (Takeuchi et al. 1992), and for ovine TRGC2, it is known that a leucine is substituted for one of the lysines (Hein et al. 1990). It has been indicated that the activation threshold for γδ T cells may be higher than for αβ T cells (Steele et al. 2000) and that the γδ T cell receptors may be of particularly low affinity because of their requirement to see only increased expression of autoantigens before they are engaged. Thus, it is a plausible hypothesis that the γδ T cells that respond to autoantigens may express a TRGC gene that does not interact particularly strongly with co-receptors or CD3. In support of this, we have found that only a small population of bovine peripheral blood γδ T cells responds to anti-CD3 monoclonal antibody stimulation (Sathiyaseelan et al. 2002b). Also it is known that different TRGC sequences have unique connecting regions (Hein and Dudler 1993) that theoretically could result in different outcomes of cellular responses once the T cell receptor has engaged antigen. That is, the KEK motif in the T cell receptor is needed to interact with CD8 and there are both $CD8^+$ and $CD8^-$ bovine γδ T cells in circulation (Machugh et al. 1997). Thus, expression of particular TRGC genes could be restricted between these two major populations. Finally, bovine γδ T cells have a unique lineage-specific co-receptor WC1 (Wijngaard et al. 1994), and expression of particular isoforms has been shown to correlate with responsiveness to particular antigens (Rogers et al. 2005). While we have shown that the $WC1.1^+$ cells respond when cultured with autologous monocytes (Sathiyaseelan et al. 2002a) and bacterial lysate (Naiman et al. 2001), Lahmers et al. (2005) have shown that the $WC1.2^+$ cells do so with *Anaplasma marginale*. Thus, different TRGC genes may be expressed by cells expressing different isoforms of WC1. Future studies will address T cell receptor gene usage by these various subpopulations of ruminant γδ T cells.

Of the two bovine TRG loci, the TRG1@ parallels the human T cell receptor gene locus placement on the chromosome based on its relationship to other genes (Miccoli et al. 2003). Interestingly, the bovine TRGC5 gene is most homologous to human TRGC as determined by proximity on a coding tree (Miccoli et al. 2003), and it is

predicted to be located in TRG1@. Moreover, the bovine TRGV3 and TRGV7 genes (Hein and Dudler 1997), which rearrange with the TRGC5 gene, also cluster with human TRGV9 (Miccoli et al. 2003). Here, we found bovine TRGC5 was the least similar to other bovine TRGC genes based on sequence identity. This is of particular interest since the human TRGV9-expressing cells are the mycobacterial IPP-reactive γδ T cell population found in peripheral blood of humans (Tanaka et al. 1994). By contrast, the human TRGV1 subgroup genes, the genes used by the other major γδ T cell population in humans, are outside any homology unit with ruminants or chickens (Miccoli et al. 2003). The IPP-reactive human cells have been compared to CD4/CD8 T cells (Chen 2002) in that are they are $CD2^+/CD5^+/CD28^+/CCR5^+/CCR7^+$, while other human γδ T cells are considered to be more like CD1-restricted T cells. Similar comparisons will have to be made in cattle as there are both $CD5^+$ and $CD5^-$ γδ T cells circulating, as well as $CD28^+$ and $CD28^-$ γδ T cells. It will be of particular interest to determine whether bovine cells expressing the bovine TRGV3–TRGC5 or TRGV7–TRGC5 are those that respond to IPP, a response reported previously (Welsh et al. 2002).

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