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CAPRINE γδ T CELL BIOLOGY

A Dissertation Presented

by

ALEHEGNE W YIRSAW

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 2021

Program in Animal Biotechnology and Biomedical Science

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CAPRINE γδ T CELL BIOLOGY

A Dissertation Presented

by

ALEHEGNE W YIRSAW

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DEDICATION

I dedicate this thesis to my wife Abeba M Zeleke and my children Amanuel and Loza A. Wubete for their encouragement words and their patience. A special feeling of gratitude to my uncle Atallel Tiruneh and his wife Emewa Mengiste for their growing me with great discipline and norm.

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ABSTRACT

CAPRINE γδ T CELL BIOLOGY

FEBRUARY 2021

ALEHEGNE W YIRSAW, DVM, ADDIS ABABA UNIVERSITY, ETHIOPIA

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Goats are important food animals and are disseminated globally. Their productivity is impacted by infectious diseases thus impacting the livestock owners and the community. γδ T lymphocytes or T cells have vital roles in immune responses in mammals and thus we identified information gaps regarding these cells in goats. We used genome annotation, PCR amplification of genomic DNA and cDNA and Sanger and PacBio sequencing for this research to evaluate the important receptors of these cells, i.e. the T cell receptor (TCR) and the hybrid pathogen pattern recognition receptor (PRR) and TCR co-receptor known as WC1. The annotated TCRγ (TRG) and TCRδ (TRD) loci were similarly organized in goats as previously reported for cattle and the gene sequences were highly conserved between these ruminant species. However, the number of genes varied slightly as a result of duplications within loci and differences occurred in mutations resulting in pseudogenes. Goats have 6 functional and 1 pseudogenes TRG gene cassettes and TRD genes have one constant gene (TRDC), 4 joining gene (TRDJ), 7 diverging genes (TRDD and 34 TRD variable genes (TRDV). In cattle, the $WC1^{+}$ γδ T cells only use TCRγ genes from one of the 6 available cassettes and we found the same conservation for goat $WC1^{+}$ γδ TCRγ gene usage. With regard to the co-receptor, we

found evidence for up to 30 caprine WC1 genes, twice that of cattle, and seven different WC1 gene structures of which 4 are unique to goats. The most distal WC1 scavenger receptor cysteine rich (SRCR) domains known as SRCR a1, based on sequence and position, were highly conserved among goat breeds but fewer were conserved between goats and cattle. Caprine WC1 genes also had multiple splice variants of transcripts coding for the intracytoplasmic domains that in some cases eliminated tyrosines shown previously to be important for signal transduction. In summary, goat WC1 molecules may have expanded functions given their expanded number and structure variations. Using immunofluorescence and flow cytometric analysis and monoclonal antibody (mAb) GB21A, reactive with the TCR δ constant region (TRDC), we found that the proportion of γδ T cells in caprine blood was not significantly less than that of CD4 or CD8 T cells, with $WC1^+$ γδ T cells ranging from ~20-90% of the total. Less than half of the $WC1^+$ cells could be classified as $WC1.1^+$ or $WC1.2^+$ subpopulations by additional mAb staining indicating a large third subpopulation in goats. Since WC1 gene expression may direct pathogen responses the WC1 genes expressed by subpopulations of $WC1^+$ γδ T cells were also identified by next generation sequencing. To experiments to evaluate responses to pathogens, we found that naïve γδ T cells proliferated in *in vitro* recall cultures stimulated with *Leptospira borgpetersenii* or *Mycobacterium avium paratuberculosis* (MAP) above that in control cultures without antigen or those with *M. bovis* BCG. The responding cells included both $WC1^+$ and $WC1^-$ γδ T cells and the $WC1^-$ γδ T cells produced IL-17. In *ex vivo* PMA-stimulated cultures $WC1^+$ γδ T cells were shown to also produce IL-17 while $WC1^-$ γδ T cells produced both IL-17 and interferon-γ (IFNγ). In the future more extensive studies of caprine γδ T cell will be needed to

evaluate the role of the various γδ T cell subpopulations to important livestock pathogens. Knowing this information will be important for understanding how to engage these cells in vaccine designs.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BCG	bacille Calmette-Guerin
BLAST	Basic Local Alignment Search Tool
BRgoat	Boer goat
Bt	*Bos taurus*
CAEV	Caprine arthritis encephalitis virus
CD	Cluster of differentiation
cDNA	Complementary DNA
ConA	Concanavalin
cRPMI	Royal Park Memorial Institute (culture medium)
FACS	Fluorescence-activated cell sorting
FSc	Forward scattered
SSc	Side scattered
GST	Gluthathione–S-transferase
γδ	Gamma delta
HLN	Hepatic lymph node
IACUC	Institutional Animal Care and Use Committee
ICD	Intracytoplasmic domain
IDs	Interadomains
IFN	Interferon
IL	Interleukin
IMGT	Immunogenetics data base

IPP	Ileal Peyer's patch
JPP	Jejunial Peyer's patch
LN	Lymph node
mAbs	Monoclonal antibodies
MAP	*Mycobacterium avium subspecies paratuberculosis*
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NCBI	National Center for Biotechnology Information's
NF	Nuclease free
NK	Natural killer
OIE	Office International des Epizooties
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCV	Packed cell volume
PMA	Phorbol 12-myristate 13-acetate
PPD	purified protein derivative
PPR	Peste Des petitis ruminants
PRR	Pattern recognition receptor
RSS	Recombination signaling sequences
RT-PCR	Reverse transcription polymerase chain reaction
SCgoat	San Clemente goat
SEM	Standard error of the mean

SRCR	Scavenger Receptor Cysteine-Rich
TGF	Transforming Growth Factor
Tγδ1	IFNγ
Tγδ17	IL-17
T19	T cell marker 19
TCR	T cell receptor
Th	T-helper
Th1	T Helper Cell Type 1
TH2	T Helper Cell Type 2
Th17	T helper 17 cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRD	TCR δ
TRDC	TCR δ constant
TRDD	TCR δ diversity
TRDJ	TCR δ joining
TRDV	TCR δ variable
Tregs	The regulatory T cells
TRG	TCR γ
TRGC	TCR γ constant
TRGJ	TCR γ joining
TRGV	TCR γ variable

WC1	Workshop cluster 1
YNgoat	Yunnan goat

1 CHAPTER I Goat γδ T cells

ABSTRACT

Goats are important food animals and are disseminated globally because of their high adaptability to varying environmental conditions and feeding regimes that provide them with a comparative advantage. Production and productivity is impacted by infectious diseases; this then contributes to societal poverty, food insecurity, and international trade restrictions. Since γδ T cells have been shown to have vital roles in immune responses in other mammals we reviewed the literature regarding what is known about their functions, distribution in tissues and organs and their responses to a variety of infections in goats. It has been shown that caprine γδ T cells produce interferon-γ and IL-17, are found in a variety of lymphoid and nonlymphoid tissues and constitute a significant population of blood mononuclear cells. Their representation in tissues and their functional responses may be altered concomitant with infection. This review summarizes caprine γδ T cell responses to *Brucella melitensis, Fasciola hepatica*, *Mycobacterium avium paratuberculosis*, caprine arthritis encephalitis virus (CAEV), and *Schistosoma bovis* in infected or vaccinated goats. Caprine γδ T cells have also been evaluated in goats infected with *M. caprae*, *Ehrilichia ruminantium*, *Haemonchus contortus* and peste des petits ruminants (PPR) virus but found to have an unknown or limited response or role in either protective immunity or immunopathogenesis in those cases.

1.1 Introduction

1.1.1 Goats

Goats are disseminated globally because of their high adaptability to varying environmental conditions including heat stress and because of their browsing and grazing feeding regimes that do not compete with humans for food. Goats also have a comparative advantage over cattle despite their small size because of their faster growth rates and thus shorter production cycles and because they require a smaller investment than do cattle. They are an important source of protein to prevent childhood stunting and promote cognitive development (Neumann et al., 2003) and mitigate environmental risks (Aziz, 2010; Tibbo, 2006b). As a result they are highly concentrated in developing countries in Africa, the Middle East, Asia, South America and Mexico as shown in Figure 1.1. The number of goats is increasing globally and estimated to be more than 1 billion in recent years (Food and Agricultural Organization of the United Nations statistic division 2013, http://faostat.org; accessed 1 Nov 2019). Altogether, goats are important for meat, milk, manure, skine, fiber, income, traction and packing (Duguma et al., 2011)

Even though goats have a comparative advantage for production, productivity is impacted by parasitic, bacterial, and viral infectious diseases. These diseases increase mortality and morbidity and abortion rates, cause weight loss, lower milk yields, increase kidding intervals and result in high treatment and vaccinations costs (Singh, 2008). In turn, societies suffer poverty, food insecurity, and malnourishment (Gewa et al., 2009), and members of those societies may become ill due to zoonotic infections acquired from goats. Most caprine infectious diseases are transboundary in nature, with significant economic and food security impacts. Moreover, these diseases hamper international trade

that seriously affects both the exporting and importing countries (Asfaw, 2003; Basagoudanavar, 2013; Seyoum, 2017).

1.1.2 Functions of γδ T cells

T cells have vital roles in immune responses for controlling infectious diseases. Based on their T-cell receptor (TCR) type, T cells are divided into two major populations: γδ and αβ T cells. αβ T cells are the classical T cells and are identified as two subpopulations, $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ T cells may act as helper T cells (T helper (Th) 2) for B cell production of antibodies, produce cytokines such as interleukin (IL)[1]-17 (Th17) that attracts neutrophils to sites of infection and interferon (IFN)γ (Th1) that activates macrophages for antimicrobial activities, or act as regulatory T cells (Tregs) producing the anti-inflammatory cytokines IL-10 and TGFβ. $CD8^+$ αβ T cells also produce cytokines including IFNγ but are distinguished by their cytotoxic activity, killing virus-infected cells and some tumor cells. In contrast, γδ T cells have characteristics of both the innate immune system, reacting to infection more rapidly than αβ T cells, and the adaptive immune system, expressing functions that overlap those of αβ T cells. As a result γδ T cells are sometimes referred to as bridging immune system cells.

γδ T cells can produce cytokines (IFNγ, IL-17 and tumor necrosis factor (TNF)-α) and chemokines, be cytotoxic, and can affect inflammation, epithelial growth, and wound healing as well as provide B cell help and assist dendritic cell maturation and, finally, they can present antigens to other T cells (Vantourout and Hayday, 2013). With regard to ruminants, bovine γδ T cell populations have also been shown to have regulatory

functions (Guzman et al., 2014; Guzman et al., 2012; Hoek et al., 2009). Thus, the responses by γδ T cells may contribute to protective immunity against infection, immune-mediated pathology (Paul et al., 2015) or be regulatory (Hahn et al., 2003; Wu et al., 1992). However, while the γδ T cells share functions with the αβ T cells, an important distinction is the way they perceive antigen and the types of antigens to which they react (Hayday et al., 2001; Hayday, 2000; Hayday et al., 1985; Melandri et al., 2018; Vantourout and Hayday, 2013). The known functions of caprine γδ T cells are reviewed below.

1.1.3 Distribution of γδ T cells

γδ T cells develop in the thymus as αβ T cells do. Once developed, γδ T cells are known for being associated with epithelial and mucosal barriers. This is thought to contribute to their perceived role as a first line of defense against infection (Hein and Dudler, 1997). However they also circulate through the blood and lymphoid system. Using lymph cannulation this has been described for both sheep and cattle (Mackay et al., 1990; Vrieling et al., 2012; Wilson et al., 1999). While γδ T cells are notable for being associated with body surfaces, many ruminant species and pigs are considered to be γδ T cell high (summarized in (Holderness et al., 2013)) meaning they have a significantly higher proportion of γδ T cells in their blood compared to humans and mice. In the γδ T cell high species, the γδ T cells may comprise up to 60% of the peripheral blood mononuclear cell (PBMC) population at birth after which the percentage decreases until the animal reaches adulthood (Davis et al., 1996b; Mackay and Hein, 1989; Mackay et al., 1986; Takamatsu et al., 2006).

93 In mice, the γδ T cells that associate with specific tissues express particular TCR
94 gene products thus dividing them into subpopulations (for review see (Paul et al., 2014)).
95 In contrast in ruminants γδ T cells are divided into subpopulations based on the
96 expression of lack thereof of a lineage-specific cell surface marker known as workshop
97 cluster 1 (WC1) or T cell marker 19 (T19) (Machugh et al., 1997; Mackay et al., 1989a;
98 Mackay and Hein, 1989; Mackay et al., 1986). WC1 is not expressed by human or
99 murine γδ T cells although many other species do have genes coding for WC1 (Herzig et
100 al., 2010b). WC1 is known to be coded for by a multigenic array and the gene products
101 are hybrid co-stimulatory and pattern recognition receptor (PRR) that act in concert with
102 the TCR (Hanby-Flarida et al., 1996; Hsu et al., 2015a; Hsu et al., 2015b). Our colleagues
103 have shown that WC1 molecules can bind pathogens resulting in activation of ruminant
104 γδ T cells when cross-linked with the TCR (Hsu et al., 2015b). $WC1^{+}$ γδ T cells make up
105 the majority of γδ T cells in the blood of cattle (Baldwin et al., 2000) and their roles have
106 been reviewed previously by us (Baldwin and Telfer, 2015a; Telfer and Baldwin, 2015).
107 Some of the $WC1^{+}$ γδ T cells in cattle, sheep and goats also express a second lineage-
108 specific molecule GD3.5 that has been postulated to designate pre-primed γδ T cells
109 because of the expression of activation and anti-apoptotic genes by cells expressing
110 GD3.5 (Jones et al., 2007). CD8 is expressed on some γδ T cells and in cattle the $WC1^{-}$
111 γδ T cells are those that express CD8 thus dividing the γδ T cells into two largely distinct
112 subpopulations (Wyatt et al., 1994). The CD8+ T cells are a small percentage of γδ T
113 cells in the blood but a major population in the spleen and uterus. The presence of γδ T
114 cell populations in various tissues and organs of goats is summarized in this review
115

116 ### 1.1.4 Responses by γδ T cells to pathogens

117 γδ T cells in humans, non-human primates and mouse models have been shown to
118 be activated in response to *Borrelia burgdorferi*, *Escherichia coli*, *Francisella tularensis*,
119 *Listeria monocytogenes* and *Mycobacterium tuberculosis*, for example (Chen, 2011,
120 2013; Chen and Letvin, 2003; Hamada et al., 2008; Henry et al., 2010; Inagaki-Ohara et
121 al., 2011; Kabelitz et al., 1991; Kaufmann and Ladel, 1994; Shi et al., 2011; Tanaka et
122 al., 1994). Also in mice, γδ T cells have been shown to play a protective role against *L.*
123 *monocytogenes* infections since there is increased multiplication of bacteria in mice that
124 are depleted of γδ T cells but not in those depleted of αβ T cells (Hiromatsu et al., 1992).
125 Depletion of γδ T cells has also been shown to affect the organization of granulomas in
126 infected mice (Kaufmann and Ladel, 1994). γδ T cells from cattle, sheep and swine have
127 been shown to respond to many pathogens including viruses, bacteria, protozoa and
128 ectoparasites (reviewed recently (Baldwin et al., 2019a)). In vivo γδ T cell depletion
129 studies in sheep has been used to show that γδ T cells inhibit protection against
130 nematodes (McClure et al., 1996) while depletion of $WC1^{+}$ γδ T cells was shown to result
131 in a more profound Th2 response and a decreased Th1 response in cattle infected with *M.*
132 *bovis* (Kennedy et al., 2002). Here we review what is known about changes in caprine γδ
133 T cells in goats infected with a variety of pathogens. The experimental designs for studies
134 cited here of goat γδ T cell responses to pathogens are shown in Table 1-1.

135

1.1.5 Functions and activation of caprine γδ T cells

In mice, γδ T cells are divided into functional subsets producing IL-17 (Tγδ17), IFNγ (Tγδ1) or IFNγ and IL-4 (γδNKT). In the thymus the two distinct innate-like populations known as Tγδ17 and Tγδ1 develop while other γδ are more adaptive and are exported to the periphery as naïve cells (Bonneville et al., 2010; Munoz-Ruiz et al., 2017; Ribot et al., 2010). Thus, these two pro-inflammatory cytokines are the principal effector functions of a large proportion of (Buus et al., 2017) γδ T cells. Caprine γδ T cells also have been shown to produce IFNγ and IL-17 (Elnaggar et al., 2018; Lybeck et al., 2009). When PMA and Ionomycin were used to stimulate caprine γδ T cells the majority of γδ T cells that made IL-17 were $WC1^+$ γδ T cells. In goats 90% of $CD8^+$ T cells that made IL-17 were γδ T cells and not αβ T cells. The IFNγ/IL-17A double positive cells in goats were largely $CD8^+$ γδ T cells with few or none expressing WC1. The identity of caprine γδ T cells that only produce IFNγ (single-positive) with regard to their surface markers (WC1 and CD8) is unknown. Based on these results we can conclude that caprine γδ T cells have the potential to respond to infectious diseases by pro-inflammatory cytokine production that could promote immunity and/or contribute to immune-mediated pathology.

It is an accepted paradigm that T cells are activated through ligation of their TCR and while this has been demonstrated directly for γδ T cells in cattle (Hanby-Flarida et al., 1996) it has not been shown for caprine cells. However, it has been shown that caprine γδ T cells may be activated in other ways. That is, they are activated by ligands for Toll-like receptor (TLR) 7/8 and TLR3 (Tourais-Esteves et al., 2008). A similar observation has been made for bovine γδ T cells and thought to be important for

159 responses to bovine respiratory syncytial virus in that case (McGill et al., 2013). TLR8
160 binds single-stranded viral RNA while TLR3 binds double-stranded DNA and TLR7
161 regulates antiviral immunity. The ligation of TLRs recruites MyD88, activates NFkβ and
162 eventually leads to antiviral activity. γδ T cell stimulation through TLR7/8 or TRL3
163 resulted in the production of IFNγ by a small percentage (1.26% and 0.74%) of caprine γδ
164 T cells, respectively, which was marginally greater than unstimulated controls at 0.23%.

165 Caprine γδ T cells have also been shown to respond to condensed tannins (CT)
166 extracted from *Grewia flava* and *Tapinanthus oleifolitus*. Exposure to these tannins
167 increased the percentage of γδ T cells expressing CD25 by two-fold and increased the
168 level of CD25 expression by individual cells following *in vitro* exposure (Tibe et al.,
169 2012). The receptor being used for activation is not known. However, given that goats
170 browse these plants it is interesting to speculate whether their ingestion of these plants
171 may activate gut-associated γδ T cells.

172

173 1.1.6 Where have γδ T cells been found in goats?

174 A variety of monoclonal antibodies have been used to detect T cell populations in
175 goats. Those used in studies reviewed here are summarized in Table 1-2.

176

177 1.1.6.1 Primary lymphoid organ

178 The thymus is the primary lymphoid organ for all subpopulations of T cells
179 including γδ T cells, while the Bursa of Fabricius, bone marrow or gut-associated
180 lymphoid tissues provides this function for B cells, dependent upon the species. It is clear
181 from studies in mice that γδ T cells can develop into functional subpopulations in the

182 thymus - in some cases predisposing the cells to the type of cytokine (IL-17 vs. IFNγ)
183 they will produce following activation (Buus et al., 2017; Munoz-Ruiz et al., 2017; Ribot
184 et al., 2010; Schmolka et al., 2013). As for other mammals, the percentages of $CD4^{+}$ and
185 $CD8^{+}$ T cells in goats was found to range from 60 to 70% in the thymus and did not show
186 any significant change at the age groups evaluated (1 week to 7 months). Goat γδ T cells
187 however were always low (<3%) despite the age of the animal examined (Caro et al.,
188 1998). A similar low percentage of γδ T cells in the thymus have been found in cattle
189 (Wilson et al., 1999). This considerably lower percentage of γδ T cells than that of αβ T
190 cells may reflect both the proportional representation of γδ T cells in the circulation and
191 the type of thymic development γδ T cells undergo relative to that of αβ T cells. That is,
192 αβ T cells undergo positive and negative selection in the thymus resulting in loss of the
193 majority of thymocytes attempting to become αβ T cells. Only a minor percentage are
194 exported to the periphery as mature αβ T cells.
195

196 **1.1.6.2 Secondary lymphoid organs and tissues**

197 In mesenteric lymph nodes (MLN), the γδ T cells are a minor population relative
198 to other T cells and are found scattered throughout the node although in 1-week old goats
199 they are more numerous (Seva et al., 1998). This difference in representation of γδ T cells
200 relative with other T cell populations increased further with age (Caro et al., 1998). The
201 percentage of γδ, $CD4^{+}$, and $CD8^{+}$ T cells in MLN reported by Caro *et al*. agreed with a
202 previous report by Navarro *et al*. (Navarro et al., 1996) as shown in Table 1-5. In
203 contrast, studies by Tourais-Esteves and co-workers (Tourais-Esteves et al., 2008)
204 reported no significant changes in γδ, $CD4^{+}$ or $CD8^{+}$ T cell populations between neonates

205 (7-13 days age) and adults (1-7 years). Thus, it is unclear whether or not statistically
206 significant changes in these T cell subpopulations do in fact occur in caprine lymph nodes
207 as goats reach adulthood. It is possible that the use of different breeds of goats and
208 exposures to environmental and/or pathogen antigens could account for these differences
209 among studies but this is only a supposition.

210 The representation of γδ T cells in jejunal Peyer's patches is lower than that of the
211 $CD4^+$ and $CD8^+$ T cells as in the MLN. Although the γδ T cell representation increases
212 from birth to 1 month of age it subsequently decreases again (Caro et al., 1998) as shown
213 in Table 1-5. This result of Caro and co-workers agrees with that of Navarro *et al*.
214 (Navarro et al., 1996) when goats were evaluated at 7 months of age. The percentages of
215 $CD4^+$ and $CD8^+$ T cells in the jejunal Peyer's patches also increases in animals between 1
216 week and 7 months of age but the increment is significant only after 1 month and thus is
217 the opposite of what was observed for the γδ T cells. As an aside, it is noteworthy that
218 during this time the $CD8^+$ T cells dominate the $CD4^+$ T cells (contrary to what is seen in
219 blood) as shown by $CD4^+/CD8^+$ T cell ratios that are less than 1 in all age groups.

220 In the case of ileal Peyer's patches (Caro et al., 1998) the percentages of γδ T
221 cells is more similar to that of $CD4^+$ and $CD8^+$ T cells at 1 week and 1 month. The γδ T
222 cells increase by about two-fold from birth to 1 month of age and then decrease at 3
223 months, remaining the same up to 7 months, the end of the study period. In contrast, the
224 $CD4^+$ T cells decrease until 3 months but then increase while the $CD8^+$ T cells increase
225 until 7 months of age. In the study by Navarro et al. (Navarro et al., 1996) the proportion
226 of TCR-$N6^+$ cells, a subset of γδ T cells, was found to have similar representation in ileal
227 Peyer's patches as that reported in the Caro *et al.* study (Caro et al., 1998).

228 Finally, in the spleen the percentage of γδ T cells (5-8%) is less than that of $CD4^+$
229 (5-14%) and $CD8^+$ T cells (28-32%) and did not show any significant change in any of
230 the four age groups evaluated, nor did the $CD8^+$ T cells. In contrast, the percentage of
231 $CD4^+$ T cells show significant differences between 1 and 3 month old goats. These
232 percentages reported by Caro and co-workers (1998) agreed with Navarro *et al*. (1996)
233 when 7-month old goats were evaluated (see Table 1-5).
234

235 **1.1.6.3 Blood**

236 In PBMC from adult goats (≥1.5 yr), γδ T cells as ascertained by staining with
237 mAb 86D have been reported to range from 6-14%, $CD4^+$ T cells from 18-37% and $CD8^+$
238 T cells from 15-31% (Table 1-3). The proportion of γδ T cells does not change with
239 increasing age once the animals reach adulthood (Jolly et al., 1997; Kaba et al., 2011;
240 Totte et al., 2002) as shown in Table 1-3. However in younger animals the percentage of
241 $CD4^+$ and $CD8^+$ T cells increases in blood as the animals' age from 1 to 7 months (Caro
242 et al., 1998; Navarro et al., 1996) whereas the percentage of γδ T cells as defined by
243 reactivity with mAb 86D decreases in blood during this time. Zafra *et al.* (Zafra et al.,
244 2013b), Baliu-Pique *et al.* (Baliu-Pique et al., 2019) and our results (Yirsaw and Baldwin,
245 unpublished data) found a higher percentage of γδ T cells in goats at 2 to 6 months of age
246 when anti-WC1 mAbs were used to detect γδ T cells (8-16%) than was reported by Caro
247 *et al.* (Caro et al., 1998) using mAb 86D (an anti-γTCR mAb) (<5%) to evaluate blood
248 from 3 to 7 month-old goats. This indicates that mAb 86D does not comprehensively
249 detect caprine γδ T cells.

250 Tourais-Esteves and co-workers (Tourais-Esteves et al., 2008) report fewer γδ T
251 cells in neonates 7 to 13 days old than other investigators have reported for adult goats 1
252 to 7 years of age when mAb 86D was used to identify them (Table 1-3). This result of
253 increasing γδ T cells with age differs from what is seen in other ruminant species (sheep
254 and cattle) for $WC1^+$ γδ T cells (Mackay and Hein, 1991; Rogers et al., 2005a). That is, in
255 sheep and cattle $WC1^+$ γδ T cells decrease with age. Again, we speculate that this
256 difference is likely to be because mAb 86D is reacting with only a proportion of γδ T
257 cells, i.e. those expressing a specific Vγ-gene, and it is this population that is lower in
258 newborns rather than all γδ T cells being lower.
259

260 **1.1.6.4 Mammary gland and milk**

261 Guiguen and colleagues found that the proportion of γδ T cells is the same in milk
262 as in blood (~4% in that study). In contrast, the proportion of $CD4^+$ T cells is lower in
263 milk than in blood while $CD8^+$ T cells are substantially higher in milk (Guiguen et al.,
264 1996). During lactation it has been shown that the percentages of T cell populations
265 ($CD4^+$, $CD8^+$ and $WC1\text{-}N2^+$) in milk changes between 1 and 63 days contrasting with
266 blood where they remain constant during this interval (Winnicka et al., 1999). That is,
267 during the first 7 days of lactation the percentage of the $WC1\text{-}N2^+$ of γδ T cells in milk
268 increases and remains higher than on the first day of lactation while the percentage of
269 $CD4^+$ T cells increases up to 14 days and that of $CD8^+$ T cells increases up to 21 days
270 (Winnicka et al., 1999) as shown in Table 1-4. However, the percentage of γδ (TCR1-
271 $N24^+$), $CD4^+$ and $CD8^+$ T cells eventually all decrease after parturition and lactation
272 (Ismail et al., 1996).

273 In the mammary gland the percentage of γδ T cells tends to increase at the drying-
274 off stage and increase significantly in late pregnancy. TCR-N6$^+$ cells, another
275 subpopulation of γδ T cells, also significantly increase at mid-pregnancy and show even
276 higher increasing increments at late-stage pregnancy but do not increase during the
277 drying-off stage (Winnicka et al., 1999). At this time it is unclear whether the γδ T cells
278 increase to control pathogens or act as regulatory cells.
279

280 **1.1.6.5 Uterus**

281 CD8$^+$ T cells are abundant in the uterine epithelium and stroma of the caruncular
282 and inter-caruncular areas whereas the CD4$^+$ and WC1$^+$ γδ T cells are rare in the stroma
283 of both the caruncular and intercaruncular areas in non-pregnant goats. In pregnant goats
284 neither CD4$^+$ nor CD8$^+$ T cells nor WC1$^+$ γδ T cells are found in the placentome nor in
285 the inter-placentome region they are infrequent. The reduction of T cells was suggested to
286 predispose the ewe to uterine infections leading to abortion (Martinez et al., 2005). While
287 γδ T cells and CD8$^+$ T cells are both rare in the uterus it would be of interest to evaluate
288 changes during intrauterine infections. Are they recruited to this organ at that time? Since
289 in sheep (Meeusen et al., 1993) it is known that the resident γδ T cells are the WC1$^-$ γδ T
290 cell population looking for WC1$^+$ γδ T cells and failing to find them in the caprine uterus
291 may not necessarily reflect the totality of the uterine γδ T cell population.
292

293 **1.1.6.6 Summary of γδ T cells in tissue and organs**

294 Overall the caprine γδ T cells are generally fewer than the CD4$^+$ and CD8$^+$ αβ T
295 cells in blood, and other tissues and organs examined. They also tend to decrease with

animal age in many tissues suggesting the γδ T cells may play a more pivotal role in younger goats. The notable increases in γδ T cell representation were seen in milk and the mammary glands relative to lactation, pregnancy and drying off status. Whether they are increasing to play a role in protection or act as regulatory T cells is unknown.

1.1.7 The role of γδ T cells in infectious diseases in goats

The involvement or changes of γδ T cells in response to nine pathogens are described below and summarized in Table 1-7.

1.1.7.1 Responses to viral infections

1.1.7.1.1 Caprine arthritis encephalitis virus (CAEV)

Caprine arthritis and encephalitis virus is a lentivirus found globally in goats. CAEV infection causes loss of condition slowly (Peacock, 1996; Stonos et al., 2014), decreasing the lifetime productivity of dairy goats and preventing export. The pathology caused by the infection includes chronic progressive arthritis, mastitis and pneumonia. In young goats it may also cause paralysis.

Several studies have evaluated the effect that CAEV infection had on the proportion of lymphocyte populations in caprine blood. In 1995, Wilkerson *et al.* (Wilkerson et al., 1995a; Wilkerson et al., 1995b) found no difference between the proportion of γδ, $CD4^+$ or $CD8^+$ T cells in blood of infected and mock-infected goats, while in the synovial fluid of arthritic joints the $CD8^+$ T cells alone increased greatly. However, Jolly *et al.* (1997) and later Ponti *et al.* and Kaba *et al.* (Jolly et al., 1997; Kaba et al., 2011; Ponti, 2008) did report differences associated with CAEV infection. The

320 Jolly *et al.* study indicated that the blood γδ T cells including the $WC1^+$ cells did
321 increment upwards (9±4 to 24±13%) during CAEV infection while there was no change
322 in $CD8^+$ T cells and a decrease in $CD4^+$ T cells. The Ponti *et al.* study also found an
323 increase in γδ T cell in blood of infected goats but no change in their proportion in milk.
324 Finally, Kaba *et al*. confirmed that there was an increase of γδ T cells (9 to 35%)
325 including the $WC1^+$ γδ T cell population in blood. The significant increase in the
326 proportion of γδ T cells in these studies suggests that these cells may either play a
327 protective role in defense and/or contribute to immune-mediated inflammation that may
328 be the core of CAEV pathogenesis (Kaba et al., 2011). It is necessary to deplete these
329 cells *in vivo* and then challenge the goats with CAEV to determine whether the response
330 by γδ T cells is largely protective and/or contributing to the pathology.
331

332 **1.1.7.1.2 Peste des petits ruminants virus (PPRV)**

333 Peste des petits ruminants (PPR) is an acute or subacute viral disease affecting
334 small ruminants but the disease is most severe in goats. It is caused by a virus in the
335 genus Morbillivirus and characterized by fever, necrotic stomatitis, gastroenteritis,
336 diarrhea, pneumonia, and death (Bailey et al., 2005; Fentahun, 2012). The economic
337 impact is very profound with global annual losses estimated at between 1.45 to 2.1
338 billion dollars (www.fao.org: FAO and OIE Present Initial Battle Plan in Global
339 Campaign to Eradicate Peste des Petits Ruminants, Oct 28, 2016; accessed Nov 2019).
340 Notably, Baron *et al*. (Baron et al., 2014) showed infection with any of the four PPR
341 virus lineages results in the same clinical signs although some isolates were slightly more
342 pathogenic than others.
343

344 With regard to the immune system following infection, no changes were observed
345 in the percentage of $WC1^+$ γδ T cells or $CD8^+$ T cells in blood at any time point evaluated
346 by Barron *et al.* (2014) suggesting these T cell populations may have a limited role in
347 either pathogenesis or protection. In contrast, the percentage of $CD4^+$ T cells was
348 transiently reduced by 60% in the first days post-infection in blood but recovered to
349 ambient levels in 7 days. It is possible that the loss of the $CD4^+$ T cells may contribute to
350 early disease establishment. Production of IL-10 that dampens inflammatory responses
351 occurred at a time when the anti-viral cytokines IFN-γ and IFN-β were decreasing. The
352 correlation may suggest a cause and effect. However the production of the IL-10 was not
353 attributed to a particular population of T cells, although in cattle γδ T cells have been
354 shown to be the Tregs (Guzman et al., 2014; Guzman et al., 2012; Hoek et al., 2009)

355

356 **1.1.7.2 Responses to bacterial infections**

357

358 **1.1.7.2.1 *Brucella melitensis***

359 Caprine brucellosis is a zoonotic bacterial infection caused by *Brucella melitensis*
360 with significant livestock and public health impacts (Tosser-Klopp et al., 2014). This
361 disease is controlled in most developed countries but still remains endemic in developing
362 countries and is considered to be one of the most economically devastating zoonotic
363 diseases in the world (Perry and Grace, 2009). Among the *Brucella* spp., *B*. melitensis is
364 the most virulent including in goats and humans. In goats, it results in abortion and is
365 shed in the milk transmitting the infection while in humans it leads to a number of
366 syndromes and may result in death.

367 Higgins and coworkers (Higgins et al., 2018) evaluated the caprine immune
368 responses to the virulent *B. melitensis* strain 16M and the vaccine strain Rev1 in pregnant
369 goats. $WC1^{+}$ γδ T cells changed little following challenge, although the response was
370 greater to infection with strain16M than with the Rev1 vaccine strain. These limited
371 responses are similar to what occurs in *Brucella*-infected cattle with regard to γδ T cells
372 (Weynants et al., 1998). In contrast, the results showed that caprine $CD4^{+}$ T cells increase
373 in representation between days 14 and 28 post-infection when goats were infected with
374 strain 16M. This parallels results of a study of human patients showing that their γδ T
375 cells dramatically expand in blood after *Brucella* infections (Bertotto et al., 1993) but
376 contrasts with results in sheep in which there are no significant changes observed in
377 $CD4^{+}$, $CD8^{+}$ or γδ T cell proportions following infection with the virulent strain of *B.*
378 *melitensis* (Suraud et al., 2008). With the less virulent Rev 1, both the percentage of
379 caprine γδ and $CD4^{+}$ T cells remained stable over the course of the study.
380 Dorneles *et al*. (Dorneles et al., 2014) found that cattle vaccinated with another
381 vaccine strain, *B. abortus* strain 19, had $CD4^{+}$ T cells that responded to brucella antigen
382 stimulation *in vitro* in cell culture assays. Such studies were not conducted with caprine
383 lymphocyte populations from 16M-vaccinated goats but would be useful to understand
384 whether the T cell populations including γδ T cells are primed to respond even though an
385 increase in representation was not evident *in vivo*.
386 Because *Brucella* are facultative intracellular bacteria, surviving and replicating in
387 host macrophages, IFNγ is important for activating macrophages for anti-brucella
388 activities (Jiang and Baldwin, 1993). Mouse models have shown that without IFNγ the
389 host may die (Goenka et al., 2011). Despite the inability to observe expansion of the γδ T

390 cells *in vivo*, the study of Higgins *et al*. (Higgins et al., 2018) showed a potential for

391 WC1$^+$ γδ T cells to produce IFNγ, although the number of IFNγ-producing WC1$^+$ cells

392 was low (<10%). However, a higher percentage of IFNγ$^+$/WC1$^+$ γδ T cells *in vivo* was

393 produced in goats infected with the highly virulent *B. melitensis* 16M strain. Thus, the

394 authors recommended further study to ascertain the functional role of WC1$^+$ γδ T cells in

395 *Brucella*-infected and vaccinated goats with regard to protective immunity.

396

397 **1.1.7.2.2 *Ehrilichia ruminantium***

398 Ehrilichiosis, also known as heartwater, nintas or cowdriosis, is caused by the

399 rickettsia *Ehrilichia* ruminantium (formerly Cowdria ruminantium), an obligate intracellular

400 bacterium. The disease got its name "heartwater" because of the accumulation of fluids

401 around the heart or in the lungs of infected animals. It is an acute tick-borne infection

402 affecting domestic ruminants in sub-Saharan Africa (Uilenberg, 1983; Uilenberg et al.,

403 1993) resulting in huge economic impact on animal production in affected areas.

404 Totte *et al.* (Totte et al., 2002) immunized goats with inactivated bacteria

405 emulsified in oil and then evaluated their T cell recall responses in vitro following several

406 booster injections *in vivo*. Following immunization, proliferation of cells cultured in

407 medium were often found to equal the proliferation measured in cultures with antigen.

408 Perhaps because of these high responses in medium cultures the specific responses by

409 WC1$^+$ γδ T cells to antigen were masked. However it is important to note that antigen-

410 specific proliferation by CD4$^+$ and CD8$^+$ T cells was, nevertheless, evident by 3 weeks

411 post-immunization. In a companion study the use of a 15kDa antigen of *Cowdria* was

412 also evaluated and found to given similar results with no proliferation by WC1$^+$ cells
413 (Gunter et al., 2002).

414 The high proliferation by cells cultured without exogenous antigens is not
415 uncommon for ruminant γδ T cells (Blumerman et al., 2007) as occurred in the above
416 study of goat γδ T cells. In that study the goats had been injected with adjuvant which
417 may have contributed to *in vitro* activation or priming that results in a propensity for *in*
418 *vitro* proliferation in medium cultures. Totte and co-workers (2002) tried several
419 approaches to reduce the proliferation in medium cultures so that antigen-specific
420 responses by γδ T cells could be evaluated. These attempts included using whole blood
421 rather than isolated PBMC and autologous serum rather than fetal bovine serum. Why
422 these responses by γδ T cells in medium cultures occur is unclear but may be an innate
423 immune response that can play a rapid role in early protection to infection. They may also
424 be bystander responses that result from co-culture with activated αβ T cells.

425 A later study by Esteves *et al*. (Esteves et al., 2004) evaluated the T cell subsets
426 expressing IFNγ transcripts in PBMC following vaccination of goats with killed *E.*
427 *ruminantium* with Montanide ISA50 adjuvant followed by a single booster dose.
428 Responses *in vitro* both in control cultures with medium only and those with antigen
429 increased following immunization. The percentage of CD4$^+$ and CD8$^+$ T cell subsets
430 expressing IFNγ increased in blood to a similar extent as one another when testing cells
431 from infected goats and was more consistent than the response by the WC1$^+$ γδ T cells.
432 After the booster, two of three animals had γδ T cells that produced IFN-γ above control
433 levels while all three animals had CD4$^+$ and CD8$^+$ T cells that did so. The amount of
434 IFNγ produced by these latter populations was substantially higher than that by the γδ T

435 cells. In conclusion, while the recombinant vaccines likely need $CD4^+$ and $CD8^+$ T cell
436 epitopes to ensure their activation, including antigenic components that stimulate $WC1^+$
437 γδ T cells could enhance vaccine efficacy.
438
439 **1.1.7.2.3 *Mycobacterium avium* subspecies paratuberculosis (MAP)**
440 Paratuberculosis, also known as Johne's disease, is caused by *Mycobacterium av.*
441 ssp. *paratuberculosis* (MAP) and affects goats, cattle, and sheep as well as other species
442 (Beard et al., 2001; de Silva et al., 2015; Reyes-Garcia et al., 2008; Windsor, 2015). It is
443 a chronic, progressive granulomatous infection mainly affecting the small intestine, liver
444 and mesenteric and hepatic lymph nodes. Infection results in weight loss, diarrhea and
445 death (Harris and Barletta, 2001; Salem et al., 2013). It occurs globally, causing
446 significant economic losses due to mortality and reduced production of livestock (Ott et
447 al., 1999)
448
449 **1.1.7.2.3.1 Presence of γδ T cells in tissues of MAP infected goats**
450 Navarro et al. (Navarro et al., 1996) conducted a study to determine the
451 lymphocyte subpopulations in the intestinal submucosa as well as regional lymph nodes
452 in goats naturally infected with MAP. The results showed that there was either no
453 difference in the number of γδ T cells in the intestinal lesions or fewer γδ T cells
454 compared to control uninfected goats, in contrast to what occurs in infected sheep (Little
455 et al., 1996). While some γδ T cells were scattered in the interfollicular and paracortical
456 areas in infected goats, they were scarce. Thus the results were interpreted to suggest a
457 limited role for γδ T cells in caprine paratuberculosis. By comparison the $CD4^+$ T cells
458 decreased and the $CD8^+$ T cells increased in number as the severity of the lesions

459 increased (tuberculoid to lepromatous). The results were considered preliminary due to
460 the low number of animals in the study but it has been suggested by others that $CD8^+$ T
461 cells may suppress γδ T cells during paratuberculosis in studies conducted in cattle
462 (Chiodini and Davis, 1992).

463 Several studies evaluated the presence of T cell subpopulations in mycobacterial
464 granulomas and the influence of γδ T cells in granuloma formation in response to
465 bacterial infections (Faisal et al., 2013b). Valheim *et al.* (Valheim et al., 2004) described
466 the paratuberculosis granulomatous lesion in detail including the occurrence of γδ, $CD4^+$
467 and $CD8^+$ T cells. The results showed a higher percentage of $CD4^+$ T cells than either γδ
468 or $CD8^+$ T cells, which were very few, in the granulomatous lesions. This was confirmed
469 by another study (Lybeck et al., 2013). The $CD4^+$ T cells were the dominant lymphocytes
470 in granulomas of both subclinically and clinically infected goats while there was no
471 difference in γδ T cells between infected and control goats. The few γδ and $CD8^+$ T cells
472 suggests their limited ability to influence the granulomatous lesions in caprine subclinical
473 paratuberculosis.

474 Schinköthe *et al.* (Schinkothe et al., 2016) also conducted a study to evaluate the
475 T cells in granuloma lesions of *M. av.* ssp. *hominisuis*-infected goats. Again the results
476 showed the presence of a large number of $CD4^+$ T cells, and this was interpreted as an
477 exuberant immune response, while γδ and $CD8^+$ T cells were present but more often as
478 singular cells or scattered cells. In contrast, in another study by that groups found that
479 when goats were infected with MAP experimentally or naturally the granulomas at three
480 months post-infection had many $CD4^+$ and γδ T cells (Kruger et al., 2015). The authors
481 interpreted this to reflect the importance of the γδ T cells.

482 In the Valheim *et al.* (2004) study, γδ T cells were found in large numbers in the
483 lamina propria outside of the granulomas with most being $WC1^-$ γδ T cells. Some $WC1^+$
484 γδ T cells were found in the epithelia, although $WC1^-$ γδ T cells made up the majority.
485 However there was no difference in the number of γδ T cells in control and infected goats
486 in non-granulomatous small intestine tissue overall. These results agree with Navarro *et*
487 *al.* (Navarro et al., 1998) and also with observations made in cattle with paratuberculosis
488 (Plattner et al., 2009; Plattner et al., 2013). However, in cattle $WC1^+$ γδ T cells have been
489 implicated to play a role in early granuloma organization with the percentage of $WC1^-$ γδ
490 T cells superseding them later. $WC1^+$ γδ T cells have also been found in Peyer's patches
491 of MAP-infected lambs (Beard et al., 2000).
492
493 **1.1.7.2.3.2 Responses to MAP antigen stimulation**
494 The response of caprine γδ T cells from blood of infected and control goats to
495 mycobacterial purified protein derivative (PPD) antigens in *in vitro* cultures was assessed
496 by measuring increased expression of the IL-2R (CD25) (Storset et al., 2000). The γδ T
497 cells made up the majority of responding cells as measured by CD25 expression in the
498 cultures at both 72 and 120 hours. This *in vitro* response was characterized as two-
499 phased. The first phase was the early response by γδ T cells from infected goats to PPD
500 stimulation in culture (as demonstrated by the higher expression of the CD25) compared
501 to that with cells from control uninfected animals. This indicates a specific recall
502 response. The second phase showed that after longer periods of *in vitro* culture the γδ T
503 cells from control animals up-regulated IL-2R expression to the same level as cells from
504 infected animals. The response differs from the more specific response by the $CD4^+$ T

505 cells although in some cases the control and infected animals' $CD4^+$ T cell responses
506 could not be distinguished just as occurred with the γδ T cells in the second phase of the
507 cultures. The response by γδ T cells included both $CD8^+$ γδ and $CD8^-$ γδ T cells which if
508 equivalent to the populations in cattle largely equates to $WC1^-$ γδ and $WC1^+$ γδ subsets of
509 T cells, respectively (Machugh et al., 1997). The response by γδ T cells from control
510 goats might occur because of cross-reactive antigens between MAP and other ubiquitous
511 mycobacteria as has been suggested by others (Koets et al., 2002). It has also been
512 suggested that γδ T cells contribute a first line of defense because they have been primed
513 by cross-reactive antigens of mycobacterium (Chan, 1984). In bovine tuberculosis, γδ T
514 cells are considered to be the first line of defense against infection followed by $CD4^+$ and
515 $CD8^+$ T cells (Pollock et al., 1996). Thus, it is reasonable to conclude that both the
516 primed and unprimed responses by the γδ T cells in goats may be important early after
517 infection.

518 Storset and co-workers. (Storset et al., 2001) showed that goats infected orally
519 had high numbers of γδ T cells in circulation that were responsive to MAP and that these
520 cells also associated with distal ileum and colon lesions. In contrast, MAP responsive
521 $CD8^+$ T cells were found in the blood one year post-inoculation in animals with no
522 detectable distal ileum and colon lesions. Thus it was suggested that γδ T cells could be
523 associated with immune-mediated pathology while the $CD8^+$ T cell response correlates
524 with control and protection. As in the other study described above, Storset *et al.* showed
525 that the γδ T cells responded to PPD with increased expression of the IL-2R with cells
526 taken from animals at >47 weeks post-infection and that these were the majority of
527 lymphoblasts in cultures stimulated with PPD. Coincident with the rise in $IL\text{-}2R^+$ cells

528 was the production of IFNγ, suggesting a type 1 immune response that would be expected
529 to be important in protective immunity against MAP.

530 Subsequently, Hasvold *et al.* (Hasvold et al., 2002) described T cell responses in
531 young goats vaccinated with a live attenuated vaccine strain that is used in many
532 countries. The study included evaluation of the effect of depleting lymphocyte subsets
533 individually on the expression of the IL-2R by the remaining cells. All vaccinated
534 animals had strong immune responses with increased IFNγ production by cells stimulated
535 with PPD in whole blood when taken 12 and 24 weeks after vaccination. The percentages
536 of $CD8^+$ and γδ T cells were higher in PBMC from the vaccinated group than from the
537 control group 24 weeks after vaccination. While the γδ T cells dominated in the cultures,
538 as in the above studies, most of the IFNγ was produced by $CD4^+$ T cells with a low
539 percentage of the IFNγ contributed by $CD8^+$ T cells. Thus, the γδ T cells did not produce
540 IFNγ after *in vitro* PPD stimulation unlike what Rhodes *et al.* (Rhodes et al., 2001)
541 showed after stimulation of bovine γδ T cells with PPD and many other *M. bovis*
542 antigens. However, it should be noted that in another study of paratuberculosis (Lybeck
543 et al., 2013) some of the caprine $CD8^+$ T cells producing IFNγ were shown to be γδ T
544 cells. If analogous to cattle, these cells are likely to be caprine $WC1^-$ γδ T cells. Hasvold
545 *et al.* (2002) found that the γδ T cells from vaccinated animals became highly activated
546 even in the control cultures with just medium. Depletion of $CD8^+$ and γδ T cells from
547 PBMC prior to culture caused an increase in $CD4^+$ T cell responses and thus $CD8^+$ and/or
548 γδ T cells might have an immunomodulatory effect (although the IL-2R expression on the
549 $CD4^+$ T cells remained the same before and after depletion of other cells). Conversely,
550 depletion of $CD4^+$ T cells before PPD stimulation led to a decrease in γδ T cells

551 suggesting a helper role for $CD4^+$ T cells for γδ T cell responses or that these γδ T cells
552 respond as bystanders to the products of activated $CD4^+$ T cells. From the small number
553 of animals, the single time point of the experiment, and because this result does not
554 correspond to the results of Storset *et al.* (Storset et al., 2003), conclusions are tentative.
555 However, the difference in this latter study might be the result of the forceful adjuvant
556 used in addition to the vaccine.

557 Later studies (Lybeck et al., 2009) showed that neutralizing IL-10 with antibodies
558 in cultures of cells from infected goats stimulated with PPD increased the percentage of
559 γδ T cells making IFNγ. Both $CD8^+$ and $CD8^-$ γδ T cells made IFNγ in these cultures
560 although only 10% of the $CD8^-$ γδ T cells did so. Interestingly, $CD4^+$ T cells continued to
561 be the major producers of IFNγ in PPD-stimulated cultures but the γδ T cells were the
562 main producers in cultures without PPD.

563 Studies using attenuated directed-gene-deletion (LeuD) mutants as vaccines
564 showed their ability to induce responses by caprine γδ T cells and that the significantly
565 higher γδ T cell levels correlated with enhanced protective efficacy (Faisal et al., 2013a).
566 This vaccine also decreased IL-10 and induced Th1 and Th17-like responses that may
567 account for the visible IFNγ responses by γδ T cells. Most importantly this vaccine was
568 efficacious, perhaps suggesting a role for type 1 γδ (Tγδ1) T cells responses in protection.
569 A similar γδ T cell response was stimulated in goats vaccinated with *Salmonella*-vectored
570 vaccines for paratuberculosis (Faisal et al., 2013b). These results suggest that although
571 natural infection may predispose a response by $CD4^+$ T cells, skewing the response to
572 include γδ T cell responses either by antigen/vaccine choice or by neutralizing IL-10 may
573 be important for developing next generation vaccines for paratuberculosis in goats.

1.1.7.2.4 *Mycobacterium tuberculosis* complex

Tuberculosis is a disease of livestock and humans that can be caused by a number of different bacterial infections. These several species of mycobacteria that cause tuberculosis are known as the tuberculosis complex. In goats it is predominantly caused by *Mycobacterium bovis* and *M. caprae* while in a few cases it is caused by *M. tuberculosis* (Cadmus et al., 2009; Hiko and Agga, 2011; Tschopp et al., 2011). Tuberculosis is a granulomatous and chronic debilitating disease that leads to significant economic loss due to decreased production, trade restrictions and a high cost to control the disease (Bezos et al., 2012; Crawshaw et al., 2008; Napp et al., 2013; Quintas et al., 2010).

Caro and colleagues (Caro et al., 2001) conducted a study to evaluate the T cell subpopulation proportions in peripheral blood and lymphoid organs in naturally tuberculosis-infected goats. They reported that there were no significant changes of any T cell populations including γδ T cells in proliferative tuberculosis (i.e., that having lesions with a small bacterial load and a high host cellular immune response). The γδ T cell proportion in blood also did not show any significant difference in ulcerative tuberculosis even though the proportion of γδ T cells decreased slightly, suggesting an overall limited response during tuberculosis. In exudative tuberculosis (i.e., having lesions with large numbers of bacilli and weak host immune responses) the $CD8^+$ T cells significantly increased in blood and lymphoid organs while the $CD4^+$ T cells significantly decreased in peripheral blood. $CD4^+$ T cells being higher in proliferative tuberculosis as compared to exudative tuberculosis may suggest they are contributing to protection in this less severe manifestation of tuberculosis (Basham and Merigan, 1983). Further studies were

597 recommended to ascertain the presence of cytokines that activate macrophages and
598 recruit monocytes to initiate granuloma formation and for the association of the higher
599 percentage of $CD8^+$ T cells in exudative tuberculosis with cytotoxic and
600 suppressor/regulatory functions. It is surprising that there is an apparent lack of a role for
601 γδ T cells in caprine tuberculosis since in cattle a wealth of studies show their response to
602 a variety of mycobacterial antigens and mycobacteria-infected dendritic cells as well as
603 showing that γδ T cells migrate into the lungs of infected or vaccinated cattle (Buza et al.,
604 2009; Hope et al., 2005; McGill et al., 2014a; Pollock et al., 1996; Pollock et al., 2005;
605 Price and Hope, 2009; Rhodes et al., 2001).
606

607 **1.1.7.3 Responses to parasitic pathogens**
608

609 **1.1.7.3.1 *Fasciola spp.***

610 Fasciolosis is one of the notable neglected tropical parasitic diseases. It is also
611 known as common liver fluke, distomatosis or liver rot. Fasciolosis is transmitted as a
612 result of food contamination by plant-borne trematodes *Fasciola hepatica* and
613 *F. gigantica*. It affects millions of ruminants as well as humans (Mas-Coma et al., 2014a;
614 Mas-Coma et al., 2014b; Nyindo and Lukambagire, 2015). This infection causes severe
615 physical wasting of infected animals and thus economic loss of over two billion US
616 dollars annually to the livestock industry in North and South America alone.
617 Zafra et al. (Zafra et al., 2010) conducted a study evaluating caprine hepatic
618 lesions and hepatic lymph nodes' local immune responses after immunizing goats with
619 glutathione–S-transferase (GST) and challenging them with *F. hepatica*. The results
620 showed that there was a severe infiltration of γδ, $CD4^+$ and $CD8^+$ T cells in the liver and

621 hepatic lymph node in both unvaccinated and vaccinated goats following challenge with
622 *F. hepatica* (Table 1-6). Thus a strong local immune response to *F. hepatica* infection
623 occurs in goats similar to that in sheep (Chauvin et al., 1995; Meeusen et al., 1995; Perez
624 et al., 1998; Perez et al., 2005). In a previous study, Zafra *et al.* (Zafra et al., 2009) had
625 shown similar responses in goats immunized with a synthetic peptide of Sm14 antigen of
626 *S. mansoni* and then challenged with *F. hepatica*; they found that the local cellular
627 immune responses decreased fluke burden but the difference was not statistically
628 significant due to the high variability of responses among animals. Additionally, the
629 study showed the absence of IFNγ in hepatic lesions and very low expression of IFNγ in
630 hepatic lymph nodes of control and vaccinated goats following challenge. This agrees
631 with the Waldvogel study (Waldvogel et al., 2004) of bovine liver and lymph nodes and
632 the Zhang *et al.* (Zhang et al., 2005) study of ovine lymph nodes following challenge. As
633 in a study in cattle by Buffoni *et al*. (Buffoni et al., 2010), the Zafra *et al.* (Zafra et al.,
634 2010) goat study showed that along with the absence or very low level of IFNγ there was
635 a high expression of IL-4 in hepatic lymph nodes and in serum one week after infection
636 of goats, suggesting a Th2 response. Although a Th2 response is considered the
637 protective response, this immune response did not cause a significant decrease in fluke
638 number, hepatic damage or fluke size.
639 Mendes *et al.* (Mendes et al., 2010) evaluated protection of young goats against *F.*
640 *hepatica* after immunization with the recombinant Schistosoma protein Sm14 adjuvant
641 with Quil A. This resulted in a significant decrease in hepatic lesions but the fluke
642 burdens and the fecal egg counts did not decrease. The results showed that γδ T cells
643 were occasionally found in hepatic lesions while in comparison there was a substantial

644 infiltration by $CD4^+$ and $CD8^+$ T cells in portal spaces in infected animals. In the
645 immunized animals there was reduced hepatic and HLN infiltration by $CD4^+$, $CD8^+$ and
646 γδ T cells as well as fewer IL-4 and IFNγ-producing cells even though the fluke burden
647 was the same. The decrease after immunization correlated with low expression of
648 IFNγ and higher expression of IL4 in hepatic lymph nodes along with lower hepatic
649 damage that was not due to the number of flukes. This implies that a Th2 response in
650 local lymph nodes and less proinflammatory IFNγ reduces the immune-mediated
651 pathology although it cannot clear the infection.
652 Finally, Zafra *et al.* (Zafra et al., 2013b) conducted a subsequent study to
653 investigate the proportions of $WC1^+$ γδ, $CD4^+$, and $CD8^+$ T cells in *F. hepatica*
654 experimentally-infected goats and in some cases those previously immunized with GST
655 and Cathepsin-L1 recombinant antigens. No protection was induced by the
656 immunizations and no differences were observed in the proportion of peripheral T cell
657 subsets between immunized and non-immunized goats except that the $CD4^+$ T cells
658 significantly decreased in the infected groups post-infection relative to the uninfected
659 controls. The decrease in $CD4^+$ T cells was associated with the marked infiltration of
660 $CD4^+$, $CD8^+$ and $WC1^+$ γδ T cells into the bile duct, hepatic lesions and hepatic lymph
661 nodes as reported previously (Zafra et al., 2009) and similar to what Perez *et al.* (Perez et
662 al., 1998) reported about the liver parenchyma in sheep.
663
664 **1.1.7.3.2 *Schistosoma bovis***
665 Schistosomiasis is a disease also known as snail fever or bilharzia, caused by
666 *Schistosoma bovis* - parasitic flatworms known as schistosomes (Chitsulo et al., 2004;

667 Vos et al., 2012). It affects a large number of livestock, including goats in Africa, causing
668 considerable production losses (McCauley, 1984), and infects more than 230 million
669 people worldwide. The pathology is predominantly a result of the host's immune response
670 to Schistosoma eggs producing granulomatous reactions (Gryseels et al., 2006). That is,
671 granulomas are formed around the Schistosoma eggs in the liver and they are vital for
672 protecting the host (Lindberg et al., 1999) but also result in compromising liver function
673 and causing pathology.

674 Lindberg *et al*. (Lindberg et al., 1999) conducted a study to provide information
675 on the immunopathology of caprine schistosomiasis. γδ T cells are known to be involved
676 in granuloma formation in response to other pathogens in mice and cattle (Plattner et al.,
677 2009; Price et al., 2010; Sandor et al., 1995). Here the authors showed that the $WC1^+$ γδ
678 T cells were predominantly found in most granulomas, especially in perioval granulomas,
679 along with $CD4^+$ and $CD8^+$ T cells agreeing with studies conducted in mice (Sandor et
680 al., 1995). Among the T cell populations in the schistosomiasis granulomas, $WC1^+$ γδ T
681 cells along with macrophages were the most plentiful in perivascular inflammatory cell
682 clusters in the submucosa. In intestinal epithelium, $WC1^+$ and $CD8^+$ cells were found but
683 not $CD4^+$ T cells, also in agreement with previous studies. This suggests γδ T cells have
684 an important, even key, role in the protective granulomas formed around the eggs but
685 thereby may also contribute to the pathogenesis of the disease.
686

687 **1.1.7.3.3** ***Haemonchus contortus***

688 *Haemonchus contortus* is a nematode that resides in the gastrointestinal tract of
689 small ruminants, specifically the abomasum. It is common in tropical and subtropical
690 environments where rainfall is frequent. It causes parasitic gastritis and enteritis and

691 death may occur rapidly if the infection is heavy. Clinical characteristics are anemia,
692 edema and weight loss. While antihelminthics are available resistance to those is
693 spreading. Goats may be segregated into more and less resistant groups (Patterson et
694 al., 1996) indicating a genetic component to control that could be exploited. Currently,
695 this infection causes large economic losses throughout the world.

696 Perez et al. (Perez et al., 2008) examined the abomasal mucosa of infected goats 3
697 to 13 days after infection with two different infective doses. In animals that received the
698 higher dose, an increase in T cells was noted by 13 days post-infection that consisted
699 primarily of a 10 to 20-fold increase in γδ T cells and 10-fold increase in $CD4^+$ T cells
700 with a less pronounced increase of $CD8^+$ T cells. In contrast, the proportion of T cells
701 decreased at 10 and 13 days in the abomasal lymph nodes due to an increase in B cell
702 numbers although the actual number of γδ T cells or $CD4^+$ T cells did not change. γδ T
703 cells were sparse especially compared to the other T cell subpopulations, representing
704 only 5-20% relatively.

705 An earlier study (Jasmer et al., 2003) explored the possibility of using parasite
706 intestinal antigens to immunize goats. When abomasal lymph node cells from the control
707 and immunized animals were compared, following challenge of the goats, for their ability
708 to proliferate in in vitro recall response to parasite antigens no difference in $CD4^+$ T cell
709 responses was found between the groups with both responding. However γδ T cells from
710 both group failed to respond to the antigens as measured by increased CD25 expression.
711 The authors interpreted this to mean that the intestinal antigens are not hidden during an
712 infection and that $CD4^+$ T cells, but not γδ T cells, are primed by them.

713

1.1.8 Conclusions and Summary

In general in mature mammals the percentage of γδ T cells are lower than other T cell subpopulations in the peripheral blood. Yet, their activation can serve as a first line of defense and bridge innate to adaptive immune responses. In goats the estimation of the proportion of γδ T cells has suffered from the use of mAbs to identify them that are not comprehensive in many studies. This is because not all γδ T cells express WC1 but anti-WC1 mAbs are often used to determine γδ T cell numbers. Also the anti-TCR mAb used (86D) reacts only with some TRG gene products while ruminants are known to have multiple cassettes of TRG genes each with their own V, J and C genes. Thus, the percentages of γδ T cells were likely underestimated in some studies and important responses by γδ T cells may have been missed. However in those studies that employed the mAb GB21A which reacts with the single TRDC gene product the measurements would be comprehensive for all γδ T cells. It has been shown that caprine γδ T cells are capable of producing both IFNγ and IL-17, the principal cytokines made by γδ T cells in mammals. It has also been shown that they can be stimulated through TLR as well as (presumably) the TCR. As reviewed here, caprine γδ T cells respond to various pathogens following infection or vaccination and these responses may either contribute to protection and/or pathogenesis, or have a regulatory role, as summarized in Table 1-7. Based on the studies reviewed here, caprine γδ T cells respond to infections with *B. melitensis, F. hepatica*, MAP and CAEV and in some cases there are indications that these responses may be important for protective immunity. However, caprine γδ T cell responses may also contribute to immune-mediated inflammation and pathogenesis of CAEV and *S. bovis* where this is known to be a primary component of the disease but a limited role in

737 either protection or pathogenesis following *M. caprae*, *E. ruminantium*, PPR virus or *H.*

738 *contortus* infections of goats. Based on the results of these studies, we conclude that the

739 ability of γδ T cells to respond to infection is not dictated by the type of pathogen

740 (bacterial, viral or helminth).

741

742 **1.1.9 Hypotheses and objectives**

743 To extend our knowledge of γδ T cells in goats we proposed to determine if

744 subpopulations of γδ T cells exist in goats as defined by their expression of cell surface

745 receptors (the TCR and the pattern recognition receptor WC1) that are multigenic

746 families in other ruminant species. We hypothesized that goats will have a multigenic

747 WC1 gene family as do cattle (Chen et al., 2012; Herzig and Baldwin, 2009) and that

748 some genes will have a high degree of identity with bovine WC1 genes while others will

749 be unique. This is predicted based on the fact that goats and cattle have shared but also

750 unique pathogens. We also hypothesize that the T cell receptor (TCR) gene loci and

751 structures among ruminants will be similar and that $WC1^{+}$ γδ T cells in goats will have

752 the same restriction of TCRγ chain gene expression as previously documented in cattle.

753 In cattle, we have shown that γδ T cells that express the co-receptor WC1 use a restricted

754 set of genes to code for the TCR γ chain while, in contrast, non-$WC1^{+}$ γδ T cells use all

755 of the TCR genes available (Blumerman et al., 2006). Finally, we expected to find γδ T

756 cell subpopulations that could be distinguished by their WC1 and TCR usage, and thus

757 likely their interaction with pathogens, as well as their functional responses to them.

758

759 The objectives of this dissertation research were:

760 1. To define the caprine WC1 gene number, chromosomal locus organization,
761 gene structures and genomic and expressed sequences
762 2. To evaluate WC1 gene similarity and differences among goat breeds and with
763 cattle
764 3. To determine the proportion of total γδ T cells, $WC1^{+}$ γδ T cells as well as
765 potentially WC1.1 and WC1.2 subpopulations of γδ T cells in PBMC and to
766 determine the WC1 genes transcribed by the subpopulations as well as their
767 functional differences with regard to responses to various pathogens and
768 cytokine production (IL-17 and IFNγ).
769 4. To define the caprine TCR γ and δ gene number, chromosomal loci
770 organization and genomic & expressed sequences including the usage of
771 multiple TRDD genes to enhance TCR diversity
772 5. To compare caprine TCR γ and δ gene similarities and differences with those
773 in cattle
774

775 Table 1-1 Experimental designs of studies of responses by goat γδ T cells to infections[a]

Disease	Goat breed or type	Age	Tissue evaluated	Design	Reference
Brucellosis	Mixed breed	≥1 yr	PBMC	Groups: 1. infected with *B. melitensis* strain 16M 2. infected with *B. melitensis* strain Rev. 1 3. uninfected	Higgins et al., 2018
Caprine arthritis encephalitis	Nubian	1.5-5 yr	PBMC	Groups: 1. uninfected 2. infected with CAEV stock virus intravenously and intracarpally	Jolly et al., 1997
	Dairy	3-5 yr	PBMC	Groups: 1. uninfected 2. naturally CAEV infected	Kaba et al., 2011
	Saanen	Birth	PBMC, synovial fluid	Groups: 1. healthy uninfected goat 2. infected with the phenotype CAEV-63 virus	Wilkerson et al., 1995a; Wilkerson et al., 1995b
	Alpine	60 day-6 mo	PBMC, milk	Uninfected and naturally infected	Ponti, 2008
Ehrilichiosis	Creole	NS	PBMC	Groups: not vaccinated or infected, subcutaneous injection of Montanide, 1. immunized with inactivated *E. ruminantium* vaccine mixed with Montanide s/c and booster after a month and then infected by virulent *E. ruminantium* culture supernatant intraveneously	Esteves et al., 2004
	Creole	NS	PBMC	Groups: 1. adjuvant only 2. immunized with killed *E. ruminantium* vaccine mixed with Montanide s/c and boosted after 2 and 12 months	Totte et al., 2002

Fasciolosis	Florida Sevillana	4 mo	Liver, HLN	Groups: 1. uninfected 2. infected with *F. hepatica* and PBS and Quil A adjuvant 3. immunized with rSm14 with Quil A	Mendes et al., 2010
	Malaguena	4 mo	Whole blood	Groups: 1. immunized with recombinant Cathepsin –L1 of *F. hepatica* 2. immunized with glutathione-S-transferase in Quil A adjuvant 3. adjuvant only	Zafra et al., 2013b
	Florida	4 mo	Liver, HLN	Groups: 1. uninfected 2. infected 3. immunized with Sm 14 + RIBI adjuvant s/c 3 doses	(Zafra et al., 2009)
	Florida	4 mo	Liver, HLN	Groups: 1. not immunized and uninfected 2. infected and immunized with adjuvant only 3. immunized with *F. hepatica* GST + Freund's adjuvant	Zafra et al., 2010
Haemonchus contortus	Florida	5 mo	Abomasal mucosa, abomasal LN	Groups: 1. uninfected 2. infected 10,000 infective dose 3. infected 20,000 infective dose	Perez et al., 2008
Paratuberculosis	Murciano-Grandina	3-5 yr	SI, LI	Goats with clinical signs of paratuberculosis and healthy controls	Navarro et al., 1998
	Norwegian	5-8 wk	Blood, liver, spleen, SI	Experimentally infected with MAP in milk replacer	Storset, 2000, 2001
	Dairy	2-3 wk	Blood	Vaccinated with MAP vaccine; IFNγ measured by ELISA at 24 weeks	Hasvold et al., 2002
	NS	NS	Granuloma	Experimentally infected with MAP	Valheim et al., 2004

	Thuringian Forest	7-16 days	Jejunum Peyer's patches	Naturally infected or experimentally infected with MAP 10 times every 2-3 days in milk replacer	Kruger et al., 2015
	Thüringer Wald ziege	10-21 days	GALT	Infected artificially 10 times every 2-4 days with MAP in milk replacer	Schinkothe et al., 2016
	Boer	8-9 wk	PBMC	Groups: 1. vaccinated with leuD mutuant M. av. ssp. paratuberculosis vaccine 2. vaccinated with PBS 3. vaccinated with M. av. ssp. paratuberculosis vaccine All were boosted at 3 wks. and challenged with M. av. ssp. paratuberculosis bacteria orally	Faisal et al., 2013
	Norwegian	2-3 wk	PBMC	Groups: 1. unvaccinated 2. vaccinated with a commercial vaccine with live attenuated bovine M. av. ssp. Paratuberculosis	Hasvold et al., 2002
	Norwegian	2.5-3.5 yr	PBMC	Groups: 1. uninfected 2. infected and exposed	Lybeck et al., 2009
PPR	Outbred British male	10-13 mo	Blood	Infected s/c or infected intranasally	Baron et al., 2014
Schistosomiasis	West African dwarf	3-4 mo	SI, MLN	Percutaneously exposed to *S. bovis*	Lindberg et al., 1999
	Florida Sevillana	4 mo	Liver, HLN	Groups: 1. unimmunized and uninfected 2. immunized with adjuvant only and infected with *F. hepatica* orally 13 wks after immunization 3. vaccinated and infected with *F. hepatica* orally 13	Zafra et al., 2010

				wks after immunization	
Tuberculosis	Murciano-Grandina	3-5 yr	Blood, lung, mediastinal LN, spleen	Goats positive for TB using comparative intradermal tuberculin test were used for the study	Caro et al., 200

a. NS, not specified, GALT, gut associated lymphatic tissue; SI, small intestine; MLN, mesenteric lymph node; HLN, hepatic lymph node; LI, large intestine; LN, lymph node

780 Table 1-2 Monoclonal antibodies used to study goat lymphocyte antigens

mAbs	Target	Isotype of mAb	Studies in which the mAbs were used
Clone 44.38	CD4	IgG2a	Baron *et al.,* 2014, Lybeck *et al*., 2009, Lybeck *et al*., 2013, Hasvold *et al*., 2002, Valheim *et al*., 2004, Higgins *et al*., 2018, Zafra *et al*., 2013
Clone 44.97	CD4	IgG2a	Valheim *et al*., 2004
GC1A	CD4	IgG2a	Schinkothe *et al*., 2016, Lybeck *et al*., 2013, Elnaggar *et al*., 2018, Davis and Ellis, 1991
GC50A1	CD4	IgM	Kaba *et al*., 2011, Jolly et al., 1997, Navarro *et al*., 1998, Caro *et al*., 2001, Esteves *et al*., 2004, Totté *et al*., 2002, Lindberg *et al*., 1998, Zafra *et al*., 2010, Mendes *et al*., 2010, Davis and Ellis, 1991
ILA11A	CD4	IgG2a	Elnaggar *et al*., 2018, Davis and Ellis, 1991
SBU-T4	CD4	IgG2a	Storset *et al*., 2001, Hasvold *et al*., 2002, Davis and Ellis, 1991
CACT80C	CD8	IgG1	Kaba *et al*., 2011, Jolly et al., 1997, Navarro *et al*., 1998, Caro *et al*., 2001, Lindberg *et al*., 1998, Zafra *et al*., 2010, Mendes *et al*., 2010, Elnaggar *et al*., 2018
Clone CC63	CD8	IgG2a	Baron et al., 2014, SchinkÖthe *et al*., 2016, Zafra *et al*., 2013, Davis and Ellis, 1991
Clone 38.65	CD8	IgG2a	Lybeck *et al*., 2009, Lybeck *et al*., 2013, Valheim *et al*., 2004
SBU-T8	CD8	IgG2a	Storset *et al*., 2001, Hasvold *et al*., 2002, Valheim *et al*., 2002
7C2B	CD8	IgG2a	Esteves *et al*., 2004, Totté *et al*., 2002, Elnaggar *et al*., 2018
86D	γδTCR	IgG1	Jolly et al., 1997, Storset *et al*., 2001, Lindberg *et al*., 1998
CACTB6A	TCR-N6	IgM	Navarro *et al*., 1998, Caro *et al*., 2001, Zafra *et al*., 2010, Mendes *et al*., 2010, Kaba *et al*., 2011, Perez et al., 2008, Seva *et al*., 1998
GB21A	δ TCR	IgG2b	Lybeck *et al*., 2009, Lybeck *et al*., 2013, Kruger et al., 2015, SchinkÖthe *et al*., 2016, Hasvold *et al*., 2002, Valheim *et al*., 2004, Elnaggar *et al*., 2018
CC15	WC1	IgG2a	Baliu-Pique *et al.,* 2019; Higgins *et al*., 2018, Esteves *et al*., 2004, Totté *et al*., 2002, Zafra *et al*., 2013
Clone 197	WC1	IgG2b	Baron et al., 2014, Lindberg *et al*., 1998
19.19	WC1	IgG1	Jolly et al., 1997, Valheim *et al*., 2004
BAQ4A	WC1-N2	IgG1	Kaba *et al*., 2011
CACTB31A	WC1-N3	IgG1	Kaba *et al*., 2011
SBU-T19	WC1	IgG	Guigeum *et al*., 1996
GD3.5	γδ marker	IgG	Jones *et al*., 2007
Clone CC302	IFNγ	IgG1	Lybeck *et al*., 2013

Clone 6.6	IFNγ	IgG1	Lybeck *et al*., 2013
MCA 1783	IFNγ	IgG1	Hasvold *et al*., 2002, Zafra *et al*., 2010, Mendes *et al*., 2010
RIF60A	IFNγ	IgG1	Elnaggar *et a*l., 2018
7B6	IFNγ	IgG1	Esteves *et al*., 2004
IL-17A2A	IL-17	IgG1	Elnaggar *et a*l., 2018
CACT116A	IL-2R	IgG1	Caro *et al*., 2001
LCTB2A	IL-2R	IgG3	Hasvold *et al*., 2002

781
782

Table 1-3 The percentage of T cells in goat blood arranged by age

Study	**Goat breed**	**Age**	**% T cell population in PBMC**[a]			
			CD4$^+$	**CD8$^+$**	**86D α-γTCR**	**WC1**
Tourais-Esteves *et al.*, 2008	Alpine goat	7-13 d	10±3	21±9	4±2	nd
Caro *et al.,* 1998	Murciano-granadina	1 wk	24.1±5.2	17.8±2.8	20.6±1.6	nd
		1 mo	18.4±2.7	12.8±5.7	10.38±1.4	nd
		3 mo	22.7±2.0	11.4±3.7	3.8±2.1	nd
		7 mo	34.0±1.2	27.3±1.5	4.9±1.6	nd
Baliu-Pique et al., 2019	White Saanen crossbred dairy goats	8 wk	~22	~17.5	nd	10.7±7.2
Zafra *et al*., 2013	Malaguena	4 mo	33-37	9-13	nd	8-16
Navarro *et al.*, 1996	Murciano-granadina	7 mo	35.5±7.0	27.4±10.0	6.6±3.4	nd
Jolly *et al*, 1997	Nubian	1.5-6 yr	33.0±7.0	22.2±6.3	8.8±4.3	12.7
Caro *et al*., 2001	Murciano-granadina		35.0±2.0	31.0±3.0	6.0±1.0	nd
Kaba *et al*., 2011[b]	Dairy	3-5 yr	37.0±12.7	21.0±11.1	9.0±8.5	nd
Wilkerson *et al*., 1995	Unknown	Adult	29.5±5.4	15.2±6.1	9.7±3.9	nd
Higgins *et al*., 2018	Mixed breed	Adult	22.9	nd	nd	4.5
			(18-37)			(1-7)
Totté *et al*.,2002	Creole goat	ns[C]	18.9	20.0	14.0	nd

a. mAbs used included mAb 86D that sees one or more of the TCRγ chains of the six available chains, but it is unknown which of those is recognized by this mAb. Mean ± SD are shown or the ranges of value if in parentheses under the mean value.

b. Kaba *et al*. (2011) also stained for WC1-N2 (13.0±9.5%) and WC1-N3 (15.0±8.4) data not shown here.

c. ns, not shown; nd, not done.

791 Table 1-4 The percentage of T cells in caprine blood and milk[a]

Day of lactation	PBMC			Milk		
	$CD4^+$	$CD8^+$	WC1-N2	$CD4^+$	$CD8^+$	WC1-N2
1	28.2±3.52	16.1±4.69	8.6±2.96	1.3±0.49	1.5±0.79	3.3±1.53
7	28.6±3.71	14.1±4.73	5.7±2.17	5.1±2.01	3.4±1.47	60.8±4.64
14	26.4±6.16	15.8±5.65	4.5±2.43	19.8±6.53	4.4±2.03	38.2±9.21
21	26.0±2.22	16.0±6.56	5.3±2.11	18.1±8.02	26.7±9.92	30.1±12.85
28	29.0±4.12	11.5±1.54	9.0±3.09	11.0±1.03	11.0±6.17	29.5±0.51
63	25.9±3.24	15.0±6.62	6.6±2.73	11.0±3.82	19.9±5.32	25.3±10.81

792 a. The mAbs used were GC50A1 for CD4, CACT80A for CD8 and BAQ4A for WC1-

793 N2; data from Winnicka et al. (1999).

794

795 Table 1-5 The percentage of T cell populations in various tissues of young goats[a]

Studies	Tissue	Age	T cell population		
			CD4$^+$	CD8$^+$	γδ
Caro *et al*. (1998)	MLN	1 wk	46.3±5.3	25.5±5.9	2.9±1.0
		1 mo	36.8±7.8	17.1±4.9	2.5±1.4
		3 mo	49.4±1.8	14.3±3.5	2.2±0.7
		7 mo	33.2±5.2	14.0±2.3	1.8±0.8
	JPP	1 wk	7.8±3.2	21.1±1.2	3.9±2.9
		1 mo	11.6±8.0	70.4±12.0	7.3±3.9
		3 mo	16.8±2.1	27.5±3.5	4.9±1.5
		7 mo	15.7±3.0	35.6±5.0	2.3±0.4
	IPP	1 wk	5.6±2.0	4.9±1.4	3.8±3.3
		1 mo	4.6±0.5	6.6±1.9	5.6±2.5
		3 mo	3.0±2.2	6.9±2.8	1.4±1.4
		7 mo	16.2±1.2	8.7±3.5	1.5±0.8
	Spleen	ns[b]	5 to 14	28 to 32	5 to 8
Navarro *et al*. (1996)	IPP	7 mo	33.3±5.3	14.3±2.8	1.8±0.8
	Spleen	7 mo	10.8±5.4	26.3±10.2	7.7±2.2

796 a. Goats used were Murciano gradina breed; mAbs used were GC50A1 for CD4,
797 CACT80A for CD8 and CACTB6A for γδ; MLN, mesenteric lymph nodes; JPP,
798 Jejunal Peyer's patches, IPP, ileal Peyer's patches.
799 b. ns, not specified
800

801 Table 1-6 T cells in hepatic tissues in *F. hepatica* infected goat after vaccination[a]

			T cell population			% of cells producing:	
Immunized with	**Challenged**	**Tissue**	**$CD4^+$**	**$CD8^+$**	**γδ**	**IL-4**	**IFNγ**
-	-	HLN[b]	69.5±11.4	30.5±7.6	12.4±3.3	0.03±0.4	0.01±0.01
Adjuvant Only	+		779.3±345.5	392.3±303.1	135.7±3.3	1.8±0.8	0.05±0.02
GST+ Adjuvant	+		664.0±267.6	134.6±16.3	105.8±28.4	1.6±0.7	0.06±0.01
-	-	Liver	6.2±2.2	6.0±2.2	0.3±0.1	-	-
Adjuvant Only	+		165.7±16.8	89±18.8[*c]	8.7±0.5[*]	-	-
GST+ Adjuvant	+		142.8±9.7	55.5±14.3[*]	2.5±0.8[*]	-	-

802 a. Data from Zafra *et al*. (2010) The mAbs used were GC50A1 for CD4, CACT80A

803 for CD8 and CACTB6A for γδ TCR. Mean ± SD is shown.

804 b. HLN = hepatic lymph node, Interfollicular

805 c. * = significant difference

806 d. +, challenged, -, not challenged and/or immunized

807

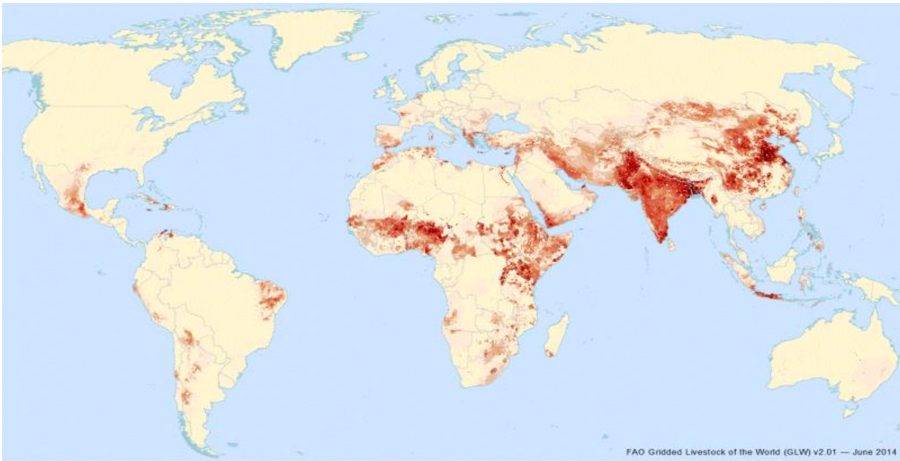
808 Table 1-7 Summary of major caprine γδ T cell responses to pathogens[a]

Pathogen	**Disease caused**	**Responses by γδ T cells**
B. melitensis	Brucellosis	A high percentage of WC1$^+$ γδ T cells make IFNγ in response to infection with the virulent strain although there is no change in their representation in blood
CAEV virus	Caprine arthritis encephalitis	γδ T cells including WC1$^+$ γδ T cells increase in representation in blood of infected goats
E. ruminantium	Cowdriosis	Increase in IL-2R on WC1$^+$ γδ T cells in vaccinated goats in *in vitro* recall responses; IFNγ is produced by some WC1$^+$ γδ T cells but not as consistently as by CD4$^+$ and CD8$^+$ T cells
F. hepatica	Fasciolosis	γδ T cells including WC1$^+$ cells infiltrate the liver and hepatic lymph nodes but make only low levels of IFNγ
H. contortus	Barber Pole worm	Increase of 10-20 fold of γδ T cells in abomasum but still a minor population of T cells
M. av. ssp. *paratuberculosis*	Paratuberculosis	γδ T cells are found in high numbers in the blood, ileum and colon with WC1$^-$ γδ T cells being the majority in the epithelia in the gut, although WC1$^+$ γδ T cells are found in the lamina propria outside of granulomas; γδ T cells make IFNγ when IL-10 is neutralized and they express IL-2R in antigen-stimulated cultures
M. caprae	Tuberculosis	Limited role in either protection or pathogenesis
PPR virus	Peste des petit ruminants	No change in the percentage of WC1$^+$ γδ T cells or other T cells with a role for IL-10 shown in dampening pro-inflammatory responses
S. bovis	Schistosomiasis	WC1$^+$ γδ T cells are found in granulomas, the submucosa and intestinal epithelium

809 a. See sections in text for the complete descriptions of the studies' results.

810

811 .



812

813

814 Figure 1.1 Global distribution of goats.

815 See http://www.fao.org.livestock-systems/en/. The Creative Commons license is found at

816 http://creativecommons.org/publicdomain/zero/1.0. Originally published in Gilbert et al.,

817 2018 (Gilbert et al., 2018)

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1351 hepatica and Fasciola gigantica: comparison of cellular response to experimental
1352 infection in sheep. Exp Parasitol 111, 154-159.
1353

1354 # 2 Chapter II DEFINING THE CAPRINE γδ T CELL WC1 MULTIGENIC ARRAY AND EVALUATION OF ITS EXPRESSED SEQUENCES AND GENE STRUCTURE CONSERVATION AMONG GOAT BREEDS AND RELATIVE TO CATTLE
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1359 ABSTRACT

1360 The purpose of this study was to evaluate the caprine γδ T cell co-receptor WC1 family
1361 with regard to the number of WC1 genes, their structures, genomic and expressed
1362 sequences and to evaluate WC1 sequence similarity among goat breeds and with cattle.
1363 While the presence of $WC1^{+}$ γδ T cells in the blood of goats had been demonstrated using
1364 monoclonal antibodies react with WC1 there was no information available about goat
1365 WC1 genes. Thus this study was conducted to fill those gaps using genome annotation,
1366 PCR amplification of genomic DNA and cDNA and subsequent Sanger and PacBio
1367 sequencing. We found evidence for up to 30 goat WC1 genes, which is more than twice
1368 that of cattle. Moreover, goats had seven different WC1 gene structures of which 4 are
1369 unique to goats. Caprine WC1 genes also had multiple splice variants of their transcripts
1370 coding for the intracytoplasmic domains, in some cases eliminating tyrosines shown
1371 previously to be important for signal transduction. The most distal WC1 SRCR domains
1372 known as SRCR a1, based on sequence and position, were highly conserved among goat
1373 breeds but fewer were conserved between goats and cattle. In summary, this work
1374 showed goats have a greater number of WC1 genes and unique WC1 gene structures
1375 relative to cattle as well as splice variants of transcripts for intracytoplasmic domains that

1376 are not found in cattle. Thus, goat WC1 molecules may have expanded functions. This

1377 finding may impact research on next generation vaccines designed to stimulate γδ T cells.

1378

2.1 Introduction

Livestock diseases contribute to poverty, food insecurity(Aziz, 2010; Duguma et al., 2011; Tibbo, 2006a), physical stunting and diminished cognitive development in children as studies have shown in sub-Saharan Africa (Gewa et al., 2009; Neumann et al., 2003; Neumann et al., 2007). Infectious diseases specifically impact goat production and productivity as a result of high treatment costs, morbidity, mortality and abortion rates as well as a result of weight loss or reduced gains and decreased carcass weight and quality (Aziz, 2010; Duguma et al., 2011; Tibbo, 2006a). Furthermore, some of the infectious diseases are zoonotic, crossing from goats to humans, reducing human capital (Kawooya, 2011). Various efforts have been conducted to combat these infectious diseases to increase the community income and reduce the public health impacts. These range from better management to vaccine development.

Vaccines are the only human intervention that has the potential to eradicate infectious diseases. However some vaccines may have limitations by targeting only conventional B cells and antibody responses thus failing to activate cell-mediated protection. Next generation vaccines targeting γδ T cells (Telfer and Baldwin, 2015) may be important for optimizing vaccination strategies due to the innate-like early responses of γδ T cells during the initiation phase of immune reactions, and because the γδ T cell receptors (TCR) are less restricted, recognizing ligands different from those recognized by αβ T cells. The latter is because γδ T cells do not rely on the recognition of classic MHC molecules with peptides to perceive infectious disease agents (Baldwin et al., 2019b; Chien, 2014; Sebestyen, 2019). In other words, there is no known antigen processing and presentation requirement for ligand recognition by γδ T cells' TCR and

1402 the antigens need not be peptides in complex with major histocompatibility complex
1403 (MHC) molecules (Davis, 2003). Hence, γδ T cells can recognize directly damaged
1404 tissues, cells and pathogens. This is believed to impart greater flexibility in γδ T cell
1405 responses than those by classical αβ T cell (Chien and Bonneville, 2006). Because γδ T
1406 cells rapidly recognize such changes they are a first line of immune defense, functioning
1407 like innate immune responses, but some populations also have immunological memory
1408 and therefore have characteristics and benefits of the adaptive arm of the immune system
1409 as well (Chien, 2014; Sebestyen, 2019). γδ T cell stimulation has been shown to
1410 contribute to protective immunity in mammals by producing the cytokines interferon-γ
1411 (IFN-γ) and IL-17 (Vantourout and Hayday, 2013). Furthermore γδ T cells may be able to
1412 direct adaptive immune responses by CD4 and CD8 αβ T cells and B cells because of
1413 their early responses and cytokine production (Baldwin et al., 2019b).

1414 In young ruminants γδ T cells are the major proportion of blood mononuclear
1415 cells and the majority of the γδ T cells in ruminant blood express the γδ T cell-specific
1416 marker Workshop Cluster 1 (WC1) on their surface (Davis, 1996; Holderness et al.,
1417 2013; Mackay et al., 1989b; Mackay and Hein, 1989; Mackay et al., 1986; Takamatsu et
1418 al., 2006). WC1is important for signaling the cell by augmenting the response through the
1419 TCR (Hanby-Flarida et al., 1996; Wang et al., 2011). In cattle, γδ T cells expressing WC1
1420 molecules ($WC1^{+}$ γδ T cells) and those not expressing WC1 molecules ($WC1^{-}$ γδ T cells)
1421 are considered as inflammatory and regulatory cells, respectively (Hedges et al., 2003;
1422 Meissner et al., 2003; Wang et al., 2009).

1423 WC1 molecules are transmembrane group B scavenger receptor cysteine rich
1424 (SRCR) superfamily members characterized by multiple cysteine residues (6 to 8) in their

1425 extracellular domains (Sarrias et al., 2004). SRCR molecules expressed on immune
1426 system cells act as pathogen recognition receptors (PRR) and include SPα, CD5,
1427 DMBT1, and CD163A. They detect pathogen associated molecular patterns (PAMPs) of
1428 bacterial, viral and fungal pathogens (End et al., 2009; Fabriek et al., 2009; Matthews et
1429 al., 2006; Sarrias et al., 2005; Vera et al., 2009). We know from previous studies that
1430 bovine WC1 molecules are coded for by a multigenic family of 13 genes each with 6 or
1431 11 SRCR domains and that different WC1 molecules' SRCR domains bind different
1432 pathogens making them also PRRs. For example, cells expressing some of the WC1.1
1433 SRCR subtypes respond to leptospira (Rogers et al., 2005b) and the BCG vaccine strain
1434 (Price et al., 2010) by proliferation and IFNγ production whereas those expressing WC1.2
1435 subtype respond to *Anaplasma* marginale (Lahmers et al., 2006; Lahmers et al., 2005).
1436 Thus, the large number of WC1 SRCR domains has the potential to increase the diversity
1437 of pathogen recognition and immune responses independent of the TCR. This recognition
1438 will be expected to augment the TCR response since when WC1 molecules are cross-
1439 linked with the TCR it results in augmentation of the TCR-induced activation (Hanby-
1440 Flarida et al., 1996; Hsu et al., 2015a).

1441 In goats the presence of WC1 on lymphocytes has been demonstrated by
1442 immunofluorescence using monoclonal antibodies (mAb) 19.19 (Jolly et al., 1997;
1443 Valheim et al., 2004; Valheim et al., 2002), Clone 197 (Baron et al., 2014; Lindberg et
1444 al., 1999) and CC15 (Esteves et al., 2004; Higgins et al., 2018; Totte et al., 2002; Zafra et
1445 al., 2013a; Zafra et al., 2013b) that react with WC1. In this study we obtained 16
1446 complete and 19 partial WC1 gene sequences using genome annotation, PCR
1447 amplification of DNA and cDNA and subsequent sequencing. The 16 complete WC1

1448 genes annotated predicted 7 unique molecular structures. We produced cDNA evidence
1449 for 10 complete WC1 genes and 2 partial WC1 genes. The majority of the most distal
1450 WC1 SRCR domains, which serve as signatures for each gene product and are known as
1451 the a1 domain, were conserved among goat breeds but fewer were conserved between
1452 goats and cattle.
1453
1454

1455 ## 2.2 Materials and Methods
1456

1457 ### 2.2.1 Genomic DNA and cDNA

1458 Female Boer goats housed at the University farm under conventional housing
1459 were used as blood donors. Blood was obtained via jugular venipuncture and collected
1460 into heparin in compliance with federal guidelines and with IACUC approval. Genomic
1461 DNA was extracted from whole blood using QIAamp DNA Mini Kit (Qiagen).
1462 Peripheral blood mononuclear cells (PBMCs) were isolated from blood via density
1463 gradient centrifugation over ficoll-hypaque (LKB-Pharmacia Biotechnology) according
1464 to the manufacturer's protocol and viable cell concentrations determined by trypan blue
1465 exclusion. PBMC was pelleted and re-suspended in Trizol (Invitrogen-Thermo Fisher
1466 Scientific) at a concentration of 5 x 10^6 cells/ml and kept at -80°C. RNA was isolated
1467 according to the manufacturer's protocol and cDNA (reverse transcription) was made
1468 using AMV reverse transcriptase (Promega, Madison, WI).

1469

1470 ### 2.2.2 Polymerase chain reaction

1471 To perform polymerase chain reaction (PCR) either genomic (gDNA) or cDNA
1472 was used as a template with primers shown in Table 2-1. For intracytoplasmic domain
1473 (ICD) transcripts primers were designed based on known bovine or swine ICD sequences
1474 at the 5' and 3' ends of the first and last exons, respectively. For full-length WC1
1475 transcripts the forward primers were designed in the untranslated region and signal
1476 sequences (Primers 6-9 in Table 2-1) for Pacific Bioscience (PacBio) sequencing or in
1477 the SRCR a1 domain for Sanger sequencing using reverse primers 3 - 4 and 10 - 14
1478 (Table 2-1). cDNA or gDNA were used as templates in PCR using Taq polymerase

1479 (Thermo Scientific) for amplification of the ICD and WC1 SRCR a1 domain transcripts.
1480 Cycling parameters for either ICD or SRCR a1 domain amplification were 30 sec at
1481 95°C, 1 min at 55°C, 45 sec at 72°C for 30 cycles with expected band sizes of 220-636
1482 bp. Two µl of cDNA was used as a template for full length WC1 transcript amplification
1483 using the Elongase Amplification system (Invitrogen) with cycling parameters of 30 sec
1484 at 94°C, 30 sec at 52°C and 4.5 min to 9 min for 35 cycles with expected band sizes of
1485 2200-6800 bp. PCR products were examined on 2% and 1.2% TAE low melt agarose gel
1486 for ICD and SRCR a1 domains or for WC1 full length transcripts, respectively, and
1487 visualized using either SYBR safe (Invitrogen) or ethidium bromide (Invitrogen).
1488
1489 **2.2.3 Gene sequencing**
1490 For Sanger sequencing the amplicons were excised and the DNA extracted using
1491 the QIAquick Gel Extraction Kit (Qiagen) and cloned into PCR 2.1 or Topo XL vectors
1492 (Invitrogen) according to the manufacture's protocol and transformed into Stable 3 or
1493 DH5α bacteria. After plasmid miniprep (QIAquick kit, Qiagen), it was sent for
1494 commercial sequencing by Sanger sequencing (GeneWiz, South Plainfield, NJ). For
1495 PacBio sequencing PCR amplicons were pooled after extracting from the agarose gel and
1496 sent to the PacBio Core Enterprise *(*UMass Medical School, Worcester, MA) for
1497 sequencing.
1498

1499 **2.2.4 Genome annotation**
1500 WC1annotation was done using the NCBI multiple alignment and Blast tools
1501 using bovine WC1 sequeces as the query (e.g., GenBank accession number for WC1-1

1502 (FJ031186)). The subject was San Clemente goat (ASM170441v1) GenBank assembly
1503 accession number GCA_001704415.1 (Bickhart, 2017a) or Yunnan goat with GenBank
1504 accession number AJP00000000 (Dong, 2013). Maker (Campbell, 2014) and Apollo
1505 (Lewis et al., 2002) were used to predict the WC1 gene location and gene structures,
1506 JBrowse (Skinner, 2009) to display the data and Inkscape (Bah, 2011) to draw the gene
1507 structures. Intron/exon boundaries were verified by comparing bovine WC1 sequences to
1508 the query and the San Clemente goat genome assembly as a subject since SRCR domain
1509 structures and sequences are highly conserved (Herzig and Baldwin, 2009; Herzig et al.,
1510 2010b)
1511

1512 **2.2.5 Sequence analysis and display**

1513 Sequence analysis and display**.** Sequencing alignment programs were used to
1514 compare gene sequences including BioEdit (Hall TA, 1999), CCL Genomics Workbench
1515 8.1.3 www.qiagenbioinformatics.com and alignment of two or more sequences using
1516 BLAST-Nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul, 1990) .
1517 Phylograms were made using CCL Workbench 8.1.3 with default parameters.
1518 WC1 gene designation**.** Boer goat WC1 SRCR a1 domains were named in the
1519 order discovered. Yunnan annotated genes were named based on NCBI's computer
1520 prediction of WC1 genes (http://www.ncbi.nlm.nih.gov\projects\mapview
1521 /maps.cgi?taxid=9925&CHR=5&MAPS=modelrna[93672503:94199726]&CMD=TXT).
1522 San Clemente goat WC1 genes were named by the order of occurrence of full length
1523 annotated genes on the chromosome except for SCgoatWC1-16 and SCgoatWC1-30.
1524 Partial WC1 genes were named following naming of the complete WC1 genes based on

1525 their location on chromosome 5 (GenBank assembly accession: GCA_001704415.1). The

1526 three goat breeds were abbreviated as BR, Boer; YN, Yunnan; and SC, San Clemente.

1527 The WC1 genes were named as BRgoatWC1-#, YNgoat-# or SCgoatWC1-#.

1528

2.3 Results

2.3.1 San Clemente goat WC1 genome annotation

As described above t is known that WC1 is a multigenic family in cattle and expressed exclusively on γδ T cells of ruminants including goats. However, in goats the WC1 gene number and their intron and exon structures were not known. Genome annotation was done to determine this since it will be important for further understanding the role of these co-receptor/pattern recognition receptors (PRR) in γδ T cell responses to various caprine pathogens. Our approach to answer these questions was to annotate the San Clement genome assembly for WC1 genes. Thirty-three full length or partial WC1 genes were identified on chromosome 5 and one gene was identified on scaffold 271 (Figure 2.1). The annotated WC1 genes were found on chromosome 5 between nt 99181668 and 102123135 and on scaffold 271 between nt 862 to 3434. Sixteen of these annotated genes were complete genes while 17 were incomplete or partial (the latter consisting of 1 to 14 exons). The complete genes were named in order of occurrence on the chromosome except for SCgoatWC1-16 and SCgoatWC1-32. The incomplete or partial genes were named subsequently in order of occurrence (Figure 2.1).

We found seven different intron-exon structures for the complete WC1 genes (Figure 2.2). They are referred to by lower case Roman numerals (i-vii) and examples of each are shown. Three of the seven types of gene structures are similar to those in cattle (Figure 2.2, i – iii; (Herzig and Baldwin, 2009)) while the other four structures found in the caprine genome are novel (Figure 2.2B).

The purported structures of the proteins that these unique genes code for are represented (Figure 2.3) using the same designations i-vii as are their sequences (Figure

1553 2.4). Goat WC1 extracellular SRCR domains are each coded for by a single exon as in
1554 cattle but the number of SRCR domains varied according to the gene structure. In all but
1555 one of the complete genes found, the first SRCR is of the "a" pattern (Herzig et al.,
1556 2010b) and thus denoted a1 as in cattle. However one of the novel gene structures in
1557 goats had a d1 domain (Figure 2.3B, v). For one gene (Figure 2.3B, vii) the a1 type
1558 domain was repeated 13 times. The most distal WC1 domain(s) in both goats and cattle
1559 are followed by SRCR domains in the pattern b-c-d-e-d (or d') usually occurring once or
1560 twice. However in one goat gene it was repeated two times (Figure 2.3B, vi). As in cattle,
1561 we found the WC1 genes had three different types of ICDs known as Type I, Type II and
1562 Type III, coded for by 4, 5 or 6 exons, respectively (Figure 2.2 and 2.3). Thus, Type II
1563 ICDs are 15-16 deduced amino acids longer than Type I while the Type III ICD is 60
1564 amino acids longer (Figure 2.4A) than Type II. The final novel structure identified in
1565 goats had a type I ICD associated with a 6-SRCR domain extracellular structure (Figure
1566 2.3B, iv). Since there was a second caprine genome assembly available, we sought to
1567 verify the SC assembly of these unique gene structures. We were able to find 5 complete
1568 WC1 genes in the Yunnan goat assembly and they corresponded to three structures, ii, iii,
1569 and vii, with vii one of the four unique structures annotated in the San Clemente
1570 assembly (data not shown. We conclude that goats have at least 7 intron-exon WC1 gene
1571 structures of which 4 are unique relative to cattle. The complete WC1 gene assemblies
1572 comprised 178 SRCR domains that is 40 SRCR domains more than the bovine WC1 gene
1573 family as shown in Table 2-3. This gives more opportunity for goat γδ T cells to bind to a
1574 variety of pathogens for better protective responses.
1575

1576 ### 2.3.2 Relationship of WC1 gene sequences among goat breeds

1577 In cattle the a1 domains of WC1 molecules are most diverse from one another and
1578 thus tend to distinguish one WC1 gene from another. The bovine WC1 a1 sequences are
1579 highly conserved among cattle breeds (Chen et al., 2012; Herzig and Baldwin, 2009).
1580 Here the WC1 sequence relationship was evaluated among three goat breeds using San
1581 Clemente and Yunnan goat WC1 a1 sequences obtained by genome annotation and Boer
1582 goat sequences obtained by PCR of genomic DNA. This comparison was also used
1583 provide a better estimate of the total variety of caprine WC1 genes. We aligned the 32 a1
1584 sequences annotated in the San Clemente genome with the 17 from the Yunnan assembly
1585 (Figure 2.5A) and prepared a phylogenetic tree (Figure 2.5B). The 17 Yunnan a1
1586 domains all had San Clemente counterparts that were ≥99.4% similar at the level of
1587 deduced amino acids with many having 100% identity including those from genes with
1588 unique structures. For the gene with 13 a1 domains (SCgoatWC1-16; see Figure 2.3B,
1589 vii) the level of identity for the domains between San Clemente and Yunnan goats was as
1590 follows: 7 (100%), 4 (99%), 1 (97%) and 1 (96%). The average of all the Yunnan goat
1591 WC1 ga1 domain sequences relative to San Clemente goat was 99.4%.

1592 Next we compared the 32 Boer goat a1 domain sequences obtained by PCR with
1593 the annotated San Clemente by aligning the deduced amino acid sequences (Figure 2.5C)
1594 and preparing a phylogram (Figure 2.5D). Twenty-two of the Boer goat a1 domains
1595 corresponded with San Clemente a1 sequences for genes with various structures (i-iii and
1596 vii) including 10 of the 13 a1 domains in structure vii with ≥97.9 % identity (Figure 2.5C
1597 and D). The other 22 Boer goat a1 domains sequence had ≥98.3% identity with San
1598 Clemente goat a1 domains. However, nine a1 domain sequences were only found among

1599 the Boer goat amplicons and 7 San Clemente a1 sequences were not found among the
1600 Boer goat sequences (Supplemental figure 2.1). Two a1 domains (BRgoatWC1-21 and
1601 BRgoatWC1-26) are nearly identical with 98.3% identity and because of this we
1602 considered they code for one gene. The WC1 gene numbers were estimated to be 30
1603 based on the number of unique San Clemente and Boer goat a1 domain sequences. These
1604 are summarized in Table 2-2.
1605

1606 2.3.3 Relationship of WC1 SRCR a1 domain sequences between goats and cattle

1607 Since some goat WC1 genes were found to have unique structures relative to
1608 cattle, we conducted further studies to determine if there was any conservation of the
1609 SRCR a1 domain sequences between goats and cattle. Since WC1 molecules are known
1610 as PRR, binding pathogens, and since goats and cattle share pathogens (Baldwin et al.,
1611 2019b) we expected to find similarities. Some bovine WC1 genes are classified as
1612 WC1.2-like genes having 4 additional amino acids at position 81-84
1613 (ESTL/ESVL/ESAL) in the a1 domain relative to those classified as WC1.1-like (see
1614 Figure 2.5E). The three cattle a1 domains classified as WC1.2 genes (BtWC1-4, WC1-7
1615 and WC1-9) cluster with three of the goat WC1.2-like genes (SCgWC1-11, SCgWC1-17
1616 and SCgWC1-19) in the phylogenetic tree (Figure 2.5F). Three of the a1 domains of
1617 goats had deduced amino acid sequence identity of 91-98% with bovine genes (BtWC1-
1618 12 and SCgoatWC1-8; BtWC1-10 and SCgoatWC1-10; BtWC1-11 and SCgoatWC1-9)
1619 and are WC1.1 types (Figure 2.5E). The remaining 7 bovine a1 sequences (BtWC1-1,
1620 BtWC1-2, BtWC1-3, BtWC1-5, BtWC1-6, BtWC1-8 and BtWC1-13) clustered together

1621 and distinctly from goat WC1 sequences (Figure 2.5F). This is interesting since these are
1622 the most highly represented in the bovine genome.
1623
1624 ### 2.3.4 CDNA evidence for annotated WC1 gene sequences.
1625 cDNA evidence to support the correctness of the assembly and annotation of the
1626 San Clemente WC1 genes was obtained by sequencing of transcripts from Boer goat
1627 PBMC (Table 2-4). WC1 PCR amplification resulted in amplicons of approximately 6,
1628 4.4 and 2.7 kB for transcripts with Type I or Type II ICDs while for transcripts with a
1629 Type III ICD the sizes were approximately 2.9, 2.2 and 1.5 kB (Supplemental figure 2.2).
1630 We obtained full-length cDNA evidence for 8 annotated WC1 genes using Sanger
1631 sequencing (Supplemental figure 2.2) and partial sequence for 4 more WC1 sequences
1632 using PacBio
1633
1634 ### 2.3.5 cDNA evidence for caprine WC1 ICDs and their splice variants
1635 We have shown previously that while cattle have three types of WC1 ICDs no
1636 splice variants have been identified (Chen et al., 2012; Herzig and Baldwin, 2009). Here
1637 we found that goats have similar ICD types and sequences with cattle. When evaluating
1638 goat WC1 cDNA sequences we discovered that splice variants occurred in the ICD. It is
1639 known from studies in cattle that there are key tyrosines in the ICD that get
1640 phosphorylated during γδ T cell activation and di-leucines that also play a role in
1641 signaling by regulating endocytosis of WC1 (Hsu et al., 2015a). Thus the splice variants
1642 were sequenced to determine the effect on maintenance of these key features of the ICDs.
1643 Using primers specific for the ICD 5' and 3' ends, eight different primers
1644 combination were evaluated of which only four gave the right amplicon size (Figure

2.6A). PCR resulted in amplicons of approximately 450, 400, 320 and 250 bp for Type I/II ICDs while for Type III ICDs the band sizes were approximately 636, 480, 330 and 300 bp (Figure 2.6A). Sequencing of these amplicons revealed that Type I ICDs had no splice variants as in cattle while Type II ICD had two splice variants missing 1 or 2 exons (Figure 2.6B and C). Both of them had similar amino acid sequences but they are different from the full construct and might be coded for by a different gene. One of these splice variant was missing the YEEL motif and it is this tyrosine (Y24) that is phosphorylated normally and one was missing the serine and di-leucine (Chen et al., 2014; Hsu et al., 2015a; Wang et al., 2009). Type III ICDs had 4 splice variants missing 1, 2 or 3 exons (Figure 2.6B and C). All of them had the motif YDDV in exon 6 that gets phosphorylated in this type of ICD (Y199) and one was missing the di-leucine needed for endocytosis. In conclusion, the splice variants of the ICD are novel relative to what is known in cattle and may affect signaling in some cases.

1660 ## 2.4 Discussion

1661 In order to extract useful information from the genome assembly, understanding
1662 of its components is essential. Because WC1 molecules are important as both co-
1663 receptors and PRR for host immune responses (Hsu et al., 2015b), genome annotation to
1664 describe caprine WC1 genes was done for both the San Clemente (Bickhart, 2017a) and
1665 Yunnan goat genome assemblies (Dong, 2013). In this study the caprine WC1 gene
1666 numbers, gene structures, cDNA evidence and ICD splice variants were defined as was
1667 WC1 gene sequence conservation among goat breeds as well as with cattle. Our results
1668 indicated that goat WC1 molecules are coded by a multigenic family as in cattle (Herzig
1669 and Baldwin, 2009) in line with our hypothesis. We identified 17 complete WC1 genes as
1670 well as 13 partial WC1 genes. The complete goat WC1 genes have 191 SRCR domains
1671 which is more than cattle (138 SRCR domains) and thus a larger variety for pathogen
1672 binding.

1673 In addition to the large number of caprine WC1 SRCR domains, the goat WC1
1674 gene number was estimated to be 30 based on the genome annotations combined with
1675 further gDNA and cDNA sequencing. This gene number is more than twice that of cattle
1676 (Chen et al., 2012; Herzig and Baldwin, 2009) although not all annotations represented a
1677 complete coding sequence. But from among the 13 goat complete WC1 genes obtained
1678 (12 annotated and 1 cDNA) there was a high degree of exon-intron structure identity with
1679 bovine WC1 genes although 4 were unique to goats. The annotated Yunnan WC1
1680 assembly was less complete than the San Clemente but it agreed with the San Clemente
1681 genome assembly with regard to the gene structures including the most unusual with 13
1682 a1 domains. Also the Yunnan and San Clemente goat WC1 a1 domains' deduced amino

1683 acid sequences had an average identity of 99.4% identity. The DNA sequences of WC1
1684 a1 domains from Boer goats also supported the correctness of the San Clemente genome
1685 sequencing, however the assembly was clearly incomplete since we obtained one
1686 complete cDNA transcript for a WC1 gene that had only an orphan a1 domain in the San
1687 Clemente assembly. Thus other partial genes including those with only a single a1
1688 domain represented in the assembled genome may in fact be a complete gene that was
1689 missed in the sequencing and/or assembly. While we made additional efforts to produce
1690 more cDNA evidence for the annotated WC1 genes by designing a variety of forward and
1691 reverse primer combinations we were unable to obtain cDNA evidence for all annotated
1692 genes or those orphan a1 domains. Those WC1 genes for which we could obtain no
1693 cDNA evidence might suggest their lower representation in the transcriptome.

1694 In cattle we know that different $WC1^+$ γδ T cell subpopulations produce cytokines
1695 and proliferate in response to different pathogens. For instance, among the 13 WC1 genes
1696 in cattle $BtWC1\text{-}3^+$ cells proliferate to the γδ T cell antigens of *Leptospira* and produce
1697 IFN-γ whereas $BtWC1\text{-}4^+$ cells proliferate to the γδ T cell antigens of *Anaplasma*
1698 *marginale* (Lahmers et al., 2006; Lahmers et al., 2005). If a specific WC1 gene
1699 expression determines responses to specific pathogens then having a larger number of
1700 WC1 genes and thus expressed SRCR domains might give a better chance of γδ T cells
1701 responding to more pathogens. We found the SRCR a1 domains are highly conserved
1702 among goat breeds like those among cattle breeds (Chen et al., 2012). This WC1
1703 conservation may suggest that most goat infectious diseases potentially threaten all
1704 breeds of goats except their variations in degree of severity and thus these are conserved
1705 through evolution like Toll-like receptors. When comparing bovine and caprine WC1 a1

1706 domains there was less conservation. However six bovine a1 domains (BtWC1-10,
1707 BtWC1-11, BtWC1-12, BtWC1-4, BtWC1-7, BtWC1-9) clustered more closely between
1708 the species. These similarities and differences may be due to the shared pathogens
1709 affecting both cattle and goats and unique pathogens affecting only goats. For example,
1710 the bovine WC1 a1 domain that binds *Mycobacteria paratuberculosis* (J. Telfer,
1711 unpublished data) differs by only 5.5% (17/310 nucleotide sequences) with SCgoatWC1-
1712 8. Both species may be infected with *M. paratuberculosis* (Baldwin et al., 2019b).
1713 The 4 goat WC1 genes with structures that do not occur in cattle might be
1714 important for stimulating responses to pathogens that only affect goats. Two of these
1715 unique structures have increased SRCR domain repetition. The presence of 13 a1 SRCR
1716 domains in one molecule might suggest their contribution in zippering with ligands of big
1717 pathogens such as helminthes. Alternatively, it might suggest that this WC1 molecule
1718 uses all SRCR a1 domains but to interact with different types of ligands of the pathogen.
1719 This latter hypothesis is based on the lower sequence identity among some of the
1720 repeated a1 domains. Similarly, the presence of 3 cassettes of the SRCR domains in
1721 another unique WC1 gene might suggest that certain pathogens require binding by a
1722 greater number of homologous SRCR domains, such as three B domains or all six D
1723 domains. The other two unique goat WC1 gene structures have features found in swine
1724 WC1 genes. The presence of an SRCR d1 domain on its distal part as occurs for some pig
1725 WC1 genes (unpublished data, L. Lepage and J.C. Telfer) suggested that this gene might
1726 be used to protect against pathogens that affect both swine and goats since feral swine
1727 can carry and transmit nearly 80% of the diseases of concern for sheep and goats
1728 (https://www.aphis.usda.gov/publications/wildlife_damage/fs-disease-risk-sheep

1729 goat.pdf). The presence of 6 SRCR domains with a type I ICD is also a unique gene
1730 structure even though swine WC1 genes only code for molecules with 6 SRCR domain
1731 (Kanan et al., 1997). However we found that in goats that the ICD was shorter than that
1732 in pigs. This type of ICD has less phosphorylation and results in less cytokine production
1733 than for example a bovine Type III ICD (Chen et al., 2014).

1734 For bovine ICD's tyrosine and serine phosphorylation are essential for signaling the
1735 cell for activation and endocytosis of WC1 molecules perhaps to limit activation (Hsu et
1736 al., 2015a). The bovine ICDs tyrosine kinase phosphorylation motif that can induce
1737 signaling differ in their locations in Type I and II ICDs compared to Type III ICDs. In
1738 goats we do not know which tyrosine are functional and get phosphorylated but the
1739 possible tyrosine phosphorylation sites in goats for Type II ICDs are Y15, Y24, Y29, Y68
1740 and Y131 and for Type III ICDs are Y23, Y56, Y84, Y116, Y133, Y168, and Y199. In
1741 cattle, we know that the tyrosine phosphorylation sites for Type II ICDs is Y24, coded for
1742 in exon 2, and for Type III ICDs is Y56 and Y199, coded for in exon 3 and 6, respectively
1743 (Chen et al., 2014). However we found goats have ICDs splice variants unlike in cattle.
1744 One of the type II ICDs splice variant transcripts had a missing second exon in which the
1745 functional tyrosine for phosphorylation is located and suggested no phosphorylation would
1746 occur although the splice variant might use other tyrosines. Similarly one of the type III
1747 ICD splice variants was missing its third exon in which one of the tyrosines for
1748 phosphorylation is located. In this case the gene might use the tyrosine that is located at
1749 199 but the response might be lower or unlike that in cattle. In general, goat ICDs might
1750 use other tyrosines at different locations but this is unlikely unless the principal one is
1751 spliced out. In future studies evaluation of the presence or the absence of the last ICD exon

1752 spliced out should be evaluated by amplification of cDNA for ICD with Rapid
1753 Amplification of cDNA Ends (RACE) PCR or by using Poly A (AAA) primers rather than
1754 primers located in the last exon as done here. Moreover an ICD signaling assay should be
1755 done to evaluate the signaling responses of IDs splice variants relative to the ICD full
1756 construct. We conclude that goats have approximately 30 WC1 genes from genome
1757 annotation combined with PCR of cDNA and genomic DNA. This gene number is more
1758 than twice that of cattle. Goats have 4 unique exon-intron structures in addition to the three
1759 classical structures of WC1 genes in cattle. We provided cDNA evidence for 12 of the
1760 annotated San Clemente WC1 genes and all of them suggested the correctness of the
1761 genome assembly as did the Yunnan annotated WC1 genes as far as they went. That is,
1762 both the annotated San Clemente partial WC1 genes as well as the additional SRCR
1763 domain sequences for orphan SRCR a1 domains suggests the incompleteness of the
1764 genome assembly. Finally we found that goats have splice variants of ICDs unlike cattle
1765 perhaps suggesting a difference in signaling initiation or sustainability following responses
1766 to some pathogens. In summary this work showed goat WC1 genes have unique features
1767 relative to cattle and this finding may impact research on next generation vaccines designed
1768 to stimulate subpopulations of γδ T cells through WC1 augmenting the TCR signal.
1769

1770 Table 2-1 Primers used for amplifying goat WC1 cDNA sequences

Primers designation	**Primer sequence**
29e1, Forward	CAG CTA CAC AGA TGG AGA GCA
BovWC1group I/II, Reverse	TCA YGA GAA AGT CAY TGK GGA TG
BovWC1group III, Reverse	CTA CAT GGT GCT AAG CTC CAC ATC
YNgoat a1, Forward	GAA GTG AAG CAC CAA GGA GAA
SCgoat a1, Forward	GAA GTGAAG YAC CAR GGA RAA TG
Long UPM no extra-Adp3	TACTAGAGTAGCACTCAAGCAGTGGTATCAACGCAGAGT
GS F UTR Com-adp3	ACTAGAGTAGCACTCGATCCTGCAGCTGGGACTG T
GS F UTR g5-adp3	TACTAGAGTAGCACTCCTGAGACTGTGACCTTGAGAAC
GT F UTR g16-adp3	TACTAGAGTAGCACTCCAATGAGATGTCATCTGACACCTGTC
WC1 RR1-adp2	GCAGAGTCATGTATAGGAGCAGCCAGRGGCTCTC
C1 RR2-adp2	GCAGAGTCATGTATAGCACTGAGTTCAACATCATCRTACC
C1 RR3-adp2	GCAGAGTCATGTATAGGRAAAGTCACTGTGGATGTTCC
C1 RR4-adp2	GCAGAGTCATGTATAGGAYCAATGAGGACTCCTTCTCC
C1 RR5-adp2	GCAGAGTCATGTATAGCAACATCATCGTATCCAGTGTCC

1771

1772

1773 Table 2-2 Relationship of San Clemente (SC) and Boar (BR) goat WC1 SRCR a1 and d1
1774 domains.

	WC1 gene name	
Gene count	**SCgoatWC1-#**	**BRgoatWC1-#**
1	1	48
2	2	25
3	3	54
4	4	82
5	5	-
6	6	-
7	7	59
8	8	-
9	9	72
10	10	6
11	11	62
12	13	-
13	14	55
14	15	-
15	16-1	83
	16-2	71
	16-3	17
	16-4	-
	16-5	30
	16-6	-
	16-7	13
	16-8	4
	16-9	90
	16-10	-
	16-11	84
	16-12	27
	16-13	107
16	17	-
17	19	-
18	20	86
19	22	-
20	23	3
21	30	44
22	-	8
23	-	21 (26)
24	-	47
25	-	56
26	-	64
27	-	77
28	-	96
29	-	99
30	-	100

1775

1776 Table 2-3 Comparison of the number of known WC1 gene structures among goats and cattle[a]

Distal domain type (a1 or d1)	# of SRCR domains	ICD type	Gene structure	# of WC1 genes identified in the genome	
				Goats	Cattle
a1	11	I	i	7	9
a1	11	II	ii	4	3
a1	6	III	iii	1	1
a1	6	I	iv	1	0
d1	11	II	v	1	0
a1	16	II	vi	1	0
a1	18	III	vii	1	0

1777 a. Only full length genes identified by genome annotation and/or cDNA sequencing are

1778 described in this table.

1779

1780

1781 Table 2-4 cDNA evidence for the annotated San Clemente goat WC1 gene sequences

	Goat cDNA sequences obtained by			
	Sanger sequencing		**PacBio sequencing**	
Annotated gene name: SCgoatWC1-#	**No sequences obtained for:**	**% of sequence identity**	**Sequences obtained for:**	**% of sequence identity**
1	c8, d6 partials	96.6	SS-c3, d'-ICD	98
2	b7, c8	97.9	SS-c3, d'-ICD	98
3	-	-	-	-
4	c8, ID3, ICD4	92	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	SS-c3, d'-ICD	99
9	C3	98.83	SS-c3, d'-ICD	99
10	-	-	SS-c3, d'-ICD	98
11	-	-	SS-c3, d'-ICD	99
13	ID1	90.4	SS-c3, d'-ICD	99
14	-	-	SS-c3, d'-ICD	98
15	B2-e10	88	SS-c3, d'-ICD	98
16	-	-	-	-
17	-	-	-	-
19	-	-	-	-
20	-	-	-	-
22	d4-tail	100 for a1domain	SS-c3	100 for a1domain
23	Complete	100 for a1domain	SS-c3	100 for a1domain
30	-	-	-	-

1782 -, no cDNA sequence available for this gene

1783

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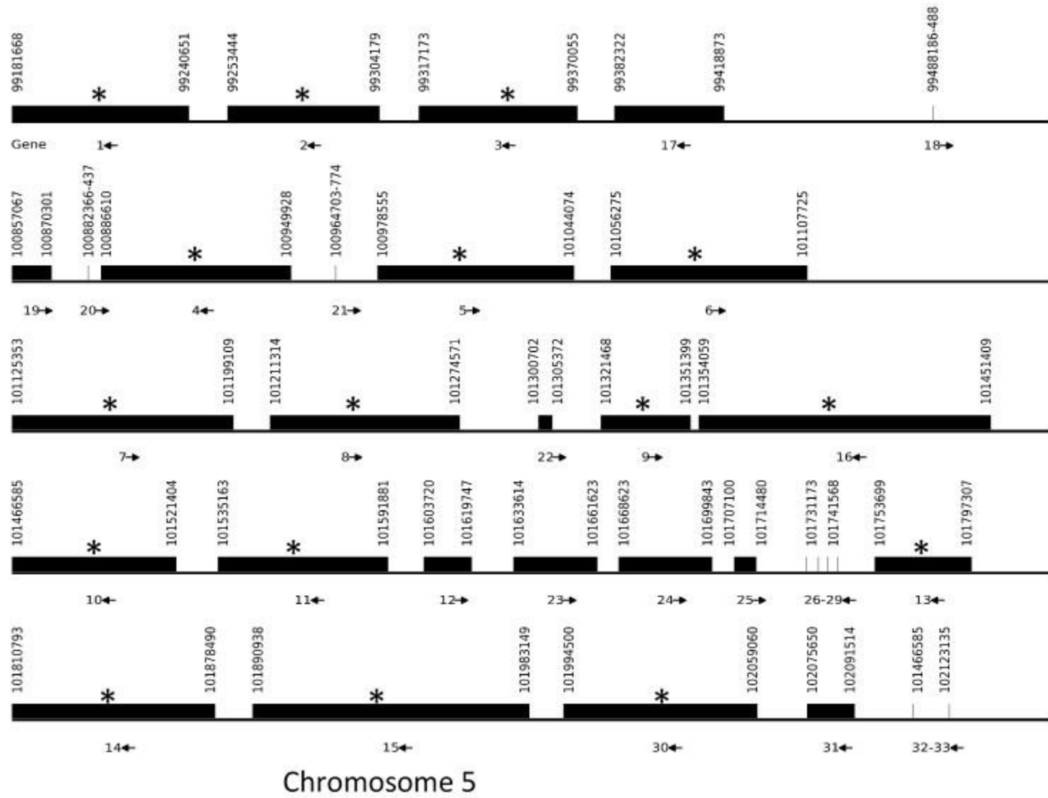

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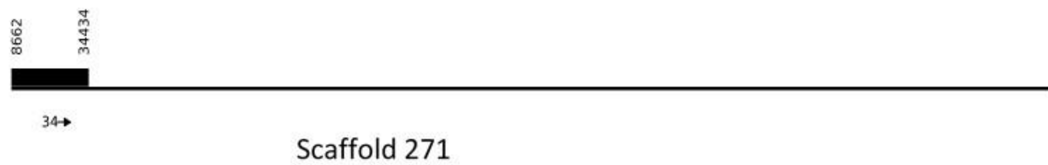

1785
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1787 Figure 2.1 Schematic representation of WC1 loci organization.

1788 (A) 33 WC1 genes were placed on chromosome 5 and (B) one unplaced gene was found

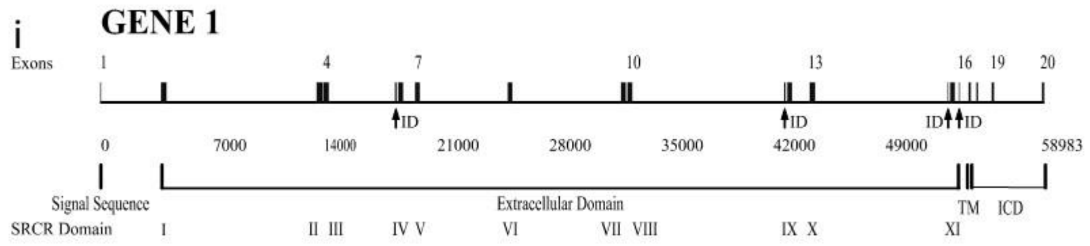
1789 on Scaffold 271. The 16 complete WC1 genes are indicated by asterisks. Gene

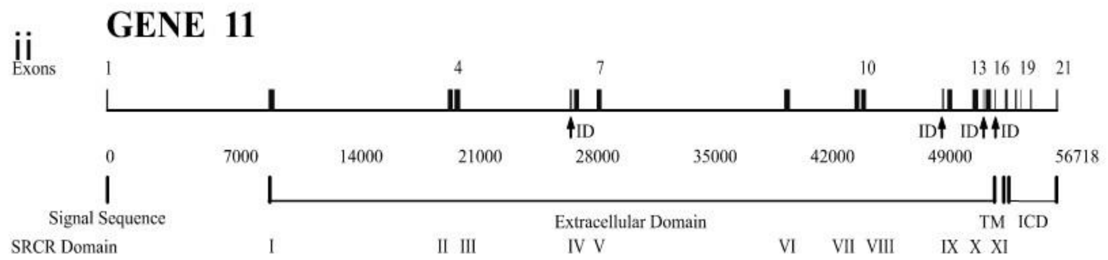
1790 designations are indicated (SCgoatWC1-1 to SCgoatWC1-34) and orientations (forward

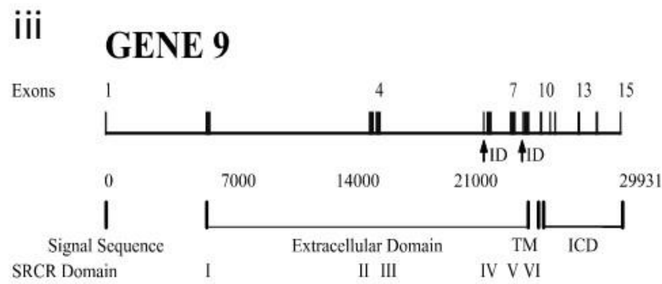
1791 or inverse) are shown by arrows

1792

A







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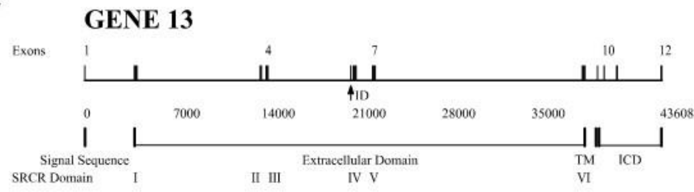

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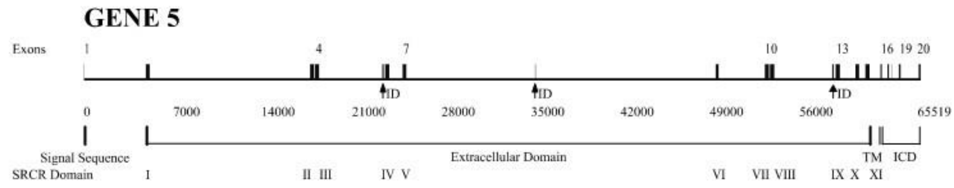

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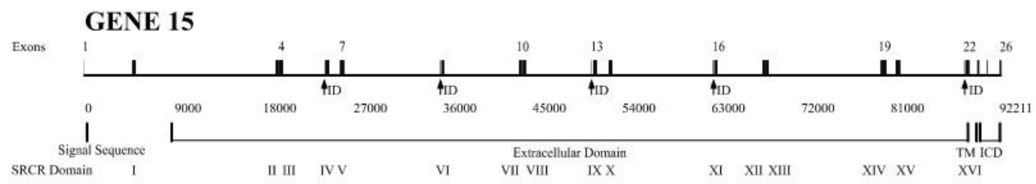

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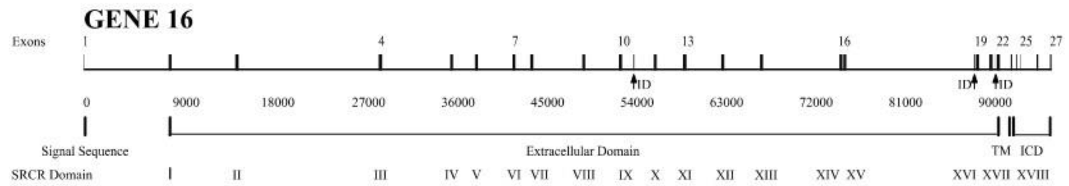

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1798 Figure 2.2 Schematic representation of WC1 intron-exon structures.

1799 Seven WC1 gene structures with variable numbers of exons were identified on
1800 chromosome 5. (A) Three structures were the same as found in cattle and representative
1801 structures of these San Clemente goat WC1 genes containing (i) 20 exons with Type I
1802 ICD (SCgoatWC1-1), 21 exons with Type II ICD (SCgoatWC1-11) or (iii) 15 exons with
1803 Type III ICD (SCgoatWC1-9) are shown. (B) 4 gene structures were unique to goats: (iv)
1804 12 exons with a Type I ICD (SCgoatWC1-13), (v) 21 exons with a Type II ICD
1805 (SCgoatWC1-5) and coding for a d1 as the most distal domain, (vi) 26 exons, with a
1806 Type II ICD (SCgoatWC1-15) and (vii) 27 exons with a Type II ICD (SCgoatWC1-16).
1807 The signal sequence, SRCR domains (I-XVIII), interdomain sequences (ID),
1808 transmembrane and intracytoplasmic domains (ICD) are indicated.

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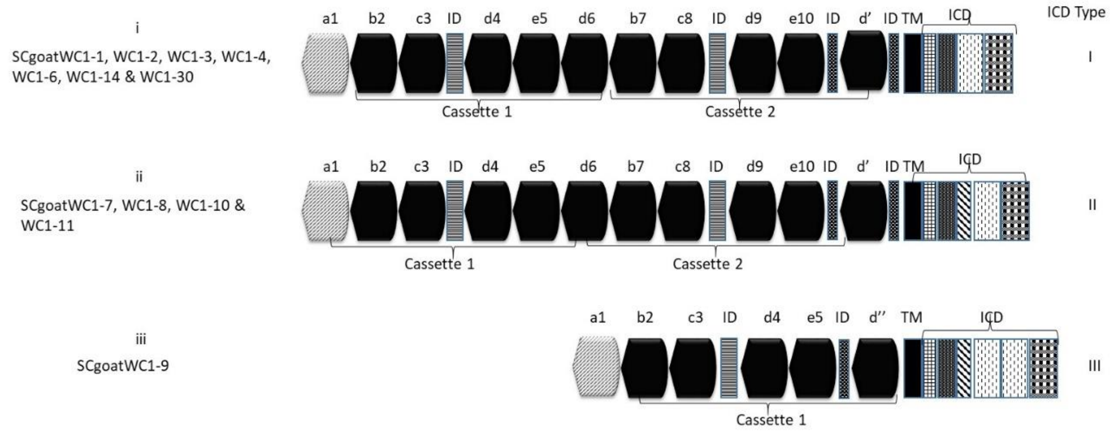

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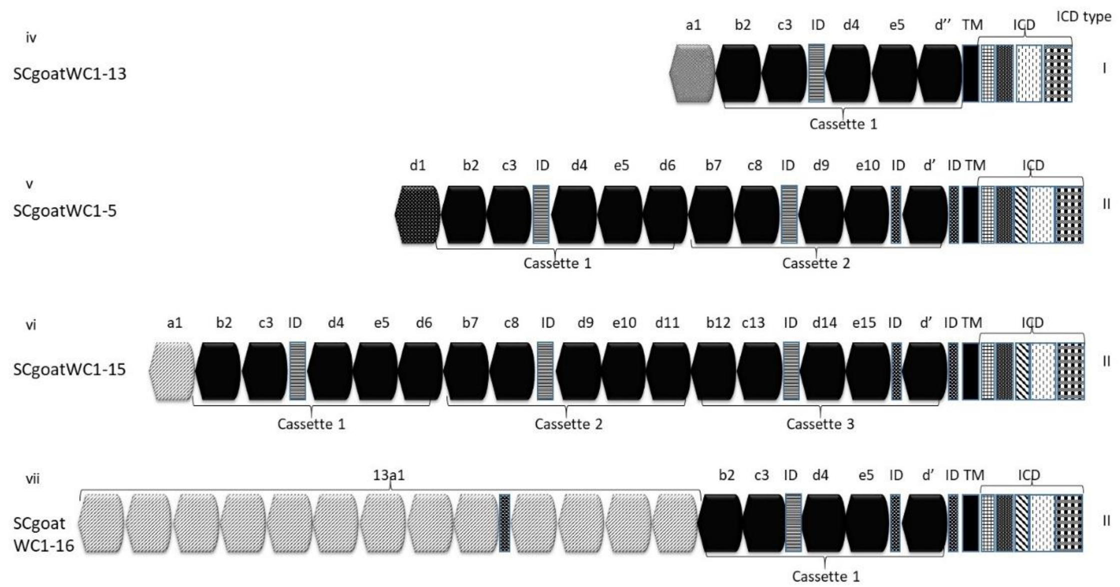

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1815 Figure 2.3 Schematic presentation of the purported structures of the goat WC1 proteins.
1816 (A) The three purported goat WC1 protein structures that resembled those in cattle (i-iii),
1817 and (B) the 4 unique goat WC1 structures (iv-vii) are shown: genes with those structures
1818 are listed. The SRCR domains (a-b-c-d-e-d), interdomains (ID), transmembrane and
1819 intracytoplasmic domains (ICD) are indicated. Also the number of repeating cassettes of
1820 SRCR domains (b-c-d-e-d) are labeled as are the most distal signatures domains as either
1821 a1 or d1. The ICD type is indicated (Type I-III), and the different ICD exon sequences
1822 are represented by various patterns. Differences in ID sequences are also denoted by
1823 patterns.

1824

A

Signal sequence

I II III ID1 IV

1825

1826

V VI VII VIII

1827

1828

1829

A cont..

ID2 IX X

ID3 XI

ID4 TM Ex Ex2

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1831

Ex3 Ex4 Ex5 Ex6

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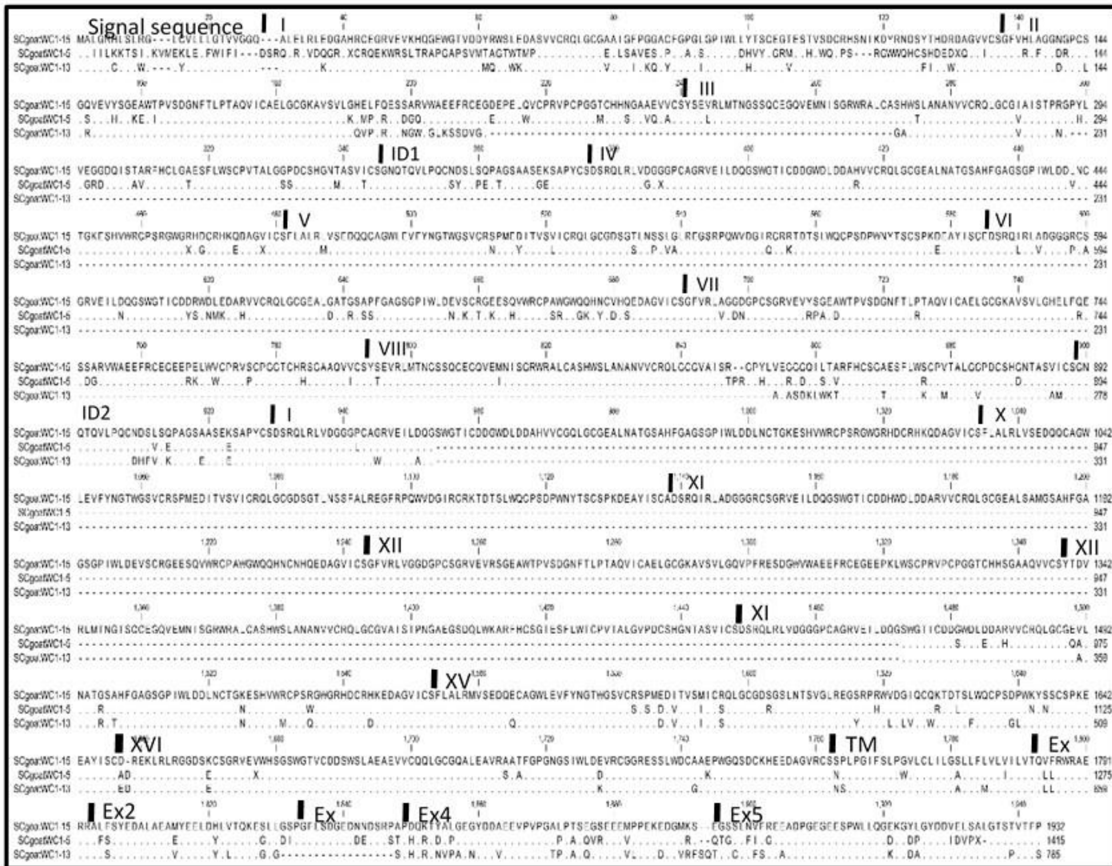

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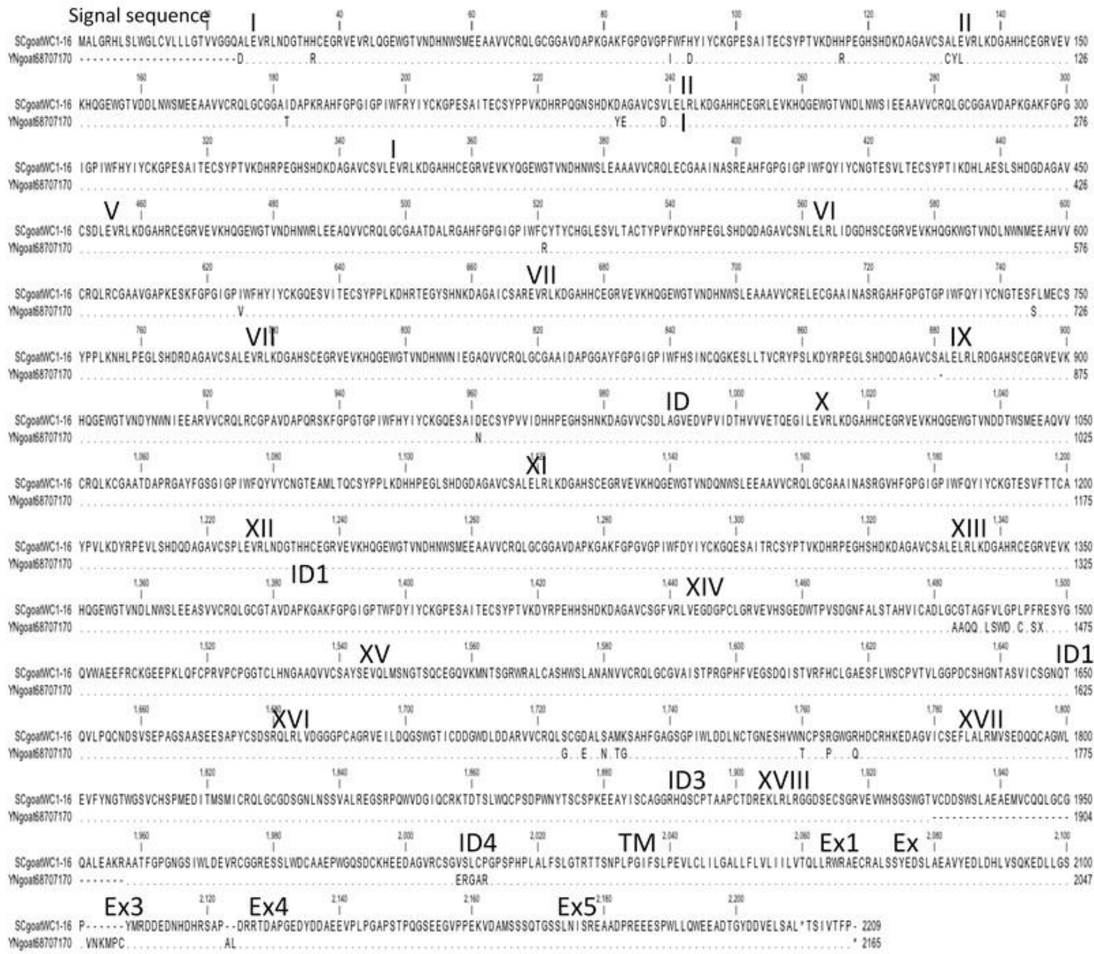

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1856 Figure 2.4 Alignment of the San Clemente goat deduced amino acid sequences for
1857 complete WC1 genes annotated in the genome.
1858 Full-length deduced amino acid sequences of the annotated WC1 genes were aligned
1859 using CLC Genomics Workbench 8.3.1 multiple sequence alignment and the default
1860 parameters. Identities are indicated by dots (.), gaps resulting from the alignment are
1861 indicated by tildes (~), gaps resulting from lack of genomic sequence are indicated by
1862 dashes (-). Signal sequence, SRCR domains (upper case Roman numerals), interdomain
1863 sequences (ID1 to ID4), transmembrane and intracytoplasmic domains' exons (ICD) are
1864 indicated. (A) Genes with similar structures as found in cattle (i-iii), (B) unique WC1
1865 gene structures (iv) SCgoatWC1-13, (v) WC1-5, (vi) WC1-15 and (C) (vii) WC1-16 are
1866 shown. For WC1-16 both San Clemente and Yunnan goat sequences are shown as
1867 verification of this unusual structure.
1868

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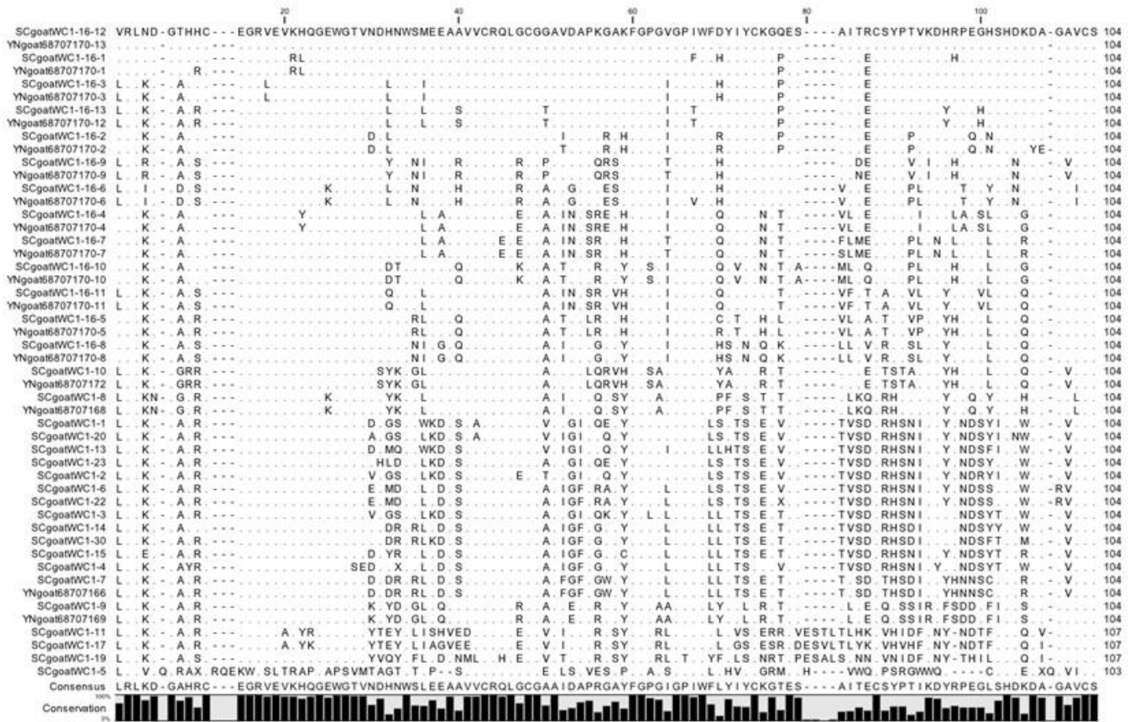

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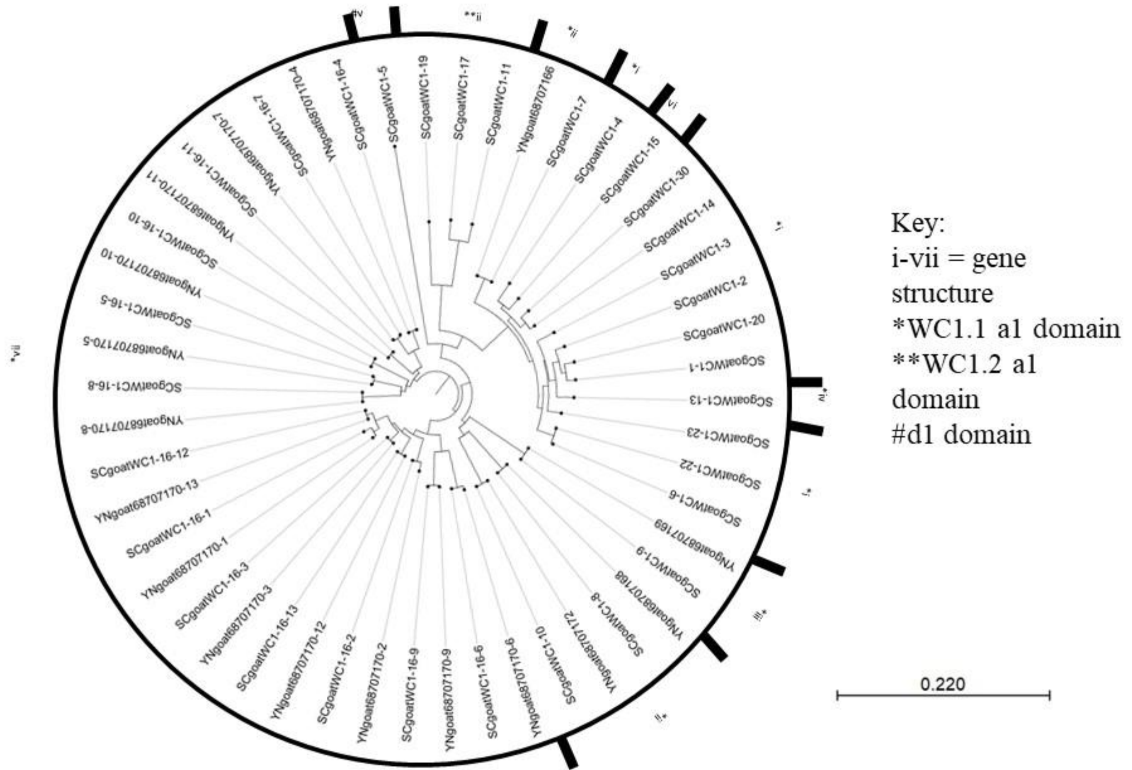

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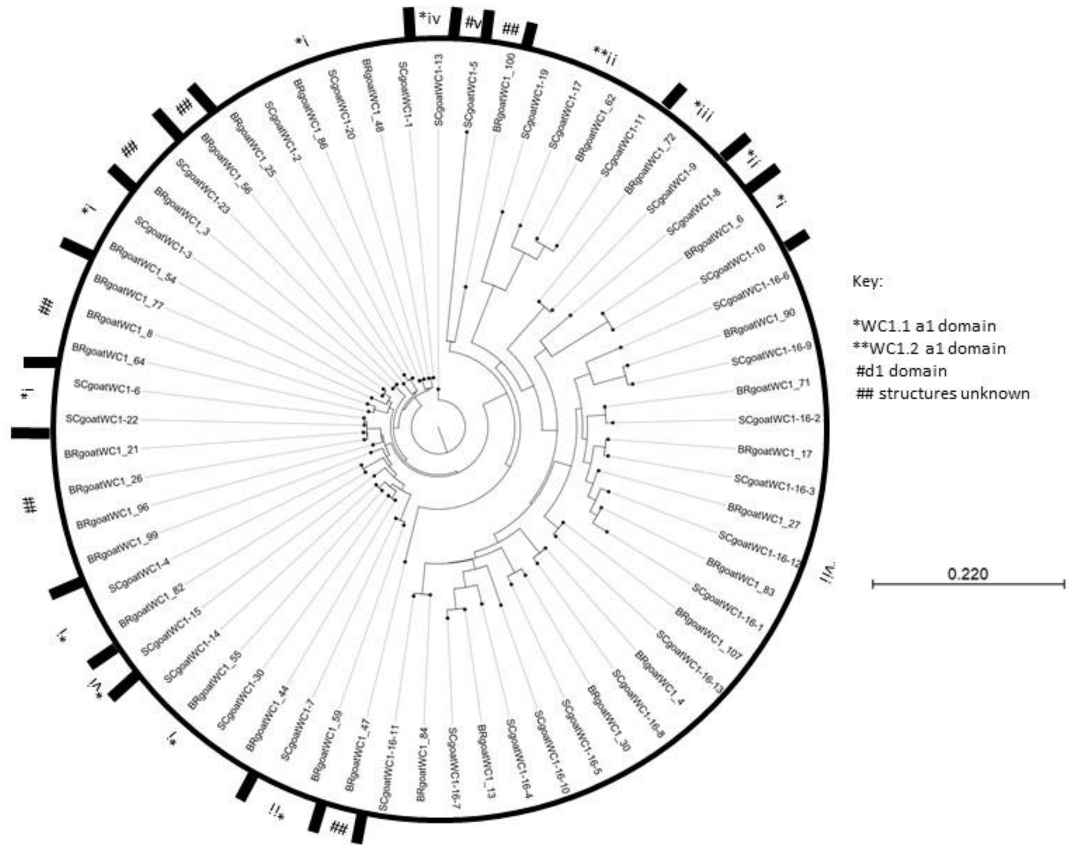

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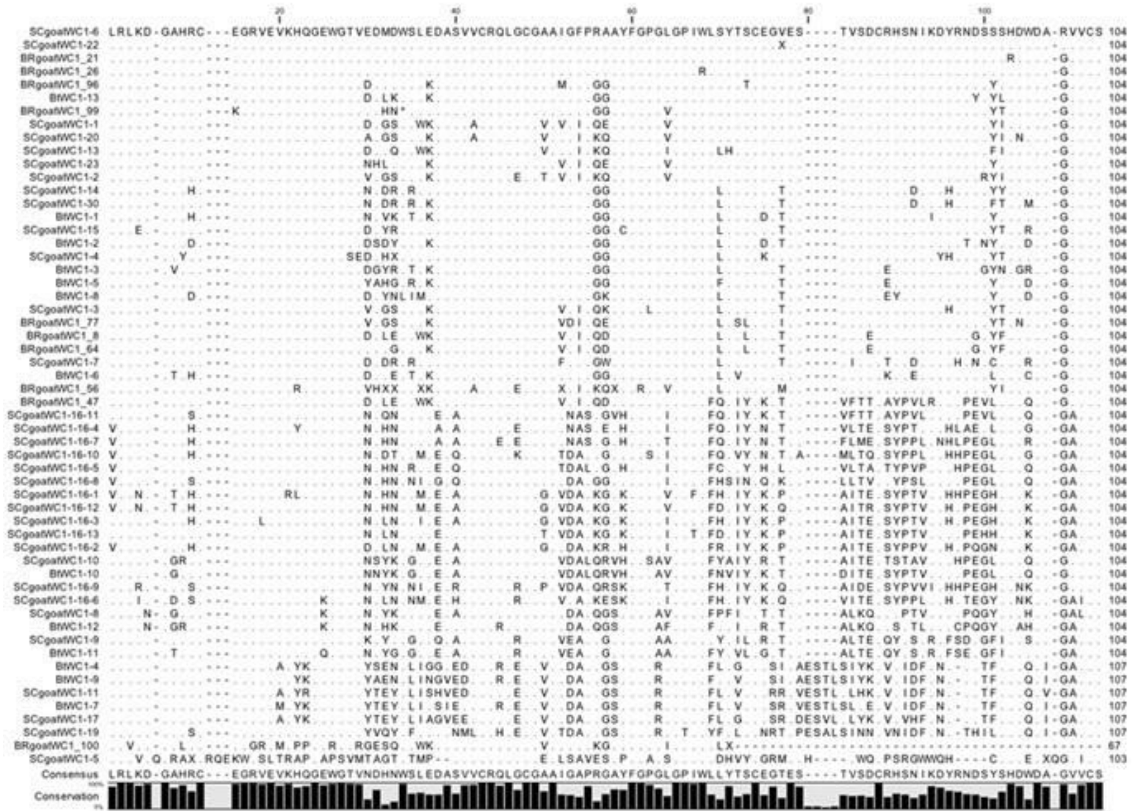

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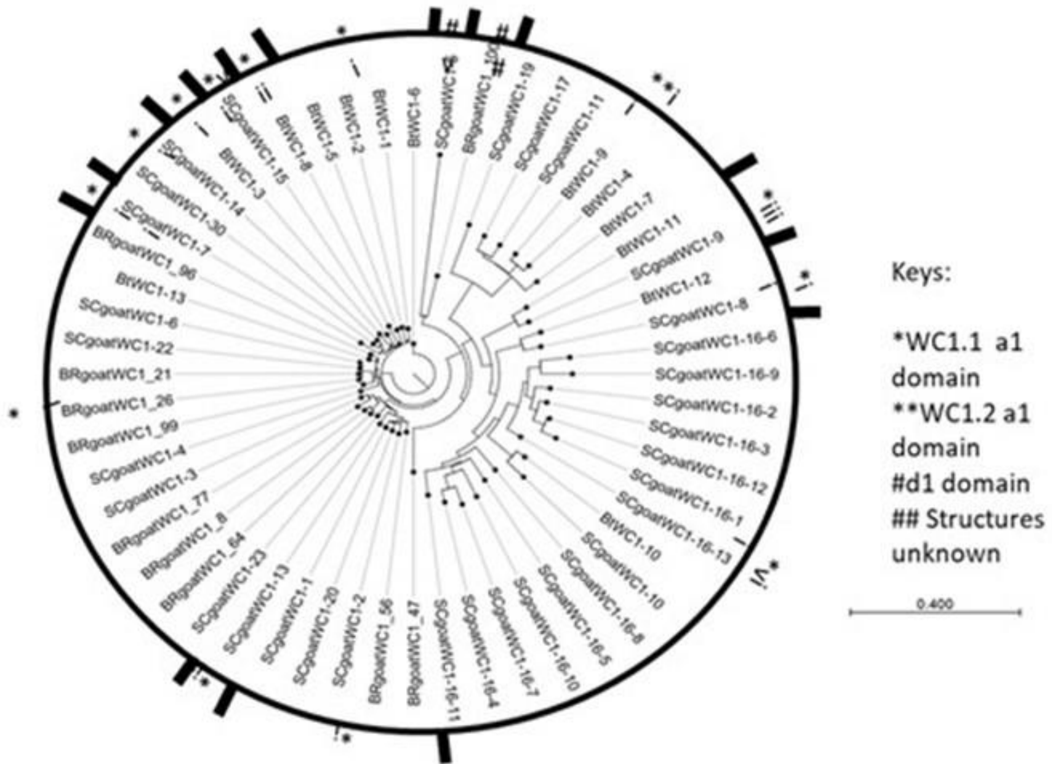

Figure 2.5 Relationship of SRCR signature domains among goat breeds and with cattle using the deduced amino acid sequences.

San Clemente and Yunnan goat WC1 a1 domain sequences obtained by genome annotation were compared by (A) an alignment and (B) a phylogenetic tree. This included the 32 San Clemente and 17 Yunnan a1 domain sequences and the single San Clemente d1 domain sequence. San Clemente and Boar goat WC1 a1 domain sequences obtained by genome annotation or PCR amplification of gDNA of Boar goats was compared by (C) an alignment and (D) a phylogenetic tree for the 32 San Clemente and 32 Boar goat a1 domain sequences and the single San Clemente d1 domain sequence. All 40 individual goat WC1 a1 domain sequences found (San Clemente and Boar goats) were compared to the 13 known bovine WC1 a1 sequences by (E) an alignment and (F) a phylogenetic tree. Gene structures from which the a1 domains were derived are indicated as i-vii, and whether the a1 domains are WC1.1 or WC1.2 types are indicated on the trees. For some genes a complete sequence was not available (SCgoatWC1-17, SCgoatWC1-19, SCgoatWC1-20 and SCgoatWC1-22 and BRgoatWC1-8, BRgoatWC1-21, BRgoatWC1-26, BRgoatWC1-47, BRgoatWC1-56, BRgoatWC1-64, BRgoatWC1-77, BRgoatWC1-96, BRgoatWC1-99 and BRgoatWC1-100) and thus their gene structures are unknown but are grouped according to a1 domain sequence similarity with genes with known gene structures. The nature of the a1 domain sequences are indicated as WC1.1, WC1.2, or unique along with the gene structures of genes whose full length sequence was known. Abbreviations: San Clemente (SC), Yunnan and Boar (BR) goats.

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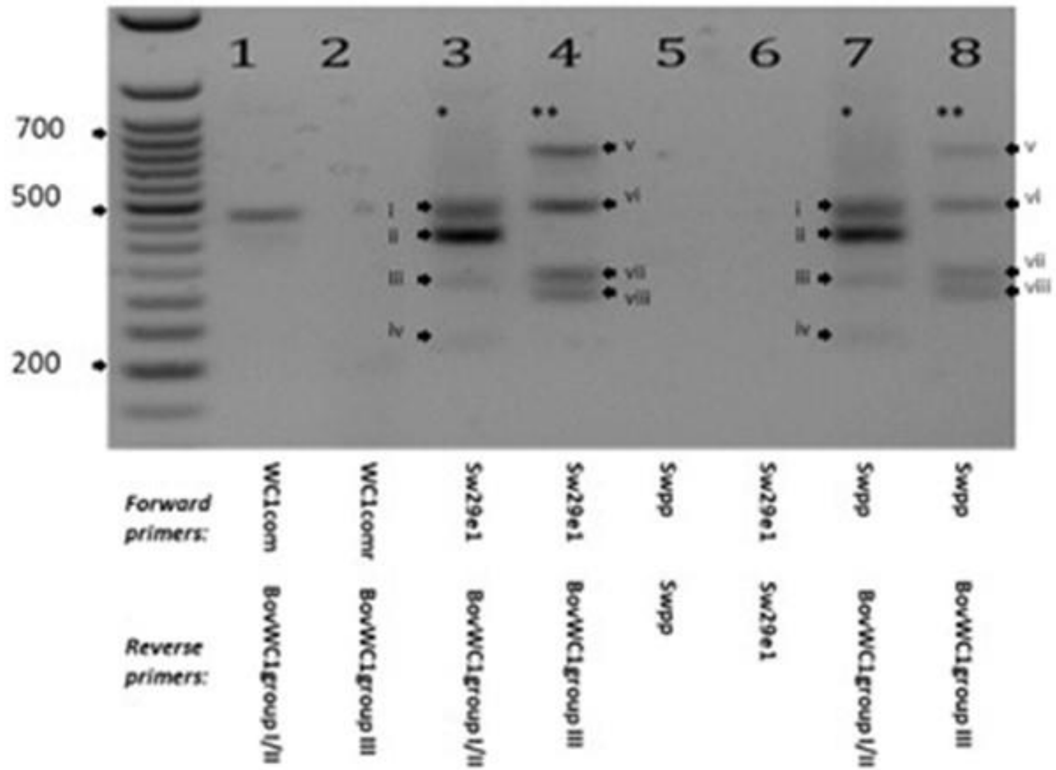

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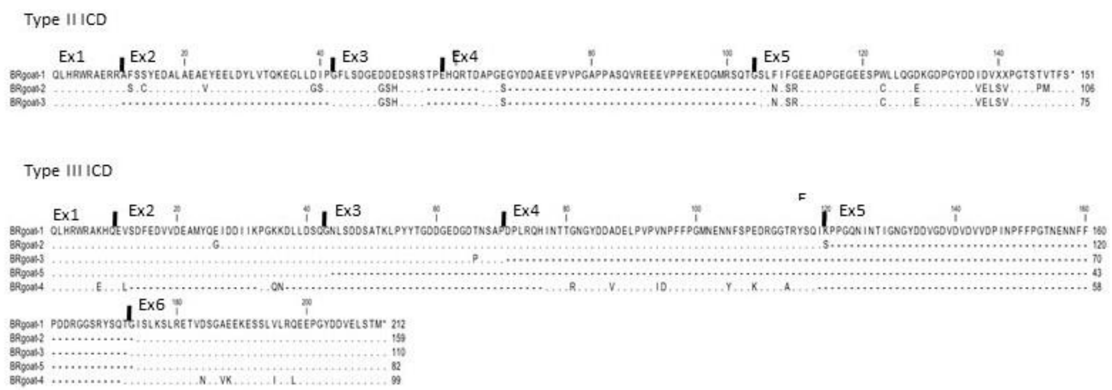

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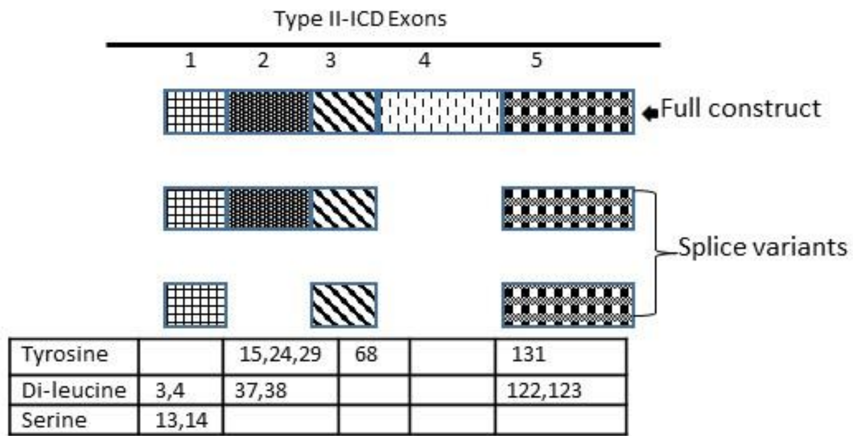
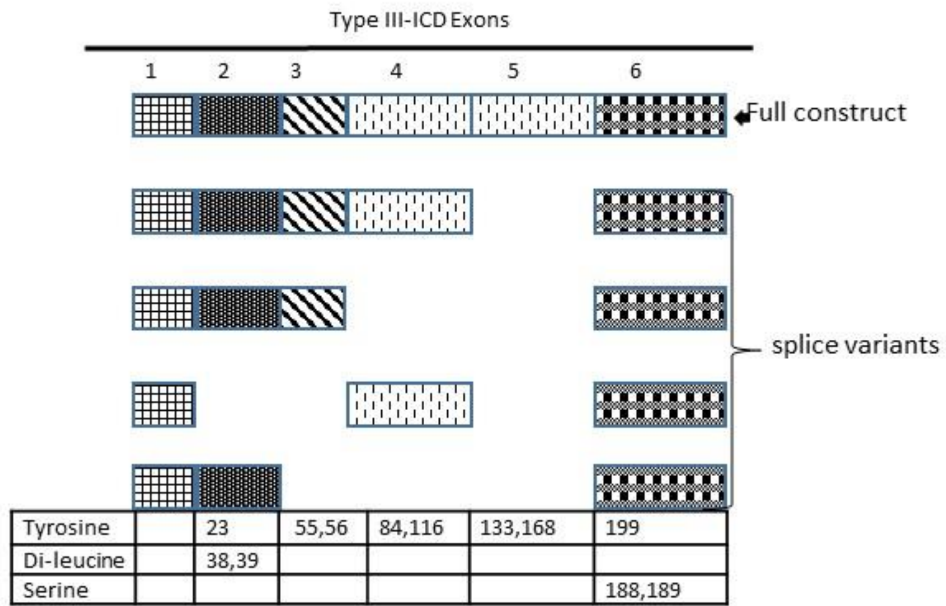


Tyrosine		15,24,29	68		131
Di-leucine	3,4	37,38			122,123
Serine	13,14				



Tyrosine		23	55,56	84,116	133,168	199
Di-leucine		38,39				
Serine						188,189

Figure 2.6 WC1 ICD splice variants.

(A) Agarose gel of amplicons of the cDNA for ICD. Primer pairs used to amplify ICDs and their splice variants are shown and products indicated as those that are Type I or II ICDs (*; lanes 3 & 7) or Type III (**, lanes 4 & 8). (B) ICD deduced amino acid sequences of the Boer goat from cDNA sequences were aligned and identities are indicated by dots (.), gaps resulting from the alignment are indicated by tildes (~), gaps resulting from lack of genomic sequence are indicated by dashes (-). Endodomain exons are indicated as ICD Ex 1 to Ex 6. (C) Eight ICD cDNA structures coded for by variable numbers of exons were illustrated with tyrosines, serine and diluceines which had been evaluated for function in bovine WC1 ICDs.

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3 CHAPTER III GOAT γδ T CELL SUBPOPULATIONS DEFINED BY WC1 GENE EXPRESSION, RESPONSES TO PATHOGENS AND CYTOKINE EXPRESSION

ABSTRACT

The major functions of γδ T cells in mammals overlap with those of αβ T cells but differ in that γδ T cells are rapid responders and see different types of antigens. While γδ T cells have been shown to be a major population of circulating lymphocytes in artiodactyl species such as cattle, sheep and pigs less is known about these cells in goats, an important agricultural species. We have recently shown that WC1, a γδ T cell specific family of hybrid pattern recognition receptors / co-receptors, is a multigenic family in goats expanded beyond what occurs in cattle. This study was conducted to address some of the limitations of previous studies in determining the proportions of γδ T cell, $WC1^+$ γδ T cells as well as the $WC1.1^+$ and $WC1.2^+$ subpopulations in blood and to evaluate their responses to various pathogens. Previously, the proportion of caprine γδ T cells was determined using monoclonal antibody (mAb) 86D but we show here does not react with all γδ T cells thereby underestimating their contribution to the lymphocyte population. Using mAb GB21a reactive with TRDC we found the proportion of γδ T cells in blood was not significantly less than CD4 or CD8 T cells. γδ T cells that expressed WC1 ranged from ~20-90% of total with less than half of the $WC1^+$ being classified as $WC1.1^+$ or $WC1.2^+$ by mAb staining. We found that naïve γδ T cells proliferated in cultures with *Leptospira* or *Mycobacterium avium paratuberculosis* (MAP) above that in control

medium cultures or those with *M. bovis* BCG and that the responding cells included both $WC1^+$ and $WC1^-$ γδ T cells. In ex vivo PMA-stimulated cultures $WC1^+$ γδ T cells produced IL-17 while $WC1^-$ γδ T cells produced both IL-17 and IFNγ. In longer-term cultures with *Leptospira* or MAP both $WC1^-$ and $WC1^+$

3.1 Introduction

Goats are distributed worldwide but are especially important in developing countries as food animals due to their adaptability to varying environmental conditions and their competitive benefits including their browsing and grazing habits (Aziz, 2010; Gilbert et al., 2018). Despite the fact that livestock are important for human nutrition, productivity is affected by parasitic, bacterial and viral pathogens (Neumann et al., 2003; Singh, 2008). T lymphocytes have vital roles in immune responses for controlling infectious diseases and among them γδ T cells are the majority in the peripheral blood mononuclear cell (PBMC) populations of young cattle, sheep and pigs (Davis et al., 1996b; Mackay and Hein, 1989; Mackay et al., 1986; Takamatsu et al., 2006). While the proportion of γδ T cells has been reported by others to be less profound in goats they are still a major population in blood (Yirsaw, 2020 (in press)-b).

The major functions of γδ T cells in mammals are varied and overlap with those of αβ T cells but differ in that the γδ T cells are rapid responders (Buza et al., 2009) and react with different types of antigens (Vermijlen et al., 2018). With regard to infectious diseases, γδ T cells have been shown in many mammalian species to produce IFNγ, IL-17 and chemokines that contribute to protective pro-inflammatory immune responses. That is, IL-17 plays a prominent role to safeguard the host from pathogens by recruiting neutrophils (Veldhoen, 2017; Weaver et al., 2007). IL-17 and IFNγ have particular roles in clearance of intracellular pathogens (Banerjee et al., 2016) as well as in cellular immunity leading to hemo-parasite elimination (Hagiwara et al., 2005). In cattle, another ruminant species, γδ T cells have been shown to respond to pathogens or their antigens including *Anaplasma marginale* (Lahmers et al., 2006; Lahmers et al., 2005), *Theileria*

parva (Daubenberger et al., 1999), *Mycobacterium avium* subsp. *paratuberculosis* (Krueger et al., 2016; Plattner et al., 2009)*, M. bovis* (Guzman et al., 2012; McGill et al., 2014b; Price and Hope, 2009; Vesosky et al., 2004) and bovine viral diarrhea virus (Endsley et al., 2002). Moreover, cattle γδ T cells have been shown to be early responders to *M. bovis* (Buza et al., 2009) and to be the first cells to produce IFNγ in response to *Leptospira borgpettersenei* serovar Hardjo-bovis vaccination (Blumerman et al., 2007; Naiman et al., 2001; Rogers et al., 2005b). They also respond to *M. bovis*-infected dendritic cells and *Brucella abortus* infected macrophages (Skyberg et al., 2011). Although studies are less extensive for goat γδ T cells they have been shown to respond to pathogens (Yirsaw, 2020 (in press)-b) including to *B. melitensis* (Higgins et al., 2018), to produce a robust local immune responses against *Fasciola hepatica* (Zafra et al., 2010) and help as an initial line of defense against *M. av.* subsp. *paratuberculosis* (Storset et al., 2001). Caprine γδ T cells also may play a role either in protection against caprine arthritis encephalitis virus (CAEV) (Jolly et al., 1997) or contribute to the pathogenesis of it (Kaba et al., 2011) as well as to that of schistosomiasis (Lindberg et al., 1999). As in cattle (Blumerman et al., 2007; McGill et al., 2014b), goat γδ T cells also produce IL-17 and interferon (IFN)γ (Elnaggar et al., 2018; Lybeck et al., 2009) signifying their potential for protection against infectious diseases.

γδ T cells can be broken into subpopulations in cattle based on expression or lack thereof of workshop cluster 1 (WC1) family members. WC1 molecules are transmembrane glycoproteins uniquely expressed on γδ T cells and belong to the scavenger receptor cysteine-rich family (SRCR) (Clevers et al., 1990; Herzig et al., 2010b; PrabhuDas et al., 2017; Wijngaard et al., 1994; Wijngaard et al., 1992). WC1

subpopulations of γδ T cells have been defined in cattle based on monoclonal antibody (mAb) reactivity with particular WC1 molecules (Chen et al., 2009a) and expression of WC1 genes (Chen et al., 2012; Chen et al., 2014; Damani-Yokota et al., 2018a; Higgins et al., 2018). Since many SRCR glycoproteins including WC1 molecules bind pathogens (Bikker et al., 2004; Brannstrom et al., 2002; Elomaa et al., 1995; Hsu et al., 2015b; Sarrias et al., 2007; Sarrias et al., 2005) the expression of particular forms of the WC1 molecules may convey the ability to respond to a particular pathogen (Rogers et al., 2005b; Telfer and Baldwin, 2015). WC1 is also involved in control of proliferation, cytokine responses and homing responses by γδ T cells (Hsu et al., 2015a; Kirkham et al., 1997; Wang et al., 2009; Wang et al., 2011; Wijngaard et al., 1994; Wijngaard et al., 1992). For example, only WC1.1 γδ T cells of cattle respond by proliferation and IFNγ production in recall responses to *Leptospira*, and WC1 molecules found on the responsive γδ T cells also bind *Leptospira* (Blumerman et al., 2007; Hsu et al., 2015b).

Our previous studies have shown that goats have a larger family of WC1 genes than cattle (Yirsaw, 2020 (in review)-a) and it has been shown by others that $WC1^{+}$ cells are found in the blood of goats (Higgins et al., 2018; Jolly et al., 1997; Zafra et al., 2013b). Although as indicated above some responses by caprine γδ T cells to particular pathogens have been evaluated, γδ T cell subpopulations including those expressing various WC1 genes are not well defined.

3.2 Materials and Methods

3.2.1 Blood and PBMC

Briefly, blood was collected from goats 6 month to 6 years of age (2 male and 26 female Boer goats) via jugular venipuncture into heparin from animals housed at the University of Massachusetts Hadley Farm in compliance with federal guidelines and with the Institutional Animal Care and Use Committee (IACUC) approval. The packed cell volume (PCV) was estimated for all goats by standard practice. Blood was collected at two time points and each sample was evaluated in duplicate for PCV; the results are expressed as the mean of the four readings. PBMCs were isolated from blood via density gradient centrifugation over ficoll-hypaque (LKB-Pharmacia Biotechnology) according to the manufacturer's protocol. Viable cell concentrations were determined by trypan blue exclusion. PBMCs were suspended in medium consisting of RPMI 1640 with 10% of either heat-inactivated adult caprine serum or fetal bovine serum (Hyclone), and 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 10 μg/ml gentamycin referred to as cRPMI. Cells were either stained by indirect immunofluoresecence immediately (ex vivo) or subjected to culture as indicated below.

3.2.2 Immunofluorescence and flow cytometric analyses

MAb used in indirect immunofluorescence and secondary antibodies with fluorochromes are shown in Table 3-1. They were used to identify cell surface differentiation antigens or intracellular cytokines. For detecting cytokine production, ex vivo cells or antigen-stimulated cells were cultured for 4-6 hr with phorbol myristic

acetate (PMA) (50 ng/ml), ionomycin (1µg/ml) and GolgiPlug protein transport inhibitor (2 µl/ml) (BD Biosciences) at 37°C prior to surface and intracellular staining using standard protocols. Cells were fixed with 4% paraformaldehyde prior to analysis by flow cytometry. In assays requiring analysis of cell division, PBMC were loaded with eFluor670 cell proliferation dye (eBioscience). For sorting, PBMC were cultured in cRPMI with 5 ug/ml ConA for 2 days at 37°C prior to surface staining with mAbs. Cells were sorted using FACS ARIA (BD Biosciences) into $WC1.1^{+}$, $WC1.2^{+}$ or $WC1^{+}/WC1.1/.2^{-}$ with 98% purity for all populations. Analysis was by FlowJo (www.flowjo.com). Live lymphocytes were identified by forward (FSc) and side scattered (SSc) in all analyses.

3.2.3 Proliferation assays

For proliferation assays, PBMC were cultured in cRPMI at 37°C with goat serum either with medium alone, ConA (5 µg/ml), *Leptospira borgpetersenii* serovar Hardjobovis (0.6 µg/ml), BCG antigens (0.6 µg/ml) or *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (0.6 µg/ml). Cultures that were performed in 96-well plates with 1.25×10^5 cells/well in a total volume of 200 µl. They were established in triplicate for each condition and incubated for 6 days including an overnight incubation with ^{3}H thymidine (New England Nuclear) at 1µCi/well added on day 5 of culture. Cells were harvested with a cell harvester and incorporation of ^{3}H thymidine determined by liquid scintillaton and results expressed as counts per minutes (CPM). For cells loaded with the cell division dye eFluor670, cultures were established in 24-well plates with 1.25×10^6 cells/well in a total volume of 2 ml but antigen concentrations and culture conditions

were as per 96-well cultures. Cells from these cultures were stained by indirect immunofluoresecence for cell surface differentiation antigens and cytokines on day 6 as described above.

3.2.4 RNA Isolation and 5’ Race cDNA synthesis

Flow cytometric sorted cells were suspended in Trizol (Invitrogen, Carlsbad, CA) and RNA extracted according to the manufacturer's instructions. RNA purity and concentration were determined by Nanodrop spectrophotometry (Thermo-Fisher). Reverse transcriptase was performed using Superscript IV (Invitrogen), a template switch oligo with unique molecular identifiers incorporated and a poly T reverse primer as outlined in Mamedov *et al*. (Mamedov et al., 2013). Primers dNTPs and RNA were first incubated at 65°C for 5 min followed by addition of enzyme, buffer, DTT, and RNAse-OUT (Invitrogen) before incubating at 50° C for 1 hr and then 80° C for 10 min. Following this, one unit of uracil deglycosylase (Invitrogen) was added and incubated at 37° C for 45 min.

3.2.5 PCR, gel electrophoresis, and amplicon purification

cDNA was used as a template for PCR with Taq polymerase (New England Biolabs). Forward (fw) primers with or without tags indicated were as follows: “universal fw: aagcagtggtatcaacgcagagt; “universal fw pb01” (for WC1.1^{+} cells) tcagacgatgcgtcatcaagcagtggtatcaacgcagagt; “universal fw pb 02” (for WC1.2^{+} cells) ctatacatgactctgccaagcagtggtatcaacgcagagt; “universal fw pb 03” (for WC1.1^{+}/WC1.2^{+} cells) tactagagtagcactccaagcagtggtatcaacgcagagt. Reverse primer used was “WC1 b

reverse” 5'-3' tctgggctgaagagttcaggtgtg. PCR was performed with the following conditions: 95ºC for 2 min, and then 30 cycles of 95ºC for 30 sec, 55-63ºC for 45 sec, and 72ºC for 30 sec. PCR products were electrophoresed using Sybr Safe (Thermo Fisher Scientific) in a 1% low melting agarose gel with a wide comb to accommodate all the product in one lane using a voltage of 110 for 30 min; bands of the expected correct size were excised and the amplicons purified with a gel extractions kit (New England Biolabs). Gel extract was then purified further using Ampure beads (New England Biolabs) before PacBio sequencing at the University of Massachusetts Medical School Deep Sequencing Core, Worcester, MA. Initially, all PCR amplicons were cloned and evaluated by Sanger sequencing to confirm primer specificity and product identity.

3.2.6 Statistical analysis

For comparison of multiple groups, an ANOVA was conducted followed by Student’s t-test when significant differences were found. For evaluation of correlations between two parameters Pearson Correlation Coefficient was used. Significant differences at 95% confidence were for $p \leq 0.05$.

3.3 Results

3.3.1 Caprine γδ T cell subpopulations in blood

While the proportion of caprine γδ T cells have been determined in PBMC using mAb 86D no in-depth studies of caprine $WC1^+$ γδ T cell subpopulations have been conducted. Thus our objective was to define γδ T cell subpopulations including their representation in goats of various ages. This information will be important for understanding the functions and roles of caprine γδ T cells in responses to infectious pathogens and potentially targeting them for activation through vaccination. Our approach was to use flow cytometry and a panel of mAbs that have been used to define T cell subpopulations in other ruminants and are known to react with goat differentiation antigens (Table 3-1; Figure 3.1).

Evaluating *ex vivo* PBMC with mAb GB21A, that reacts with the TCR δ constant (TRDC) chain (Machugh et al., 1997) and thus stains all γδ T cells, indicated that the proportion of γδ T cells was not significantly less than that of CD4 and CD8 T cells (Figure 3.2A). The proportion of $WC1^+$ γδ T cells in PBMC determined using a pan-WC1 mAb CC15 (Figure 3.2B) showed they were less than the total γδ T cells and that the proportion varied substantially among animals (Figure 3.2C and 3.2D). Within the $WC1^+$ population, mAbs previously used to define subpopulations in cattle known as WC1.1 and WC1.2 (Wijngaard et al., 1994) showed that three goat subpopulations could also be defined with these mAb (Figure 3. 3B). $WC1.1^+$ and $WC1.2^+$ cells had similar relative proportions to one another and as found in cattle PBMC but generally accounted for fewer of the total $WC1^+$ cells than occurs in cattle. There was a significant correlation

between the proportion of total γδ T cells and $WC1^+$ T cells (Figure 3.2C) but no correlation with age for either population in animals 6 months of age or older (Figure 3.2 E and Figure 3-2F). There was also no correlation with PCV (Supplemental figure 3.1).

Previous studies have used mAb 86D to define caprine γδ T cells (for review see (Yirsaw and Baldwin, 2020 (in pres)-b)); this mAb that reacts with one of the TCR γ chains (Davis et al., 1996a). To evaluate mAb 86D's ligand expression on $WC1^+$ and $WC1^-$ γδ T cells relative to TRDC expression we conducted multicolor immunofluorescence. The results showed that mAb 86D sees only a portion of the γδ T cells whether they are $WC1^-$ or $WC1^+$ PBMC (Figure 3.3A). To further define the caprine γδ T cells, the expression of CD2 and CD8 on $WC1^+$ and $WC1^-$ γδ T cells from PBMC was evaluated since in pigs and cattle, these cell surface differentiation antigens are used to define subpopulations. Caprine γδ T cells had similar CD2 and CD8 expression as these other artiodactyls, e.g. cattle shown here for comparison, with most or all $WC1^+$ γδ T cells being negative for both (Figure 3.3 B).

3.3.2 Defining WC1 genes in WC1 subpopulations

The mAbs used to define the WC1.1 and WC1.2 subpopulations in cattle react with specific and unique WC1 gene products (Chen et al., 2009a). These $WC1.1^+$ and $WC1.2^+$ subpopulations have some overlap with regard to the WC1 genes expressed by them (Chen et al., 2014; Damani-Yokota et al., 2018b). Nevertheless, expression of particular WC1 gene products correlates with the ability of bovine γδ T cells to respond to pathogens (Baldwin and Telfer, 2015b; Rogers et al., 2005b). To determine the WC1 genes expressed by the caprine $WC1.1^+$ and $WC1.2^+$ subpopulations and $WC1^+$ cells that

did not react with the mAbs that define WC1.1 and WC1.2 cells, ex vivo PBMC were sorted by flow cytometry (Figure 3.4) and used as templates for RT-PCR and PacBio next generation sequencing (Table 3-2). This work is ongoing.

3.3.3 Cytokine production by WC1 subpopulations

It is known that caprine γδ T cells can produce the important pro-inflammatory cytokines IFNγ and IL-17 (Elnaggar et al., 2018; Lybeck et al., 2009). Here we evaluated the ability of the various γδ T cell subpopulations to produce these cytokines following short-term stimulation of *ex vivo* cells with PMA/ionomycin. Responses by CD4 and CD8 T cells were included for comparison (Figure 3.5). We found that caprine γδ T cells produced both IL-17 and IFNγ although the majority was attributable to $WC1^-$ γδ T cells than by the $WC1^-$ population of γδ T cells; CD4 and CD8 T cells produced more of both cytokines ex vivo (Figure 3.5). The production of cytokines by the $WC1.1^+$ subpopulation was extremely low and $WC1.2^+$ cells could not be identified because of the limitations of the isotypes of mAbs to pair with the anti-cytokine mAbs.

3.3.4 Responses to pathogens

The response by caprine γδ T cells to a variety of pathogens has been evaluated previously (reviewed in Yirsaw and Baldwin, submitted). For example, both primed and unprimed caprine γδ T cells have been shown to respond to MAP (Storset et al., 2000). Here we investigated the response to three bacterial pathogens using PBMC. Using ^{3}H-thymidine incorporation as an initial analysis of proliferation, we found that PBMC from all four animals tested responded to *Leptospira* while three of the four also responded to

BCG and MAP with the two adult goats responding more vigorously than the immature goats (Figure 3.6A). To determine which cell populations were responding in these cultures eFluor670-loaded cells were cultured with pathogens and cells stained with mAb after 6 days of culture (Figure 3.6B). PBMC cultures from the two adult goats (#611 and 621) showed that δTCR^{+} cells including WC1^{+} cells divided more to *Leptospira* and MAP than in medium cultures or BCG-containing cultures. There was no cell division to pathogen antigens by γδ T cells when PBMC were from the two kids (data not shown).

Evaluation of cytokine production by the cells in pathogen-stimulated cultures found IL-17 was being produced by γδ T cells to all three pathogens tested (Figure 7) but that was by the WC1^{-} γδ T cells only. A second animal had WC1^{-} γδ T cells that made IL-17 to *Leptospira* but not the other pathogens. No IFNγ production by γδ T cells occurred in these cultures (data not shown).

3.4 Discussion

In previous studies the proportion of caprine γδ T cells in PBMC was determined using mAb 86D (reviewed in Yirsaw and Baldwin, submitted) (Caro et al., 1998) but as we showed here this mAb does not react with all caprine γδ T cells, generally reacting with about half or fewer. Furthermore, $WC1^+$ γδ T cell subpopulations were not studied previously. The aims of this work were to determine the proportions of total γδ T cells and $WC1^+$ and $WC1^-$ γδ T cell subpopulations and their responses to various pathogens by proliferation and cytokine production (IL-17 and IFNγ). The results showed that additional major $WC1^+$ subpopulation was found in goats not previously found in cattle and that both caprine $WC1^+$ cells and $WC1^-$ γδ T cells respond to the pathogens MAP and *Leptospira*.

In sheep and cattle the proportion of γδ T cells decreases as the animals age from birth to adulthood (Ayoub and Yang, 1996; Washington et al., 1992). Our studies with goats agreed with previous studies (Jolly *et al*, 1997, Caro *et al*., 2001, Kaba *et al*., 2011[b] and Wilkerson *et al*., 1995) in that they and we showed that the proportion of γδ T cells did not change with age when animals were greater than 6 months. This occurred even though the proportion of γδ T cells detected in PBMC in our study was generally higher than in previous studies since we used GB21A mAb that reacts with the TCR δ chain constant region (Machugh et al., 1997). While there is only one TCR δ constant gene, mAb 86D sees one of the TCR γ chains (Davis et al., 1996a) of which there are multiple V and C gene [Gillespie, #68]. Our preliminary analyses suggest that mAb 86D is reacting with one of the TCR Vγ chains based on its distribution between both $WC1^+$ and $WC1^-$ γδ T cells as shown here. While our results do not show a decrease in γδ T cells

after 6 months of age, Caro *et al.* (Caro et al., 1998) showed that a decrease of the γδ T cells occurs between one week and 7 months of age; that is consistent with what occurs in other ruminants [Ayoub, 1996 #73]. Goats have been reported by others to not be a 'γδ T cell high' species (Baliu-Pique et al., 2019), a designation frequently given to other ruminants whose proportion of γδ T cells can comprise 60% of PBMC in very young animals (Mackay and Hein, 1989; Mackay et al., 1986; Pieper et al., 2011; Sinkora and Butler, 2009; Walker et al., 1994). We found that while the proportion of γδ T cells in goats in our study indeed did not rise to the proportions reported for other ruminants they nevertheless were not considerably less than that of CD4 and CD8 T cells in PBMC. And while the proportion of total γδ T cells did not decline after 6 months of age the proportion of $WC1^+$ γδ T cells in PBMC did show a trend towards decline after 6 months of age but it was not significant.

We showed here that goats have more $WC1^+/WC1.1^-/WC1.2^-$ cells than cattle. In cattle, essentially all $WC1^+$ γδ T cells can be defined as expressing either or both of the molecules recognized by α-WC1.1 and α-WC1.2 mAbs (Blumerman et al., 2007; Herzig and Baldwin, 2009; Rogers et al., 2005b). We have recently found that goats have a more extensive WC1 family than cattle (Yirsaw, 2020 (in review)-a), and thus the potential for additional WC1 subpopulations is logical. Our subpopulation sorting studies and evaluation of WC1 gene expression will answer this question. This work is ongoing. Since we know that WC1 molecules act as receptors to interact with pathogens (PRR) (Hsu et al., 2015b) goats are likely to have a greater variety of pathogen-interaction possibilities than cattle. Having expression of these PRRs segregated on T cell subpopulations also means that cells can specialize. Subpopulations of γδ T cells in cattle

based on their WC1 gene expression can differ in their cytokine responses (Guzman et al., 2012; Rogers et al., 2005b) and we expect to find the same for caprine cells once we have more clearly defined the subpopulations by WC1 gene expression. Such differences in functions by responsive subpopulations may affect the outcome of an infection.

To begin to address the question of subpopulation diversity we evaluated ex vivo cells stimulated with PMA for 6 hours as well as cells cultured for 6 days with pathogens. The γδ T cells that showed responses by IL-17 and IFNγ production in *ex vivo* cultures were largely or entirely $WC1^-$ γδ T cells. In the bacterial-stimulated cultures of several days, the $WC1^-$ γδ T cells responded to *M. bovis* BCG, *Leptospira* and MAP antigens by producing IL-17 although no IFNγ production was detected. The results of the proliferation assays suggested that innate responses to MAP could be cross-reactive with other *M. avium* species or are truly innate responses without any priming of the γδ T cells since our goats are MAP negative. The proliferative response to MAP by $WC1^-$ γδ T cells is consistent with responses found in cattle although some $WC1^+$ γδ T cells are also found in granulomas in gut tissue of infected cattle (Plattner et al., 2009) consistent with the response by the caprine $WC1^+$ cells here. The proliferative response by $WC1^+$ goat cells to *Leptospira* corresponds with observations in cattle using previously vaccinated animals (Blumerman et al., 2007). The γδ T cells of those animals also made IFNγ that we did not find here with the naïve caprine γδ T cells.

It was interesting that there was no cell division by γδ T cells to pathogen antigens when PBMC were from the immature goats. Generally, it is postulated that the higher number of γδ T cells in young ruminants and pigs signifies the enhanced or primary role of these cells in young animals. The paradigm put forth is that the γδ T cells bridge innate

and adaptive immune responses by being early rapid responders prior to sensitization of αβ T cells and induction of long-term memory response by those cells (Krueger et al., 2016). However we also know that some populations of γδ T cells both in mice and other ruminants have memory responses (Blumerman et al., 2007). In mice, this is a function of specific subpopulations of γδ T cells (Munoz-Ruiz et al., 2017; Ribot et al., 2010). It is possible that the responses we are measuring in our in vitro cultures reflect memory responses by the γδ T cells in the adult goats, perhaps due to cross-reactive antigens, and that the immature goats have not yet had exposure.

More extensive studies of caprine γδ T cell responses will be needed to evaluate the role of the various γδ T cell subpopulations to pathogens. Having the more thoroughly defined populations by WC1 gene expression will be a crucial part of this. Overall having this information will be important for understanding how to engage γδ T cells in vaccine designs against important livestock pathogens.

Table 3-1 Antibodies used to define goat lymphocytes and cytokines

mAb clone	**Isotype**	**Specificity**	**Source of mAb**	**Secondary Ab[c] (α-isotype-fluorochrome)**
MUC2A	IgG2a	CD2	WSUMAC[a]	IgG2a-PE-Cy7
17D	IgG1	CD4	WSUMAC	IgG1-PE
GC1A	IgG2a	CD4	WSUMAC	IgG2a-PE
ILA51	IgG1	CD8	ILRAD[b]	IgG1-PE
7C2B	IgG2a	CD8	WSUMAC	IgG2a-AlexFluor647
86D	IgG1	γ TCR	WSUMAC	IgG1-PE
GB21A	IgG2b	δ TCR	WSUMAC	IgG2b-AlexFluor488, IgG2b-PE
CC15	IgG2a	WC1+	BioRad	IgG2a-FITC, IgG2a-PE
BAG25A	IgM	WC1.1	WSUMAC	IgM-PE
CACTB32A	IgG1	WC1.2	WSUMAC	IgG1-PE
RIF60A	IgG1	IFNγ	WSUMAC	IgG1-PE-Cy7, IgG1-AlexFluor647
IL-17A2A	IgG1	IL-17	WSUMAC	IgG1-PE-Cy7, IgG1-AlexFluor 647

WSUMAC, Washington State University Monoclonal Antibody Center International Laboratory for Research on Animal Diseases, Nairobi, Kenya. All purchased from Invitrogen.

Table 3-2 WC1 gene usage by $WC1^+$ subpopulations. (it is ongoing)

WC1 gene (WC1-x)	Purified cell population analyzed		
	$WC1.1^+$	$WC1.2^+$	$WC1^+/WC1.1^-/WC1.2^-$

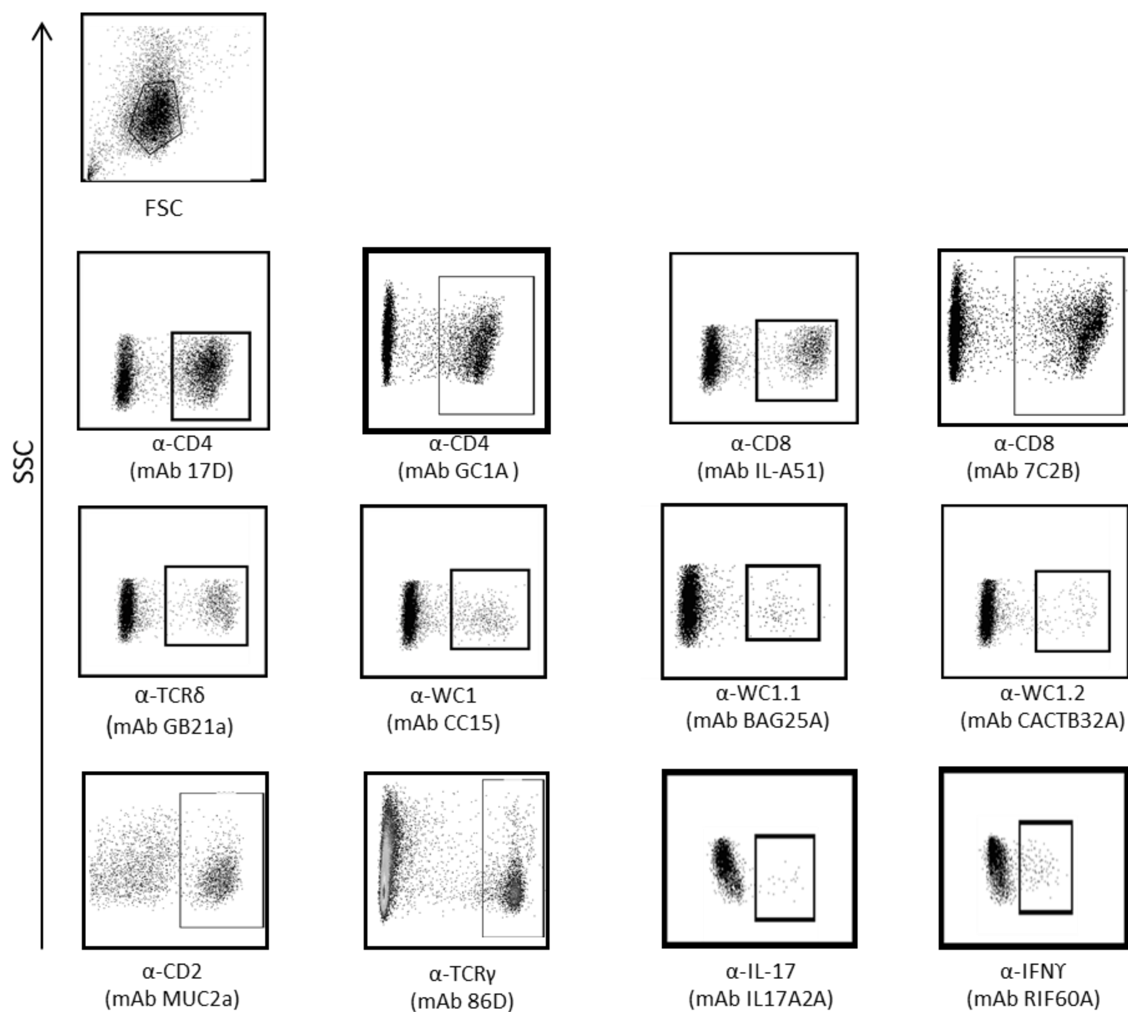
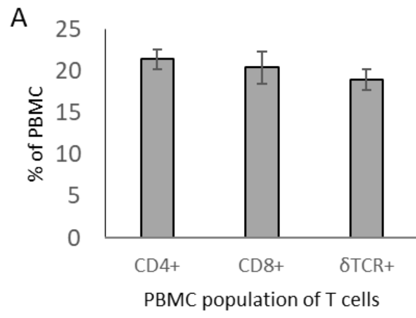
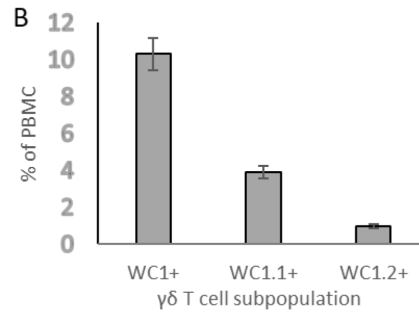
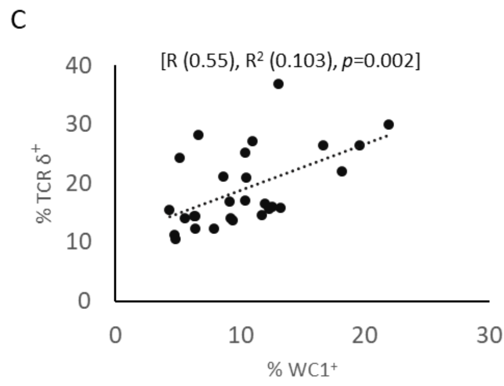


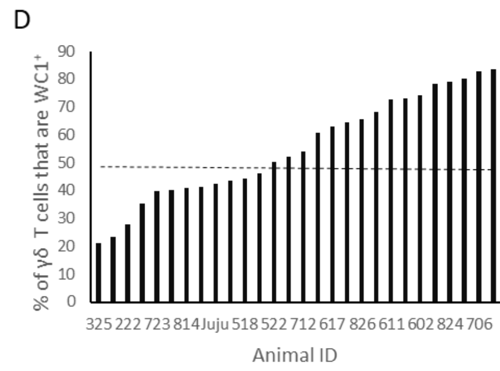
Figure 3.1 Flow cytometry strategy.

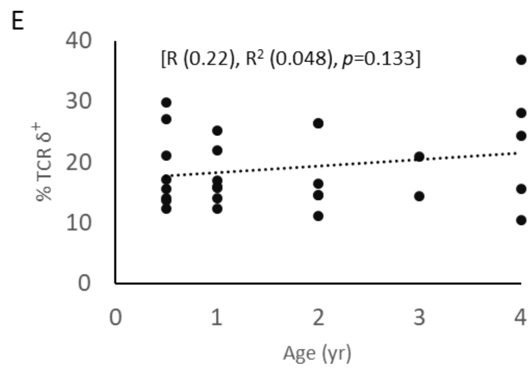
PBMC were stained with mAbs shown as indicated in Table 3-1. Controls with secondary antibodies only are shown for background staining. Cells were analyzed by flow cytometry with cells gated on side scatter (SSc) and forward scatter (FSc) as shown.


A
25
20
15
10
5
0
% of PBMC
CD4+
CD8+
δTCR+
PBMC population of T cells


B
12
10
8
6
4
2
0
% of PBMC
WC1+
WC1.1+
WC1.2+
γδ T cell subpopulation


C
[R (0.55), R² (0.103), p=0.002]
40
30
20
10
0
% TCR δ+
0
10
20
30
% WC1+


D
90
80
70
60
50
40
30
20
10
0
% of γδ T cells that are WC1+
325 222 723 814 Juju 518 522 712 617 826 611 602 824 706
Animal ID


E
[R (0.22), R² (0.048), p=0.133]
40
30
20
10
0
% TCR δ+
0
1
2
3
4
Age (yr)

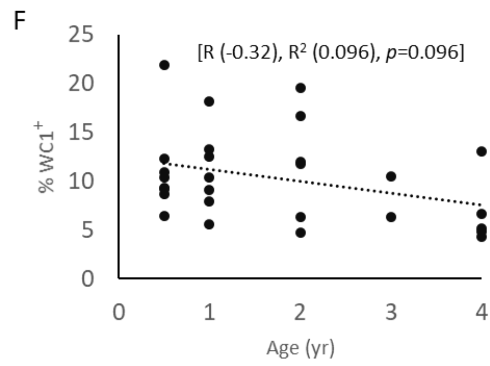
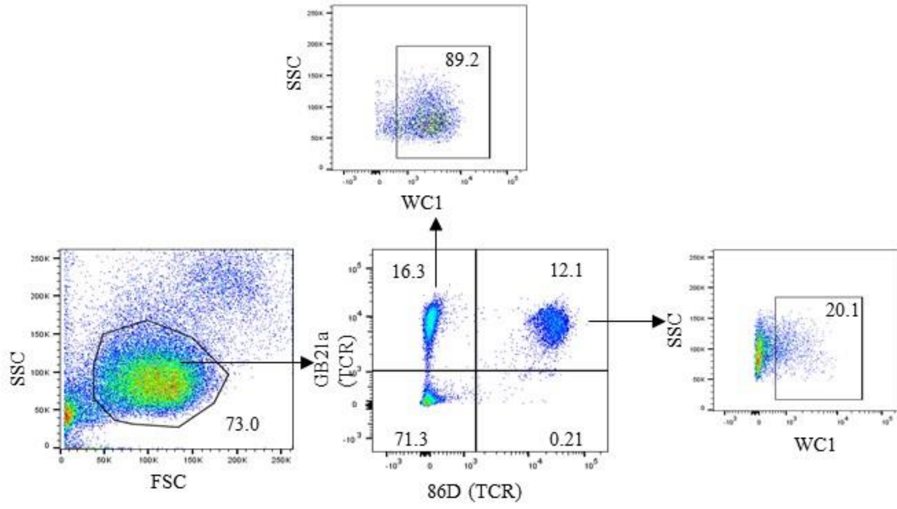

F
[R (-0.32), R² (0.096), p=0.096]
25
20
15
10
5
0
% WC1+
0
1
2
3
4
Age (yr)

Figure 3.2 γδ T cells subpopulations.

PBMC isolated from goats were stained by indirect immunofluorescence using mAb to define (a) CD4, CD8, δTCR^+ T cells; and (b) pan$WC1^+$ (mAb CC15), $WC1.1^+$ and $WC1.2^+$ T cells; the results shown are the mean (±SEM) from 28 animals repeated one time. Statistical significance was done by Analysis of Variance (ANOVA). (c) Correlation was calculated using Pearson Correlation Coefficient for the relationship of $WC1^+$ to δTCR^+ cells [R (0.55), R^2 (0.103), p=0.002] for results from one experiment. (d) The percentage of δTCR^+ T cells that are $WC1^+$ is shown for representative individual animals. (e) Correlation of animal age with percentage of δTCR^+ cells [R (0.22), R^2 (0.048), p=0.133] and (f) animal age with percentage of $WC1^+$ cells [R (-0.32), R^2 (0.096), p=0.096] are shown from one experiment.


A
SSC
WC1
89.2
FSC
73.0
GB21a (TCR)
86D (TCR)
16.3
12.1
71.3
0.21
20.1

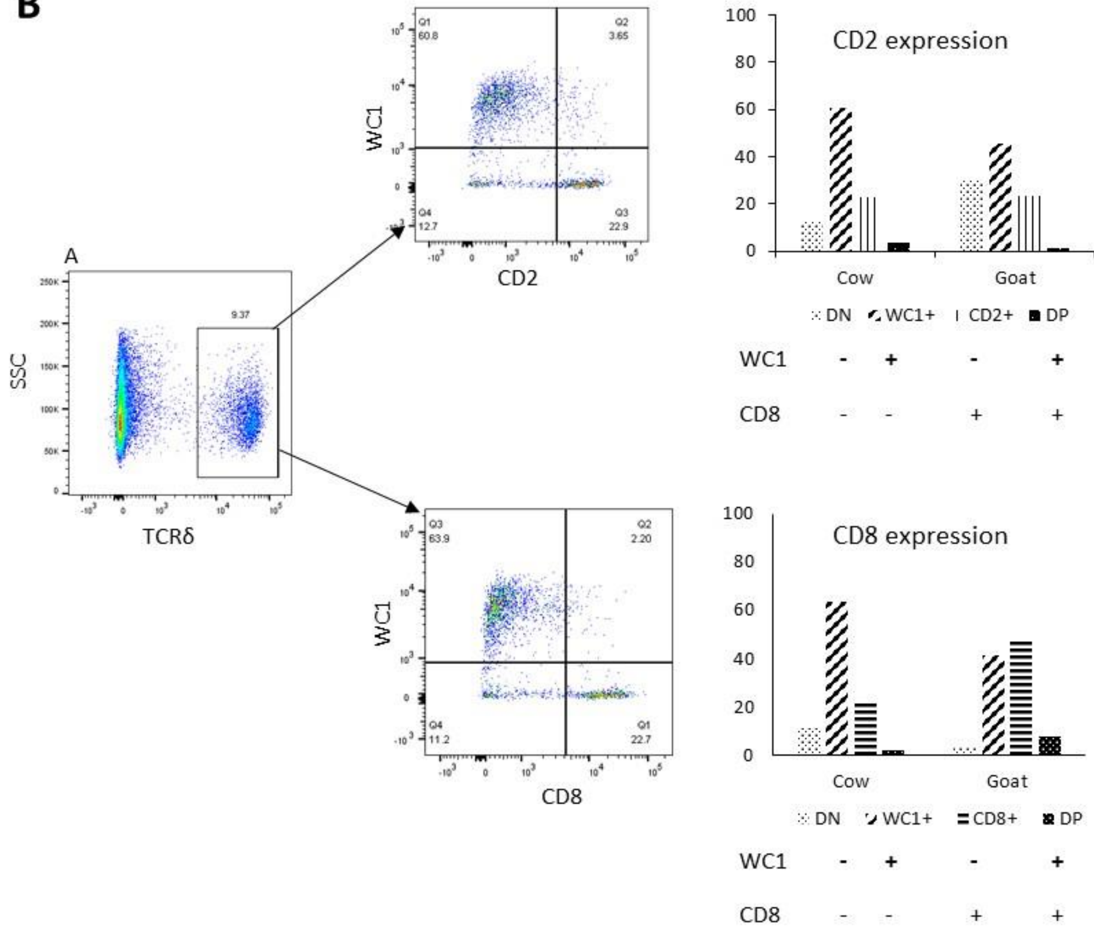

B
SSC
TCRδ
9.37
WC1
CD2
CD8
CD2 expression
CD8 expression
Cow
Goat
DN
WC1+
CD2+
CD8+
DP
WC1 - + - +
CD8 - - + +

Figure 3.3 Expression of CD8 and CD2 by γδ T cells.

Example of immunofluorescence staining of goat PBMC with mAbs indicated to evaluate expression of (a) the ligand of 86D by $WC1^{+}$ and $WC1^{-}$ γδ T cells and (b) for CD2 and CD8; bovine cells were included for comparison for CD2 and CD8 expression and shown in bar graph.

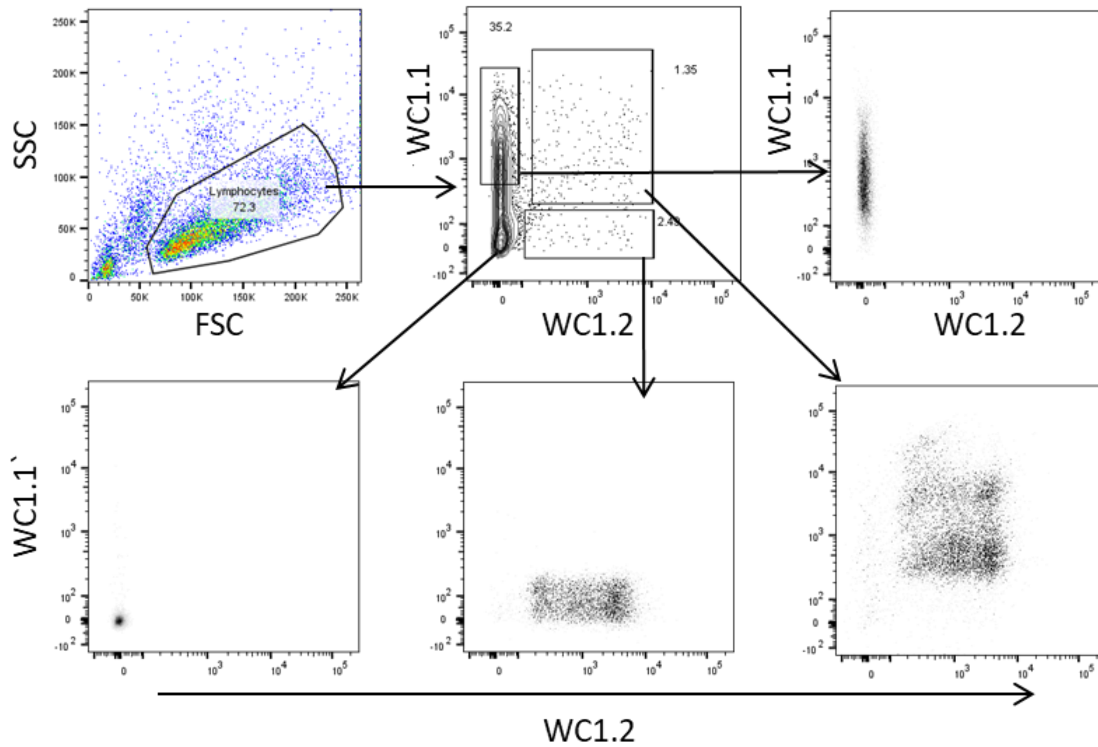


Figure 3.4 Defining WC1 genes in WC1^{+} subpopulations.

Following surface staining of caprine PBMC, cells were purified by flow cytometric sorting. The sorting strategy and result are shown for isolation of WC1^{+}/WC1.1^{-}/WC1.2^{-}, WC1.1^{+} and WC1.2^{+} subpopulations.

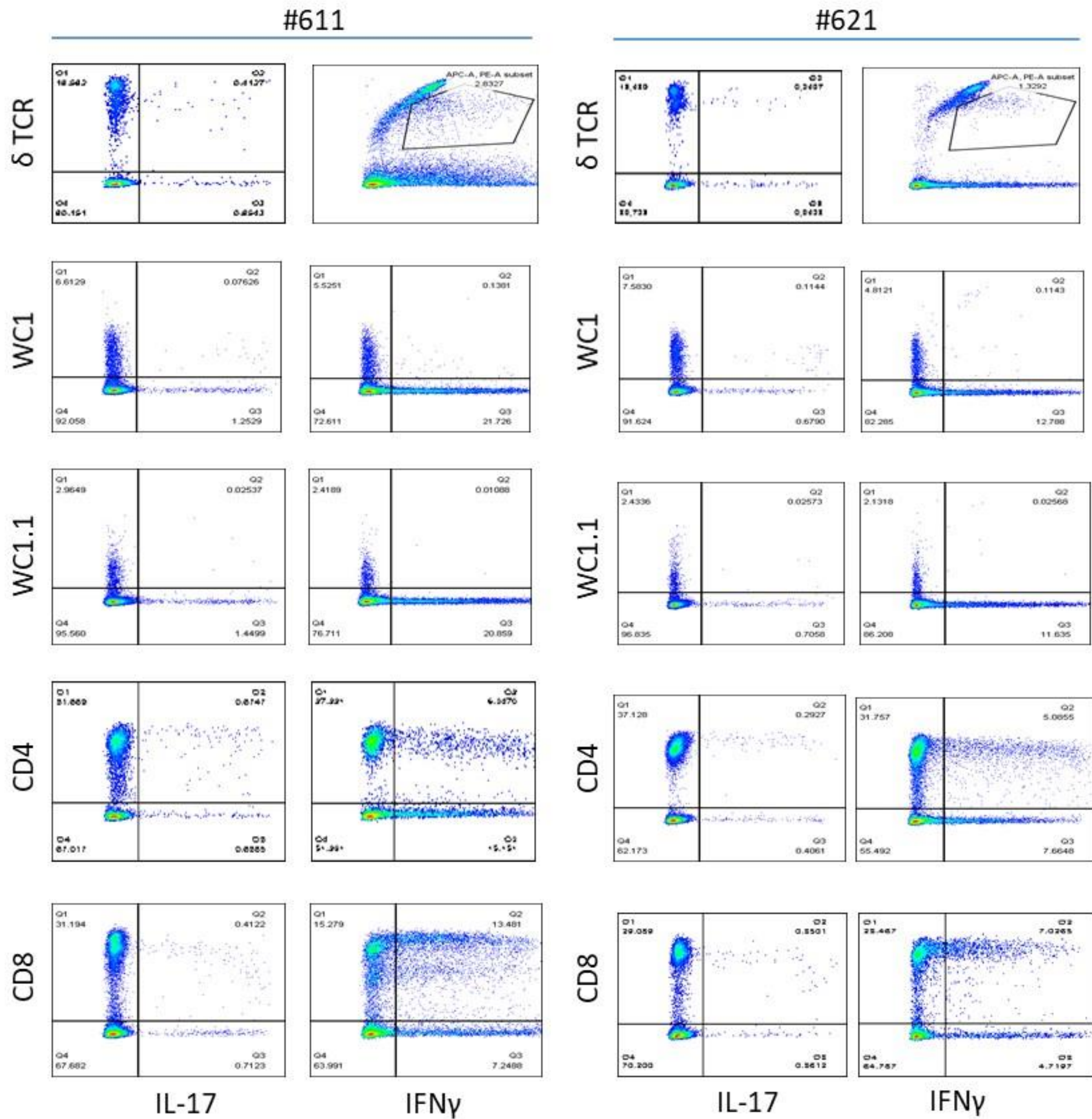
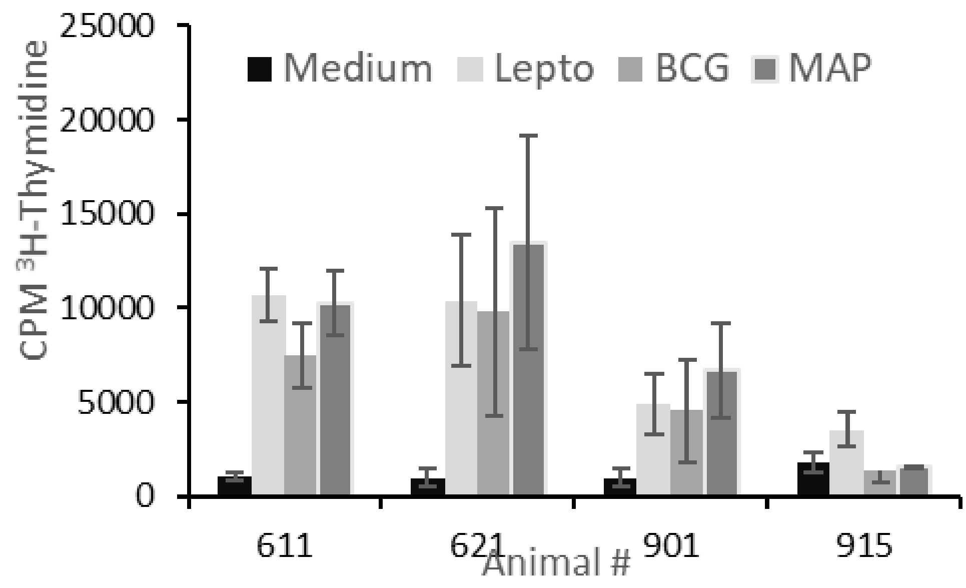


Figure 3.5 Cytokine production by γδ T cell subpopulations.

PBMC were isolated from two 3-year old goats and stained by indirect immunofluorescence for expression of surface molecules and intracellular cytokine expression after 6 hr culture with PMA/ionomycin using mAb in Table 3-1. Example of two experiments performed is presented.


A
CPM ³H-Thymidine
25000
20000
15000
10000
5000
0
Medium
Lepto
BCG
MAP
611
621
901
915
Animal #

B

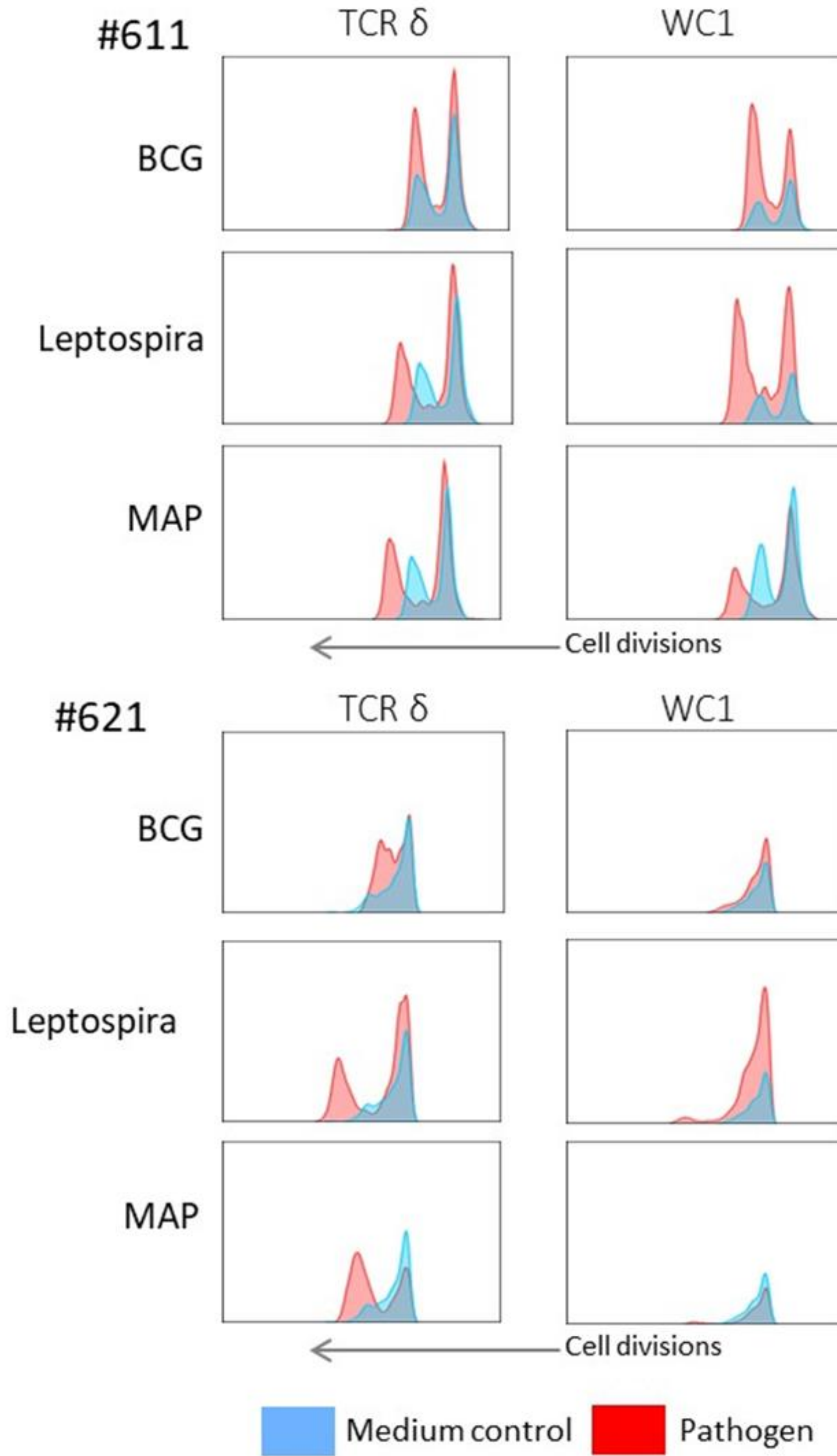


Figure 3.6 Pathogen response.

(a) PBMC from two three-year old and two 4-month old female Boer goats were cultured for 6 days either with medium only or with Leptospira, BCG or MAP antigens. Each condition was set-up in triplicate wells and the experiment repeated. The results are expressed as the mean ($\pm$SEM) of 6 readings. An example of two experiments is shown. (b) PBMC were stained with eFluor670 cell division dye and cultured as in "a" and then surface stained with mAbs as indicated. The results with antigen is overlapped with medium for each pathogen's histogram. Examples of two experiments performed.

Evaluation of cytokine production by the cells in pathogen-stimulated cultures found IL-17 was being produced by γδ T cells to all three pathogens tested (Figure 8) but that was by the $WC1^-$ γδ T cells only. A second animal had $WC1^-$ γδ T cells that made IL-17 to *Leptospira* but not the other pathogens.

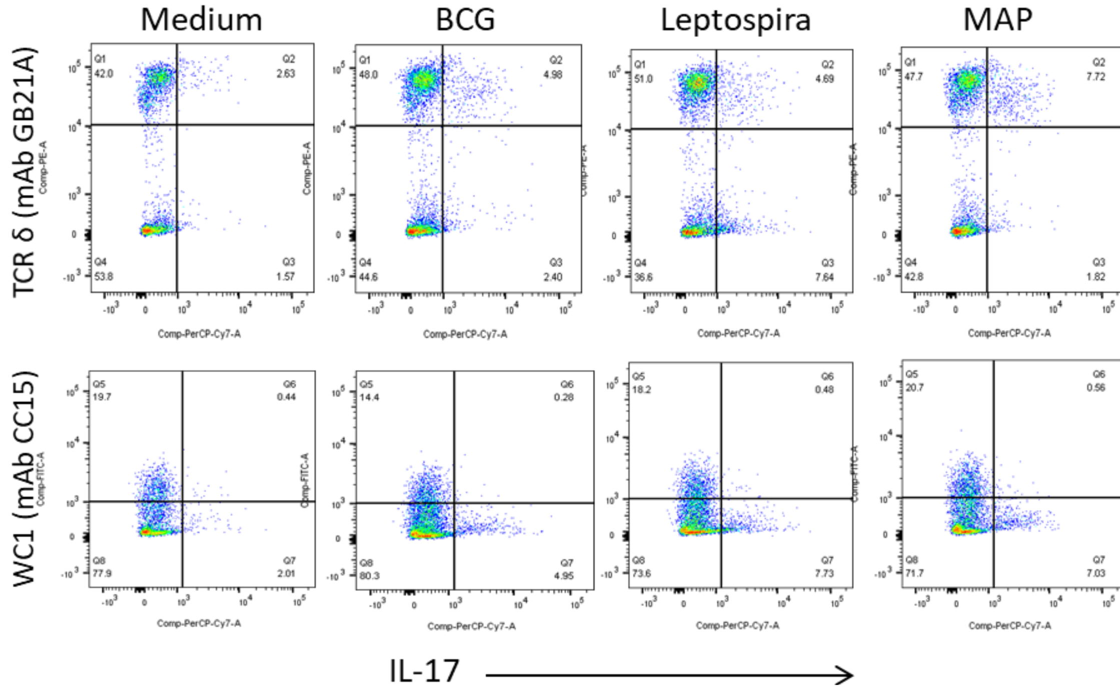


Figure 3.7 Cytokine production in response to pathogens.

PBMC isolated from two 3-year old goats and stained by indirect immunofluorescence for expression of surface molecules and intracellular cytokine expression after 6 days of culture with BCG, *Leptospira* and MAP. Example of 2 experiments performed shown.

Bibilography

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4 CHAPTER IV CHARACTERIZATION OF THE DOMESTIC GOAT γδ T CELL RECEPTOR GENE LOCI AND GENE USAGE

ABSTRCT

We hypothesize that the T cell receptor (TCR) gene loci and structures among ruminants will be similar and that $WC1^{+}$ γδ T cells in goats will have the same restriction of TCRγ chain gene expression as previous ly documented in cattle. While goats and cattle diverged 30 million of years ago through evolution, they have many similarities in gene sequences. We sought to characterize the TCR loci coding for both the γ and δ chains using an improved assembly (ARS1) of the highly homozygous San Clemente goat breed [12]. Annotation of this assembly has already proven useful for describing other immune system genes including antibody and leucocyte receptors. We annotated the TRG and TRD loci and compared usage of these genes with that in cattle. In general, both the TRG and TRD loci were similarly organized in goats and cattle and the gene sequences were highly conserved. However, the number of genes varied slightly as a result of duplications within loci and differences occurred in mutations resulting in pseudogenes. It is known in cattle that $WC1^{+}$ γδ T cells only use TCRγ genes from one of the 6 available cassettes. We hypothesize this is because the structure of that Cγ gene product is quite different from that of the other 5 Cγ genes and this difference is necessary to interact with WC1 for signal transduction following antigen ligation. Thus, we expected to find the same conservation for goat $WC1^{+}$ γδ TCRγ gene usage. In cattle we found that a single TCRδ transcript could incorporate sequence from more than one diversity gene (TRDD) which is an unusual feature of TCR δ chains among mammals. Evaluation of

caprine γδ T cells for this feature showed more than one diversity gene (TRDD) usage similar to cattle.

4.1 Introduction

Goats are an important component of agriculture in developing countries serving as a source of food and other commodities. Therefore, the generation of immunity to pathogens to maintain animal health is pertinent to both human nutrition and economic stability. Ruminants as well as pigs and poultry have a large proportion of γδ T cells in their peripheral blood mononuclear cell (PBMC) populations and thus these species are considered γδ T cell high (Hein and Mackay, 1991; Mackay and Hein, 1989; Sowder et al., 1988; Takamatsu et al., 2006; Yirsaw, Submitted for publication.-b). In young cattle and sheep up to 60% of PBMC can be γδ T cells, supporting the notion that they are important for immunity early in life (Davis et al., 1996b; Washington et al., 1992). It has been shown that caprine γδ T cells respond to various pathogens and in some cases are thought to contribute to protection (Vermijlen et al., 2018; Yirsaw, Submitted for publication.-a). The T cell receptor (TCR) is important for activating γδ T cell responses to pathogens in cattle (Baldwin et al., 2002; Blumerman et al., 2006; Lahmers et al., 2005; McGill et al., 2014b) and thus this is likely to be so in goats. Although γ and δ TCR genes are well characterized in cattle (Connelley et al., 2014; Conrad et al., 2007; Damani-Yokota et al., 2018a; Herzig et al., 2006; Herzig et al., 2010a) and sheep (Piccinni et al., 2015; Vaccarelli et al., 2005) they have yet to be described in goats. Here we seek to fill this gap.

The γδ TCR is made up of two polypeptides, the γ and δ chains. In some species the diversity of genes coding for these chains is less than that of the αβ TCR in that there are many fewer variable region genes (Krangel, 2009). However, with regard to the TCR δ chain cattle, sheep, and pigs have been found to have an expanded TRDV1 repertoire

relative to humans and mice with up to 50 TRDV1 genes available for rearrangement (Antonacci et al., 2005; Piccinni et al., 2015; Uenishi et al., 2009). A single TCR δ chain has also been shown in cattle to include sequence from all five diversity genes generating long CDR3 regions (Herzig et al., 2010a; Van Rhijn et al., 2007) while in mice and humans only one or two TRDD gene sequences are incorporated (Van Rhijn et al., 2007). With regard to the TCRγ chain, in many species the loci coding for them contain cassettes with V, J, and C genes in each while this does not occur in humans or poultry (Antonacci et al., 2020). Generation of the γ chain favors gene rearrangement within a cassette (Conrad et al., 2007; Miccoli et al., 2003). γδ T cells in cattle that express the lineage-specific hybrid pattern recognition receptor (PRR)/TCR co-receptor called Workshop Cluster 1 (WC1) (Hanby-Flarida et al., 1996; Hsu et al., 2015b) only express TCRγ genes from the TRGC5 cassette. As a result, it limits the diversity of the γ chain to five V genes paired with the single J and C genes (Blumerman et al., 2006). We wished to determine if these peculiarities found in cattle also occur in goats.

Here we sought to characterize the TCR loci of genes coding for both the γ and δ chains using a caprine genome assembly ARS1 that was more robust than those previously available. This particular assembly employed the San Clemente goat breed that is a highly homozygous (Bickhart et al., 2017). It was the most contiguous genome assembly for a non-model organism reported to date (Bickhart, 2017a) by combining longread PacBio single molecule real-time sequencing and physical mapping methods with error correction using shorter Illumina reads. Annotation of this assembly has already proven useful for other immune genes (Schwartz et al., 2018; Schwartz et al., 2019). As such, we looked to completely annotate the TCRγ (TRG) and δ (TRD) loci

while also comparing usage of these genes with what is already known in another ruminant species, cattle. Goats and cattle diverged 30 million of years ago through evolution establishing differences among polymorphic genes such as MHC and leukocyte receptor complexes but also consistencies in expansion of certain gene families (Schwartz et al., 2019). TCR genes are not polymorphic among individuals within a species and therefore may be more conserved on a genomic level among artiodactyl species.. We highlight many similarities of organization and expression of goat and cattle TCR genes but also key differences.

4.2 Materials and Methods

4.2.1 Genome annotation

The long-read genome sequence assembly ARS1 from a San Clemente goat was retrieved from GenBank where it is deposited under BioProject accession code PRJNA290100 (Bickhart et al., 2017). The TRG genes are contained on chromosome 4 (GenBank: NC_030811.1) and TRD genes on chromosome 10 (GenBank: NC_030817.1). Caprine TRG and TRD genes were annotated manually based on cattle TRG and TRD gene sequences (Conrad et al., 2007; Herzig et al., 2006; Herzig et al., 2010a; Ishiguro et al., 1993) that can be found in the IMGT database (www.imgt.org). This was done using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) after placing the bovine TRG and TRD sequences as query. The accession number for sequences used for bovine TRDC, TRDD and TRDJ genes in IMGT database is Btau_Chr10.28 whereas the accession number for sequences of TRDV genes are multiple, e.g., Btau_Chr10.28, Btau_un22, Btau_un41, Btau_un129, Btau_un139 etc. Similarly the accession numbers of sequences used for bovine TRGC are D90409, D90411, D90414, X63680, AY735449, AY644518 and AY644517, for TRGJ are AY6445187, AY644518, AC172685, AY644517, 937068 and AC172685, and for TRGV are AY644517 and NW_937068. Computer-based annotation was done using Maker (Campbell, 2014) and Apollo (Lewis et al., 2002) to verify the manually annotated TRG and TRD gene sequences as well as to search for additional exons coding for TCR. Maker Snap was trained multiple times to increase the accuracy and reliability of the predicted genes by inputting the annotated caprine TRG and TRD

gene nucleotide and deduced amino acid sequences. The manual and the computer-based annotations were compared for gene exon locations and sequences. Pseudogenes were defined based on the presence of a truncation, nonsense mutation, frameshift or defective initiation codon. Nomenclature of the annotated caprine TRG and TRD genes was based on similarity to the bovine gene sequences (Damani-Yokota et al., 2018a). Recombination signaling sequences (RSS) were defined by locating the borders of each V, D and J rearranging gene using expected canonical sequences and intervening spacing for these (Gellert, 2002).

4.2.2 Phylogenetic trees and alignments

Trees comparing archived bovine TCR sequences with the identified caprine sequences were generated in two ways. First sequences were aligned in BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) with ClustalW then analyzed in a neighbor-joining tree. Other trees were generated using Phylogeny.fr (www.phylogeny.fr) (Dereeper et al., 2010; Dereeper et al., 2008) with sequences aligned with MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/).

4.2.3 γδ T cell subpopulation purification

Blood was collected from the jugular veins of Boer goats ranging from 2-3 years of age and housed at the University of Massachusetts' farm as approved by the University of Massachusetts IACUC. PBMCs were isolated from blood by centrifugation over ficoll hypaque and cultured in complete RPMI medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 200 mM l-glutamine (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma) and 10 mg/ml gentamycin (Invitrogen)) with 5ug/ml concanavalin A (ConA) for 2 days. Cells were stained with primary monoclonal

antibodies (mAb) GB21A (anti-δ TCR) and CC15 (anti-WC1) purchased from Washington State University Monoclonal Antibody Center (Pullman, WA, USA) and secondary antibodies goat anti-mouse IgG2b-AF488 and IgG2a-AF647 (Invitrogen). Live lymphocytes were identified by forward and side scatter and were sorted using a FACS ARIA (Becton Dickinson) into TCRδ^{+}/WC1^{+} and TCRδ^{+}/WC1^{-} cells with 99% purity for both populations.

4.2.4 RNA isolation and RACE cDNA synthesis

γδ T cells were solubilized in Trizol (Invitrogen, Carlsbad, CA) and RNA extracted according to the manufacturer's instructions. RNA purity and concentration were determined by Nanodrop spectrophotometry (Thermo-Fisher). Reverse transcriptase was performed using Superscript IV (Invitrogen), a template switch oligo with unique molecular identifiers incorporated and a poly T reverse primer as outlined in Mamedov *et al*. 2013 (Mamedov et al., 2013). Primers, dNTPs and RNA were first incubated at 65C for 5 min., then enzyme, buffer, DTT, and *RNase OUT* (Invitrogen) were added before incubating at 50C for 1 hr then 80C for 10 min. One unit of uracil deglycosylase (Invitrogen) was added and the mixture incubated at 37C for 45 min. For analysis of the CDR3, cDNA was made without unique molecular identifiers by using the AMV RT kit(Promega) and the oligodt primer.

4.2.5 PCR and gene sequencing

cDNA was used as a template for PCR with Taq polymerase (New England Biolabs) and the primers outlined in Table 4-1. PCR was performed with the following conditions: 1) 95C for 2 min, 2) 95C for 30 sec, 3) 55-63C for 45 sec, 4) 72C for 30 sec,

repeating steps 2-4 for 30 cycles. To confirm primer specificity amplicons were cloned into a sequencing vector by standard techniques and sent for commercial Sanger sequencing. For PacBio sequencing, products were run on a Sybersafe 1% low melt agarose gel with a taped comb to fit all of the product into one lane. After running at a voltage of 110 for 30 min amplicons of the correct size were extracted and purified with the New England Biolabs gel extraction and then purified further with Ampure beads (Beckman Coulter) and sequenced at the University of Massachusetts Medical School for PacBio sequencing (https://www.umassmed.edu/nemo/pacific-biosciences/). FastA files of sequences were analyzed by filtering sequences based on PacBio tags applied to different input populations during PCR. Sequences were then aligned as described above. Any repeated base pairs that likely occurred due to the PacBio sequencing were resolved based on comparison with known sequences of TCR.

4.3 Results

4.3.1 Chromosomal organization of caprine γδ TCR genes

Here we annotated the loci of the TCRγ (TRG) and TCRδ (TRD) genes in a recent assembly of the goat genome (Schwartz et al., 2018; Schwartz et al., 2019). Caprine TRG genes were identified on chromosome 4 at two loci (Antonacci et al., 2007) while the TRD locus was located on chromosome 10. V, D, J, and C genes were named according to their homology with bovine genes.

The TRG genes were clustered within 7 cassettes or units named according to the functional Cγ gene included (Figure 4.1A). The first locus TRG1@ was found in an inverted orientation and contained 3 cassettes named C4, C3, and C5; both C3 and C5 cassettes had multiple V and J genes while the C4 cassette lacked a TRGJ gene. The C3 cassette also had an additional constant gene TRGC7 between TRGV9 and TRGV2 and was classified as a pseudogene due to a frame shift. The second locus, TRG2@, was assigned in a forward orientation. It contained 4 cassettes, C1, C8, C2 and C6 each with at least one V and J gene but TRGC8 was a pseudogene. Compared to cattle, the order of the cassettes and the contents of each were similar (Supplemental figure 4.2) although cattle only have 6 TRG cassettes and TRG1@ is in a forward orientation. A bovine homologue of TRGC8 is not reported in the literature but sequence for a homologous pseudogene is found in the IMGT database. Most caprine TRGV genes found were predicted to be functional except for TRGV10 which has a point mutation that induces a stop codon (Supplemental figure 4.1) (although this is known to be a functional gene in cattle).

To further evaluate the conservation within the caprine TCR loci with other mammals, RSS sequences were identified upstream and downstream of each gene. RSS spacers are organized as a heptamer, a 23 bp spacer, and a nanomer 3' downstream of gene with a nonamer, 12 bp spacer, and heptamer 5' upstream with these sequences for TCR and immunoglobulin gene rearrangement being highly conserved across species (Chen et al., 2009b; Parra et al., 2008; Yang et al., 2017). Generally, the first 3 bp of heptamers are canonical (i.e., CAC) whereas nonamers have conserved adenines at bp 5 and 6. A change in gene expression can occur if the heptamer and nanomer are noncanonical (Schwartz et al., 2018). There is adherence to these RSS rules in the goat assembly especially among TRDV genes (Table 4-2). Of the TRGV genes, three deviate from the conserved heptamer sequence and four have different nonamers of 17 total genes (Table 4-3). Something else interesting to note is that some duplicated genes such as TRGV3-1 and TRGV3-2 had differences in both heptamer and nanomer sequences where only one had the canonical heptamer. When caprine TRGV3 genes were compared to the bovine homologues, bTRGV3-1 nanomer was identical with the caprine equivalent whereas TRGV3-2 was not only different from caprine TRGV3-1 but was also different from the bovine homologues. Other bovine RSS sequences were included (such as bTRGV4) for comparison of variations. For examining the RSS sequences of genes that comprise the CDR3 (i.e., TRDD TRDJ and TRGJ), the end of the heptamer shown is where the reverse complement of the canonical sequence occurs (…GTG) and this varied among genes. While most of the genes had a conserved tyrosine at bp 5 and 6 some variation occurred (Table4-4). Since noncanonical or mutated RSS has been shown to result in reduced expression of caprine immunoglobulin J genes (Schwartz et al., 2018).

4.3.2 Comparison of caprine and bovine TCR gene sequences

Given the conservation of TCRγ loci organization between cattle and goats, we expected that the respective caprine and bovine TRGV, TRGC and TRGV genes would assort based on locus location. All TRGV genes corresponded to a bovine gene in a maximum likelihood tree except that there was no caprine homologue of bovine TRGV11, a pseudogene in both cattle and sheep (Figure 4-2A). There was also more duplication of the TRGV5 gene in goats than in cattle. In most instances duplicated genes clustered more closely within a species than between species. Most Cγ gene sequences clustered between species in an expected way (Figure 4.2B). There is not a bovine equivalent to TRGC8 but that caprine TRGC8 gene clustered with its neighboring TRGCs, i.e., TRGC1 and TRGC2. This may indicate that this is a duplication within the caprine cassette given the similar organization and homology of the other V and J genes found there but that this did not occur in cattle. The caprine pseudogene TRGC7 clusters with the other C gene in that cassette, i.e., TRGC3, indicating this too may be a duplication event. While sequence for a bovine equivalent may be found at IMGT as indicated above, it has not been placed in any available bovine genome assemblies to date.

Caprine annotated sequences and bovine nucleotide sequences from IMGT were aligned with MUSCLE and displayed in a Maximum likelihood phylogenetic tree A) TRGV B) TRGC. Numbers indicate branch support values. c before a gene name indicates a Caprine TCR sequence and b before a gene name indicates a bovine TCR sequence. Bovine sequences follow naming conventions from previous annotations.

To compare TRDV genes between ruminant species we used representative bovine TRDV1 genes from each of the 11 TRDV1 subfamily groups described previously along with the other TRDV genes (Damani-Yokota et al., 2018a; Herzig et al., 2010a) and a bovine TRAV gene that was classified based on homology with human TRAV genes as a benchmark. When aligned and displayed in a neighbor-joining tree caprine TRDV1 genes also clustered into 11 subclades (Supplemental figure 4.3) but not all of them clustered with representative bovine TRDV1 genes. There are 2 groups (2 and 10) of caprine TRDV1 genes that segregated away from the bovine TRDV1 genes thus leaving group 3 with only two bovine genes and with bovine TRDV1-c outside of all caprine groups. Since the bTRDV1-c may not have been the best "representative" of the subclade in cattle, we also looked at all of the bovine TRDV1 genes with the caprine genes in a phylogenetic tree (Supplemental figure 4.3). This analysis showed 13 different subclades with only 12 containing goat TRDV1 genes. Again, the group that bTRDV1-c was representing clustered separately and did not have any caprine equivalents. Thus, not all subclades of bovine TRDV1 genes clustered with goat genes indicating different duplication events of the two species. Interestingly, the caprine copies of TRDV3 (cTRDV3-1, cTRDV3-2, and cTRDV3-3) clustered with bTRDVb3 and bTRAV33 but not with bTRDV3 again showing that goats do not have a bTRDVY or bTRDV2 homologue at least in this assembly as no gene clustered with them.

We next evaluated the genes that code for the CDR3s of these TCR chains and aligned the J and D genes. Interestingly, although goats have more TRG cassettes than cattle both species had the same number of TRGJ genes but arising from different duplication events (Figure 4.3A). Alignment of both TRDJ and TRGJ genes between

species revealed that bovine and caprine corresponding genes based on location within the loci have a high degree of similarity (Figure 4.3C). Although goats have fewer TRDV1 genes than cattle, goats have more TRDD and TRDJ genes available for rearrangement to add diversity. Some TRDD genes that have similar placement in the locus are similar between cattle and goats while others are quite distinct (Figure 4.3B). Considering how short these sequences are and the fact that they are subjected to N and P addition, it is difficult to reach a conclusion on the impact of this.

4.3.3 TCR gene expression among γδ T cell subpopulations

After identifying the conservation of γ and δ TCR genes between goats and cattle, the question still remained whether they have the same expression patterns among bovine γδ T cell subpopulations. To achieve a shallow level of quantification of the usage of TCR genes, a 5' RACE method was used (Mamedov et al., 2013). This was achieved with a polyT primer during first strand synthesis that adds a Smart Oligo tag to the 3' end. During second strand synthesis different molecular identifiers are generated by random insertion, thus uniquely tagging each individual transcript with up to ~17 x 10^6 unique variants. In this way TCR reads obtained following PCR amplification and sequencing can be attributed to individual mRNA molecules. Using this approach we addressed the hypothesis that γδ T cells that express WC1 will have a restricted expression of genes found in the TRGC5 cassette as occurs in cattle. To test this, we purified $WC1^+$ γδ T cells and $WC1^-$ γδ T cells by flow cytometric sorting (Figure 4.4A) and subjected them to the 5' RACE cDNA production. PCR reactions performed with $WC1^+$ cells' cDNA yielded no product except for TRGC5 whereas $WC1^-$ γδ T cells

displayed expression of all TRGC genes (Figure 4.4B). This indicated that caprine WC1$^+$ cells are indeed restricted as are bovine cells. To confirm these sequences PCR amplicons were sent for PacBio sequencing. Sequences that are shown (Table 4-5) also confirmed that expression of TCRγ genes are indeed limited to rearrangement within that cassette... The second question we addressed was whether the products of multiple TRDD genes were incorporated into TCRδ chains as occurs in cattle (Herzig et al., 2010a; Herzig et al., 2015; Van Rhijn et al., 2007). The usage of TRG genes by the WC1$^+$ and WC1$^-$ γδ T cells (Table 4-6) and thetranscription of TRDV1 genes by γδ T cells[a] (Table 4-7) are ongoing the data not yet analized.

4.3.4 cDNA analysis as evidence for TRD gene usage

The caprine TRDD genes were identified using genome annotation and these allowed us to evaluate TRDD gene usage in cDNA sequences that had been currently sequenced in our laboratory. CDR3 of TRDV1, TRDV3 and TRDV4 containing sequences were analyzed abut not for CDR3 of TRDV2 since we could not get any transcripts for it. All D genes were represented in transcripts containing genes of the three TRDV subgroups. P nucleotides were observed flanking untrimmed TRDD regions and extensive N-regions were frequently found between TRDD regions. Some cases were ambiguous because nucleotides could be attributed to either P- or N-region but also to the presence of more than one TRDD gene. Therefore, we applied the criteria that at least five nucleotides of a particular TRDD gene must be present to claim usage of that gene with reasonable confidence even though Herzig et al., 2010 used 6 neucleotides. .

Examples of TRDD gene usage with nucleotide and deduced amino acid sequences are shown (Figure 4.6).

4.4 Discussion

While cattle and goats diverged evolutionarily several million years ago our data indicated that they share a high degree of similarity in the organization of their TRG and TRD genome loci and TRG and TRD gene sequences as well as gene transcription patterns by γδ T cell subpopulations.with regard to the TRD locus, one of the striking features for more than one species of artiodactyls is the expanded TRDV1 repertoire compared to humans and mice, as a result of extensive duplication events (Antonacci et al., 2005; Yang et al., 1995). Although goats were found to have fewer TRDV1 genes than cattle, with only 30 caprine TRDV1 genes, sheep also have fewer than cattle with 40 TRDV1 genes. When evaluated in a phylogenetic tree, 35 of the sheep TRDV1 genes cluster into 5 groups (Antonacci et al., 2005; Piccinni et al., 2015) while we previously showed that the over 60 bovine TRDV1 genes segregate into 11 phylogenetic groups or subclades (Herzig et al., 2010a). Caprine TRDV1 genes also clustered into 11 groups with the bovine TRDV1 subclade representatives with only bTRDV1-c excluded from clustering with caprine genes. So the relationship of caprine TRDV1 genes appears to be more similar to that of cattle than to sheep. There is the possibility that more caprine TRDV1 genes are located upstream within the TRA locus although we found no evidence for that in this particular assembly. While the organization of the caprine TRG loci were similar to those of both cattle and sheep there were some unique aspects in that caprine TRG1 locus was in an inverted orientation (Antonacci et al., 2020b; Conrad et al., 2007). Additional nonfunctional caprine TRGC genes were found, i.e., TRGC7 and TRGC8, and these do not have ovine equivalents, and only sequence for TRGC7 has been found in cattle in which is also a pseudogene (IMGT www.imgt.org) and not yet placed in the

locus. Based on the their location and homology to neighboring TRGC genes we assume that these pseudogenes are duplications of functional C genes. Most of the differences found among ruminant species evaluated here appear to be due to duplication events through evolution.

The CDR3 regions of TCRs are important because of its unique ability to add untemplated nucleotides thus giving vast diversity utilized in the binding of unique pathogen epitopes. Some CDR3 sequences are considered "public" and are shared among T cells from individuals where more "private" sequences indicate clonal expansion of a particular CDR3 expressing group of cells that are specific to a infecting pathogen(Madi et al., 2014). Many of the caprine TCR genes that make up the CDR3 had homologues with bovine genes but there were also unique sequences. Goats were found to have more TRDJ and TRDD genes that could add CDR3 diversity especially since there are fewer caprine TRDV1s and similar to cattle, Similar to goats, pigs also have more TRDD genes than cattle, all with functional RSS upstream and downstream sequences (Uenishi et al., 2009). However, the addition of more caprine TRGJs may not add diversity since they appear to be duplication events rather than unique genes. Depending on the antigen gamma delta TCR can bind using one of the chains CDR3 more predominantly. The δ chain of the TCR in crystal structures has been shown to be the dominant chain in mouse γδ T cell interactions with MHC like molecules such as T22 and CD1d (Adams et al., 2005; Luoma et al., 2013). Where the γ chain has been shown to be important for interaction with butyrophilin-like molecules although this is dependent on a variable region not on the CDR3(Willcox et al., 2019).

While having a large number of genes is clearly important for generating a repertoire of antigen-binding potentials gene expression can be controlled by the RSS such that some genes are much less frequently incorporated into viable TCRs. That is, canonical RSS sequences are expected to influence the ability of RAG-mediated rearrangement to occur (Wei and Lieber, 1993). The RSS of TCR and immunoglobulin genes have been characterized in a range of species from sandbar sharks to opossums (Chen et al., 2009b; Hassanin et al., 2000; Parra et al., 2008; Yang et al., 2017). RSS is widely studied in species to classify functional genes over ones that may not be rearranged by establishing canonical or non-canonical RSS sequences. It is expected that genes found without the canonical heptamer sequence will not be rearranged or will be rearranged at a lower frequency than genes that have it. This has been shown for caprine immunoglobulin genes for which noncanonical or mutated RSS resulted in less expression of the particular J genes (Schwartz et al., 2018). In agreement with mice and human, caprine TCR genes are flagged in the same way with the same canonical starting sequence of CAC within the heptamer with very few genes having a non-canonical sequence. The conservation of RSS sequences among caprine TRDV1 genes supports the idea that these genes underwent large-scale duplication events throughout evolution. Overall, the caprine TCR genes of both TRD and TRG loci follow the same RSS conventions in canonical sequence within the nanomer and heptamer as those for other species with few caprine TCR genes having changes within the conserved regions.

Much like cattle, goats have been found to express WC1 on subpopulations of γδ T cells and it has been shown that these cells participate in immune responses to pathogens (Yirsaw, Submitted for publication.-a; Yirsaw, Submitted for publication.-b).

In cattle, we have shown that γδ T cells that express the co-receptor WC1 use a restricted set of genes to code for the TCR γ chain while, in contrast, non-WC1$^+$ γδ T cells use all of the TCR genes available (Blumerman et al., 2006). This implies that the restriction is important for WC1$^+$ cell function especially since the predicted structure of the gene product expressed by WC1$^+$ γδ T cells (i.e., TRGC5) is substantially different from those of the other TRGC genes. We showed here that goats have similar organization of the TRGC5 cassette as cattle and sheep (Vaccarelli et al., 2005). Organizational similarities imply the conservation of the TRGC5 cassette throughout evolution and the potential of an important role for its genes' products in immune function to combat shared pathogens. Validating our thoughts on TRGC5 others have referred to it as an ancient cassette among bovine, ovine, and dromedary implying that other cassettes of the gamma chain are duplications of it(Antonacci et al., 2020a; Antonacci et al., 2020b; Vaccarelli et al., 2008). We have hypothesized that the restricted use of a particular TCR chain is because of the need for it to physically interact with WC1 to augment cell signaling in response to pathogens since WC1 and the TCR need to be co-ligated for there to be augmented signaling (Chen et al., 2014; Hanby-Flarida et al., 1996). If true we would also expect other species that express WC1, such as pigs, to have an analogous if not homolgous TRGC5 in terms of gene product. A similar paradigm has been proven for the interaction of the TCRα chain with the accessory signaling molecules CD3 where the membrane proximal portion of the TRAC region controls signaling with the CD3 complex(Backstrom et al., 1996).

Our work with the caprine γδ TCR genes continues to fill gaps in our knowledge regarding the evolution of the γδ TCR among artiodactyls and, more specifically, among

ruminant species. The large number of duplication events in both the TRG and TRD loci that are analogous in the bovine and caprine genomes is striking. The γδ TCR gene repertoire diversity is much greater in ruminants compared to humans and mice and mirrors the γδ T cell expansion in the blood of ruminants. γδ T cells may also play different roles in humans and mice than they play in ruminants although much is still unknown about the function of γδ T cells in goats particularly. Our work here with the goat γδ TCR loci will allow us to form hypotheses regarding the functional equivalence between goats and bovine γδ T cells since there are many more basic studies with the bovine γδ T cells available to model this on (Baldwin et al., 2002; Blumerman et al., 2007; Meraviglia et al., 2011; Naiman et al., 2001; Naiman et al., 2002; Rogers et al., 2005b; Wang et al., 2011).

Table 4-1 Primers used for PCR[a]

Primer Name	Primer sequences
TRGC1+2 rev	GATTGTTTGACTGATGAAAGCGGTGCC
TRGC2 rev	CAAAGGCACGTCTGGAAGGTGAAAA
TRGC3 rev	GACATGCTTTTGTAGAATTTGCAACAGGGAC
TRGC4 rev	GGCTCCCAGTGACTGTTTAAACCAG
TRGC5 rev	GAGGTTGCTACACGTGCCTGCATG
TRGC6 rev	CTTGTTGACTTCTAGAAGATTCACCTCTTG
TRDC rev	CATGTCACCGAATCGGGATCG
TRDV FWD1-1, 2, 8, 13, 20, 21, 24, 25, 28 fwd	CAAGGAATGGCCGCTACTCTG
TRDV FWD1-6, 11, 15, 16, 30 fwd	CCATCAGCCTCACCATTTCAGC
TRDV FWD1-3, 4, 18, 19 fwd	GCTGGAAGACTCTGCAAAGTACTTCTG
RDV FWD1-5, 9, 17, 27 fwd	CCCAGTGGAGAGATGATTTTCCTTAC
TRDV FWD1-7, 10, 12, 14, 22 fwd	GCTTCCCAGTGGAGAGATGAC
TRDV FWD1-23, 29 fwd	GTCCATCAGCCTCACCATTTCG
TRDV FWD3-1, 3-2, 3-3 fwd	CACCATCTCTTCCTTGCACCTG
TRDV4 fwd	GAGATCCTGAAGGCATCAGAGAGAG
WC1b rev	TCTGGGCTGAAGAGTTCAGGTGTG
Universal fw	AAGCAGTGTATCAACGCAGAGT
Universal fw pb01	TCAGACGATGCGTCATCAAGCAGTGGTATCAACGCAG
Universal fw pb02	CTATACATGACTCTGCCAAGCAGTGGTATCAACGCAG
Universal fw pb03	TACTAGAGTAGCACTCCAAGCAGTGGTATCAACGCAG

a. fw, forward primer; rev, reverse primer

Table 4-2 RSS genomic features downstream of the caprine TRDV gene

Gene	Heptamer	Spacer (23 nt)	Nonamer
TRDV4	CAC CCT G	CTG CAG ACT CAC TTC TAA GCA AC	TCA AAA GAC
TRDV3-1	CAC GGT G	AGG GTA GTG ACA GGA AGC ACC TT	ACA AAA ACC
TRDV3-2	CAC AGT G	ATA GAA ATG ATA GTA GAG GAT AA	TCA AAA ACC
TRDV3-3	AAC AGT G	AGA GAA ATG ACA ATT CAA GTC AG	TTC AGT AGC
TRDV2	ATG ACT A	CAG AAA AAA ATA AAA ACA TGA TA	GAA GAA CTT
TRDV1-1, 1-21, 1-24	CAC AGT G	CTT GAA GTA GTG GGA ATA GCT AA	ACA AAA ACC
TRDV1-2, 1-20, 1-28	//	CTT GAA GTA ATA GGA AAA GCT GT	//
TRDV1-4, 1-14	//	CTT GAA GTA ATA GGA AAA GCT GA	//
TRDV1-6	//	CTT GAA GTA ATA TGA AAG GCT GA	//
TRDV1-8, 1-13	//	CTT GAA GTG ATA GGA AAA GCT GT	//
TRDV1-10, 1-22	//	TTT GAA GTA ATA GGA AAA GCT GT	//
TRDV1-12	//	TTT GAG TTA ATA GGA AAA GCT GA	//
TRDV1-23	//	GTT GAA GTA ATG GGA AAA GCT GA	//
TRDV1-25	//	CTT GAA GTA ATG GAA AAA GCT GT	//
TRDV1-29	//	ATT GAA GTA ATG GGA AAA GCT GA	//
TRDV1-30	//	CTT GAA GTA ATG AGA AAA GCT GT	//
TRDV1-5	//	CCC GAG GTA ATG GAA AAA GCT GA	ACA AAA GCC
TRDV1-9	//	CTT GAA GTA ATA GGA AAA GCT GA	//
TRDV1-7	//	//	ACA TAA ACC
TRDV1-18, 1-27	//	//	ACA AAA CCC
TRDV1-11	//	CTT GAA GTA ATA GGA AAA GCT GA	//
TRDV1-16	//	//	ACA AAA ACA
TRDV1-17	//	CTC GAG ATA TGG AAA AAG CTG AA	CAA GAA CCC
TRDV1-19	//	GTT GAA GTG ATA GGA AAA CTT TG	AAA ATT TGA
TRDV1-3	//	GTT GAA GTG ATA GGA AAA GCT GA	ACA AAA ACT
TRDV1-15	CAC AAT G	CTT GAA GTA ATG GGA AAA GCT GT	ACA AAA ACC

TRDV1-26	CTC AGT G	CTT GAA GTA ATA GAA AAG GCT GA	ACA AAA TTC
TRDD1	CAC AGT A	TTA CAA ACC TCA AAG AGA CCT CT	ACA GAA ACT
TRDD2	CAC ATT G	CTG CAA TAC CCA AAG AGA CCG GT	//
TRDD4	//	CTA CAA AAC CCA CAG GGA ACC AT	//
TRDD3	CAC GAA G	GGT GAA GTG TAT TAA ACC CTT GT	TCA AAA ACC
TRDD6	//	GTT GAA GTG TAT TAA ATC CTT GT	//
TRDD5	CAC CAC A	ATA CAA ACA TCA CAC AGA CCT GT	ACA GGA ACT
RDD7	CAC GGT G	ATA CAA AAC CCA CAG AGA CCT GT	ACA AAA ACT

Table 4-3 RSS genomic features downstream of the caprine TRGV TRGJ genes

Gene	Heptamer	Spacer (23 nt)	Nonamer
TRGV1	CAC GTC A	GTT CAG ACT TCC TGT ACA CTG CA	CTG GAA ATT
bTRGV1	CAC ATT G	GTT CAG ACT TCC TGT ACA CTG CA	CTG GAA ATT
TRGV5-2	CAC GTT G	GTT CAG ACT TCC TGT ACA CTG CA	CTG GAA ATT
TRGV2	//	GTT TAG ACT TCC TTT ACA CTG CA	//
TRGV5-1	//	GTT CAG ACT TCC TGT ACA CTG CA	//
TRGV5-3	//	GTT CAG ACT TCC TGT ACA CTG CA	//
TRGV8-2	//	GTT TAG ACT TCC TTT ACA CTG CA	//
TRGV9	//	GTT CAG CCC TCC TTT ACA CTG CA	CTG AAA TTT
TRGV3-1	CAC AGT A	-TA GAA CTG TGG AGA CAA TCC AC	ACA AGA TCC
bTRGV3-1	CAC AGT A	CTA GAA CTG TGG AGA TAA CCC AT	ACA AGA TCC
TRGV3-2	CAC GGC A	CTA GAA CTA TGG AGA TAA CCC AC	ACA AGA TCC
bTRGV3-2	CAC AGT A	CTA GAA CTG TGG AGA TAA CCC AT	ACA AGA TCC
TRGV4	CAA GGT A	TTA CAG AGT CAG AAG GAA CGT GG	AAG AAA ACT
bTRGV4	CAC GGT A	TTA CAG AGT CAG AAG GAA CAT GG	AAG AAA ACT
TRGV6	CGC ACA G	CAG AGT GCG CAG CCC CGG CAT CT	GCA CAA AAA
TRGV7	CAC AGT G	TGG GAG TTT CTT GGA TAA CAC AC	ACA AGA ACC
TRGV10	CAT GTT G	CAG AAG AGA TCA GCT GTC ATT G	AAC AAA AAC

Table 4-4 RSS genomic features upstream of the caprine TRDD, TRDJ and TRGJ genes

Gene	Nonamer	Spacer (12 nt)	Heptamer
TRDD1, TRDD4	GGT TTT TGT	AAA GCT CTG TAG	CAC TGT G
TRDD2	//	AAA GCT CTG CAG	//
TRDD7	//	AAA GGA CTG TAG	//
TRDD3	GGT TGT TGT	AAA ACT GTG CTT	//
TRDD5	GGT TTT TAT	AAA GCT CTG TGA	TAC TGT G
TRDD6	GGT TAT TGT	AAA GCT ATG CTT	//
TRDJ1	GGT TTT TGG	AAA GCC CTC AAG	CTC TGT G
TRDJ2	AGT TTT TAG	ACT GGG TTT ATC	AGC TGT G
TRDJ3	GTT ACT TGT	CAG GCA GTG TCA	CAA TGT G
TRDJ4	ATG ATG TCT	GAA ATG GTG TAC	TGA TAA C
TRGJ1-1	GAT TAT TGT	AGG AGC ATT TAT	CAT TGT G
TRGJ1-3	//	CGG AGC ATT TAT	//
TRGJ2-1	//	AGG AGC ATT TAT	//
TRGJ1-2	AGT TTT TGA	TAT GAC TTG AAT	CAC TGT G
TRGJ2-2	//	TAT GAC TTG AAT	//
TRGJ3-1	ATT ATT GTA	GGA GCT TCA ACC	AGT GTG A
TRGJ5-1	AGG CTG AGA	TTT TTG TAG AAG	CTC CAA T
TRGJ6-1	AGT TTT TGC	CAC AGG TTG AAT	CAC TGT G

Table 4-5 Identity of bovine and caprine genes in the TRGC5 cassette

Corresponding Bovine/ Caprine gene name	**% Identity (nucleotide)**
TRGV3	94
TRGV4	91
TRGV7	92
TRGV10	95
TRGJ5	87
TRGC5	96

Table 4-6 Usage of TRG genes by the $WC1^+$ and $WC1^-$ γδ T cells[a.]

TRGC cassette that the Vγ genes are associated with	TCR Vγ gene name	$WC1^+$ γδ T cells	$WC1^-$ γδ T cells
TRGC 1	TRGV5-3	-	✓
TRGC 2	TRGV 5-1	-	✓
	TRGV 5-2	-	✓
TRGC 3	TRGV 2	-	✓
	TRGV 8-1	-	✓
	TRGV 8-2	-	✓
	TRGV 9	-	✓
TRGC4	TRGV 1	-	✓
TRGC5	TRGV3-1	✓	✓
	TRGV3-2	✓	✓
	TRGV4	✓	✓
	TRGV7	✓	✓
	TRGV10	✓	✓

a. Following flow cytometric purification of γδ T cell populations, RT-PCR and PacBio sequencing was used to assess gene transcription.

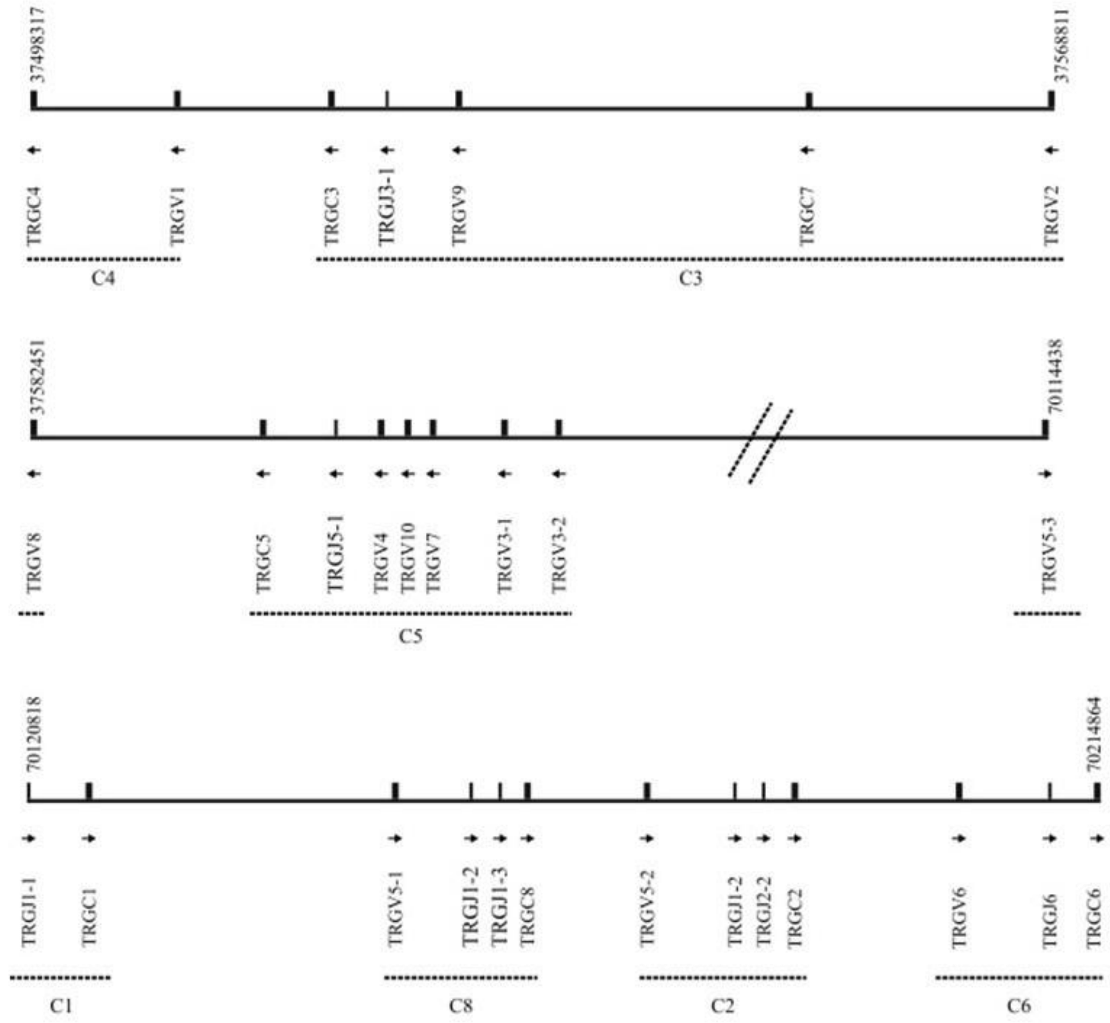
Table 4-7 Transcription of TRDV1 genes by γδ T cells[a]

TCR Vδ clades	WC1^{+} γδ T cells	WC1^{-} γδ T cells
TRDV1-1	✓	✓
1-2	✓	✓
1-3	✓	✓
1-4	✓	✓
1-5	✓	✓
1-6	✓	✓
1-7	✓	✓
1-8	✓	✓
1-9	✓	✓
1-10	✓	✓
1-11	✓	✓
1-12	✓	✓
1-13	✓	✓

Following flow cytometric purification of γδ T cell populations, RT-PCR and PacBio sequencing was used to assess gene transcriptio

A

TRG - 1 and 2



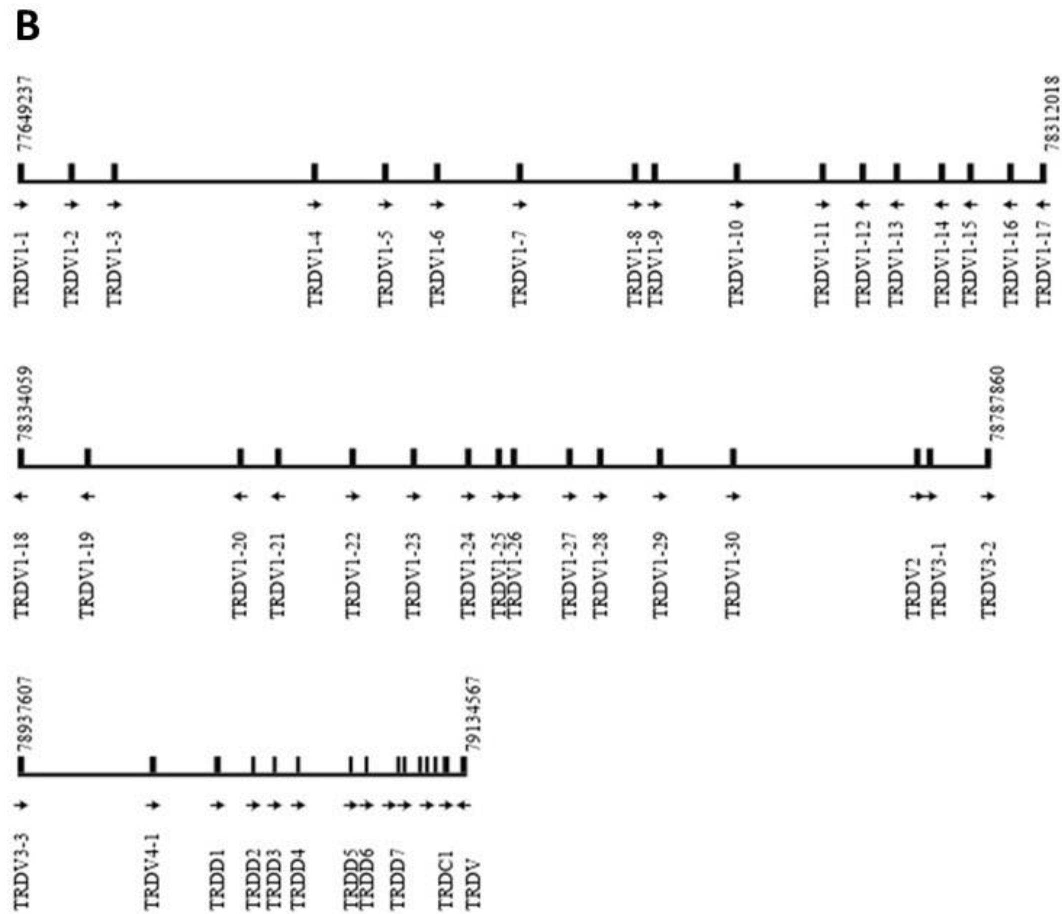
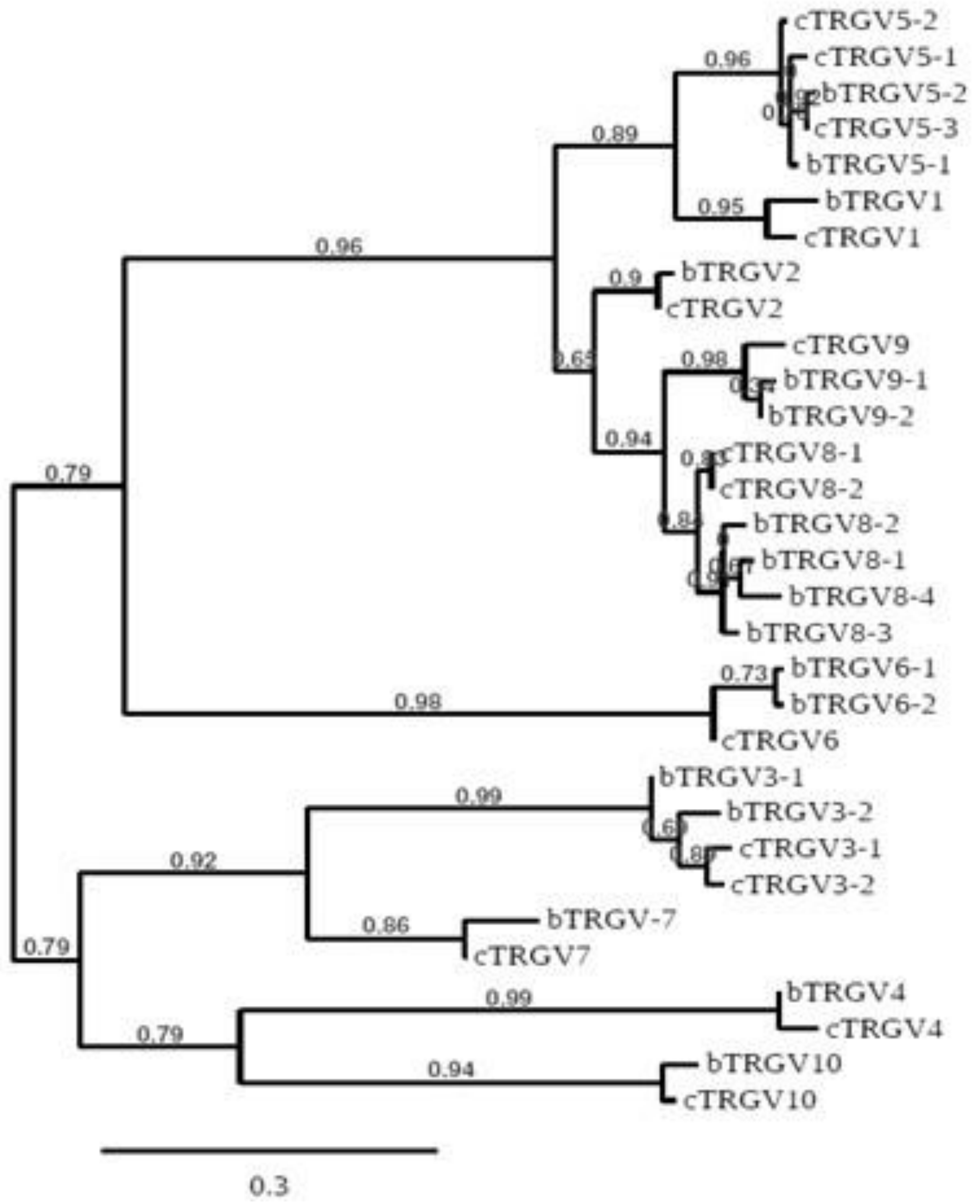


Figure 4.1 Organization of the goat TCR γ and δ loci.

Arrows indicate the orientation of the genes. Genes were named based on equivalent genes in cattle. (A) The TRG genes displayed at two loci on chromosome 4 separated by a double slash. TRG1@ locus was found between position 37,498,317 and 37,637,483 whereas the TRG2@ locus was between position 70,114,164 and 70,214,864. Dotted lines represent predicted gene rearrangements within cassettes. (B) The TRD genes displayed on chromosome 10 between 77,649,737 and 79,134,567.

A



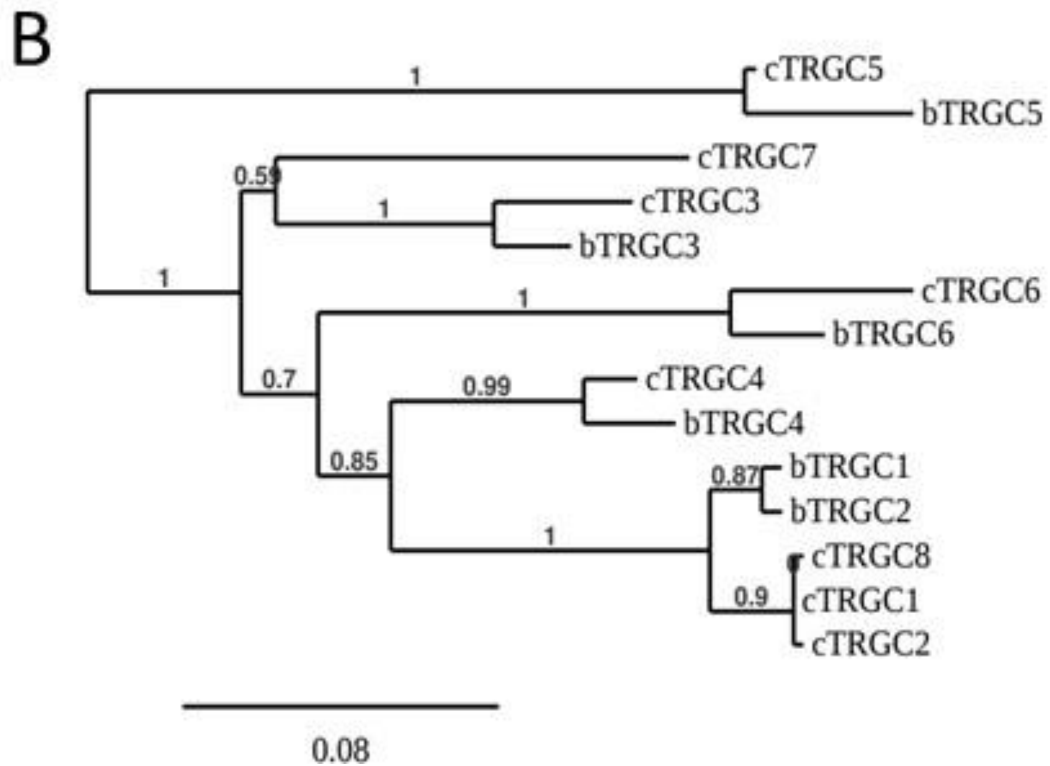


Figure 4.2 Caprine annotated sequences and bovine nucleotide sequences from IMGT lined up with MUSCLE and displayed in a Maximum likelihood phylogenetic tree A) TRGV B) TRGC. Numbers indicate branch support values. c before a gene name indicates a Caprine TCR sequence and b before a gene name indicates a bovine TCR sequence. Bovine sequences follow naming conventions from previous annotations. Lines indicate cluster names based on cattle TRDV1 groups (Herzig et al., 2010).

Figure 4.3 Phylogenetic comparison of caprine and bovine TRDV genes.

TRDV genes were lined up with ClustalW using default parameters and displayed in a neighbor-joining phylogenetic tree. ‘Cap’ before a gene name indicates a caprine TCR sequence and b before a gene name indicates a bovine TCR sequence. Bovine sequences follow naming conventions from previous annotations. Lines indicate subclades assigned but a clustering of 2 nodes of difference.

A

```
                 10        20        30        40        50        60
        ....|....|....|....|....|....|....|....|....|....|....|....|.
cTRGJ1-1  ACAGCTCGGGCTGG-AAGAAGATATTTGGAGAAGGAGCTAACATCATAGTAACTCCTTCCG
bTRGJ1-1  .......A......-.G........................................C.T.
cTRGJ1-2  ..............-...................A......................C...
bTRGJ1-2  -----GAATAA.TAC.TA..A.AC..CAAT.TT.....A..AC...T..C..AG------
cTRGJ1-3  -----GAATAA.TAC.TA..A.AC..CAAT.TT.....A..AC...T..C..AG------
cTRGJ2-1  ..............-..........................................C..-
bTRGJ2-1  .......A...G..-.G......T.................................C.T-
cTRGJ2-2  -----GAATAA.TAC.TA..A.AC..CAAT.TT.....A..AC...T..C..AG------
bTRGJ2-2  -----GAATAG.TACTTA..A.AC..CAAT.TT.....A..AC...T..C..AG------
cTRGJ3-1  -T..TG.A......-......C........A...C.A..G.GC........G..T.CC.T-
bTRGJ3-1  -T..TG.A......-...............A.....A..G.GC........G.....C.T-
cTRGJ3-2  -...TG.A......-......C........A...C.A..G.GC........G..T.CC.T-
bTRGJ4-1  --------------A-..............A.....A....GC.....A..G....CCAT.
bTRGJ4-2  -----.AATAA.TAC..A..A.AC..CAAT.TT...A.A..AC.TG.T..C..AG------
cTRGJ5-1  -..TTGT.ACAA..G.TC...G.......T......A....GC..G......TA...C.T.
bTRGJ5-1  ....T..AAC....-.TC...G.......T......A....GC..G......TA...C.T.
cTRGJ6-1  -----GAATTTAAAC..C.T...C.....T.G....A.A..AG.TT.C..CCAGG------
bTRGJ6-1  -----GAATTTAAAC..C.T...C.....T......A.A..AG.TT.C..CCTAG------
```

B

```
                10
         ....|....|....|
cTRDD1   ATTGG-TTGGAGC--
bTRDD1   G....AC...G.GG-
cTRDD2   GG...GA.TT.CGAG
bTRDD2   GG...GA.TT.CGAG
cTRDD3   -ACTACA.AT.C---
bTRDD3   GG...GG.ACTA.--
cTRDD4   GCG..AG.ACTCG--
bTRDD4   -ACAACG.AC-----
cTRDD5   GG...GG.ACTA.--
bTRDD5   GG...GA.ACG----
cTRDD6   -A.TACG.AC-----
cTRDD7   GG...GA.AC.----
```

C

```
                10        20        30        40        50        60
         ....|....|....|....|....|....|....|....|....|....|....|....|
cTRDJ1   -----TACTGATAACAC-AACTCGTCTTTGGAAATGAGACTCTACTCATCATGGAACCGA
bTRDJ1   ----A.GAGAC.G...--.G...A..........A.G...C.GC......G.......A.
cTRDJ2   GCTCC.GGGACACC.GGC.GA.GT.T......GC..GC..CAA....T..G....G..CC
bTRDJ2   --------CAG..T.C---...AA.A.....C..A.GA..CTAT..G.A.G.......AG
cTRDJ3   --------CAGA.T.C---...AA.A.....C..A.GA..CTAT..G.A.G.......A-
bTRDJ3   -CTCC.GGGACACC.GAC.GA.AT.T......GC..GC..CAA....T..G....G..CC
cTRDJ4   -----.GAGAC.G...--.G...AC.........A.G...C.GC......G.......A. 1
```

Figure 4.4 Aligned comparison of caprine and bovine joining and diversity gene sequences.

Nucleotide lineups of caprine and bovine derived sequences with ClustalW using default parameters A) TRGJ B) TRDD C) TRDJ; dashes (-) indicate gaps put in place by the program to give a better alignment. Periods (.) represent the same nucleotide as what is shown in the top sequence.

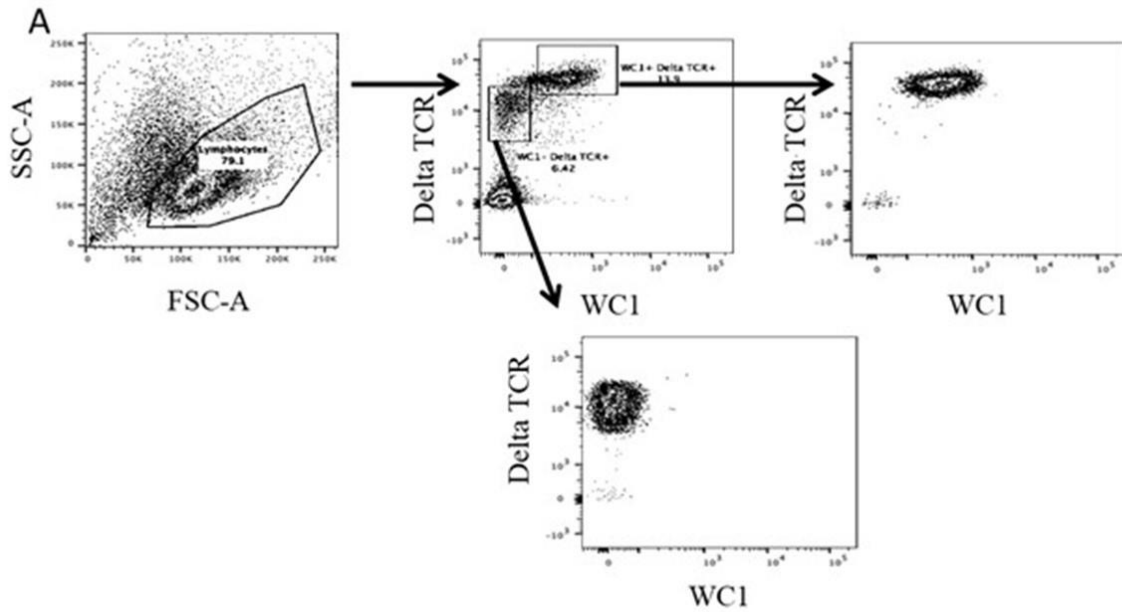


Figure 4.5 Sort Strategy and PCR of γ δ T cell subpopulations.

A) Flow cytometry sort strategy of goat PBMCs that were first gated on live lymphocytes based on forward (fsc) and side scatter (ssc). Cells had been stained by indirect immunofluorescence using mAbs reactive with WC1 or TCR δ. Numbers displayed indicate percent cells within gates. B) Gel electrophoresis of sorte cells.

TRDV			PN	TRDD1	P/N	TRDD2	P/N	TRDD3	P/N	TRDD4	P/N	TRDD5	P/N	RDD6	P/N	TRDD7	P/N	TRDJ
Germline				ATGGTTGGAGC		GGTGGGATTTACGAG		GCGGGAGTACTCG		ACTACATATAC		GGTGGGGTACTAC		ATTACGTAC		GGTGGGATAC		
TRDV1	14	ACTTCTGTGCTCTCTGTGTAC T S V L S V Y	TACCCCTAC T P			TGGGATTTAC W D L	A	AGTA S										CCGAATCCACTAATATTT P N P L I F
TRDV1	21	ATTTCTGTGCTCTCTGTGACT I S V L S V T	GTC V	TTGGAG L E	AC	GTCGG V	T											GAGAGACTGACAAGCTCACCT E R L T S S P
TRDV1	22	ACTTCTGTGCTCTCTGTCTTG T S V L S V L	GT	GTTGGAG V G		GGATTTACGA G F T		GCGGGAGTA A G V										CTCAATCCACTAATATTT P N P L I F
TRDV1	23	ACTTCTGCGCTCTCTCTGTTC T S A L S L F	CCAAGG P R	TGGTTGGA W L										ATTACGT I T				CCGAATCCACTAATATTT T N P L I F
TRDV3	02	TTTCTGTGCTCTCTGGGGCTA F L C S L G L	CGTTCCT R S	TTGGA L								GGTGGGGTACTA V G Y		TTACG Y				GTGGGACTGACAAGCTCACCT V G L T S S P
TRDV3	04	TTTCTGTGCTCTCTCCCAGGG F L C S L P G				TGGGAT W D									CTCCC L	GGATAC G Y	CGGCG R	CAGAATCCACTAATATTT Q N P L I F
TRDV3	06	TTTCTGTGCTCTCTGGGAATA F L C S L G I											CGGG R	TACGTAC Y V	G	GGGAT G		ACCCTACTGACAAGCTCACCT T L L T S S P
TRDV3	09	TTTCTGTGCTCTCTGGGAATA F L C S L G I	CAAGTTTG Q V								CT	GTGGGGTACTA V G F	T	TTACGTA L R	TT			TGGGATCCACTAATATTT W D P L I F
TRDV3	10	TTTCTGTGCTCTCTGGGAATG F L C S L G M							GCTAAT					ATTACGTA I T				CTGAGACTGACAAGCTCACCT L R L T S S P
TRDV3	11	TTTCTGTGCTCTCTGGGAACC F L C S L G T			CTACGT L R	TTTACG F T	GT											GGGATACTGACAGGCTCACCT G I L T G S P
TRDV3	16	TTTCTGTGCTCTCTGGGAATA F L C S L G I	CGTATCTCCT R I S	GTTGGAGC V G													GATAA D	GTGGGACTGACAAGCTCACCT V G L T S S P
TRDV4	08	TTTTACTACTGTGCCAGT F Y Y C A S										GATCCCT	GATCCCT D P	TTACG L	CCCCC P	TGGG W		ATACATCCACTAATATTT I H P L I F
TRDV4	12	TTTTACTACTGTGCCAGC F Y Y C A S										GGGGT G	T	TTACGT L R		GGTG G		GTCTCACTGACAAGCTCACCT V S L T S S P
TRDV4	17	TTTTACTACTGTGCCAGT F Y Y C A S						GCGGGAGTACT A G V							GGTC G	GGGAT G		ATGAGACTGACAAGCTCACCT M R L T S S P

Figure 4.6 TRD rearranged CDR3 sequences and TRDD gene usage.

TRD rearranged CDR3nucleotide sequences, and their corresponding deduced amino acid sequences, derived from caprine cDNA from peripheral blood mononuclear cells, were aligned to demonstrate TRDD usage. Germline TRDD sequences are shown above representative TRDV1, TRDV3 and TRDV4 sequences containing between one and three TRDD genes. TRDD genes are shaded and gene usage was determined by the presence of at least five nucleotides of a particular TRDD gene. Sufficient cDNA sequence was analyzed to determine TRDV gene usage; however, all the sequences of TRDV and TRDJ genes were not shown, only some of the end sequences of TRDV genes and the beginning of TRDJ genes presented.

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5 CHAPTER V CONCLUSIONS AND FUTURE STUDIES

WC1 molecules are important as both co-receptors and PRR for host immune responses (Hsu, 2013; Hsu et al., 2015b; Telfer and Baldwin, 2015). In this study the caprine WC1 gene numbers, gene structures, cDNA evidence and ICD splice variants were defined as was WC1 gene sequence conservation among goat breeds as well as with cattle (Chen, 2012). Our results indicated that goat WC1 molecules are coded by a multigenic family as in cattle (Herzig and Baldwin, 2009) in line with our hypothesis. We identified 17 complete WC1 genes as well as 13 partial WC1 genes. The complete goat WC1 genes have 191 SRCR domains which is more than cattle (138 SRCR domains) (Herzig and Baldwin, 2009). In general the gene number results suggest that goats have approximately 30 WC1 genes from genome annotation combined with PCR of cDNA and genomic DNA. This gene number is more than twice that of cattle and thus a larger variety for pathogen binding. Goats have 4 unique exon-intron structures in addition to the three classical structures of WC1 genes in cattle. We provided cDNA evidence for 12 of the annotated San Clemente WC1 genes and all of them suggested the correctness of the genome assembly as did the Yunnan annotated WC1 genes as far as it went. Both the annotated San Clemente partial WC1 genes as well as the obtained additional SRCR domain transcript sequences for orphan SRCR a1 domains suggests the incompleteness of the genome assembly. We found the SRCR a1 domains are highly conserved among goat breeds like those among cattle breeds (Chen et al., 2009a). This WC1 conservation may suggest that most goat infectious diseases potentially threaten all breeds of goats except their variations in degree of severity and thus these are conserved through evolution like Toll-like receptors are (Rolls et al., 2007). When comparing bovine and

caprine WC1 a1 domains there was less conservation. However six bovine a1 domains (BtWC1-10, BtWC1-11, BtWC1-12, BtWC1-4, BtWC1-7, BtWC1-9) clustered more closely between the species. These similarities and differences may be due to the shared pathogens affecting both cattle and goats and unique pathogens affecting only goats. For example, the bovine WC1 a1 domain that binds *Mycobacteria paratuberculosis* (J. Telfer, unpublished data) differs by only 5.5% (17/310 nucleotide sequences) with SCgoatWC1-8. Both species may be infected with *M. paratuberculosis* (Baldwin et al., 2019c). Finally we found that goats have splice variants of ICDs unlike cattle perhaps suggesting a difference in signaling initiation or sustainability following responses to some pathogens. In summary this work showed goat WC1 genes have unique features relative to cattle and this finding may impact research on next generation vaccines designed to stimulate subpopulations of γδ T cells through WC1 augmenting the TCR.

We defined γδ T cell subpopulations including their representation in goats of various ages since this information will be important for understanding the functions and roles of caprine γδ T cells in responses to infectious pathogens and potentially targeting them for activation through vaccination. The proportion of γδ T cells in the PBMC were measured in goats ≥6 months of age using flow cytometry and a panel of mAbs. In sheep and cattle the proportion of γδ T cells decreases as the animal age from birth to adulthood. Our studies with goats agreed with Jolly *et al*, 1997, Caro *et al*., 2001, Kaba *et al*., 2011[b] and Wilkerson *et al*., 1995 showing that the proportion of γδ T cells did not change with age when animals were greater than 6 months even though the γδ T cell proportion in the PBMC in our study was generally higher than in previous studies since we used GB21A mAb that reacts with the T cell receptor delta constant (TRDC) chain

(Machugh et al., 1997) and thus stains all γδ T cells while mAb 86D sees one of the γ TCR chains. We suspect it is reacting with one of the TCR Vγ chains based on its distribution among both $WC1^+$ and $WC1^-$ γδ T cells. While our results do not show a decrease after 6 months, Caro *et al.,* 1998 showed a decrease of the γδ T cells occurs between one week and 7 months of age. While the proportion of γδ T cells in goats in our study was less than that of CD4 and CD8 T cells it was not considerably so. And while the proportion of $WC1^+$ γδ T cells in PBMC was less than that of the total γδ T cells, after 6 months the proportion of neither γδ T cells nor the $WC1^+$ γδ T cells changed. Goats seem to have more $WC1^+/WC1.1^-/WC1.2^-$ cells than cattle since goats have potentially twice as many genes as cattle, and thus the potential for additional WC1 subpopulations is logical. The γδ T cells that showed responses by IL-17 and IFγ production in *ex vivo* cultures were largely or entirely $WC1^-$ γδ T cells. In the bacteria-stimulated cultures of several days, the $WC1^+$ γδ T cells responded to *M. bovis* BCG, *Leptospira* and MAP antigens by producing IL-17 although no IFNγ production was detected. For another animal the $WC1^-$ γδ T cells made IL-17 to *Leptospira*. Proliferation assays showed that innate responses to MAP could be cross-reactive to other *M. avium* species or are truly innate responses without any priming of the γδ T cells since our goats are MAP negative. The response to MAP by $WC1^-$ γδ T cells is consistent with responses found in cattle although some $WC1^+$ γδ T cells are found in granulomas in gut tissue of infected cattle (Plattner et al., 2009). The response by $WC1^+$ goat cells to *Leptospira* corresponds with observations in cattle (Blumerman et al., 2007). It was interesting that there was no cell division by γδ T cells to pathogen antigens when PBMC were from the two kids. It is

possible that the responses we are measuring in our in vitro cultures reflect memory responses by the γδ T cells in the adult goats, perhaps due to cross-reactive antigens, and that the kids simply have not yet had exposure.

We have characterized the goat TRG and TRD loci and compared them to the loci of other ruminants. Based on our comparisons with the bovine loci share an astounding amount of similarities in gene organization and sequences. In cattle, we have shown that γδ T cells that express the co-receptor WC1 have a restricted TCR γ chain implying that this is important for WC1+ cell function. Since Bovine γδ T cells that express WC1 only use TCR genes within the TRGC5 cassette (Blumerman et al., 2006). We have shown that goats have similar organization of this cassette and it also has been shown that sheep do as well (Vaccarelli et al., 2005). We hypothesize that the TCR chain expressing the TRGC5 gene physically is interacting with WC1 to augment cell signaling in response to pathogens since they need to be co-ligated in order for there to be augmented signaling. It has been shown with more than one species that artiodactyls have expanded TRDV1 repertoires having undergone extensive duplication events (Antonacci et al., 2005; Yang et al., 1995). Although goats were found to have fewer TRDV1 genes than cattle, and sheep. When put caprine and ovine TRDV genes into a neighbor joining tree, all clustered into 10 and 5 groups respectively (Antonacci et al., 2005; Piccinni et al., 2015). Goat only one less than bovine TRDV1 genes. In addition to this there is the possibility of more TRDV1 genes being located upstream or incorporated within the TRA locus. Since we used bovine genes as a query this may have biased us against finding any unique TRDV genes in goat. Moreover the γ locus TRGC1 was found in the reverse orientation which is different from what has been shown in both cattle and sheep (Conrad

et al., 2007). Additional nonfunctional C genes, TRGC7 and TRGC8, were found in goat that do not have sheep equivalent but TRGC7 has been found in bovine to be a pseudogene (IMGT) and aligns accordingly with the goat TRGC7 (data not shown). Based on the location and homology to surrounding C genes we assume that these pseudogenes are duplications of functional C genes. Some of the goat TRGJ, TRDD and TRDJ genes had homologues in the bovine genes but also had a few unique sequences. Goats have more TRDJ and TRDD genes which could add CDR3 diversity especially since there are fewer TRDV1s.

In the future more extensive studies of caprine γδ T cell will be needed:

1. To evaluate the role of the various γδ T cell subpopulations to various important livestock pathogens including viral, bacterial and parasitic worms since knowing this information will be important for understanding how to engage these cells in vaccine designs. In this regard some of the activities to be conducted are:
    - 1.1 Additional proliferation and cytokine production assay needed for many globally important caprine diseases by obtaining pathogens from OIE reference laboratories and / or vaccine institutes since in this study we included fewer of the pathogens..
    - 1.2 Focusing on goat unique WC1 genes are important
        - 1.2.1 To obtain gene transcript for SCg-WC1-5, SCg-WC1-13, SCg-WC1-15 and SCg-WC1-16.
        - 1.2.2 Conducting gene expression assay to many small ruminants pathogens after obtaining the unique goat WC1 gene transcripts

1.3 Detailed study on γδ T cell subpopulations response profiles soon before and after challenging with important pathogens in order to generate important information for next generation vaccines.

1.4 Detailed study on γδ T cell subpopulations response profiles soon before and after vaccinated to protect with important pathogens in order to know the contribution of γδ T cell subpopulations on the current vaccines

2. To evaluate the intracytoplasmic tail splice variant functions since we know in the bovine ICD's tyrosine and serine phosphorylation are essential for signaling the cell for activation and endocytosis of WC1 molecules perhaps to limit activation (Herzig and Baldwin, 2009; Wang et al., 2009). The bovine ICDs tyrosine kinase phosphorylation motif that can induce signaling differ in their locations in Type I and II ICDs compared to Type III ICDs but in goats we do not know which tyrosine are functional and get phosphorylated

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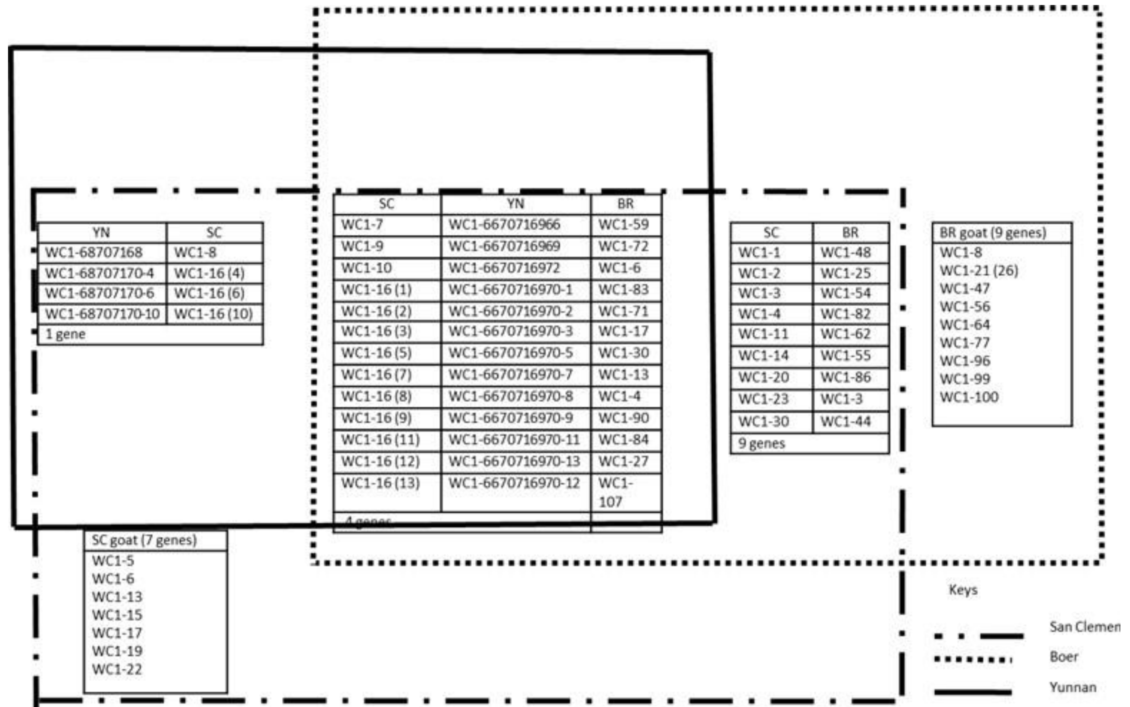
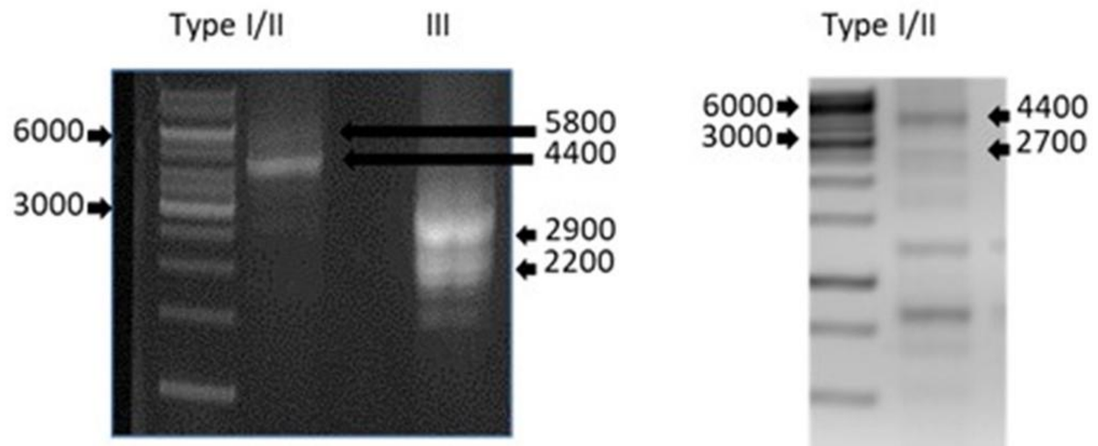
APPENDICES



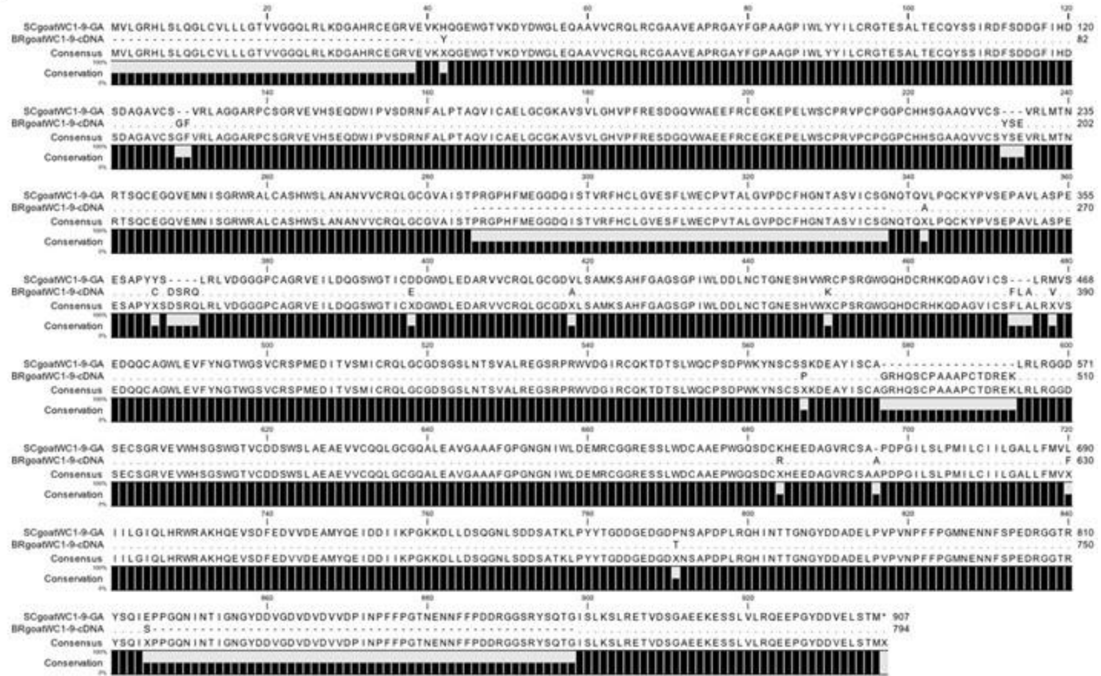
A. Supplemental figure 2.1 Goat WC1 gene number estimation using Venn diagram summary.

All the 81 unique a1 domains and the single d1 domain sequences were grouped as found in each breed with corresponding names in the compartments within this figure. Yunnan (YN), San Clemente (SC) and Boer (BR) goats. The relationships of a1 domains among goat breeds was made using sequence alignment and phylogenetic trees as shown.


A
Type I/II
III
6000
3000
5800
4400
2900
2200
Type I/II
6000
3000
4400
2700

B

C



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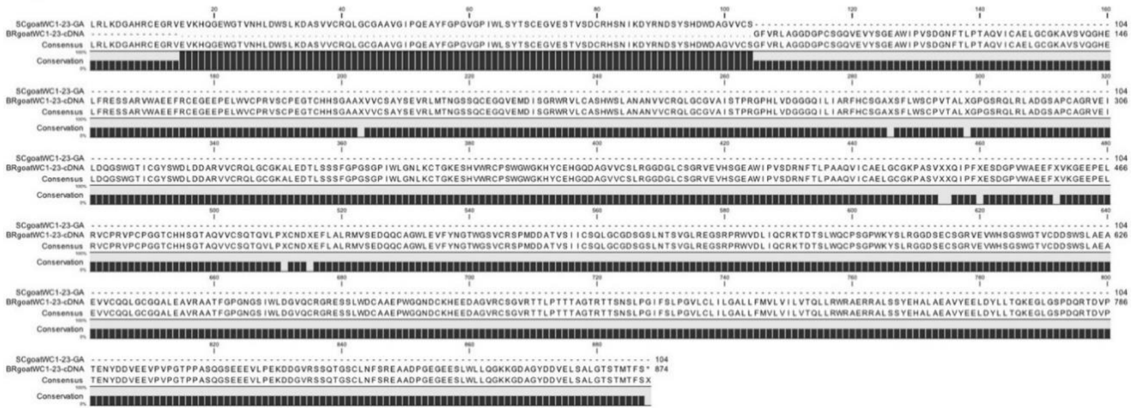

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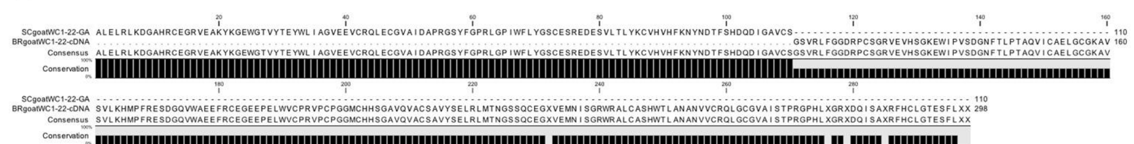

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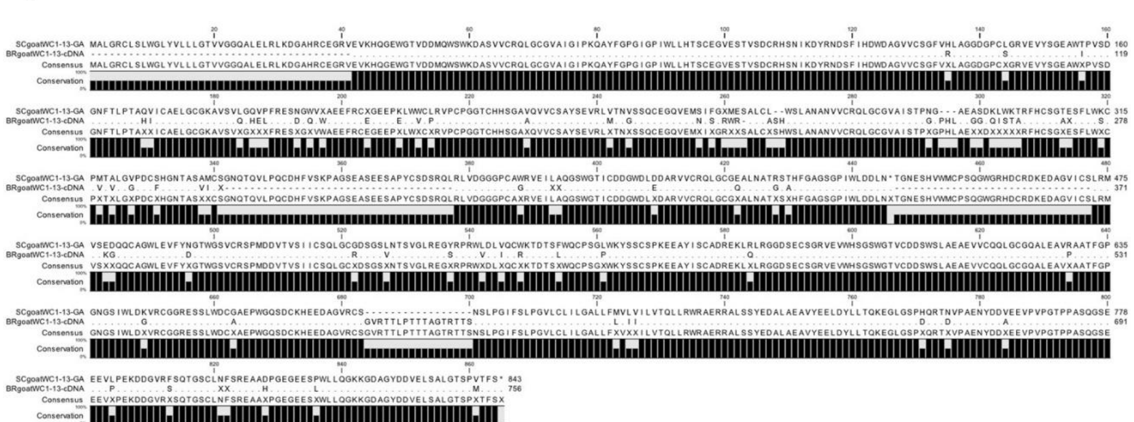
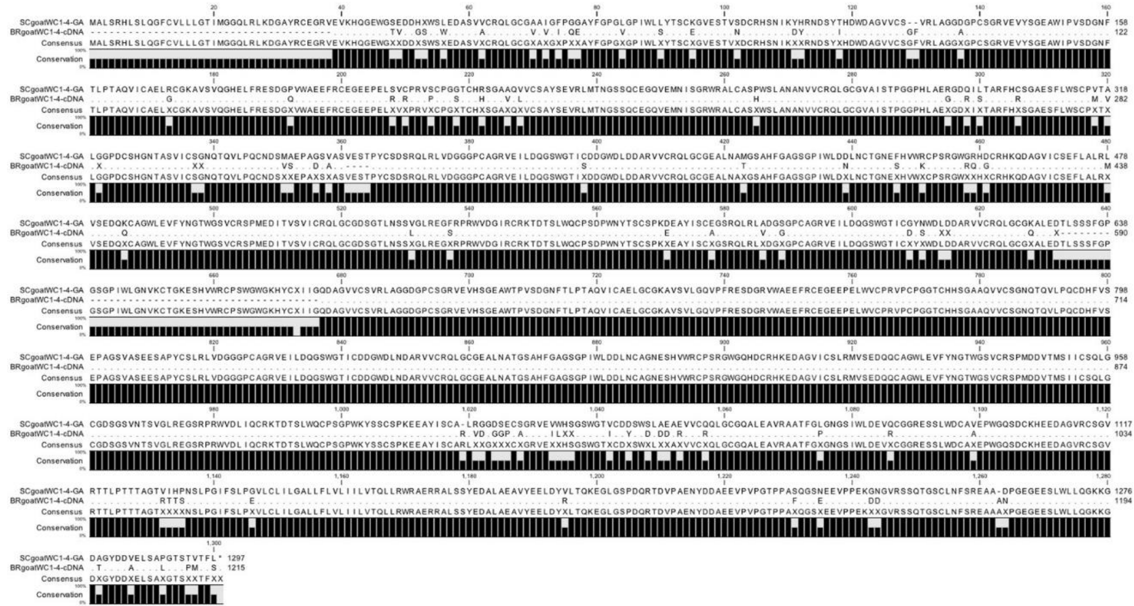
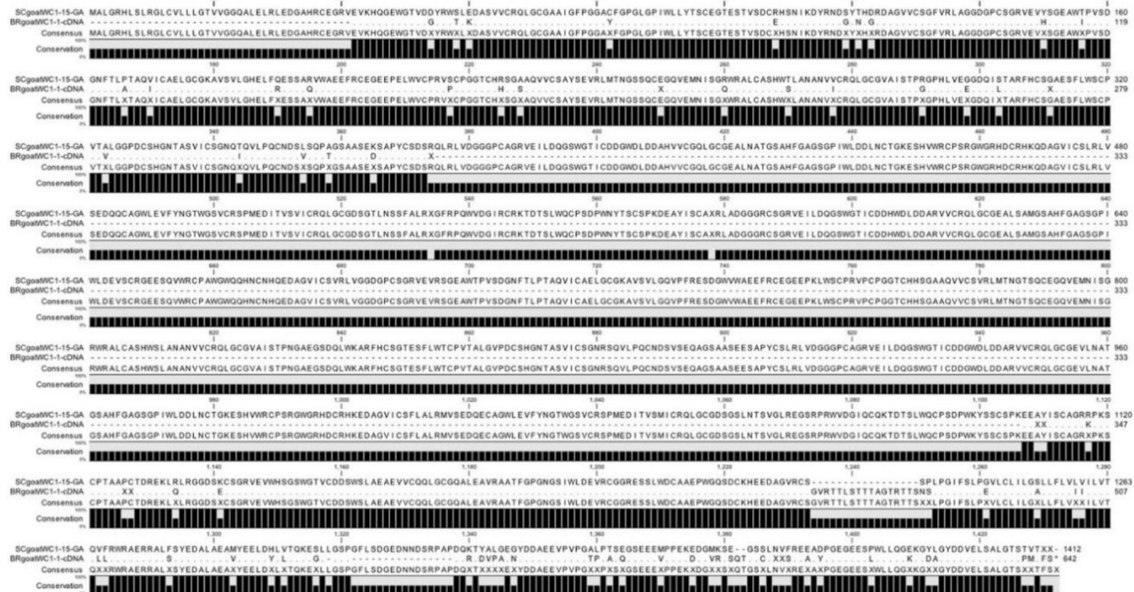


G



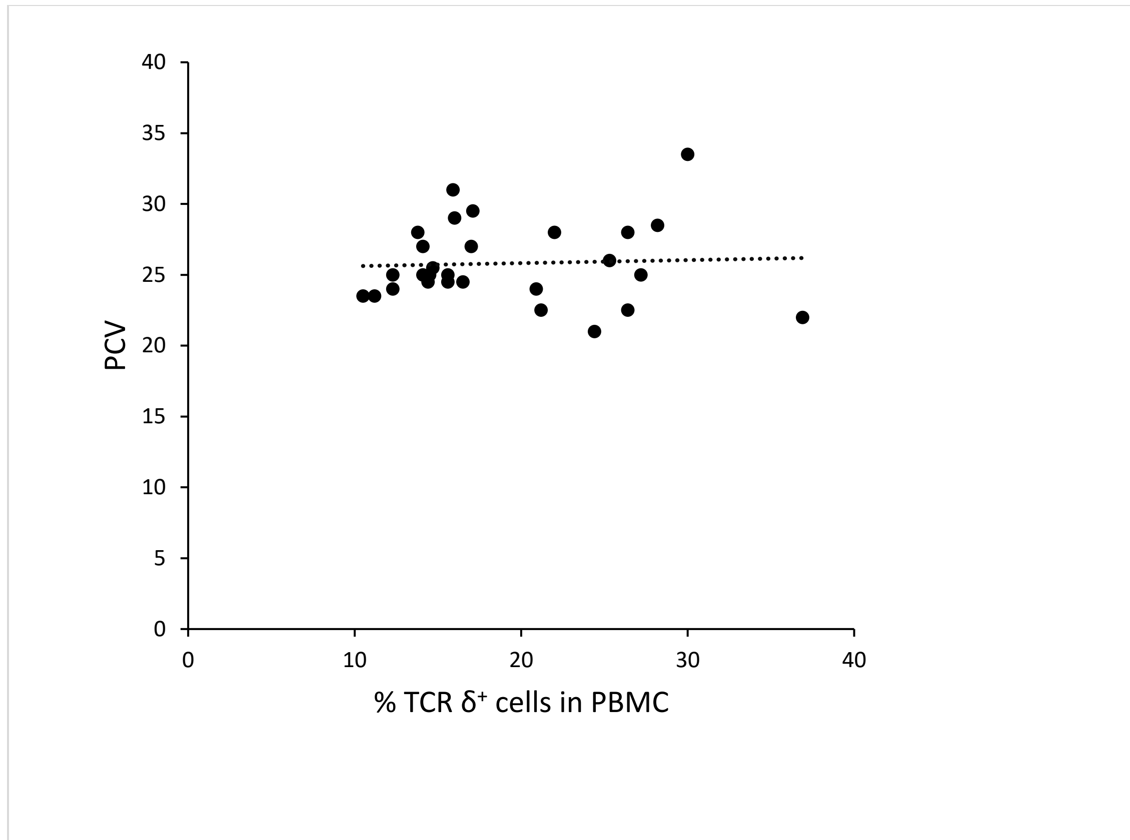
H



I

B. Supplemental figure 2.2 Alignment of the deduced amino acid sequences of the complete WC1 cDNA sequences.

(A) Agarose gels of the amplicons obtained by RT-PCR for WC1 transcripts. Significant bands are indicated with an asterisk on the left-hand gel: lane 1, size markers; lane 2, ICD Type I/II, 5800 bp (faint band) and 4400 bp; lane 4, ICD Type III, 2900 bp and 2200 bp; right-hand gel: lane 1, size markers; lane 2, ICD Type I/II, 4400 bp and 2700 bp. Full-length deduced amino acid sequences of the annotated WC1 genes from the San Clemente assembly and WC1 transcript sequences from Boer goat DNA were aligned. Identities are indicated by dots (.), gaps resulting from the alignment are indicated by tildes (~), gaps resulting from lack of cDNA are indicated by dashes (-) and the N nucleotide sequences show as "x" when converted to deduced amino acids. Sequences shown are (B) SCgoatWC1-1-GA vs BRgoatWC1-1-cDNA sequences, (C) SCgoatWC1-9-GA vs BRgoatWC1-9-cDNA sequences, (D) SCgoatWC1-23-GA vs BRgoatWC1-23-cDNA sequences, (E) SCgoatWC1-22-GA vs BRgoatWC1-22-cDNA sequences, (F) SCgoatWC1-13-GA vs BRgoatWC1-13-cDNA sequences, (G) SCgoatWC1-4-GA vs BRgoatWC1-4-cDNA sequences, (H) SCgoatWC1-15-GA vs BRgoatWC1-15-cDNA sequences, and (I) SCgoatWC1-2-GA vs BRgoatWC1-2-cDNA sequences.



C. Supplemental figure 3.1 PCV relative to percentage γδ T cells.

PCV was determined for all goats by standard methods. The blood was collected two times and each sample was tested in duplicate and the result expressed here as the mean of the four readings. The percentage of total γδ T cells was established with α-δ TCR mAb by immunofluorescence. Pearson Correlation Coefficient showed weak correlation that was not significant [R 0.049), R^2 (0.002), p=0.086].

A

```
                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
cTRGV10 DLPITQRITSITKKKGNMAPLECQIKIDKLKKNVYMHWYRQKPEQPLKRILYISSNENVIHEQGISEERYEARKWPSNALVSLRIH*ATEEEAGLYYCACWLGX
bTRGV10 .I..............T..............................V.........V.................S.......Q...D.........VE.

                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
cTRGV7  QLTLEQPELSVTGTREKSIIMTCKVFSKDFSKDYIHWYRQKPDQGLEQLLYVSTAPAQNELGGKKNKLEARKDAPSSTSTLKISPLEKEDEATYYCAGWL
bTRGV7  ....V.T.V............S...........................F.LD...L.D.............K.........................LA

                10        20        30        40        50        60        70        80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
cTRGV4  STRRPDRTVHISCKLSGVPLENAIVHWYQEKEGEPLKRILYGSANSYKLDKPNSRLEMDNKKNGIFYLVINNVVKSDEATYYCACWDX
bTRGV4  ........................M...............................I..........I..............

                                                                                                   90       100
        ..........................................................................................|....|....|..
cTRGV3-1 LSKVEQAQISLSTEVKKSIDIECKTESTNFESETVYWYWQKRNQALEHLVYVISTTTAARNQVDGKNKIEARKDARMFTSTLTVNFVEKEDVGIYCCAGWDX
cTRGV3-2 .........................I.............R..................................................D.....Y....-.
bTRGV3-1 X.............A....N...I........I...R..K.........T...........................................I........Y...S.
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B

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                10        20        30        40        50        60        70        80        90
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
cTRGC5  DRRLDGDLPPKPTIFPPSVEEVKRHSAGTHLCLLQNFFPDAIKVQWKEKNGNTILESHQGNIIKTNDTYMKFSWLTLTKKAMEKEHVCIV
bTRGC5  .......................L.G.................I......V......Y.........................D.......

               100       110       120       130       140       150       160
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
cTRGC5  KHENNKGGRDQEILPSFVNKEVATRACMKKESDTLQLQPASTSAYYTYLLLLLKSMIYPSIIAPCVFWRTGIPSNGKIP
bTRGC5  ...........Q......K....H...............N...............................D....
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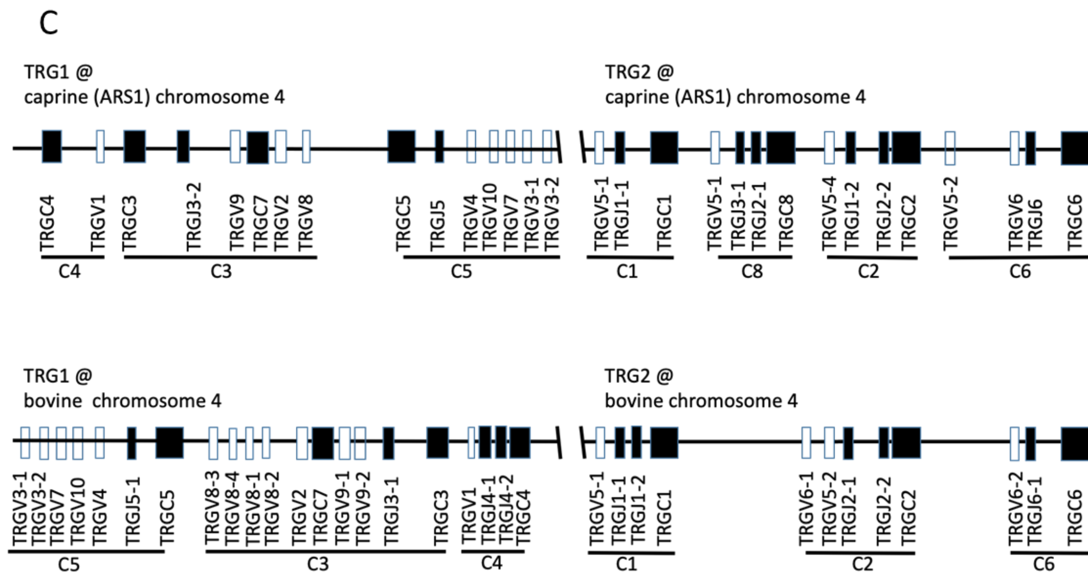
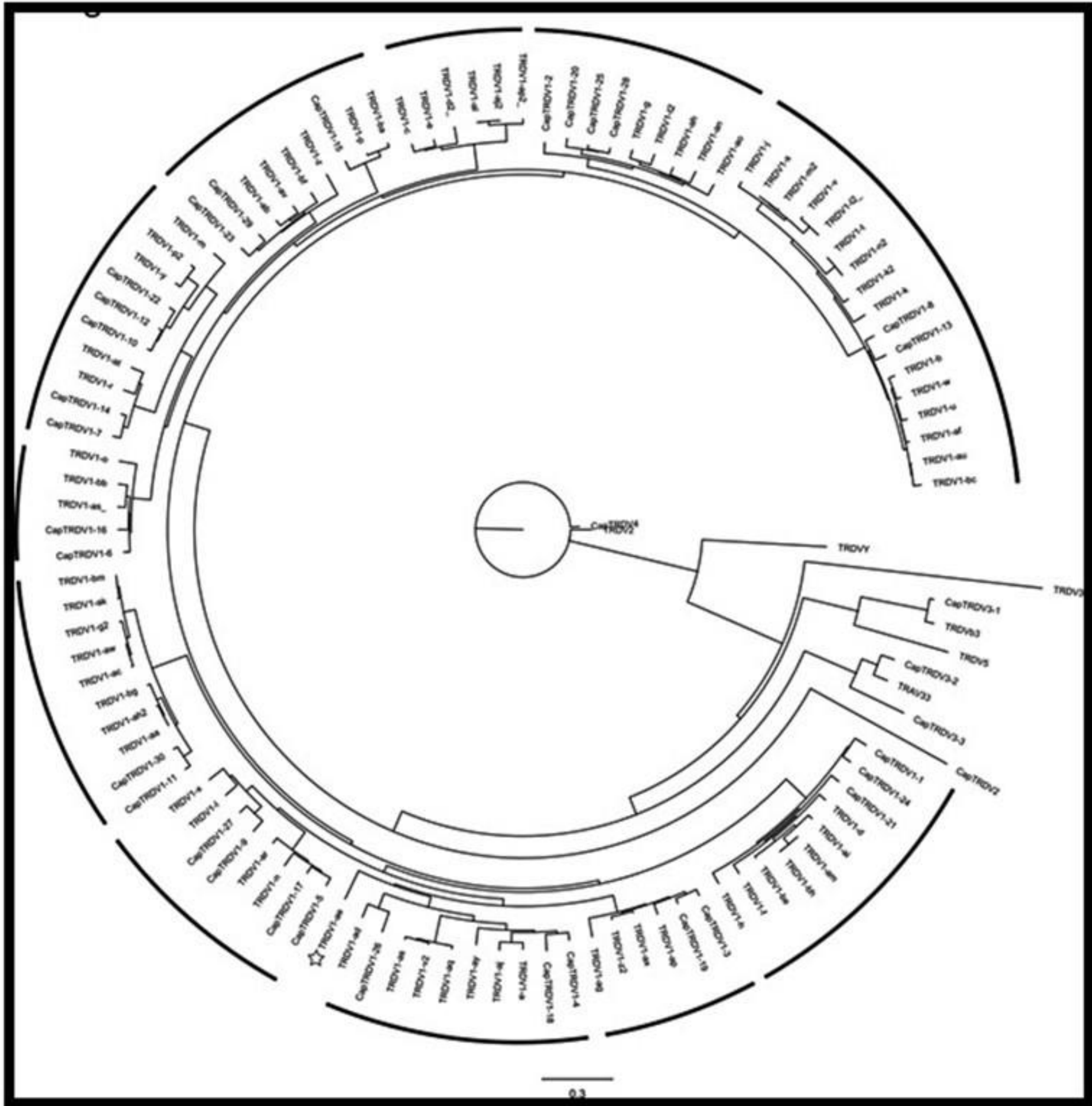


D. Supplemental figure 4.1. Deduced amino acid alignment of TRGC5 cassette's genes. (A, B) Deduced amino acid sequence alignment of genes in the bovine and caprine TRGC5 cassette using ClustalW. Dashes (-) are gaps introduced for preferred alignment. Periods (.) represent the same nucleotide as shown for the first sequence. (C) Location of the caprine and bovine TRG genes at two loci on chromosome 4 (substantial gap indicated as a double slash). The caprine TRG1@ locus is inverted. Although the structures are similar between the species it is notable that there are several duplication or deletion events including the addition of the C8 cassette in caprine TRG2@.


TRDVY
CapTRDV3-1
TRDV5
CapTRDV3-2
CapTRDV3-3
CapTRDV2
CapTRDV1-1
CapTRDV1-24
CapTRDV1-21
0.3

E. Supplemental figure 4.2 Phylogenetic Tree of Goat TRDV and all Bovine TRDV genes.

TRDV genes were lined up with ClustalW using default parameters and displayed in a neighbor-joining phylogenetic tree. 'Cap' before a gene name indicates a caprine TCR sequence and nothing before indicates a bovine TRDV gene. Bovine sequences follow naming conventions from previous annotations. Lines indicate 11 subclades of bovine TRDV1 genes. The star indicates where bovine TRDV genes did not cluster as expected.

A

```
                  10        20        30        40        50        60        70        80        90        100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
bTRGV1    SSNAKGAQMSVTGKT~~WETASFTCDLTQD~AT~~~YIHLYKQQEGMAPQRLFYYDVYYTKIQFESGTKKEKYSVYK~VTGRSYRFAILNLEDSDSGMYYCAVWDK~
cTRGV1    ...............~~...T.....F...~VK~~~.............R.........S..E....VN.A......GAA............H....T........~
bTRGV2    ...TE.EK..I.KA.GSGSSVEII...ITQTLK~~~...W..Y...T..R..L...IS.S.VVL...VSEG..K...~E~~K..T......QE.........A.E.~
cTRGV2    ...ME.EK..I.KAAVPGAFVEII...ITQTVK~~~...W..Y...T..R..L...IS.S..VL...ISEG..K...~E~~K..T...S..QE..........E.~
bTRGV3-1  L.KVEQ..I.LSTEV~~KKSINIR.KIESTNFES~ETVYW.R.KKDQ.LEH.V.VTSTT.AARNQVDG.~N.IEAR.DARMFTSTLTVNFI.KE.V.I....G..~
cTRGV3-1  L.KVEQ..I.LSTEV~~KKSIDIH.KTESTNFES~ETVYW.W.KRNQ.LEH.V.VISTT.AARNQVDG.~N.IEAR.DARMFTSTLTVNFV.KE.V.I.C..G..~
bTRGV3-2  L.KVEQ..I.LSTEA~~KKSIDIH.KIESTNFES~DTV.W.R.KLNQVLEH.V.VTSIT.AARNQVDG.~N.IEAR.DARMFTSTLTVNFI.KE.V.I....G..~
cTRGV3-2  L.KVEQ..I.LSTEV~~KKSIDIH.KIESTNFES~ETVYW.R.KRNQ.LEH.V.VISTT.AARNQVDG.~N.IEAR.DARMFTSTLTVNFV.KD.V.I....G.~~~
bTRGV4    ~~~~~~~~~.TRRPD~~~R.VHIS.K.SGVPLEN~AIM.W.QEK..EPLK.IL.GSAN~~SYKLDK~~PNSRLEIDN~KKNGIFYLI.N.VVK..EAT...~~~~~~
cTRGV4    ~~~~~~~~~.TRRPD~~~R.VHIS.K.SGVPLEN~AIV.W.QEK..EPLK.IL.GSAN~~SYKLDK~~PNSRLEMDN~KKNGIFYLV.N.VVK..EAT....C..~
bTRGV5-1  ...~~~~L.LI.S..~~G.....P.....G~..~~~......HE..T..R..L...S.NS.AVL...ISGT..H...~G.....T......QA....I....I...~
cTRGV5-1  ...~~~~L.LI.S..~~G.....P....RR~..~~~......HE.....R..L..HS.NS.PVL...ISGT..H...~G.....T...Q..QA....I....I...~
bTRGV5-2  ...~~~~L.LI.SN.~~G.....P.....G~..~~~......HE.....R..L...S.NS.AVL...ISGT..H...~G.....T.T.V..QA....I....I..N~
cTRGV5-2  ...~~~~L.LI.S..~~G.....P.....G~..~~~......HE.....R..L...S.NS.PVL...ISGA..H...~G.....T......QA....I....I...~
cTRGV5-3  ...~~~~L.LI.S..~~G.....P.....G~..~~~......HE.....R..L...S.NS.PVL...ISGT..H...~G..M..T.T.V..QA....I....I...~
bTRGV6-1  EIRVFQRSVM.GHAG~~~GAITLP.QISRS~VD~~~.V.WFR.L..Q..E..L.LALSKRDV.WD.VLRGD.VNAARGAD.K..TMSLRK.AK..E.L....T.GS
cTRGV6    EIRLSQRSVM.GSAG~~~GAMTMP.QVSRS~VN~~~.V.WFR.L..Q..E..L.LALSKRDV.WD.VLGGD.V.AARGGD.K.CTMSLRK.AK..E.L....A..~
bTRGV6-2  EIRVFQRSVM.GHAG~~~GALTMP.QISKS~VD~~~.V.WFR.L..Q..E..L.LALSKRDV.WD.VLRGD.VNAARGTD.K.CTMSLRK.AK..E.L....T~~~
bTRGV8-1  ...MEEDKL..MRA.~~GSSIVID....GS~~~~~~...W..F...AV.R..L....SNS.AVLD..ISEG..HA..~G..K..T.V.S..QE....T......E.~
cTRGV8-1  ...MEEDKL...RA.~~GSSVVID.....N~~~~~~...W..F...TV.R..L......S.VVL...ISEG..H...~G..K..T.V.S..QE....T......E.~
bTRGV8-2  ...MEEDKL..MRA.~~GSSVVID....GS~~~~~~...W..F...AV.R..L......S.VELD..ISER..R...~G~~~~~KIL.S..QE....T......E.~
cTRGV8-2  ...MEEDKL...RA.~~GSSVVID.....N~~~~~~...W..F...TV.R..L......S.VVL...ISEG..H...~G..K..T.V.S..QE....T......E.~
bTRGV8-3  ...MEEDKL..MRA.~~GSSVVIE....GS~~~~~~...W..F...AV.G..L......S.VVLG..ISEG..H...~G..K..T.V.S..QE....T......E.~
bTRGV8-4  ...MEEDKL..MRA.~~GSSVVMD....GS~~~~~~...W..F.D.AV.R..LH.I.SNS.VVLD..ISEG..H...~G..K..T.V.S..QE....T......E.~
bTRGV9-1  ...ME.DK..I.RA.~~GSS.VIP...PTQNKQ~~~...W..F...TV.R..L......S.VVL.P.ISPG..HG.E~D.DKI.T.V.S..QE....V.R....E.~
cTRGV9    ...ME.NR..I.RAI~~GSS.VIP...PTQNIK~~~...W..F...TI.R..L....S.S.VVL...ISPG..HC.E~G.DKI.K.V.SS.QE....V.R....E.~
bTRGV9-2  ...MEDER..I.RA.~~GSS.VIP...PTQNKQ~~~...W..F...TV.R..L....S.S.VVL...ISPG..HG.E~G.DKM.K.V.S..QE....V.R....E.~
bTRGV10   DIPITQRIT.I.K.K~~GN..FLE.QIKI.KLKKNV.M.W.R.KPEQPLK.VL.ISSNENVVHEQGISE~.R.EAR.WPSNSLVSLR.HQATEE.A.L....C.VE
cTRGV10   DLPITQRIT.I.K.K~~GNM.FLE.QIKI.KLKKNV.M.W.R.KPEQPLK.IL.ISSNENV.HEQGISE~.R.EAR.WPSNALVSLR.H*ATEEEA.L....C.LG
bTRGV-7   QLTLVQTEV....TR~~EKSIIMS.KVFSKDFSK~D...W.R.KPDQGLEQ.LFVL~~DAPALNDL.G..N.LEAR.DKPSSTSTLK.SF..KE.EAT....G.LLA
cTRGV7    QLTLEQPEL....TR~~EKSIIM..KVFSKDFSK~D...W.R.KPDQGLEQ.L.VS~~TAPA.NHL.G..N.LEAR.DAPSSTSTLK.SF..KE.EAT....G.L~~
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B

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              10        20        30        40        50        60        70        80        90        100       110
CapTRDV1-1   -----AQRVIQDQPDISQQLQQSVTLNCQYA-TSWSY---------N--YLFWYKLLPNGEMIYLISQV---SSQQNARNGRYSVNIQKAHKSISLTISDLQLEDSAEYFCALWX-
CapTRDV1-2   -----..K.T...S.V.S.V........R.E-...G----------AY......Q..S.Q.T...Q.Y---.GYS..........F.R.D.....I..S.......K....FC.-
CapTRDV1-3   -----..K.T......AS.V.E.AAM....E-..R-----------.SYNI....Q..S.......G.N---.YSP...D....I.F.RSL.A...I..A.K.....K......EX
CapTRDV1-4   -----....T.......S.V.EV...S.R.E-.IQ-----------SRYNI....Q..S.......G.G---..S....Y....L.L.RSR...........E......K......EX
CapTRDV1-5   -----..K.........FT.I.EA..M..RCE-...-----------SSYNI....QP.S....F.T------------.D.H..I.F.RSC..S.F...T.......K.S...FEX
CapTRDV1-6   -----..S.T......LSEV.KT.....R.E-...NM---------LSY.I....Q..S.Q.T...R.H---.DDG...D......F.............A.E......K...V.R.-
CapTRDV1-7   -----X.K.T.Y....TS.ARNV.I...R.E-...NV---------YTCWI....Q..S...T...P.Y---.ADG.E.D.H....F.....F......S.K..H.GK...T..EX
CapTRDV1-8   -----..K.T...S.V.S..........R.E-....-----------TY..Y...Q..S.Q.T.V.H.G---.QVT...KD.....FK..D.........A.......K.....---
CapTRDV1-9   -----..K....P....NRI.E......R.E-..Q-----------SRY-I....H..S....F.T------------KD..F.IHFDRVR..S.....T.......K......EX
CapTRDV1-10  -----..N.T.N..V.IS.V.KI.......E-I.RNV---------HDYWI...QQ..S...T...H.Y---.EDR.E.D......F...R.........F.K..H..K.....---
CapTRDV1-11  -----..K.T....Y.TS.I....I...R.E-V...G---------YTH......H..S...TF..R.E---...P..........F.R.RN.......A..P....T....V.EX
CapTRDV1-12  -----..N.T.N.TV.IS.V.KI.......E-I.RNV---------HDYWI...QQ..S...T...H.Y---.EDR.E.D......F...R.........F.K..H..K.....---
CapTRDV1-13  -----..K.T...S.V.S.V......S.R.E-....-----------VY..Y...Q..S.Q.T.V.R.G---.EMT...KE.....FK..D...K....A.......K.....---
CapTRDV1-14  -----..K.T.Y....TS.VRNV.I...R.E-...NV---------YTYWI....Q..S...T...H.Y---.EDG.E.D.H....F...Q.F.......S.K..H.GK......EX
CapTRDV1-15  -----..K.T.E.S..IR.V....IFD...G-IG.FT---------YYYSIY...Q..R.Q.TL..H.H---.EHG..A.......F...Y.........A.......K.....X--
CapTRDV1-16  -----..S.T......LSEV.KTI......E-...DM---------LRY.I....Q..S.Q.T...R.Y---.D.G...D......F.............A.E......M...V.RVX
CapTRDV1-17  -----..K.........FT.I.EA..I..RCE-...-----------.SYNI....QP.S....F.T------------.D.H..I.F.RSLI.S.....T....G..K.....FE-
CapTRDV1-18  -----....T.N.....T.V.EI...S.R.E-.VQ-----------SHYHI....Q..S.......G.G---..S....Y......FKVSR.F.........E.......K......EX
CapTRDV1-19  -----..K.T.......S.V.E.A.M....E-..Q-----------.NYNI....Q..S..T....G.N---.YSP...D....I.F.RSR.A...I..A.K.....K......EX
CapTRDV1-20  -----..K.T.N.......V........R.E-...T-----------YY..V...Q..S.Q.T...Q.Y---.ENS..........F...D.....I..S.......K...X----
CapTRDV1-21  ------.K.....Q...S..........R..-.A.T.---------Y--.........................---.............F...R..V...................EX
CapTRDV1-22  -----..N.T.N..V.IS.V.KI....S..E-I.RNV---------HDYQI..CQQ..S...T...H.Y---.EDR.E.D......F...R.........F.K..H..K.....---
CapTRDV1-23  -----..K.T......TR.VE.........E-V..YM---------EYYSI....Q..R.Q.T...R.Y---.EDG...D......F...D.........A..P..T.K.....---
CapTRDV1-24  -----............S..........R.G-..T..S---------Y--...........S.......H..---.............F...R.........................-
CapTRDV1-25  -----..K.T.....V.S.V........R.E-....-----------YY..V...Q..S.Q.T...Q.Y---.ENS.T.........F...D.....I..S.......K....X---
CapTRDV1-26  -----.HT.T.......SXV.EG.....R.K-.R.-----------RSY-V....Q.LS..I.....L.G---..TH..VDVC....F.RS...L.....N.......K...S..EX
CapTRDV1-27  -----..K....P...PSRV.E......R.E-..Q-----------SSYSI....H..S....F.T------------.D....I.FVRSR..S.....T.......K......EX
CapTRDV1-28  -----..K.T.N...V.S.V........R.E-....-----------YY..V...Q..S.Q.T...Q.Y---.GHS..........F.R.D.....I..S.......K...X----
CapTRDV1-29  -----X.K.T......TR.VE.......L.E-V..YM---------SFYSI....Q....Q.T...R.Y---.DVG...D......F...D.........A....T.K.....---
CapTRDV1-30  -----..K.T....Y.TS.I....I...R.E-VRS.G---------YTH......Q..S...TF..R.E---...P..........F.R.RN.......A.......K....V.EX
bTRDV1-aq    -----....A.......S.V.EV...R...E-.RL-----------SSFRI....Q..S.......G..---..S.K..D...TTDL.ISR..................K....V.EX
bTRDV1-ae    -----..K.T.......SEA.EA.....R.E-...-----------SSYNT.....FAS...TF..G.G---.YSL...D.C..I.F..SR.A.................K...T..EX
bTRDV1-x     -----..K.....AG..S.V.E......R.E-..Q.NNILQVVIVTTSYNI..F.Q..S.K..F.T------------.D.H..I.FERSR..S.....N.......K......EX
bTRDV1-be    -----..N.......T............R.G-..I...--------------M.................A---.............F...R.........................EX
bTRDV1-c     -----..Q.T.V.TA..S.V..T.....R.E-V..TT---------DYY.I....Q..R...TL..R.Y---.EVG..........F...D.....I..A.......K......EX
bTRDV1-r     -----..K.T.Y...TTS.VWNI.I...R.E-...NV---------YIYWI....Q..SR..T...H.Y---.ENG.E.D.HH...F.....F......P.K..H.GK......EX
bTRDV1-bb    -----..S.T......LSEV.KA.....R.E-...DM---------FHY.I...TQ..S.Q.T...R.Y---.DAG...D......F...Q.........A.E......M...V.RAX
bTRDV1-aa    -----..K.T....YVTS.I....I...R.E-V.L.R---------YTH..Y...Q..S...TF..R.E---...P..........F...QN........A.......K....V.EX
bTRDV1-ba    -----..K.T...S..IS.V....IF....G-IG.FT---------YYYSIY...Q..R.Q.TP..H.N---.EHG..GY.......F...Y.........A.......K...G.REX
bTRDV1-g     -----..K.T....A..T.V........R.E-....-----------YYN.....Q..S.Q.T...Q.Y---.EHG..........F...D.....I..S.......K.....C.X
bTRDV1-s     -----..K.T...S.V.S.V........R.E-.N.D-----------VY......Q..S.Q.T.V.R.G---.LVT..KED.....FK..D.........A.......K.....CEX
CapTRDV3-1   -----XNVESA.V.TVYKKE.E...VE.KFS-V.YT-----------YYMMY..RQPSS.....M.NIY---.QSKQT.E.....EFY.PNQMLK....A.T.S...I....IREF
bTRDVb3      ---XSNNVESA.V.TVFKKE.E...VE.KFS-V.YT-----------YYMMY..RQPSS.....M.NIY---.QNKHS.E.....EFY.PNQMLK....A.T.S...I....VREF
CapTRDV3-2   -----.DK.T.A.TTVT..E.EAA.IG.T.E-..RT-----------YYT....RQF.G.R.EF..Y.D---.NQA...RD.....F..GK.T......S.H.A...K......EY
bTRAV33      -XSSM.DK.TEA.TTVTAREREA..IG.T.E-..RT-----------YYT....RQF.G.R.EF..H.D---.NNA...RD.....F..GK.I......S.Y.A...K......ED
CapTRDV3-3   ------XWQRNPSSEQ.SST.RRPWTA.VKPHRESR------------LCTGTESF.VE.WNSLF.RMI-----IEQMQG.MVTLEFHWEK.F......S...A..ET...G.LEY
CapTRDV4     ------VVLVPQDQEKTVPV.K....S.SMKGAMFISK----------H.I...RKT.GNT.TFIYREE---GVYGPGFEDNFRGK.DNSTNQAM.E.LKASER.EGF.Y..SDY-
bTRDV2       -XVMS.VVLVPQDQEKTVVV.K.A..S.SMKGATFISK----------Y.I...RKT.GNT.TFIYQEW---GLYGPGFKDNFRGK.VNLTNQAV.EVLKASER.EGF.Y..TDY-
bTRDV3       XKGVLCNQ.T.SS.EQRVAS.SE...L.TFQ-.TY.D-----------PD.Y..RKR.D.VFQFVLYRDNTR.YDADFAQ..FT.LHSMSQ.TFH.V..SVRP..T.T.Y...RLX
CapTRDV2     ~~XSRLSQI..V•.TTVS.ESKNEE.DSS.D~N.LIC~~~~~~~~~~~FV.YCT.V..VRR•LSPN.AH~~~.AEAD.TE.QNF.KF...T.M.R.I.LSS......I.I.C.REX
bTRDVY       -XVSLDVILESETKTLTVL..EPA.FR.NVTGGN.KN-----------YQMS...RNE.NVLTLVYRLS---NNSNENVR.GFKGK.DTSKNQYV.N.QKTTIK.VGT.Y.GSDT-
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C

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                    10        20        30        40        50        60        70        80        90        100       110       120
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CapTRGC1   ERNLAADTSPKPTVFLPSIAEINHDNAGTYLCLLENFFPDVITVSWRVKNDKRALPSQQGNTMKTKDTYMKLSW~LTVTENSMDKQHVCVVK~~~HQKNIGGIDQEIIFPSIKEVVTSAV
bTRGC1     ....E...............................K...........A.........................F..~.................~~~.K......................L.
CapTRGC2   ............................................................................~.................~~~.......................S...
bTRGC2     ....E...............................K...........A...........................~.................~~~.K......................L.
CapTRGC3   D.D.DK.M.....M.....T..KR..N........D...H..K.Y..E.RGNKV....E.K.I..D........~...SG.....E.M.I..~~~.E..KR.TN...L..AVN...S.V.
bTRGC3     D.D.DI.M.....M.....T..KRE.T............H..K.Y..E.RGN.V........V..A.....F..~...SG.....E.I.I..~~~.E..KT.DN...L..PVN.....V.
CapTRGC4   D....T.L.....I..........NKT........K....I.K.Y.KE.DGN...........N.T........~..........E.I...Q~~~.ER..R..N...L....N...S.I.
bTRGC4     D....T.I.....I..........SKT........K....I.K.Y.KE.DGNK............T........~..........E.I...Q~~~.ER.....N...L....N...S.I.
CapTRGC5   D.R.DG.LF....I.F..VE.VKRHS...H....Q.....A.K.Q.KE..GNTI.E.H...II..N.....F..~..L.KKA.E.E...I..~~~.EN.K..R....L.SPVNK~~~~E.
bTRGC5     D.R.DG.LF....I.F..VE.VKLHG...H....Q.....A.KIQ.KE..VNTI.E.Y...II..N.....F..~..L.KKA...E...I..~~~.EN.K..R..Q.L.SPV.K~~~~E.
CapTRGC6   DK..PT.II....I.....N.V..QQTA..............K...KE..GN.V...........NN....F..~.........K.E.M.I.R~~~LE..A..K....L..AVN.~FSPV.
bTRGC6     DKK.P........I.....N.V..QQ.A.......K......K...KE...N.V...........N....TF..~.........KEE.M.I..~~~.E..TR.K....L..AVN..F.PV.
CapTRGC7   D.DFDV.M.....M.....P...C...........KK.X.H..K.Y..E..GN.V.........T..T.I.L.F.*~..M...AWR.NTYVSSNM~RKI.EEKIRRSFFLQLVKSLQLSLLL
CapTRGC8   .........................................................................PDRDRKLYG*TTC..CPN~~~...................~~~~~~

                    130       140       150       160       170       180       190       200       210       220       230
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.....
CapTRGC1   TTTEPPTTEP~~~~~~~~~~~~~PNDCLTDES~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GALQLQLT~TTSAYYTYLLLLLLS~~~~~~AVYFVVIISCVFRRTGVCCDGKIS
bTRGC1     P.......K.PTTEP~~~PTTEP.........KLTGTGSKKACLKDGS~~~~DTNSTKACLEGESST.....MN.SA.......F.F..~~~~~~T................V~~~~~~
CapTRGC2   ..........P~~~~~~~~TTEP.........EVTGTGSKKACLKDESEVTGDTNSTKARLEGENC.......~..............R.~~~~~~......................~
bTRGC2     P....~~~~~~~~~~~~~PTAEP.........~ITDTGSKKACLKDGS~~~~DTNSTKACLEGKSST.....MN.SA............~~~~~~T...A................R...
CapTRGC3   ...K..~~~~~~~~~~~~~~~~~~..G.K.R.EKQVP~~~~~~~~~~~~~~~VANSTKACLKDENDT.....M~N...........IK.~~~~~~T...AI.T..........GNQ.S.
bTRGC3     .A.K..~~~~~~~~~~~~~~~~~~..G.K.K~KKQVP~~~~~~~~~~~~~~~VVNSTKACLKDENNT...H.M~N...........IT.~~~~~~T..L.I.T..........GIQ.S.
CapTRGC4   P...A~~~~~~~~~~~~~~~~~~~.S...NQ~~~~~~~~~~~~~~~~ESEVTADNNSTKVCLEDESDT.....M~N............K.~~~~~~V...II.T........I.....N.
bTRGC4     P...S~~~~~~~~~~~~~~~~~~~.S...NHD.KVTGTGSKKACLKDESEVTADNNSTKVCLKDESNT.....M~N............K.~~~~~~V..CII.T........I.....NL
CapTRGC5   A.~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~RACMKKESDT....FA~S............K.~~~~~~MI..SI.AF...W...IFSN...F
bTRGC5     A.~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~HACMKKESDT....FA~N............K.~~~~~~MI..SI.AF...W...IFS....F
CapTRGC6   A..G..~~~~~~~~~~~~~~~~~~D...Q...~VTDTDFTKVCSRGES~~~~~SRSQQFYKSMSEDTVE...X~YN...........K.~~~~~~.....TTFC.........DT.DSK
bTRGC6     ......~~~~~~~~~~~~~~~~~~....K.G.EVTDTDLTKACARDESE~~~FANSTKACLKDENNTV...F.~YN...........K.~~~~~~TI.~~~~~~~~~~~~~~~~~~~~~
CapTRGC7   NFQIMV*~~~~~~~~~~~~~~~~~~WMKLMFILQKHV*~~~~~~~~~TMETVPKSXXMFILQKHV*TMETIPCSCS.XXTPLPXT.TS.SSSRGQSTLSSLPSASTREQVS.TTGRAX
CapTRGC8   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~........~...............~~~~~~......................
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F. Supplemental figure 4.3 All TCR genes of caprine and bovine aligned.

Deduced amino acid sequence alignment using ClustalW of bovine and caprine TCR genes. A) TRGV, B) TRDV, and C) TRGC. Dashes (-) are gaps introduced for preferred alignment. Periods (.) represent the same nucleotide as shown for the first sequence

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