TCRs Function as Innate-like Receptors in the Bovine T Cell Response against Leptospira

γδ TCRs function as innate-like receptors in the bovine γδ T cell response against *Leptospira*

Running title: γδ TCR repertoire in cattle response to Leptospirosis

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Abbreviations: CDR, complementarity determining region; D, diversity; J, joining; mAb, monoclonal antibody; NGS, next generation sequencing; PE, pycoerythrin; PBMC, peripheral blood mononuclear cells; PRR, pattern recognition receptor; RT-PCR, reverse transcriptase polymerase chain reaction; TCR, T cell receptor; TRD, T cell receptor delta; TRG, T cell receptor gamma; V, variable; WC1, workshop cluster 1.

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Abstract

Leptospira serovar Hardjo are bacterial pathogens of cattle that also cause zoonotic disease in humans. Vaccine-mediated protection against *Leptopspira* serovar Hardjo in cattle is associated with a $WC1^+$ γδ T cell response that can be recalled *in vitro* from PBMC by antigenic stimulation. This provides a model system in which to examine protective vaccine-induced γδ T cell responses in a 'γδ T cell high' species. Only a small proportion (5-10%) of $WC1^+$ γδ T cells from immunized cattle are *Leptospira*-responders, implying that antigen-specificity is determined by clonally-distributed receptors. Both WC1 and TCR are known to be required for *Leptospira*-specific responses by bovine $WC1^+$ γδ T cells. Through variegated expression patterns and V(D)J recombination, respectively, they have the capacity to confer antigen-specificity. In this study we develop and use a high-throughput TCR sequencing approach to study the TRG and TRD repertoires of naïve *ex vivo* PBMC, *Leptospira*-responding and *Leptospira* non-responding $WC1^+$ γδ T cells to examine the potential role of γδ TCR in determining antigen specificity. Our results provide novel insights into the PBMC γδ TCR repertoires in cattle, demonstrating the TRG repertoire to be clonally stratified and essentially 'public' whilst the TRD repertoire shows much higher levels of clonal diversity and is essentially 'private'. TCR repertoire analysis of *Leptospira*-responding $WC1^+$ γδ T cells identifies no signature of TCR-mediated selection, suggesting that TCR functions largely as an 'innate-like' receptor and does not act as a primary determinant of antigen specificity in the response to this pathogen.

Key-points:

- First description of bovine γδ TCR repertoires using NGS approach
- In γδ T cell response against *Leptospira* TCR functional as 'innate-like' receptors

Introduction

Leptospirosis is a re-emerging zoonotic disease of global importance resulting from infection with spirochete bacteria of the genus *Leptospira* (1). Estimates of global disease burdens in humans are of ~1 million cases and ~58,900 deaths per year, although these figures are likely to represent a significant under-estimate due to the high prevalence of leptospirosis in resource-poor tropical low-and-middle income countries (2). Leptospirosis can also affect a range of livestock species including cattle where infection can be present at high frequency and cause substantial losses due to reduced fertility and productivity (3-6). Although a number of Leptospira serovars can cause disease in cattle, those of most concern in many geographical regions are *Leptospira borgpetersenii serovar Hardjo* and *L. interrogans serovar Hardjo (7),* which can be transmitted to humans and, thus, bovine leptospirosis is important as both an economically significant veterinary disease and a public health disease.

Vaccines that are safe and provide effective protection against *Leptospira* are not currently available for use in humans (8). In contrast, a number of commercially-available vaccines for cattle have been developed and are widely used in high-income countries as a method of controlling the disease (9), although most vaccines do not provide protection against the *Leptospira* Hardjo serovars (10). Although antibodies have long been considered as the primary mediators of anti-*Leptospira* immunity and antibody titre remains a parameter used to assess potential vaccine efficacy (8), analysis of the immune responses induced by the bovine vaccines have demonstrated that protection against *Leptospira* Hardjo serovars is dependent on the induction of a cell-mediated immunity associated with potent interferon-γ producing γδ T cell responses (11, 12). This has provided a model system in which to study a pathogen-relevant

protective γδ T cell response in a 'γδ T cell high species' (11-17). Human studies have also shown that γδ T cells constitute a significant proportion of the response to *Leptospira* infection, with the responding cells expressing Vγ9/Vδ2 TCR (18), implying the response to be 'innate-like' (as this is a characteristic feature of this subset of human γδ T cells (19)). This suggests that studies to achieve a better understanding of the γδ T cell responses using the bovine model may assist in the generation of improved vaccines not only for cattle but also humans.

Our previous studies analyzing the role of different γδ T cell receptors in conferring *Leptospira*-specificity, including WC1 and γδ TCR, have shown that bovine *Leptospira*-specific γδ T cells are almost exclusively in the subset that express WC1. WC1 is a pattern recognition receptor that is a member of the scavenger receptor cysteine rich (SRCR) superfamily (11, 12). WC1 expression has been confirmed to be essential for the capacity of γδ T cells to mount *Leptospira*-specific responses, acting as a co-receptor that potentiates TCR mediated signaling (20); however only a small proportion (5-10%) of $WC1^+$ γδ T cells are *Leptospira*-responsive (11, 12). WC1 is a multi-genic family, with 13 different WC1 genes identified in the bovine genome (21), and exhibits variegated expression with individual γδ T cells expressing different numbers and permutations of WC1 genes (16). This has been proposed to provide a mechanism by which WC1 expression can diversify the antigen-specificity of γδ T cell populations and direct the specificity of individual cells. Although there is clear evidence that WC1 expression can influence the pathogen-specificity of γδ T cells (13) and that only a subset of WC1 molecules have the capacity to bind directly to *Leptospira* (14), recent work at single-cell resolution failed to identify a pattern of WC1 expression that could fully account for the *Leptospira*-specificity of a subset of $WC1^+$ γδ T cells (16).

The role of TCR in determining antigen-specificity of γδ T cells is complex with some γδ TCR appearing to function as 'innate-like' receptors (19), others as genuine adaptive receptors (22), whilst others appear to combine both 'innate-like' and adaptive characteristics depending on the ligand (termed 'adaptate'(23)). High-throughput sequencing (HTS) approaches used in a number of recent studies have provided novel insights into how γδ TCRs influence antigen-specificity and function of human γδ T cells (24-28). The high resolution of the TCR repertoire analyses conducted using HTS approaches has also provided greater detail on the spectrum of 'public' vs. 'private' repertoires (i.e. TCR clonotypes shared by multiple individuals and those that are restricted to individuals only) of the γ and δ TR chains (27). Antibody-blocking experiments have confirmed that TCR engagement is essential for *Leptospira*-specific responses by bovine γδ T cells (17), however very little is known about the γδ TCR repertoire of the responding cells and how significant a role TCR plays in determining specificity. Previous studies using low-resolution approaches (V subgroup-specific semi-quantitative PCR, low throughput TCR chain sequencing and CDR3δ spectratyping) have not identified any features that discriminate the TCR repertoires of *Leptospira*-responding and non-responding $WC1^+$ γδ T cells (15, 17).

In this study we developed and applied an Illumina-based HTS approach to study the γδ TCR repertoires of unstimulated naïve *ex vivo* PBMC, *Leptospira*-responding and non-*Leptospira*-responding γδ T cells derived from *Leptospira*-vaccinated cattle to provide high-resolution TCR data that could be used to examine the role of TCR in determining the antigen-specificity of bovine γδ T cells in the response to this pathogen. This study is the first to generate high-volume γδ TCR data for cattle and provides novel information about the TCRγ (TRG) and TCRδ (TRD) repertoires expressed in *ex vivo* bovine PBMC. Comparative analysis of the TRD and TRG

repertoires of *Leptospira*-responsive and non-responsive populations showed no signatures of TCR mediated selection for antigen-specificity. The data suggests that in the response to *Leptospira,* the bovine γδ TCR functions as an 'innate-like' receptor, with antigen-specificity deriving partially from the WC1 co-receptor and perhaps in coordination with other factors that are yet to be elucidated.

Material and Methods

Blood and isolation of PBMC.

Four Holstein cattle, kept in conventional housing at the South Deerfield Farm (University of Massachusetts, US) were vaccinated with *Spirovac* (Zoetis, Parsipanny, NJ, US) at 6 and 7 months of age (two doses given 4 weeks apart according to manufacturer's instructions). Blood samples were collected from the jugular vein of cattle and mixed with heparin as approved by the University of Massachusetts IACUC prior to the first vaccination (used for assessment of *ex vivo* PBMC γδ TCR repertoire) and 2 weeks after the second dose of vaccine had been administered (used for *in vitro* stimulation with *Leptospira* - see below). PBMC were isolated by density gradient centrifugation (Ficoll-Paque Plus, Cytvia, Marlborough, MA, US) according to the manufacturer's instructions.

In vitro stimulation with *Leptospira*.

PBMC extracted from animals 2 weeks after immunization were dye-loaded with efluor670 at a concentration of 5mM/2x10^7 cells for 10 min at 37°C and then washed with serum-containing medium at 4°C. Cells were then cultured at a density of 2.5x10^5/ml in complete-RPMI medium (RPMI-1640 (Gibco, Thermo-fisher, Waltham, MA, US) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, US), 200 mM l-glutamine (Sigma, Saint Louis, MO, US), 5×10^{-5} M 2-mercaptoethanol (Sigma) and 10 mg/ml gentamycin (Invitrogen, Carlsbad, CA, US) with or without 10μg/ml *Leptospira* antigen (sonicated whole cells of *L. borgpetersenii* serovar hardjo-bovis clone RZ33). After culture for 7 days, cells were harvested and stained for WC1 using FITC-conjugated CC15 (anti-panWC1 - Biorad, Hercules, CA, US). Cells were then subjected to flow cytometry for analysis and cell sorting based on a combination

147 of cell phenotype and cell division (as determined by the dilution of the efluor670 dye) using a
148 FACS DIVA (Becton Dickinson, Franklin Lakes, NJ, US). Populations of $WC1^+$ γδ T cells that
149 responded to *Leptospira* by cell division (defined as $WC1^+$/efluor670^{lo}) and those that did not
150 (defined as $WC1^+$/efluor670^{hi}) were isolated by sorting and confirmed to have purity of >95%.
151
152 **Preparation of γ and δ TCR chain libraries.**
153 Flow cytometrically sorted cells were put into Trizol (Invitrogen) and RNA was extracted
154 according to the manufacturer's instructions. RNA purity and concentration were determined by
155 Nanodrop spectrophotometry (Thermo-Fisher). Reverse transcription was performed with
156 Superscript IV (Invitrogen), using a combination of a template switch oligo (AAG CAG TGG
157 TAT CAA CGC AGA GTA CTC TT (ggggg); g bases in parentheses are RNA) incorporating a
158 primer annealing site and a Poly T oligo (TTT TTT TTT TTT TTT TTT TTT TTT TVN), based
159 on the protocol described in Mamedov et. al. 2013 (29). The oligos, dNTPs and RNA were
160 incubated at 65°C for 5 min, immediately placed on ice, followed by addition of RT enzyme,
161 SSIV buffer, DTT, and RNAse OUT (Invitrogen) and incubation of the complete reaction
162 mixture at 50°C for 1 hr and then, 80°C for 10 min.
163
164 Aliquots of cDNA from each sample was amplified by RACE PCR using a universal 5' primer
165 for the sequence incorporated in the switch oligo during cDNA synthesis (AltUPM: GCA GTG
166 GTA TCA ACG CAG AGT) in combination with either a TRD-specific (CTG GCA GCA GGT
167 TGA CTT T) or TRGC5-specific (AAT AAG TCT CCA TCA AGC CTT CTA TC) 3' primer.
168 For each primer a series of primers incorporating different i5 and i7 indices and Nextera
169 ligators/adaptors to facilitate direct loading of PCR amplicons onto an Ilumina platform were

used to allow pooling and subsequent de-multiplexing of data. All primers were ordered from IDT, Redwood City, CA, US. PCR amplification was completed using the Phusion HF system (NEB, Ipswich, MA, US) with reaction mixtures composed of: Phusion HF 5x buffer, 3%DMSO, 10mM dNTPs, 10mM of both 5' and 3' primers, cDNA (1.25µl/50µl reaction), Phusion Hot Start DNA Polymerase (1U/50µl reaction) and dH_2O to final volume. Reaction conditions for both TRG and TRD amplification included an initial denaturation period of 30s at 98 °C, 30 cycles at 98°C for 10s, 65°C for 30s, and 72°C for 30s, and a final extension period of 5 min at 72 °C. After PCR successful amplification was confirmed using gel electrophoresis, products were quantified using D5000 TapeStation analysis (Agilent, Stockport, UK) and normalized quantities of DNA for the TCR amplicon pooled. Pooled amplicons were electrophoresed and the TCR band excised and purified using a Qiagen gel extraction kit (Qiagen, Manchester, UK) and further purified using Ampure beads (Beckman Coulter, High Wycombe, UK) - both according to the manufacturer's instructions. Following an evaluation for quantity and purity by TapeStation analysis the pooled samples were submitted to Edinburgh Genomics (University of Edinburgh, UK) for sequencing using the Illumina MiSeq v3. Platform, with PhiX added at 10% to introduce complexity into the DNA.

Analysis of NGS data.

Following de-multiplexing and initial quality control and trimming (Cutadapt(30)), processing of TCR repertoire data was completed using the MiTCR algorithm (31) with bespoke bovine TRG and TRA/D sequence databases. The TRD database was constructed from the TRA/D gene sequence data described by Connelley *et al.* 2014 (32) and the TRG database from the data in the IMGT homepage (www.IMGT.org) (33) supplemented with additional sequence data for

193 TRGV11_1 and TRGJ5_2 (34, 35). Subsequent analysis of the TCR repertoire data used a

194 combination of functions available through the Immunarch (36) and VDJtools packages (37)

195 **Results**

196 **TCR transcript repertoires of γ and δ chains from PBMC.**

197 To date characterization of the bovine TRG and TRD repertoires has been conducted using low-
198 resolution, low-throughput approaches (15, 17). To enable a more comprehensive assessment, a
199 MiSeq-based 5'-RACE approach, similar to those that have been employed to analyze TCR
200 repertoires in other species including humans (24-28) was designed and implemented.

201

202 Since our primary interest was in the TCR repertoires expressed by $WC1^+$ cells, the TRG chain
203 protocol was designed to specifically analyze the expression of TRG genes found in the TRGC5
204 cassette because these are the only genes expressed by $WC1^+$ γδ T cells in cattle (17). The
205 approach was applied to provide data on the TRG/TRD repertoires expressed within the PBMC
206 of 4 animals (a summary of the read data is provided in Supplementary Table I). Analysis of the
207 TRG transcriptome showed that in all 4 individuals bTRGV3_2 and bTRGV7_1 were dominant,
208 with bTRGV3_1 constituting a smaller proportion of the TRGC5-cassette represented genes, and
209 TRGV4, TRGV10, and the nonfunctional gene TRGV11 at low frequencies (Figure 1a and
210 Supplementary Figure 1a). Of the two TRGJ genes expressed TRGJ5_1 was expressed at a
211 higher level, although in some animals this was only marginally greater than the levels observed
212 for TRGJ5_2.

213

214 The TRD repertoire utilized a much larger range of genes with a different repertoire of TRDV
215 expressed in the 4 individuals examined. TRDV gene usage exhibited a hierarchical structure,
216 with a limited number of dominant genes and a large number of genes expressed at only low
217 levels in the repertoire of each individual (Figure 1b and Supplementary Figure 1a). Although

there was variation among individuals with regard to which TRDV genes were most dominantly transcribed, it was evident that there was a subset of TRDV genes that were consistently over-represented in all or most of the individuals examined (Figure 1c and Supplementary Figure 1b). The proportion of the TRD repertoire that each TRDV subgroup comprised was consistent between the 4 individuals studied - TRDV1 was dominant (82.7-87.4%), TRDVb3 also made a substantial contribution (6-10.9%), but all of the other TRDV subgroups were present at very low frequencies: TRDV2 (~0.2%), TRDV3 (0.1-0.2%) and TRDVY (0.03-0.07%). Notably, the TRD repertoire expressed a number of TRAV genes. This included TRAV33, which has been previously described as a gene expressed in TRD chains (32), and was found to represent 2-3% of the repertoire. Other TRAV genes included members of a large number of TRAV subgroups (TRAV3, 8, 9, 14, 19, 22, 23, 24, 25, 26, 28, 29, 33, 36, 38, 41, X and Y) - however these were all represented at very low levels (total representation of other TRAV genes ranged from 0.1%-0.2%). In all 4 animals, TRDJ1 and TRDJ3 were the dominant TRDJ genes used, with TRDJ2 under-represented.

Analysis of the CDR3 lengths coded for by the TRG and TRD transcripts showed that both adhered to a Gaussian/near-Gaussian distribution (Figure 2). The TRG CDR3 regions were predominantly between 8 and 18 deduced amino acids long whilst, as anticipated from previous data (15), the CDR3 lengths of TRD chains were longer and of a greater size range, predominantly being between 11 and 32 deduced amino acids long (Figure 2). Analysis of the clonality of the TRG and TRD repertoires revealed that they had fundamentally different clonotypic structures (Figure 3a). The TRG repertoire in all 4 animals showed relatively equal proportions of clonotypes that were considered to be small, medium, large or hyper-expanded,

whereas the TRD repertoire was predominantly composed of small clonotypes, with medium, large and hyper-expanded clonotypes constituting a much smaller fraction of the repertoire (Figure 3a). This difference in composition of the TRG and TRD repertoires was reflected in the values obtained from a suite of diversity indices (Figure 3b); Chao1 values for the TRD and TRG repertoires ranged from 60377 to 118686 and 7452 to 13826, respectively, the Hill diversity profile for the TRD and TRG repertoires showed divergence for q values <4, with the TRD and TRG ranging from 21810 to 83830 and 682 to 1029 at q=1 (corresponding to the exponential Shannon-Weiner index), and the D50 diversity index for the TRD repertoires ranged from 10970 to 28334, in contrast to the equivalent values for the TRG repertoires which had a range of 68-156. This contrast in diversity was also seen in other measures such as the Gini-Simpson index (for the TRD this was >0.999 for all 4 repertoires characterized, whilst for the TRG repertoires the value was between 0.979 and 0.987) and the Inverse-Simpson index (ranging from 1177 to 38401 for TRD and 47 to 80 for TRG repertoires). As such, by all measures utilized, the TRG repertoires were found to be substantially less diverse than the TRD repertoires characterized from the same individuals.

Another parameter by which the expressed TRD and TRG repertoires markedly differed was the degree by which they were classified as 'private' (i.e., TCR clonotypes unique to an individual). The TRG repertoires were characterized by high levels of overlap between individuals, with pairwise comparisons showing that ~20% of clonotypes were shared in pairwise comparisons (Table I), representing between 1565 and 2636 shared or 'public' clonotypes (Table II) As a consequence, classical measures of overlap such as the Jaccard index (overlap of clonotypes without any weighting for size of clonotypes) and Morisita-Horn index (overlap of clonotypes

with weighting for clonotype size) were both high; with values of ~0.08 and ~0.94 respectively (Table III and IV - for both indices a value of 0 represents no overlap and a value of 1 represents total overlap). In particular, the Morisita-Horn values indicate a near complete overlap of the TRG repertoires between the individuals. In contrast the values for the overlap observed between the TRD repertoires were considerably lower - the pairwise overlap between individuals was ~0.03% (range = 0.02-0.05% - Table I), the number of shared clonotypes was limited (range = 12-31 - Table II) and the Jaccard and Morisita-Horn indices were ~0.0001 and ~0.0005, respectively (Table III and IV); together indicating that the TRD repertoires were largely 'private'. Further examination of the clonotypic sharing between the individuals showed that 1262 TRG clonotypes (based on deduced amino acid sequences of the CDR3) were identified in all 4 individuals (data not shown). This included many of the largest clonotypes, including TRGV3_2-CAGWDSSTWIKVF-TRGJ5_1, which was the largest in all 4 samples, representing 8.9-13.0% of the repertoire. In contrast, only 4 of the TRD clonotypes were identified in all 4 repertoires and these clonotypes were not the numerically dominant clonotype in any sample (data not shown).

In summary, analysis of the TRG and TRD repertoires expressed by γδ T cells in *ex vivo* PBMC showed that the TRG repertoire, which is formed from a limited number of V and J genes, to have a high level of clonotypic stratification (and, as a consequence, reduced diversity) and to be largely 'public'. In contrast the TRD repertoire utilizes a large number of V genes, has a much less stratified clonotypic structure (and consequently higher diversity) and is largely 'private'. These observations on the contrasting features of the TRG and TRD repertoires were confirmed on analysis of duplicated samples from the same 4 individuals (data not shown).

Analysis of the TCR repertoires of *Leptospira* responding and *Leptospira* non-responding WC1$^+$ γδ T cells.

In previous studies, we have demonstrated that immunization against *Leptospira* generates a population of 'memory' WC1$^+$ γδ T cells that proliferate following *in vitro* stimulation with *Leptospira* antigen (11, 12). Only ~5-10% of WC1$^+$ γδ T cells from immunized animals respond to *Leptospira*, suggesting that TCR may be determining the capacity of individual WC1$^+$ γδ T cells to respond to *Leptospira*. To investigate this the 4 animals in the study were immunized with *Leptospira* and 2 weeks after completion of the immunization course PBMC were isolated, loaded with a cell proliferation dye (eFluor670) and stimulated *in vitro* with *Leptospira* antigen. Following 7 days of culture, subpopulations of dividing and non-dividing WC1$^+$ γδ T cells (as determined by dilution of the proliferation dye - Supplementary Figure 2) were isolated by flow cytometry sorting and their TCR repertoires examined.

Analysis of the relative expression frequencies of different V-J gene permutations in the TRD repertoires of the proliferating γδ T cells (i.e. *Leptospira*-responding) demonstrated apparent differences relative to the repertoires found in the *ex vivo* PBMC (Figure 4a - over- or under-representation identified as divergence from a diagonal line that marks equal frequency of V-J combinations in the responding populations and PBMC). However, the V-J combinations that deviated from the line were generally observed to behave the same way in the equivalent plot comparing representation between the non-dividing γδ T cells and PBMC (Figure 4b). As a consequence, when comparing the representation of different V-J combinations in *Leptospira*-responding and non-responding γδ T cells (Figure 4c) there was a very high-level of correlation (0.955, $p < 2.2 \times 10^{-16}$), suggesting that these changes in V-J usage were not associated with the

antigen-specificity of γδ T cells. Furthermore, when comparing across the 4 individuals there was no V-J combination that was consistently over-/under-represented (e.g., in Figure 4c TRDV1au-TRDJ3 was over-represented in the non-responding γδ T cells in Animal_2, under-represented in Animal_1 and Animal_3 but equally represented in the responding and non-responding populations in Animal_4). The equivalent analysis comparing the TRG repertoires in PBMC, responding and non-responding γδ T cells resulted in similar observations (Supplementary Figure 3). Based on these data there is no clear indication that the specificity of *Leptospira* responses was dependent on TCR utilizing specific combinations of either TRD or TRG V-J genes.

CDR3 spectratyping analysis of the responding and non-responding populations demonstrated that they still largely adhered to a Gaussian distribution in each individual. Although, this 'normal' distribution was retained, it was notable that for the TRD repertoires in the dividing T cells of Animal_3 and Animal_4 there were 'shifts' in CDR3 length to the left and right to give modal CDR3 lengths of 15 and 22 deduced amino acids, respectively (Figure 5a). The absence of major distortion of the CDR3 length distribution indicated that the responding and non-responding populations were broadly representative of the initial $WC1^+$ γδ T cell population and that specificity for *Leptospira* was not driven by TCRs bearing CDR3s of specific lengths. Similarly, analysis showed that the clonotypic structure of TRG and TRD repertoires of both the *Leptospira*-responding and *Leptospira* non-responding populations were comparable to those observed in the PBMC (Figure 5b). The TRG repertoires were composed of roughly equal proportions of small, medium, large and hyper-expanded clonotypes, whilst the TRD repertoires were predominantly composed of small clonotypes with larger clonotypes still only constituting

small fractions of the population (Figure 5b). The fact that a greater fraction of the repertoires in the *Leptospira*-responding $WC1^+$ γδ T cells were not occupied by larger clonotypes indicates an absence of selective proliferation of a small subset of T cells bearing a specific repertoire of TCRs conferring specificity for *Leptopsira*.

This apparent lack of any TCR-mediated selection for *Leptospira*-responsiveness during *in vitro* re-stimulation with antigen was reflected in the absence of any convergence of the repertoires in the *Leptospira*-responding populations (as may have been anticipated in TCR-dependent antigen recognition). Analysis of the overlap, Jaccard and Morisita-Horn indices showed that the degree of similarity between the *Leptospira*-responding TRG repertoires was not different from the degrees of similarity seen between *Leptospira*-non-responding populations or *ex vivo* PBMC or in comparisons across these conditions (Figure 6a). For example, the Morisita-Horn indices for similarity between the *Leptospira*-responding repertoires (n=6) between the *Leptospira* responding and non-responding populations from the same individuals (n=4) and pairwise between all of the *Leptospira* responsive and non-responsive populations (n=16) were 0.9825, 0.9815 and 0.9720, respectively. The values from the other indices supported the observation that the *Leptospira*-responding populations exhibited no evidence of antigen-driven TRG convergence (Figure 6a). This pattern was more marked in the analysis of the TRD repertoires where, for example, the Morisita-Horn value for similarity between the *Leptospira* responding repertoires (0.0018, n=6) was lower than those observed when comparing the similarity between the *Leptospira*-responding $WC1^+$ γδ T cells and the PBMC or *Leptospira* non-responding γδ T cells (0.0059 and 0.0218 for pairwise comparison between all individuals, n=16). Lack of enhanced similarity between the TCR repertoires expressed by *Leptospira* responding $WC1^+$ γδ

T cell populations is evident in the lack of clustering of these repertoires in multi-dimensional scale plotting of similarity index scores (e.g. Figure 6b).

In summary, comparative analysis of the TRG and TRD repertoires demonstrated that antigen-specificity of *Leptospira*-responding $WC1^+$ γδ T cells was independent of TCR with specific characteristics and that TCR, although required for *Leptospira*-specific responses, did not have a definitive role in conferring antigen specificity.

Discussion

The objective of this study was to develop a NGS approach to sequencing the bovine TRG and TRD repertoire and then apply it to the analysis of the γδ TCR repertoires of *Leptospira*-specific $WC1^+$ γδ T cells to determine what role γδ TCR had in conferring antigenic-specificity. T cell receptors are considered to be pivotal in determining the antigen-specificity of T cells, with somatic V(D)J recombination key to generating a highly diverse TCR repertoire that enables individuals to respond to the potentially limitless range of antigens to which they may be exposed. However, this concept of TCR repertoire diversity is largely derived from analysis of αβ TCR repertoires and as yet there has been limited work using high-throughput approaches to study γδ TCR repertoires in 'γδ T cell high' species such as cattle (38).

In the initial part of this study the 'baseline' repertoires of TRG and TRD in bovine WC1+ γδ T cells from PBMC were characterized. We found that the TRG repertoire had a highly stratified clonotypic structure with roughly equal representation of hyper-expanded, large, medium and small clonotypes. As a consequence of the large proportion of the repertoire being occupied by expanded clonotypes, the TRG repertoire had limited diversity. In addition, similarity indices demonstrated that the TRG repertoires expressed in the 4 individuals were virtually identical, with Morisita-Horn values ranging between 0.91-0.95 (where a value of 1 represents complete overlap and 0 no overlap), thus the TRG repertoire in bovine PBMC appears to be essentially 'public'. It was notable that many of the hyper-expanded clonotypes were shared by all 4 animals, including the TRGV3_2-CAGWDSSTWIKVF-TRGJ5_1 clonotype, which was the most dominant in all 4 animals, accounting for 8.9 - 13.0% of TRG sequences. As yet the antigen-specificity of this, or other dominant 'public' clonotypes, and if they have specific

386 biological functions equivalent to that observed for other TCR-defined γδ T cell subsets (such as
387 the human Vγ9+/Vδ2+ population) is unknown, but will be an area of future investigation. The
388 limited diversity and the associated high level of 'publicity', is perhaps to be anticipated as
389 within the TRGC5 cassette (the only TRGC cassette utilized by $WC1^+$ γδ T cells and studied
390 herein) there are only 5 putatively functional TRGV and 2 functional TRGJ genes. Furthermore,
391 although designated as functional, both TRGV4_1 and TRGV10_1 constituted less than 0.5% of
392 the TRG sequence reads in all 4 animals (average of 0.17% and 0.07%) - consistently lower than
393 that recorded for the non-functional TRGV11_1 gene (average 0.77%). This suggests that
394 TRGV4_1 and TRGV10_1 make insubstantial contributions to the TRG repertoire and may
395 actually only be present as co-expressed non-functional TRG chains (in cells that have
396 rearranged and expressed a second, functional TRG chain, as must be assumed for cells
397 expressing TRGV11_1^+ TRG chains). Consequently, it is conceivable that the diversity of the
398 functional TRG repertoire of bovine $WC1^+$ γδ T cells is actually restricted to the products of only
399 3 TRGV genes - TRGV3_1, TRGV3_2 and TRGV7_1, which notably, are phylogenetically
400 closely related (35).
401
402 In contrast, the TRD repertoire was composed predominantly of small, non-expanded clonotypes
403 and had high levels of diversity. For example, the D50 index (the number of the largest
404 clonotypes required to account for 50% of the repertoire) for TRD ranged from 10970 to 28334
405 (in contrast to the equivalent values for the TRG repertoires which were only 68-156). This high
406 level of diversity was accompanied by the TRD repertoires being essentially 'private', exhibiting
407 limited sharing of clonotypes between individuals, with Morisita-Horn values ranging from
408 0.00014 to 0.0011. The capacity to generate highly diverse TRD repertoires is a product of both

409 the large number of TRDV genes in the TRDA/D locus and the capacity to utilize multiple TRD
410 genes in the formation of the 'junctional' region that constitutes the CDR3δ (32, 39, 40). In
411 cattle, most of the expansion of the TRDV genomic repertoire has occurred within the TRDV1
412 subgroup which includes a minimum of 60 TRDV1 genes (32, 41). Previous work has shown
413 TRDV1 to dominate the expressed TRD repertoire in PBMC (32) but the data presented herein
414 provides the first high resolution quantification of the relative expression of the different TRDV
415 subgroups, and of the different members of the TRDV1 subgroup. The representation of the
416 different TRDV subgroups was very consistent between individuals, with TRDV1 dominant
417 (~87-90%) and TRDVb3 and TRAV33 the only other 2 subgroups represented to any substantial
418 degree (~5-10% and 2-4% of TRD sequence reads, respectively). In addition to TRAV33, genes
419 from a large number of other TRAV subgroups were identified in the TRD repertoire. However,
420 these TRAV subgroups combined only represented ~0.1-0.2% of the TRD repertoire, suggesting
421 that, with the exception of TRAV33, no 'dual' TRA/DV genes make a substantial contribution to
422 the bovine TRD repertoire. TRDV2, which in humans is expressed by the dominant γδ T cell
423 subset in the PBMC, is present at only a low frequency (~0.2%) as are TRDV3 and TRDVY
424 (average frequency of 0.16% and 0.05% respectively). Notably, >50% of the TRD repertoire in
425 all 4 individuals was accounted for by the same 9 TRDV1 genes (TRDV1af, ai, am, as, au, bb, e
426 and w); indicating that not only is there strong bias in the utilization of the TRDV1 subgroup by
427 TRD chains, but within TRDV1 there is a strong preferential expression of a limited number of
428 TRDV1 genes.
429
430 The combination of 'public' TRG and 'private' TRD repertoires observed in cattle is similar to
431 the characteristics of the γδ TCR repertoires described in human PBMC (27). Similarly, the

clonotypic structure of the bovine and human TRG repertoires are comparable. One marked difference between the human and bovine γδ TCR repertoires is the clonal structure of the TRD. Recent high-throughput sequencing of the adult human TRD repertoires has demonstrated that in the majority of adults the TRD repertoire of $V\delta1^+$ (i.e. $TRDV1^+$) T cells is highly 'focused', exhibiting very low D_{75} values (i.e. the percentage of the most abundant clonotypes that occupy 75% of the TCR repertoire) of <6 (28). In contrast the $V\delta1^+$ TRD repertoires observed in the umbilical cord-blood of neonates show less clonotypic dominance (described as 'unfocused') and consequently higher D_{75} values (mean of ~14). Evidence that a number of pathogens, such a HCMV (27) and *Plasmodium* (24) can drive clonotypic expansions of $V\delta1^+$ T cells leading to substantial changes in the TCR repertoire, has been used to infer that the increasing clonal dominance observed in human $V\delta1^+$ TRD repertoires is a consequence of post-natal antigenic stimulation driving the clonotypic expansion of γδ T cells bearing cognate TCR (22). Clonally expanded γδ T cells generally transition to an activated $CD27^{lo/neg}/CD45RA^+$ phenotype (28), and there is a correlation between the proportion of γδ T cells with this phenotype and the clonotypic narrowing of the TRD repertoire. The $CD27^{lo/neg}/CD45RA^+$ subset within PBMC γδ T cells is generally expanded by 2 years of age, implying that the 'focusing' of the $V\delta1^+$ TCR repertoire occurs very early in life (42). Strikingly, the equivalent D_{75} values for the bovine TRD repertoires ranged from 42 to 50 (data not shown); consistent with the very small representation of expanded clonotypes observed. Although relatively young (~6 months old at the point of sampling), the calves included in this study would be considered as juveniles and were housed and managed in conventional systems (including routine vaccinations), consequently it would be anticipated that they would have been exposed to a wide range of antigenic stimuli. Thus, although there are similarities between the TCR repertoires of humans and cattle, there are also

455 noticeable differences such as the disparity in the clonotypic structures of the TRD repertoire in
456 humans and cattle and, the absence of a large Vδ2^{+} γδ T cell population in cattle, implying that
457 there may be significant differences in the biology, ligand specificity and function of the TCR
458 repertoires in human and cattle γδ T cell subsets present within the PBMC.
459
460 To study the role of TCRs in determining the specificity of *Leptospira*-specific γδ T cell
461 responses we employed a well-established *in vitro* stimulation model that has been used
462 previously to study various aspects of the γδ T cell response to this pathogen induced by
463 vaccination. This has included studies that have confirmed that bovine γδ T cells proliferating *in*
464 *vitro* after stimulation with *Leptospira* express IFNG, upregulate various chemokine receptors
465 (CCR5 and CXCR3) and effector molecules (e.g. fas, fasL, perforin and GZMB) and have a
466 phenotype reflecting activation and transition to an effector phenotype (CD44^{hi}, CD25^{hi},
467 CD62L^{lo}) (12, 43-45). Consequently, TCR repertoire analyses on proliferating vs. non-
468 proliferating γδ T cells populations provide a robust model to compare the TCR repertoires of
469 *Leptospira*-specific and non-specific γδ T cells.
470
471 Our results demonstrated that the TCR repertoire of *Leptospira*-specific γδ T cells exhibited no
472 signatures associated with TCR-mediated antigenic selection. There was no preferential usage of
473 specific V-J permutations in responding populations compared to non-specific populations, no
474 large expansion of clonotypes leading to alteration in the clonotypic structure and no
475 convergence of the TRG or TRD repertoires between individuals that could be used to infer
476 antigenic-selection of TCR with specific features.
477

Previous studies using spectratpying analysis and semi-quantitative TRDV- and TRGV-subgroup specific PCRs and low through-put sequencing had previously failed to identify any significant changes in TCR repertoire associated with *Leptospira*-specific populations (15, 17); our data provide high resolution TCR analysis to support and confirm these findings. However, antibody blocking experiments have clearly demonstrated that *Leptospira*-specificity is γδ-TCR dependent (17). A prominent features of γδ TCRs is the wide variety in modalities in which they can bind ligands - with structural analysis showing the relative significance of germline and non-germline CDRs varying depending on the γδ TCR/ligand under consideration and if the recognition conforms to an 'innate-' or 'adaptive-' like pattern (reviewed in (46, 47). Recent work has shown that individual γδ TCRs have the capacity to exhibit both 'innate-like' and 'adaptive'-like binding to different ligands, using different components of the TCR (HV4 in the Vγ FR3 region and the Vδ CDR1/CDR3 respectively) to achieve this (26). Notably, γδ T cell responses elicited by 'innate'-like TCR ligation, such as those seen in human Vγ9Vδ2^{+} T cell responses to butyrophilin (BTN) molecules in response to phospho-antigens, drive highly polyclonal responses which, similar to the results presented herein, do not cause any modification of the TCR repertoire (25). Previous data has suggested the WC1^{+} γδ T cell response in *Leptospira*-immunized animals is highly polyclonal - with 5-15% of the *ex vivo* population proliferating following *in vitro* stimulation (11, 12). Intriguingly, the germline encoded HV4 regions of TRGV3-1, 3-2 and 7-1 (i.e. the only TRGV genes that contribute substantially to the WC1^{+} TRG repertoire) show high levels of similarity, with a conserved KIEARKDxxxxTSTLxx motif; conservation of the HV4 region, which has been shown to enable 'innate'-like responses by human Vγ4^{+} T cells (26), suggests a possible mechanism by which a similar 'innate'-like response against *Leptospira* by WC1^{+} γδ T cells may be facilitated. Recent re-consideration of

TCR repertoire data has suggested that such 'innate'-like responses may lead to the formation of 'memory' populations that can provide protection against secondary exposure to a number of pathogens including *Plasmodium*, *Listeria monocytogenes* and *Bacillus subtilis,* however confirmation of this requires higher resolution TCR profiling data being generated for these models (19).

Although previous data has shown that $WC1^+$ expression is required for *Leptospira*-responses by bovine γδ T cells, the fact that only a fraction of $WC1^+$ γδ T cells respond indicates that other factors must contribute to the determination of antigen-specificity. Previous single-cell analyses have indicated that this is not due to the number or permutations of WC1 molecules expressed by individual γδ T cells (16) and our data suggests that it is not the γδ TCR. Consequently, the factors that justify why only a small fraction of bovine $WC1^+$ γδ T cells respond to *Leptospira* are yet to be fully elucidated. γδ T cells are known to be able to express a wide variety of other activating receptors including NKG2D, 2B4, NKp46 and other NK-like receptors (48-50) - the capacity of γδ T cells to respond to *Leptospira* may depend on the cumulative effect of different permutations of such receptors, the TCR and WC1 to achieve the threshold required for activation. Further investigation of this may require the use of high-resolution analyses that are now possible through technologies such as single-cell transcriptomics and CyTOF mass cytometry.

The other outstanding question regarding γδ TCR recognition of *Leptospira* is the identity of the cognate ligand(s). Identification of γδ TCR ligands is notoriously difficult due to the highly diverse nature of the potential candidates and also the different modalities by which the γδ TCR

524 can bind to them (46, 47). In previous studies we have demonstrated that extra-cellular domains
525 of the $WC1^+$ can bind directly to *Leptospira* (14) and recent work using imaging flow cytometry
526 and STORM approaches has demonstrated that following activation, the γδ TCR and $WC1^+$
527 molecules on *Leptospira*-specific γδ T cells co-localise on the cell surface (51). The spatial
528 restrictions indicated by co-localisation of the γδ TCR and $WC1^+$ imply that the two receptors
529 have ligands that are also spatially linked and so we speculate that the γδ TCR ligand is also the
530 *Leptospira*, rather than any MHC-like restriction or self-molecule indicating cellular-stress. A
531 number of microbial molecules have been demonstrated to serve as ligands for γδ TCR,
532 including proteins from Mycobacteria and the SEA (Staphylococcal enterotoxin A) super-
533 antigen, the latter of which activated γδ T cells via germline-encoded components of the Vγ
534 chain (19, 47, 52). Identification of the *Leptospira* molecule(s) that act as γδ TCR ligands would
535 be a critical next step in furthering our understanding of γδ T cell recognition of the bacteria;
536 although non-trivial, application of tools such as TCR-transduced reporter cell lines and protein
537 arrays offer feasible routes to address this challenge. Integrating high resolution analysis of the
538 γδ TCR repertoires employed in the response to defined *Leptospira* antigens will complement
539 other analyses and provide further opportunities to examine the role in γδ TCR in mediating
540 antigen-specificity in the response to this pathogen.
541
542 In summary, in this study we have developed a HTS approach to studying the γδ TCR repertoire
543 in cattle and applied this to analyze the response of γδ T cells to *Leptospira*. Our results indicate
544 that the response is genuinely polyclonal and conforms to an 'innate'-like γδ TCR response with
545 no evidence of TCR-mediated selection. We speculate this response may be mediated through
546 the HV4 domain of the Vγ chain which is relatively conserved in the $WC1^+$ subset in which

Leptospira-specific γδ T cells are found. Further work will be needed to identify what other receptors are contributing to the specificity of γδ T cells for *Leptospira* and to also identify the epitope(s) recognized by the γδ TCR (and $WC1^{+}$). Although the bovine γδ TCR repertoire showed similarities with human γδ TCR, there are a number of fundamental differences which potentially have major functional implications (e.g. the near-absence of any $V\delta2^{+}$ subset in the PBMC). This is similar to other facets of 'innate-like' lymphocytic components of the bovine immune system (e.g. the apparent absence of iNKT cells in cattle), highlighting that although there is often the capacity to translate scientific findings between species, this may not always possible, and there is a need to maintain species-specific research in this ever-increasing important area of immunology.

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Figure Legends

Figure 1. V and J gene usage in TRD and TRG repertoires expressed in naïve *ex vivo* PBMC. Circos plot representation of (A) TRGV and TRGJ gene utilization in the expressed TRG repertoire and (B) TRDV and TRDJ utilization in the expressed TRD repertoire. To enable visualization of the data presented in these plots data from only 2 individuals for the TRG and TRD repertoires is shown (Animal_1 and Animal_2). Data from all 4 animals is shown in Supplementary Figure 1a. For the TRD images the circumferential line indicates the TRDV repertoire; due to the large number of TRDV genes annotation of individual genes requires observation of the figures in Supplementary Figure 1a. (C) Heat map representation of TRDV gene utilization in the expressed TRD repertoire. Although there is variation between individuals in the hierarchy of different TRDV gene usage, a subset of TRDV genes appears to be consistently over-represented in the repertoire including TRDV1w, TRDV1au, TRDV1af and TRDV1ai, as shown. For clarity, only specific genes have been labeled in this figure; a version of the same image with all of the TRDV/AV genes labeled is provided in Supplementary Figure 1b. Circos plots and the heatmap were generated using the PlotFancyVJUsage and CalcSegmentUsage functions in the VDJtools package.

Figure 2. CDR3 virtual spectratype profiles of TRD and TRG repertoires identified in PBMC from 4 individuals. The CDR3 length is shown (in deduced amino acids - x-axis) with the number of clonotypes with CDR3 of each length represented on the y-axis. Each panel shows the data from 4 individuals as detailed in the legend. Spectratypes were generated using the Immunarch package

Figure 3. Clonotypic structure and diversity of TRG and TRD repertoires in PBMC. A) The clonotypic structure of the expressed TRG and TRD repertoires as described by the proportion composed of small, medium, large and hyper-expanded clonotypes in the 4 individuals included in this study is shown. B) Diversity of the expressed TRG and TRD repertoires as described by (B.1) Chao1 values, (B.2) Hill's Numbers - for q = 1 the values for the TRD repertoires are all >20,000, whereas the equivalent values for the TRG repertoires are <200 and (B.3) D50 values. For each of the diversity parameters reported higher values represent a more diverse TCR repertoire. All images generated using the Immunarch package.

Figure 4. Relative frequency of different V-J combinations in the TRD repertoires expressed in PBMC, *Leptospira*-responding and *Leptospira*-non-responding populations from 4 different animals. The relative abundance of reads for TRD chains using different V-J combinations is shown on the x- and y- axes. The diagonal lines represent equal representation in the two conditions being compared (i.e., they have an x, y intercept at 0, 0 and a slope of 1); deviation from these lines reflect discrepancy in the representation of V-J combinations between the compared conditions. Comparison of TRD repertoires expressed in A) PBMC and *Leptospira* responding γδ T cells, B) PBMC and *Leptospira* non-responding γδ T cells and C) *Leptospira* responding and non-responding γδ T cells. In panel C the positions of the TRDV1au_DJ3 combinations on the graph have been highlighted. The correlation co-efficient and p-value of the correlation value are shown for each comparison. Each point on the graph is color-coded to individual animals according to the legend shown.

Figure 5. Clonotypic structure and diversity of TRG and TRD repertoires in PBMC, *Leptospira*-responding and *Leptospira* non-responding WC1^{+} γδ T cells. A) Spectratyping - the CDR3 length is shown (in deduced amino acids on the x-axis) and the y-axis represent the number of clonotypes with CDR3 of each length. Each panel shows the data from 4 individuals as detailed in the legend. B) The clonotypic structure of the expressed TRG (upper panel) and TRD repertoires (lower panel) as described by the proportion composed of small, medium, large and hyper-expanded clonotypes in the 4 individuals included in this study is shown (each animal is shown by a dot, the average by the height of the bar and the SD by the error bars). All figure components generated using the Immunarch package.

Figure 6. Similarity of TRG and TRD repertoires expressed in PBMC, and in *Leptospira* responding and non-responding populations. A) The values of overlap, Jaccard and Morisita-Horn indices calculated for the similarity between the TCR repertoires expressed by these populations are shown for TRG (upper panel) and TRD (lower panel). Comparisons between conditions that are suffixed with 'auto' refer to comparison between the repertoires from the same individual (n=4), those suffixed with 'all' refer to all pairwise comparisons among the 4 animals in the study (n=16) and those without suffixes refer to comparisons between the same conditions across all 4 individuals (n=6). B) Multi-dimensional scaling of the Jaccard index values for comparison of the TRD repertoires. Lack of clustering of the TRD repertoires for *Leptospira* responding γδ T cell populations indicates an absence of selection for TRD sequences in the *Leptospira*-specific responses. Similar MDS profiles were obtained using the other indices for both the TRD and TRG repertoires (data not shown). MDS scaling component of the figure generated using the Immunarch package.