Next generation sequencing of transcribed genes in ruminant γδ T cell populations

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Alexandria Gillespie[1], Kathleen Loonie [1], Fengqiu Zhang[1], James Prendergast[2], Timothy Connelley[2], and Cynthia L. Baldwin[1,3*]

[1] Department of Veterinary & Animal Sciences, University of Massachusetts, Amherst, MA

[2]Roslin Institute, University of Edinburgh, Scotland, UK

[3] Program in Molecular & Cellular Biology, University of Massachusetts, Amherst, MA

[*]**Corresponding Author**: Integrated Sciences Building, 661 North Pleasant Street, University of Massachusetts, Amherst, MA 01003; cbaldwin@umass.edu, Phone 413-687-2864; Fax 413-545-6326.

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Abbreviations: AF, Alexa fluor; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NGS, next generation sequencing; PBMC, peripheral blood mononuclear cells; PRR, pattern recognition receptor; SAGE, serial analysis of gene expression; SRCR, scavenger receptor cysteine rich; TCR, T cell receptor; WC1, workshop cluster 1.

Abstract

Bovine γδ T cells are distinguished by expression of WC1, hybrid pattern recognition receptors and co-receptors to the T cell receptor (TCR), or their absence. WC1 molecules bind pathogens and the ability of γδ T cells to respond to pathogens largely correlates with their expression of particular WC1 genes. Following activation, the TCR and WC1 molecules co-localize and knocking down WC1 abrogates the ability of WC1-expressing γδ T cells to respond to antigen. It is known that these two major populations, $WC1^+$ and $WC1^-$, differ in their TCR gene expression and previous studies showed other differences using semi-quantitative RT-PCR and serial analysis of gene expression. Differences in genes expressed would influence the functional outcome when $WC1^+$ vs. $WC1^-$ γδ T cells respond to pathogens. To identify unique aspects of their transcriptome, here we performed RNA-Seq of flow cytometrically sorted bovine $WC1^+$ and $WC1^-$ γδ T cells and compared them to all mononuclear cells in blood. The greatest differences in gene expression were found between γδ T cells and other mononuclear cells and included those involved in lymphocyte activation and effector processes. Only minor differences occurred between ex vivo $WC1^+$ vs. $WC1^-$ γδ T cells with those gene products being involved in cell adhesion and chemotaxis. After culturing cells from primed animals with *Leptospira* antigens major difference in the transcriptome was evident, with over 600 genes significantly differentially expressed including those focused on cytokine signaling. Unexpectedly, antigen-responding and non-responding populations of $WC1^+$ γδ T cells had few differences in their transcriptomes outside of cytotoxic factors although they had more WC1-1, WC1-2 and WC1-13 transcripts. Through differential gene expression we were able to define properties of ex vivo and stimulated $WC1^+$ cells which will be useful in understanding their functional biology.

1.1 Introduction

γδ T cells have several important differences with regard to how they interact with antigen when compared to αβ T cells. They do not need to see antigen in the context of MHC on an antigen-presenting cell and they express particular pattern recognition receptors (PRR) that act as another mechanism for pathogen recognition. One such PRR is the T cell receptor (TCR) co-receptor known as Workshop Cluster 1 (WC1) found exclusively on bovine γδ T cells. WC1 is a member of the scavenger receptor cysteine rich (SRCR) superfamily and like other SRCR molecules has been shown to physically bind to pathogens [1-4]. In cattle, WC1 is encoded by 13 genes [5] with variegated expression creating subpopulations of γδ T cells [6, 7]. Based on the WC1 genes expressed, the resulting γδ T cell subpopulations respond to different pathogens [8-10] showing the importance of these receptors for directing γδ T cell responses. Moreover, we have recently shown that the TCR and WC1 co-localize following cell activation [11], complementing previous findings showing that when WC1 is knocked-down it abrogates the ability of γδ T cells to respond to antigen [12] while co-crosslinking WC1 with the TCR augments the T cell response [12-14].

However, there is a second population of γδ T cells that do not express WC1 and which are the prominent subpopulation in many tissues including the gut, skin and uterus [15]. Serial analysis of gene expression (SAGE) has been performed on $CD8^-$ and $CD8^+$ γδ T cells which are largely reciprocal to WC1 expression and in that study, it was found that $CD8^-$ (~$WC1^+$) γδ T cells had a more resting but activated phenotype than the other γδ T cells [16]. In pigs,

another artiodactyl species, gene expression analysis using RNA-Seq and single cell analysis has shown that the population of $CD2^+$ γδ T cells that also largely corresponds to the $WC1^-$ cells in cattle [17] are more similar to human γδ T cells [18] while swine $CD2^-$ γδ T cells that correspond to bovine $WC1^+$ γδ T cells do not have a human counterpart. This is logical since humans do not have genes encoding WC1. What was not known is the differences in gene expression between bovine $WC1^+$ and $WC1^-$ γδ T cells or unique features of the $WC1^+$ γδ T cells that proliferate to antigen in recall cultures when cells from vaccinated animals are used.

Using RNA-Seq, here we compared the transcriptome of all blood γδ T cells ($WC1^+$ and $WC1^-$) to peripheral blood mononuclear cells (PBMC) as a baseline to determine if a profile of unique genes is expressed by γδ T cells relative to other lymphocyte populations. To determine differences between γδ T cell subpopulations, ex vivo $WC1^+$ and $WC1^-$ bovine γδ T cells were compared. Differences in gene expression between these major populations of γδ T cells could influence their ability to respond to pathogens and the functional outcome that would ensue. We also determined which genes are expressed by $WC1^+$ γδ T cells when cultured with our model *Leptospira* antigen in recall cultures using cells from primed cattle and asked whether the cells that undergo cell division in response to stimulation have a unique gene expression profile relative to those that do not. While major differences were found between PBMC and all γδ T cells as well as between activated and ex vivo $WC1^+$ γδ T cells, fewer differences were found between ex vivo $WC1^+$ and $WC1^-$ γδ T cells and even fewer key differences between those $WC1^+$ cells that proliferate in recall cultures with *Leptospira* and those that do not.

2.0 Material and Methods

2.1 Blood and cells. Bovine blood was collected from the jugular vein of cattle ages 4-7 years old as approved by the University of Massachusetts Institutional Animal Care and Use Committee. Samples from two adult cattle immunized with the *Leptospira* vaccine *Spirovac* (Zoetis) were used. Animals were vaccinated with 2 doses 1-month apart and boosted in the future if recall responses were found to wane. PBMC were isolated from blood over ficoll-hypaque gradients and suspended 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)). Fresh PBMC to be cultured were washed with phosphate buffered saline 3 to 4 times and then dye-loaded with efluor670 (ThermoFisher) by incubating at a concentration of 5mM/2x10^7 cells for 10 min at 37C and then washed with serum-containing medium at 4C before being put in culture with medium alone or with sonicated *Leptospira borgpetersenii* serovar hardjobovis as described by us previously [19]. At 7 days, cells were assessed by flow cytometry for cell divisions after staining by indirect immunofluorescence for cell surface markers.

For indirect immunofluorescence staining, cells were stained with monoclonal antibody (mAb) CC15-FITC (α-pan-WC1, Biorad) and GB21A (α-TCRδ) with anti-mouse IgG2b conjugated to Alexa fluor 647 as the secondary antibody (Thermofisher). Cells were

analyzed by flow cytometry using a FACS DIVA (Becton Dickinson) and gated according to forward and side scatter. Sorting was performed with a FACS ARIA (Becton Dickinson) into $WC1^+$ and $WC1^-$ ex vivo populations and into $WC1^+$ dividing and non-dividing populations following culture with antigen, with purities ranging from 96-99% for each.

2.2 Library preparation. Preparation was similar to that previously used for a porcine RNA-Seq study [18]. That is, cells were suspended in Trizol (Invitrogen) and RNA was extracted according to the manufacturer's instructions with RNA purity and concentration were determined by Nanodrop spectrophotometry (Thermo-Fisher). Following this, RNA with an RNA integrity number (RIN) of 8 or higher was enriched for mRNA using NEB's Next Poly(A) mRNA Magnetic Isolation and then used to create libraries using NEB Next Ultra II DNA library preparation kit according to the manufacturer's instructions. Samples were multiplexed with the NEB Next Multiple Oligos for Illumina kit using single end indexes (i7) 1-10 and analyzed with a bioanalyzer for molarity. Samples were combined at a 1:1 molar ratio (total of 10nM) before sequencing with a Illumina Next Seq-500 paired-end using the Mid-150 cycle kit.

2.3 Analysis of next generation sequencing data. Data from RNA-Seq runs were analyzed using publicly available Illumina BaseSpace apps to check quality, trim adaptors, align with either the bovine genome (Btau6.0) or human genome (UCSC hg19 PAR-masked), and determine differential gene expression. The following BaseSpace apps were used in the analysis: FASTQ Toolkit, FastQC, RNA-seq Alignment, and RNA-seq

Differential Expression. Sequencing results were processed through FASTQC and adaptor trimming before genome alignment and differential gene expression apps. All samples passed quality check and reads that were low quality with lengths below 75 base pairs were not included in downstream analysis. For analysis of WC1 gene expression, a customized library of the 13 WC1 genes was made for alignment (Roslin Institute). Heatmaps were directly from the Illumina BaseSpace apps indicated or made by using open-source software (http://www.heatmapper.ca/expression/). Ontology graphs were produced from RNA-Seq differential expression profiles and mapped to hallmark gene set phenotypes with Metascape [20].

3.0 Results

3.1 Part I: RNA-Seq of γδ T cell subpopulations: experimental design and quality control.

Others have previously performed comparisons of populations similar to $WC1^-$ and $WC1^+$ γδ T cell gene expression using SAGE to evaluate $CD8^+$ or $CD8^-$ γδ T cells (which largely correspond to $WC1^-$ and $WC1^+$ cells, respectively) [16] and we have used microarrays comparing $WC1^+$ γδ T cells to $CD4^+$ T cells [21]. We expanded on those results here with this more in-depth experimental approach.

To identify unique genes expressed by γδ T cells we made several comparisons using RNA-Seq (Fig 1). First, we compared all mononuclear populations in PBMC (Sample 1) with ex vivo $WC1^+$ and $WC1^-$ γδ T cells (Samples 2 & 3) and then compared these two ex vivo γδ T

156 cell subpopulations to one another. We also compared the ex vivo $WC1^+$ cells (Sample 2)
157 with that same cell subpopulation following culture with antigen in vitro in recall responses.
158 The latter were assessed as those that had undergone cell division and those that had not
159 divided (Samples 4 and 5). Finally, we compared just those $WC1^+$ γδ T cells that proliferated
160 in recall cultures with antigen (Sample 4) with those that had not divided (Sample 5) to one
161 another. These cell populations described in Fig 1A were flow cytometrically sorted as
162 shown in Fig. 1B.
163
164 RNA-Seq results where mapped to both the bovine and human genomes (Fig 1C). This was
165 done since while the human genome annotation is more comprehensive and established than
166 the bovine genome, cattle have some multigenic families of immune system genes that differ
167 from those of humans. For example, the WC1 gene family is absent from humans. However,
168 when the results were mapped to the bovine genome only ~30% of transcripts mapped to
169 coding regions with a large proportion mapping to intergenic sequences (Fig 1C). When
170 mapped to the human genome the percentage corresponding to coding regions rose to over
171 50% with fewer transcripts mapping to intergenic regions. This change presumably resulted
172 from insufficient and incomplete annotation of the bovine Btau6.0 assembly. Thus, going
173 forward we mapped results to both the human and bovine genome annotation to get the most
174 comprehensive representation of transcriptional differences.
175
176 When all samples were compared using Pearson's correlation mapping, we saw clear
177 differences among the transcript profiles of the five sample populations (Fig 2A). This was
178 also affirmed by the Principal Component Analysis (Fig 2B). Unexpectedly, we found that

179 the dividing and nondividing cultured cell populations clustered more closely within an
180 individual animal than by dividing vs non-dividing. This indicated that differences between
181 these populations would be more specific and targeted to cellular functions.
182
183 **3.2 Part II: Comparison of the transcriptomes of ex vivo cells.**
184 **3.2.1 γδ T cells vs PBMC.** We first determined the transcriptional differences
185 between ex vivo γδ T cells and PBMC (Fig 3), the latter of which would include other
186 lymphocyte populations such as CD4 and CD8 αβ T cells, NK cells and B cells. When
187 differential gene expression was analyzed by mapping to the human genome, 1173 genes
188 where significantly different (Supplemental Figure 1). A gene ontology map of those
189 differentially transcribed genes is shown in Fig 3B. Those most significantly differently
190 transcribed were genes involved in lymphocyte activation and immune effector processes.
191 The curated list of genes indicate a mix of functions that are differentially expressed between
192 the two groups (Fig 3A). When focused on genes associated with immune cells and their
193 function, we found more transcripts for *CXCR4, SOX4, RORA* and *ID3* in both $WC1^+$ and
194 $WC1^-$ γδ T cell populations relative to PBMC. Also, some genes were found more highly
195 expressed by $WC1^+$ cells compared to all PBMC including *GATA3, BLK,* and *RUNX2*. This
196 result was similar when we mapped the transcriptome to the bovine genome and indicated
197 that many of the same genes that had higher expression levels in γδ T cells relative to PBMC
198 included *CXCR4,* and *RORA* (Supplemental Fig. 2) and that $WC1^+$ cells specifically had
199 more transcripts of *BLK and ZAP70*.
200

3.2.2 WC1$^+$ vs. WC1$^-$ γδ T cells. Differences in gene expression of ex vivo cells between WC1$^+$ and WC1$^-$ γδ T cells was then evaluated in more depth. Others have shown that resting WC1$^+$ cells had greater expression of *ILR2* and *CD44*, associated with a more activated cell state while WC1$^-$ γδ T cells expressed more *IL-10* and *IL-1β* which could have a role in immune response regulation and mediation of inflammatory responses [16]. We found many fewer genes that were differentially transcribed (86 genes; Supplemental Figure 3) between these two γδ T cell subpopulations than were found when comparing both subpopulations of γδ T cells with PBMC. However, we also found genes that coincided with expression differences between swine CD2$^+$ and CD2$^-$ γδ T cell subpopulations that others had recently reported, subpopulations that largely correlate with WC1$^-$ and WC1$^+$ γδ T cells, respectively [17]. These genes included *IKZF2* and *LCK* being more highly expressed by swine CD2$^+$ γδ T cells and here by the bovine WC1$^-$ γδ T cells (the largely corresponding subpopulation), and greater transcription of *PIK3AP1, TIMP1, S100A4, SAMSN1,* and *ANXA1* in swine CD2$^-$ γδ T cells as well as bovine WC1$^+$ cells here (Fig 4A).

Gene ontology mapping showed that genes significantly differently expressed between WC1$^-$ and WC1$^+$ γδ T cells are involved in several pathways including cell adhesion and chemotaxis (Fig 4B). Genes such as *L1CAM* that encodes a transmembrane protein important for cell adhesion and migration were found more highly transcribed in WC1$^+$ cells. Transcripts coding for *EOMES* and *LCK* were found at higher levels in WC1$^-$ γδ T cells and not found in WC1$^+$ cells. In the WC1$^+$ γδ T cell population we found greater *SOX13* transcription, a transcription factor we have shown to be involved in WC1 expression [22], and higher levels of *BLK* transcripts, a src family kinase that is a candidate for

phosphorylation of WC1 during signaling [23]. In Fig 4C we mapped the transcriptome to the bovine genome and the only genes found to differ in expression levels were *WC1* and *CD163L1* which were higher in the $WC1^+$ cells, as expected. *CD163L1* is either closely related to WC1 or is a WC1 variant and mis-named in the bovine genome since these share many SRCR domains (Fig 4C). While this served as a good control for the sorting of the cell populations it did not provide us with information needed to discern functional differences between these two subpopulations.

3.3 PART III – Comparison of the transcriptome of antigen-cultured cells:

3.3.1 Ex vivo γδ T cells vs. cultured γδ T cells. We next evaluated differential gene expression between $WC1^+$ γδ T cells that were ex vivo with those that had been cultured with *Leptospira* and found 523 significantly differentially transcribed genes when mapped to the human genome (Supplemental Figure 4A). We curated this to a list of genes associated with immune function (Fig 5A) Ontology mapping showed that those most significantly different in transcription included genes involved in cytokine signaling, including IFNγ signaling, as well as cellular responses to stress (Fig 5B). When that group was curated to include genes relevant to immune function, ex vivo $WC1^+$ cells had more transcripts for *EOMES* and *ZBTB16 (*i.e., *PLZF)* than cultured $WC1^+$ γδ T cells (Fig 5A), agreeing with previous results using qRT-PCR [24]. In contrast, cultured $WC1^+$ γδ T cells had more transcripts for *STAT1, STAT3, STAT5A* and *NFKB* and various MHC class I genes. This was consistent when samples were mapped to the bovine genome (Supplemental Figure 4B, Fig 5C) but this latter mapping also revealed more *CCR6* and *IFNAR2* transcription in the cultured $WC1^+$ cell population. When the differential gene expression from mapping to the bovine genome was

247 put into an ontology display the most significant pathways where response to amino acid
248 deficiency, regulation of cell adhesion, response to lipopolysaccharide, and cytokine
249 signaling (Fig 5D).
250
251 **3.3.2 WC1$^+$ γδ T cells proliferating vs. non-proliferating in antigen recall**
252 **cultures.** When we evaluated the overall differences in gene transcription between WC1$^+$ γδ
253 T cells that responded to *Leptospira* stimulation by cell division in *in vitro* recall cultures and
254 those that did not divide (Fig 2B), unlike what we expected WC1$^+$ non-dividing cells closely
255 resembled the dividing cells with regard to their transcriptome profiles. When mapped to the
256 human genome there were only 28 genes that were significantly different (Fig 6A). Those
257 genes with differences in transcription levels were those related to cell division, with the
258 exception of cathepsin W (*CTSW*) which is important for cytotoxic function [25] and was
259 higher in the dividing population, as well as a few unknown genes that related to functions
260 outside of the immune system. The ontology map showed that the difference in transcription
261 levels were not highly significant and the pathways included cold-induced thermogenesis and
262 muscle development which are not obviously related to immune function (Fig 6B). When
263 mapped to the bovine genome, more significant transcriptional differences were found (104
264 genes) including those coding for perforin, granzyme A, CXCR6, and CTSW being higher
265 among dividing WC1$^+$ γδ T cells (Supplemental Figure 5). These genes were curated to those
266 associated with immune functions (Fig 6C). When significantly different genes were put into
267 an ontology map the most significant pathways were found to be NK cell interactions, cell
268 division, organophosphate biosynthetic process, and cytokine signaling (Fig 6D). Based on
269 this, we concluded that cultured and dividing WC1$^+$ γδ T cells would be expected to have

greater cytotoxic function relative to both ex vivo $WC1^+$ γδ T cells and non-dividing cultured $WC1^+$ γδ T cells.

Because expression of particular WC1 genes is associated with responses to particular pathogens [8, 10, 26, 27] we wished to determine if differential transcription of WC1 genes could be detected when comparing the $WC1^+$ γδ T cells from vaccinated animals that had divided in recall cultures with leptospira antigens with those that did not. To do this, a library of the 13 bovine WC1 expressed gene sequences [5, 7] was built since this is not appropriately annotated in the bovine genome used in the Illumina software nor are these genes found in the human genome. When compared, we found that most of the WC1 genes were not differentially expressed consistently by the two animals except for WC1-1, WC1-2 and WC1-13 that had greater expression by the dividing cells from both animals (Supplemental Fig 6).

4.0 Discussion

The goal of this study was to further our understanding of the characteristics of bovine $WC1^+$ γδ T cells including their unique gene programs relative to other mononuclear cell populations in blood and their distinct functional responses, particularly to pathogens. Using RNA-Seq we were able to confirm and extend results from previous studies of bovine γδ T cells [16, 21]. Those studies used the more limited RT-PCR and SAGE techniques that have been shown to be inferior to newer next generation sequencing (NGS) technologies [28]. While those studies detected differences among γδ T cell subpopulations they had less depth

293 or a narrower focus. One shortcoming of this study is the sample size which may have
294 impacted how many genes have been found to be significantly different as well as how
295 different the transcription levels are. Though much of our results coincide with what others
296 have found using different methods. Perhaps, as expected, we found the greatest
297 transcriptional differences were between γδ T cells and all PBMC from blood with
298 differences including genes controlling how the cells are activated and the immune effector
299 processes. Following that, the greatest difference was between ex vivo $WC1^+$ γδ T cells and
300 those cultured in vitro for seven days, with cytokine signaling being the most different. The
301 fewest differences were found between the two major subpopulations of γδ T cells ($WC1^+$
302 and $WC1^-$) although the greatest differences focused on genes whose products are involved in
303 cell adhesion and chemotaxis. This is consistent with the difference in their tissue distribution
304 [29]. Finally, similarly few differences were found when comparing $WC1^+$ γδ T cells that had
305 divided in recall cultures with *Leptospira* and those that had not but here they were
306 associated with cell division, as expected, and immune effector functions including cytokine
307 signaling and cytotoxicity being more highly expressed by the dividing population as well as
308 glycolysis (Table 1).
309
310 When the transcriptome of γδ T cells from blood in humans have been compared to αβ T
311 cells or NK cells through single cell RNA-seq a very similar total number of differentially
312 expressed genes was found (400-500 for each of the two comparison, respectively) as that
313 which we report here between γδ T cells compared to PBMC [30]. Much like for human
314 cells, the results here indicated the functional heterogenicity between ex vivo γδ T cells and
315 PBMC was transcripts that indicate γδ T cells have a more cytotoxic profile. In human single

316 cell RNA-Seq the γδ T cells were clustered most closely with cells associated with cytotoxic
317 function, being localized between NK and CD8 T cells, and they also had a significantly
318 higher expression of cytotoxicity genes agreeing with our results. Our differences included
319 higher expression of *ID3*, a DNA binding inhibitor known to be important for CD8 T cell
320 differentiation into effector and memory T cells, in the γδ T cells.
321
322 The ex vivo bovine γδ T cells from blood had transcripts of genes consistent with different
323 functional lineages. This included *GATA3*, a transcription factor associated with Th2
324 differentiation and response, as well as *RORA,* a gene associated with type 2 innate lymphoid
325 cell development, potentially indicating that the γδ T cell populations have multiple functions
326 or have subpopulations with distinct functions. This supposition agrees with what we know
327 about $WC1^+$ and $WC1^-$ γδ T cells as they are usually found in abundance in different organs
328 and tissues and express different cell surface differentiation antigens including CD8 and CD2
329 [29]. A study by Jutila and colleagues using SAGE [31, 32] evaluated the transcriptome of
330 $CD8^+$ and $CD8^-$ γδ T cells, which coincide largely with $WC1^-$ and $WC1^+$ γδ T cell
331 populations, respectively, and found that resting $CD8^-$ (~$WC1^+$) γδ T cells seemed to be more
332 readily activated with higher transcription of genes coding for IL-2R and when activated with
333 a mitogen had greater transcription of genes such as *galectin-1,* which is associated with
334 apoptotic regulation of thymocytes and activated T cells, and *BLIMP1*, which is primarily
335 found in myeloid cells as a transcription and differentiation factor. Although we did not see
336 differences in transcription of those genes in our studies, we did find that generally $WC1^-$ γδ
337 T cells had more transcripts for *EOMES,* a transcription factor important for CD8 and NK
338 differentiation and function, *CTSW*, a gene that encodes protein associated with the

membrane of natural killer and cytotoxic cells, and *LCK*, a src family kinase that associates with CD4 and CD8. *LCK* expression in WC1$^-$ γδ T cells is consistent with the dichotomy of CD8 expression on WC1$^-$ γδ T cells whereas WC1$^+$ cells usually do not express CD8. As an alternative to *LCK*, WC1$^+$ cells expressed more *BLK*, a src family kinase that we have implicated in WC1 signaling previously [23]. In a recent publication a group looked at the transcriptome of porcine γδ T cells and found differences between CD2$^-$ and CD2$^+$ γδ T cells, a distinction that has largely been made across species as equivalent to WC1$^+$ and WC1$^-$ γδ T cells, respectively [17, 29, 33]. Here we affirmed that several of those genes also have different transcriptional levels among WC1$^+$ and WC1$^-$ γδ T cells in cattle.

Similar to the subpopulations defined by WC1, in mice, research has been performed using single cell RNA-seq that showed some γδ T cell subpopulations are differentiated by expression of SCART1 a close relative of WC1 [2, 34] and that those cells are pre-programmed to become Tγδ17 cells. Another marker that differentiates γδ T cell subpopulations that has been found to be important in thymic fate determination is *SOX13* which has been implicated in murine γδ T cell development into Tγδ17 cells [22, 35]. Here we reaffirmed that transcripts for SOX13 are found at significantly higher levels in WC1$^+$ γδ T cells. In those studies, we had shown that SOX13 may be important for differentiation of WC1.1 or WC1.2 subpopulation in the thymus by controlling WC1 gene expression [24]. Overall, we found that ex vivo WC1$^+$ and WC1$^-$ γδ T cells were the most transcriptionally similar of any of the comparisons we made yet have key gene expression differences consistent with their localization in different tissues [15].

Both appropriate functional differentiation and clonal expansion are necessary to control infections. Following antigen activation in the periphery, CD4 αβ T cell undergo cell division and differentiate to form various functional Th subsets. The differentiation is dependent upon many factors including dwell time with antigen and the cytokine milieux and is dependent on upregulation of key transcription factors such as GATA3 and T-bet. Some subpopulations of γδ T cells differ in fundamental ways from this paradigm. That is, in mice two major γδ T cell subpopulations are pre-programmed in the thymus to be Tγδ17 or Tγδ1 (IFNγ producers). However, there are other murine γδ T cells that are exported from the thymus as functionally naïve and differentiate in the periphery similar to αβ T cells [36, 37]. Here we analyzed $WC1^+$ γδ T cells that had divided in response to culture with antigen and those that had not divided to ascertain if they were transcriptionally unique from one another and whether there were identifiable genes that were driving or associated with the response. The 104 genes we found to be differentially expressed was similar in magnitude to the RNA-Seq data evaluating transcriptional differences among human Th subsets [38]. This included genes that were expected to define the functional subpopulations including *FOXP3, IFNG* and *EOMES*. In an earlier study Blumerman et al. showed that when compared to ex vivo bovine cells, antigen-stimulated $WC1^+$ γδ T cells produced IFNγ and had increased expression of CCR5 and CXCR3 [21, 39], chemokine receptors preferentially expressed on Th1 differentiated CD4 T cells. Together these results implied that the activated $WC1^+$ cells may have a Tγδ1-like function [40]. Although we did not find significantly more transcripts of these chemokine receptors following culture with *Leptospira* here we did find more transcripts for a number of other chemokine receptors such as *CXCR5, CXCR6,* and *CCR8*. However, surprisingly we did not detect more transcripts for IFNγ, which could be due to the bovine

genome used was insufficiently annotated, that these bovine gene sequences were not able to map correctly to the human library, that the sequencing performed here was not deep enough to detect the cytokine transcripts or that the culture time was too long to detect the cytokine transcripts. However, $WC1^+$ γδ T cells that had divided had more transcripts of *ICOS*, a protein associated with co-stimulation and cell to cell signaling; *gzmA*, which is important for cytotoxic function; and *NKG7*, a natural killer granule protein that has been implicated in inflammation. Interestingly, most of these genes were only identified when the library was mapped to the bovine genome and not the human. This could be due to a difference in sequence of genes between human and cattle which would limit what genes are present in data mapped to the human genome. In general, these results agree with those showing that stimulated $WC1^+$ γδ T cells have also been shown to have increased expression of cytotoxic related factors such as Fas, FasL, perforin, and granzyme B when compared to whole PBMC in previous studies [21, 41, 42] in that here we found $WC1^+$ γδ T cells that divided had more transcription of *PFN1*, *GNLY, gzmA* and *CTSW*, genes associated with cytotoxic function. These results showing cytotoxic function are in agreement with single cell RNA-Seq studies of human γδ T cells [43].

Finally, understanding how the γδ T cells recognize pathogens is equally important to understanding their biology. Since we have shown that specific WC1 molecules can bind particular pathogens[14] and that their expression is associated with responses to that pathogen [8, 44], our results here that the dividing cells have a greater expression of three particular WC1 genes is in agreement. Of the three more greatly expressed, only one (WC1-13) has an a1 domain that is known to bind to *Leptospira*, the model pathogen used in these

studies [14]. However, other internal SRCR domains can actually be better binders of leptospires, than the most distal and unique a1 SRCR domains and those 10 internal domains of WC1-1, WC1-2 or WC1-13 have not yet been tested for binding. In other studies, we have shown using STORM that the WC1 and TCR come together in microdomains following cell activation [11]. In other studies, we were unable to find a distinct TCRγ or δ V gene usage or CDR3 sequence associated with dividing $WC1^+$ γδ T cells [45]. Thus, our finding here add support to the hypothesis that WC1 molecules contribute substantially to antigen specificity.

5.0. Conclusions

Defining activation mechanisms and functional outcomes of γδ T cell subpopulation responses is important for understanding the overall role of γδ T cells in combating infectious diseases. It is also key information if γδ T cell activation were to be included as part of vaccine constructs. This later is a reasonable goal given that in many situations γδ T cells develop memory responses [15] including the $WC1^+$ cells in cattle [46]. Overall, we found their gene programs to be most different from other mononuclear populations in blood rather than between the γδ T cell subpopulations defined by WC1 expression. Culture resulted in a significant difference in gene expression relative to ex vivo, with those $WC1^+$ cells that divide in response to antigen stimulation having increased gene expression for chemokine receptors and cytotoxic function along with specific WC1 genes that may be important for pathogen binding and subsequent cell activation.

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8.0 Conflict of interest

There are no known conflicts of interest.

9.0 Author contributions

AG contributed to the design of experiments, acquisition, analysis, interpretation of the data and drafting the manuscript. KL and FZ contributed to building the NGS library. JP built and analyzed the data relative to the WC1 NGS library. CB and TC contributed to the conception and design of the research and obtained funding, interpreted data and drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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589 Table 1. Summary of differences in gene transcription among the various cell types and
590 treatments.

591

	Reference Genome Used for Analysis			
	Human		**Bovine**	
Samples compared	**Pathways that differed most significantly (Ontology map)**	**Examples of notable genes with significantly higher transcription levels**	**Pathways that differed most significantly (Ontology map)**	**Examples of notable genes with significantly higher transcription levels**
Ex vivo: PBMC vs γδ T cells	• lymphocyte activation • immune effector processes	*GATA3, CXCR4, SOX4, RORA, ID3*	--	*SOX4, ID3, CXCR4, BLK, WC1-12, ZAP70, CCL5*
Ex vivo γδ T cells: $WC1^+$ vs $WC1^-$	• cell adhesion • chemotaxis	*EOMES, LCK* in $WC1^-$; *SOX13* and *BLK* in $WC1^+$	--	*WC1, CD163L1*
$WC1^+$ γδ T cells: Ex vivo vs cultured	• cytokine signaling • cellular response to stress • IFNγ signaling	*EOMES, ZBTB16* (Plzf) in ex vivo; *STAT1, STAT3, STAT5, NFKB,* MHC I in cultured cells	• cytokine signaling • NFκB signaling • adaptive immune system • IFN signaling	*NFKAB1, STAT1, CCR6, CXCR5, Ly9, SYK, TLR4, CSF1*
Cultured $WC1^+$ γδ T cells: Divided vs non-divided	• positive regulation of cell death	*CTSW*	• aerobic glycolysis • cytokine signaling • cell division • immune effector process • cell killing	*PRF1, CXCR6, CCL4 & CCL5, gzmaA, ICOS, GNLY, CTSW, NKG7* in divided cells

Figure Legends

Fig. 1 Experimental design. (A) Samples for RNA-Seq were isolated from 5 different cell populations as shown, some of which were ex vivo while others were cultured with antigen for 7 days. (B) Ex vivo bovine PBMC (Sample 1) were sorted based on immunofluorescent staining using anti-pan WC1 mAb (CC15-FITC), anti-δ TCR mAb (GB21A) with secondary α-mouse-IgG2b-AF647 to obtain $WC1^+$ and $WC1^-$ γδ T cells (Sample 2 and 3). PBMC were dye loaded and put in culture with *Leptospira* antigen for 7 days before flow cytometrically sorting, based on the cell division dye and anti-pan WC1 mAb (CC15-FITC) staining, into dividing (Sample 4) and non-dividing (Sample 5) γδ T cell populations. (C) Alignment to different genome when sequences were mapped to either the bovine genome (top) or human genome (bottom). 9 of the 10 samples are shown here (5 samples for each animal)

Fig. 2 RNA-Seq results. (A) Pearson's pairwise correlation of all samples sequenced. (B) Principal Component Analysis comparing all 5 types of samples (see Fig 1 for sample types) from 2 different animals to one another.

Fig. 3 RNA-Seq results of differential transcript expression of PBMC vs. $WC1^+$ and $WC1^-$ γδ T cells. (A) Heatmap of curated immune function associated genes that were significantly different between the two types of samples when mapped to the human genome. (B) Ontology graph showing the level of significance of gene transcription differences and pathways to which they belong.

615 **Fig. 4** RNA-Seq results of differential expression analysis of ex vivo $WC1^+$ and $WC1^-$ γδ T
616 cells. (A) Heatmap of curated immune function associated that were significantly different
617 between the two types of samples when mapped to the human genome. (B) Ontology graph
618 showing the level of significance of gene transcription differences and pathways to which
619 they belong. (C) Heatmap showing the genes with significant differences in transcription
620 when mapped to the bovine genome.

621

622 **Fig. 5** RNA-Seq results of differential expression analysis of $WC1^+$ γδ T cells ex vivo and
623 following culture. (A) Heatmap of curated immune function associated that were
624 significantly different between the ex vivo $WC1^+$ γδ T cells and both dividing and non-
625 dividing $WC1^+$ γδ T cells following culture for 7 days with *Leptospira* when mapped to the
626 human genome. (B) Ontology graph showing the level of significance of gene transcription
627 differences and pathways to which they belong using data from mapping to the human
628 genome. (C) Heatmap of curated immune function associated that were significantly
629 different between the ex vivo $WC1^+$ γδ T cells and both dividing and non-dividing $WC1^+$ γδ
630 T cells following culture for 7 days with *Leptospira* when mapped to the bovine genome. (D)
631 Ontology graph showing the level of significance of gene transcription differences and
632 pathways to which they belong using data from mapping to the bovine genome.

633

634 **Fig. 6** RNA-Seq results of differential expression analysis of $WC1^+$ γδ T cells that had
635 proliferated in culture and those that did not. (A) Heatmap of all genes that were significantly
636 different between the two types of samples when mapped to the human genome. (B)

Ontology graph showing the level of significance of gene transcription differences and pathways to which they belong using data from mapping to the human genome. (C) Heatmap showing curated genes with significant differences in transcript when mapped to the bovine genome. (D) Ontology graph showing the level of significance of gene transcription differences and pathways to which they belong using data from mapping to the bovine genome.

Supplemental Fig. 1 Full heat map displaying RNA-seq differential gene expression results comparing PBMC to WC1+ and WC1- samples when mapped to the human genome.

Supplemental Fig. 2 RNA-Seq results of differential expression analysis of $WC1^{+}$ γδ T cells that had proliferated in culture and those that did not. (A) Heatmap of all genes that were significantly different between the two types of samples when mapped to the bovine genome. (B) Heatmap showing the genes with significant differences in transcription when mapped to the bovine genome with a curated list of genes of particular interest.

Supplemental Fig. 3 Full heat map displaying RNA-seq differential gene expression results comparing WC1+ and WC1- samples when mapped to the human genome.

Supplemental Fig. 4 RNA-Seq results of differential expression analysis of $WC1^+$ γδ T cells ex vivo and following culture. (A) All genes that are significantly different when mapped to the human genome. (B) All genes that are significantly different when mapped to the bovine genome.

Supplemental Fig. 5 RNA-Seq results of differential expression analysis of $WC1^+$ γδ T cells that had proliferated in culture and those that did not. Heatmap shows all genes that were found to be significantly different when mapped to the bovine genome.

Supplemental Fig. 6 Expression of WC1 genes by dividing and nondividing γδ T cells. Using a custom library of bovine WC1 SRCR a1 domain sequences. RNA-Seq results of the sorted populations from 2 animals were analyzed. Red is relatively higher expression while blue is less.