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Research paper

Inverted $CD4^+/CD8^+$ T cell ratio in Boran (*Bos indicus*) cattle

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ABSTRACT

The $CD4^+/CD8^+$ ratio is used as a marker of the immune regulation of T cell balance. When the ratio in peripheral blood is less than 1, this is considered an indication of immune suppression in an individual. Previous work on bovine Peripheral Blood Mononuclear Cells (PBMC) has consistently reported a ratio ≥1 as seen in other mammalian hosts, i.e. higher circulating $CD4^+$ cell numbers than $CD8^+$ cell numbers. However, a consistent inverted $CD4^+/CD8^+$ ratio (<1) was observed in Boran cattle, an African *Bos indicus* breed. The T cell populations were characterized in Boran cattle (n = 52), revealing higher percentages of circulating $CD8^+$ cells (31.9 % average) than $CD4^+$ cells (19.1 % average), thus resulting in the inversion of the expected T cell homeostasis in these animals. The results show that this inversion is not an effect of age or relatedness of the cattle, rather, it was shared by almost all Boran cattle used in this study. Despite this inversion being a feature shared by both males and females, the female cattle had significantly higher $CD4^+/CD8^+$ ratios than the male Boran.

This paper describes the characteristics of the T cell fractions in the study animals and compares the findings to those of other Boran cattle in Kenya, and four other cattle breeds representing African indicine, African taurine, Brazilian indicine and European taurine cattle. We demonstrate that the consistent observation of inverted $CD4^+/CD8^+$ cell ratio was restricted to the Boran.

1. Introduction

T lymphocytes are crucial in adaptive immunity to infectious diseases, where they are involved in the regulation of the immune response and as effector cells. T lymphocytes comprise $CD4^+$ cells, $CD8^+$ cells, gamma delta (γδ) T cells and a small subset of natural killer (NK) T cells. $CD8^+$ T cells are a subpopulation of MHC class I-restricted T cells and are important mediators of adaptive immunity. They include $CD8^+$ cytotoxic T cells and $CD8^+$ suppressor T cells (Farhood et al., 2019; Wissinger, 2017). The CD8 molecule is generally expressed as either an α/α homodimer or an α/β heterodimer and functions as a co-receptor with the T cell receptor (TCR) for binding major histocompatibility complexes (MHC) (Sun and Kavathas, 1997). The CD8 α/β heterodimer plays an active role in the effector functions of $CD8^+$ T cells, whereas the α/α chain is thought to be involved in cell differentiation and in down-modulating the functional avidity of the $CD8^+$ cell (Cheroutre and Lambolez, 2008; Walker et al. (2013)). $CD8^+$ T cells can also co-express γδ receptors and NKp46 receptors, all of which play different roles in immune function. $CD4^+$ T cells are also part of adaptive immunity where they assist in coordinating immune response by stimulating other immune cells such as macrophages, B cells, and $CD8^+$ cells to fight infection. The CD4 molecule is typically not co-expressed with either the γδ or NK receptors.

Immune function cannot be measured by a single biomarker, but instead several parameters are relied on to assess the robustness of an immune system in an individual at a particular timepoint (Gnjatic et al. (2017)). This usually involves measuring different leukocyte numbers, percentages or ratios in circulating blood (Calder, 2007). Ratios commonly used in measuring immune activation include the ratio of memory T cells to naïve T cells (CD45RO: CD45RA) (Yang et al. (2017)), the T cell to B cell ratio (Horny et al. (1993); Steel et al. (1974)) and lymphocyte to monocyte ratio (Li et al. (2012)). Most of these cells can

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be conveniently differentiated using well characterized and described cell surface markers that identify them based on their lineage and stage of differentiation. The ratio between helper and cytotoxic T cells ($CD4^+$ and $CD8^+$, respectively) is used to assess the immunoregulatory balance of T cells (Lu et al., 2015). An abnormal ratio may indicate predisposition to autoimmune disease, cancer or impairment of the body's ability to respond to certain infections (Calder, 2007; McBrien et al. (2018)). For example, it has been shown that in humans, healthy individuals have a $CD4^+/CD8^+$ ratio of ≥ 1, whereas a ratio of < 1 (inverted ratio) has been described as a marker of persistent immune dysfunction, particularly in HIV positive individuals (Gojak et al. (2019)), as well as being an indicator of increased morbidity and death from non-HIV related diseases (McBride and Striker, 2017).

Several studies have reported the $CD4^+/CD8^+$ ratio in bovine blood as >1 (Byoung et al., 2005; Ohtsuka et al. (2004); Rivas et al. (2000)) but this can fluctuate with disease (Ohtsuka et al. (2004)). Different tissues have different $CD4^+/CD8^+$ ratios; however, a change in the peripheral ratio does not affect the ratio in the other tissues (Lee and Woodward, 1996). Byoung et al. (2005) showed that there is a temporary inversion in the circulating $CD4^+/CD8^+$ ratio of cattle infected with *Staphylococcus* super-antigen. This temporary inversion is also seen in cattle infected with Blue Tongue Virus (Ellis et al., 1990) and *Mycobacterium paratuberculosis* (Johne's disease) (Bassey and Collins, 1997). A comparison of Boran and N'Dama $CD4^+/CD8^+$ ratio has been undertaken in trypanotolerance studies, which reported ratios >1, although N'Dama cattle which are tolerant to trypanosomiasis displayed a slightly lower ratio than susceptible Boran cattle in the study (Williams et al., 1991). A study by Flynn et al. (1994) investigated the $CD4^+/CD8^+$ ratio in Kapiti Boran cattle before and during infection with *Trypanosoma congolense* and reported a ratio >1 in healthy state which did not fluctuate greatly during the infection.

A cell phenotyping exercise on healthy Boran cattle that had previously been involved in an experiment that exposed them to *Theileria parva* infection (Sitt et al., 2015) revealed an inverted $CD4^+/CD8^+$ ratio in circulating blood. This was an unexpected finding since these cattle had been free from *T.parva* infection for seven years prior to this experiment. As various forms of theileriosis typically increase the $CD4^+$ T cell population, thereby raising the $CD4^+/CD8^+$ ratio (Aziz et al., 2019), either a normal or increased phenotype (>1 ratio) was expected. With the aid of cell phenotyping techniques, we estimated the abundance of $CD4^+$ and $CD8^+$ cells, including different fractions of $CD8^+$ cells in Boran cattle, considering age, sex, and infection status. This paper reports our findings and compares the cell compositions of five breeds of cattle representing African indicine, African taurine, Brazilian indicine and European taurine breeds of cattle.

2. Methods

2.1. Animals

The Boran cattle used for this study originated from the ILRI ranch, Kapiti, Kenya (n = 52). At the time of sampling, the adult cattle were aged between 3–7 years old, whereas the calves were under 12 months of age. Twenty of these Boran cattle were progeny descended from the same sire, Y, which were initially selected through farm records and subsequently confirmed by genotyping, and were shown by ELISA (Katende et al., 1998) to have no history of *Theileria parva* infection before leaving the Kapiti ranch. The animals were neither tested for exposure to other diseases nor were they suffering any known clinical disease. A further 15 Boran cattle used were unrelated to sire Y, while the relatedness status for seven of the Boran cattle tested was unknown. East African Short-horn Zebu (EASZ) (n = 3), N'Dama (n = 3) and Holstein-Friesian cattle (n = 3) used as comparison groups were from the ILRI Nairobi campus farm. The N'Dama were 4 years old while the Friesians and EASZ were yearlings. An additional 28 Holstein-Friesian cattle (< 12 months old) from the UK (Roslin Institute, Edinburgh) and 3 Nelore cattle (12–14 years old) from Brazil (the Instituto de Zootecnia Faz Experimental de Sertãozinho, Sao Paulo) were included as part of the comparison animals.

2.2. PBMC isolation protocol

The animals were bled by jugular venipuncture and the blood was collected in one of the following ways: in EDTA tubes (10 mL); 60 mL syringes with Alsever's solution in 1:2 Alsever's solution:blood ratio or in 60 mL syringes with citrate phosphate dextrose (CDP) in 1:9 CPD: blood ratio. PBMCs were isolated using the density gradient centrifugation technique. In brief, blood was layered onto Ficoll® Paque Plus (GE Healthcare) solution at a 3:2 ratio of blood:Ficoll and centrifuged at 800−1000x*g* for 30 min at room temperature, with the brake off. The PBMC-rich interface was aspirated using a sterile pipette and transferred to a sterile falcon tube, which was topped up with sterile magnesium-free phosphate buffered saline (PBS) solution. The PBMCs were centrifuged at 500x*g* for 10 min at room temperature with brake on. The supernatant was discarded, and the remaining pellet resuspended in 3 mL of tris-ammonium chloride lysis buffer (5 mL of 0.175 M TRIS pH 7.4, 5 mL of 1.44 M ammonium chloride and 40 mL of distilled H_20) to lyse any remaining red blood cells (RBCs). Following incubation for 5 min at room temperature, the tube was topped up with PBS before centrifugation at 174−300x*g* for 10 min. Two more washes were performed by discarding the supernatant and resuspending the PBMC pellet in PBS followed by centrifugation at 174−300x*g* at room temperature to remove platelets.

After the second wash the PBMCs were resuspended in either complete tissue culture medium (RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 100 units/mL penicillin, 50 g/mL streptomycin, and 50ug/mL gentamicin and 5×10^{-5} β -mercaptoethanol) or FACS buffer (PBS/0.5 % BSA/2 mM EDTA). They were counted on a hemocytometer and pelleted into pre-labelled wells of a 96 well plate at a density of 5×10^5/well (1×10^6/well for the UK Holstein-Friesian and Nelore cattle).

2.3. Cell subtype phenotyping by fluorescent activated cell sorting (FACS)

Surface staining of cells: the cells in the 96 well plates were incubated at 4 °C for 20−30 min with 50−100 µl of each corresponding monoclonal antibody (MAb) – anti-CD4 MAb (IL-A12 [IgG2a]), anti-CD8 α chain MAb (IL-A105 [IgG2a] or IL-A51 MAb [IgG1]), anti-CD8 α/β heterodimer MAb (CC58 [IgG1]), anti-CD3 MAb (MMIA [IgG1]), anti-gamma delta TCR1-N24 delta chain-specific MAb (GB21A [IgG2b]), and anti- NKp46 MAb (GR13 [IgG1]).

At the end of the incubation period the cells were rinsed twice with either pre-chilled RPMI medium or FACS buffer and centrifuged for 3 min at 500−700x*g*. Staining with 50−100 µl fluorescent conjugated isotype-specific secondary antibodies (southern Biotech and Invitrogen Molecular Probes) as specified above was undertaken and the cells were incubated as above before a final rinse with pre-chilled sterile PBS. The secondary antibodies were conjugated with either R-phycoerythrin, fluorescein, allophycocyanin, Alexa Fluor 647 or Alexa Fluor 488, The cells were fixed with 200 µl of 2% paraformaldehyde (fixing was not done for the N'Dama, Roslin Holstein-Friesian and Nelore cattle).

Flow Cytometry was performed on the BD FACSCanto II flow cytometer (Becton Dickinson, Erembodegem, Belgium) for all Kenyan samples. The UK Holstein-Friesian samples were analyzed on a BD LSR Fortessa (Becton Dickinson (BD Biosciences, San Jose, USA)) while the Nelore samples were analyzed using a FACSAria II (Becton Dickinson (BD Biosciences, San Jose, USA)).

All the results were analyzed with FlowJo V10 software. Gating was initially done around the cell population of interest (lymphocytes) on a forward scatter (FSC) by side scatter (SSC) plot. Single cells were then gated from the lymphocyte population using a FSC-A by FSC-H plot. A threshold for each fluorochrome used was then set using the respective

isotype controls, and the percentage of cells showing fluorescence above that of the controls was recorded as the percentage of cells positive for each cell type. For double-stained experiments, i.e. $CD4^+$ and $CD8^+$ cells stained with both MAb MMIA (anti-bovine CD3) and either MAb IL-A12 (anti-bovine CD4) or MAb IL-A51(anti-bovine CD8), a gate was placed on the single cells to select $CD3^+$ cells first followed by detection of either $CD4^+$ or $CD8^+$ cells. The same was done for triple-stained populations where $CD8^+$ cells were selected first before plotting for NK versus γδ cells.

To determine the absolute numbers of $CD4^+$ and $CD8^+$ cells, the cells were double-stained with MAb MMIA (anti-bovine CD3) and either MAb IL-A12 (anti-bovine CD4) or MAb IL-A51(anti-bovine CD8) before gating on FACS. The estimated percentages of $CD4^+$ and $CD8^+$ cells were used to calculate the absolute number of each subpopulation.

2.4. Comparison of mean $CD4^+/CD8^+$ ratio of different demographic groups

Multiple linear regression models were used to test whether the mean $CD4^+/CD8^+$ ratio was different between sexes, adults versus yearlings, sire Y progeny versus unrelated, and breeds.

2.5. Estimation of absolute $CD4^+$ and $CD8^+$ cell counts

Lymphocytes were quantified using a hematology analyzer (Nihon Kohden Hematology analyzer Celltac MEK-6450 for veterinary use). The absolute numbers of circulating $CD4^+$ and $CD8^+$ cells were then calculated by multiplying the respective percentages from the FACS analysis above with the lymphocyte counts from the hematology analyzer.

3. Results

3.1. $CD4^+/CD8^+$ ratio in Boran

In a routine cell phenotyping experiment it was observed that the three Boran cattle had a higher percentage of circulating $CD8^+$ T cells than $CD4^+$ T cells as identified by the MAbs IL-A51 and IL-A12, respectively (Table 1, experiment 1). This being the first report of inverted peripheral $CD4^+/CD8^+$ ratio in healthy cattle, we sought to determine if this cellular phenotype was a feature of animals of this lineage, age group, gender, *T. parva* infection history or a combination thereof. We phenotyped lymphocytes from 48 additional Boran cattle (Table 1; experiment 2). The three animals used in experiment 1 (Table 1) were adult male siblings from the same bull, Y, and had previously been infected with *T. parva* in a vaccine field trial held seven years prior to this experiment (Sitt et al., 2015), but have since been kept in a *T. parva* non-endemic area with strict vector control. The 48 additional cattle used in experiment 2 comprised both adult cattle and weaned calves, some of which were descended from the same sire, Y, while others were unrelated. These animals had no history of *T. parva* infection according to the farm records.

FACS analysis using MAb IL-A12 and IL-A51 revealed that 34 of the 48 animals had an inverted $CD4^+/CD8^+$ ratio (Table 1, experiment 2). The remaining 14 that had a ratio ≥1 were mainly female (only four males) and included both age groups tested in the study. The inverted ratio was observed in both adults and calves, in progeny of the same sire and those of other bulls, and in both sexes. These additional cattle (experiment 2) had no history of exposure to *T. parva* suggesting that the inverted ratio was not a consequence of the past exposure, as could potentially have been the case for the animals used in experiment 1.

A total of 51 Boran cattle had been tested up to this point (Experiments 1 and 2 combined; Table 1) of which 37 (72.55 %) had an inverted $CD4^+/CD8^+$ ratio. The mean $CD4^+/CD8^+$ ratio for all the Boran cattle was 0.87, suggesting that the low $CD4^+/CD8^+$ ratio is a feature of the breed. Although most cattle had an inverted ratio, there was a significant difference in the ratio between sexes after accounting for the effect of age and parentage using a multivariate linear regression model. Female Boran cattle had a significantly higher average $CD4^+/CD8^+$ ratio than that observed for males; 0.99 and 0.76 for females and males, respectively (p value = 0.02).

When the $CD4^+/CD8^+$ ratios of these animals were plotted together (Fig. 1), most of the young cattle clustered together with a lower percentage of both of these cell types in the blood than adults, regardless of sex. Multivariate linear regression modeling showed that although calves had on average lower $CD4^+/CD8^+$ ratios than adults, the cellular phenotype was not related to age (p value = 0.66, having accounted for

Table 1
Percentages of circulating $CD4^+$ and $CD8^+$ lymphocytes and CD4/CD8 ratios in Boran cattle. The results are the means of duplicate samples from each animal.

	Animal	Sex	% $CD4^+$	% $CD8^+$	CD4/CD8 ratio
*Expt. 1					
Adult prog.[a]	2124	m	26.4	29.2	0.9
	2166	m	18.6	30	0.62
	2227	m	23.4	30.7	0.76
					0.76[b]
*Expt. 2					
Adult prog.	919	m	20.8	46.8	0.44
	1138	m	24.8	47.8	0.52
	1148	m	28.8	41.9	0.69
	1456	m	31.6	33.6	0.94
	1481	m	14.2	42.8	0.33
	2674	f	32.8	24.9	1.32
	2727	f	32.8	31.5	1.04
	2762	f	28.4	20.7	1.38
	2888	f	19.4	16.4	1.18
	4772	f	20.9	15.45	1.35
	4947	f	18.4	20.95	0.88
	4526	f	10.3	7.2	1.4
	4830	f	12	12.7	0.9
	4851	m	9.2	14.5	0.6
	4981	m	7.9	16.8	0.5
Calf prog.	4772c	f	11.2	11.4	0.98
	4947c	f	11.5	34.7	0.33
	4978c	m	18.3	22.5	0.81
	5032c	m	11.6	10.8	1.07
	5065c	m	19.7	26.9	0.73
					0.87[b]
	931	m	23.3	39.4	0.59
	946	m	22.4	41.2	0.54
Adult unrel.[a]	764	m	26.8	43.2	0.62
	1146	m	21.1	29.3	0.72
	1183	m	24.2	40.5	0.6
	1275	m	31.9	24.4	1.31
	2737	f	42.6	19.7	2.17
	2888	f	29.3	35	0.84
	2976	f	27.1	16.3	1.66
	4545	f	15.7	33.2	0.47
	4747	f	32	29.9	1.07
	4761	f	21.9	33.1	0.66
	4835	f	23.2	32.2	0.72
	4965	f	24.6	32.6	0.75
	4994	m	12.8	16.4	0.8
	4774	f	14	8.2	1.7
	4795	m	6.7	6.7	1
	4808	f	9.4	10.4	0.9
	4545c	f	11.2	24.7	0.45
	4747c	f	20.3	29.9	0.68
	4761c	f	18.1	22.9	0.79
Calf unrel.	4835c	f	14.3	23	0.62
	4965c	f	8.8	14.5	0.6
	4754c	m	24.3	36.6	0.66
	4756c	m	16.3	18.2	0.9
	4782c	m	20.8	15.5	1.34
	4805c	m	12.4	16	0.77
	4867c	m	24.9	26.8	0.93
					0.89[b]

[a] Prog. indicates progeny of the sire Y and unrel. refers to progeny of other sires.
[b] The mean CD4/CD8 ratio of the respective groups.
* Expt1 refers to experiment 1 while Expt2 is experiment2.

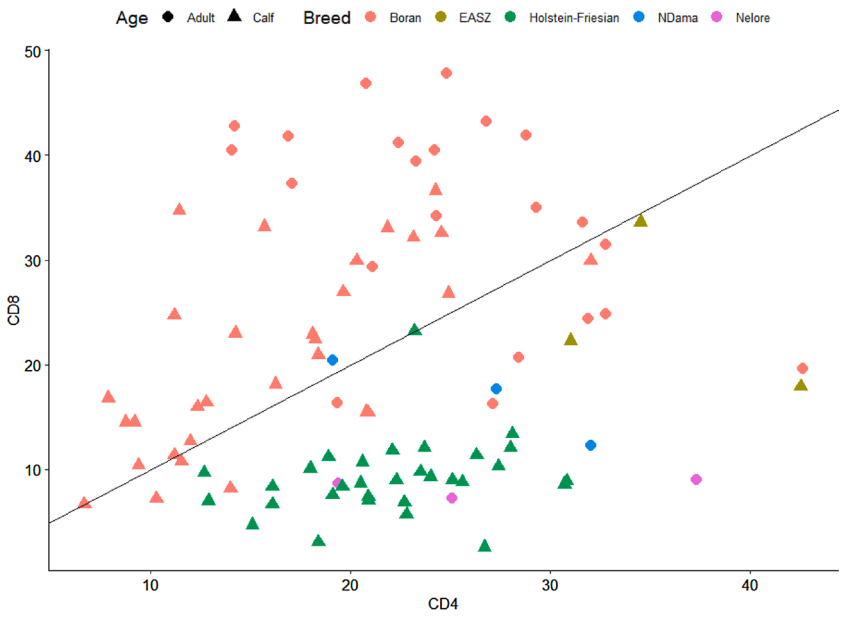

Fig. 1. Plot showing how the percentage $CD8^+$ versus percentage $CD4^+$ cells in the different breeds of cattle tested differ/ cluster from each other. The identity line indicates a CD4/CD8 ratio of 1, above it the ratio is inverted while below the line, the ratio is >1.

the effects of sex and parentage).

Seven female animals were selected whose cells had been cryopreserved when they were weaned calves, and the percentages of $CD4^+$ and $CD8^+$ cells of the same animals as 3-year old adult cows were compared. The mean percentage of $CD4^+$ cells almost doubled from calves to adult age (13.6 % as calves to 22.4 % as adults) while the $CD8^+$ percentage also increased from calf to adulthood (23.0%–28.2% respectively). Despite the increase in $CD4^+$ cell percentages, the $CD4^+$/$CD8^+$ ratio remained inverted in both cases (0.59 and 0.79 on average as calves and as adults respectively).

Progeny of sire Y had lower $CD4^+/CD8^+$ ratio than the unrelated animals (effects of sex and age accounted for in the model), but this was not a significant difference (p value 0.72).

The percentage of $CD4^+CD8^+$ double positive T cells in circulating blood was very small compared to the percentage of circulating $CD4^+$ and $CD8^+$ cells (1.43 % and 1.16 % in three Boran and three Holstein-Friesian cattle, respectively). This population did not substantively affect the CD4/CD8 ratios of these cattle. The $CD4^+CD8^+$ double positive cell has not been widely reported in cattle, however, Maślanka et al. (2018) showed a small population present in bovine peripheral lymphocytes.

3.2. $CD4^+/CD8^+$ ratio comparison in different cattle breeds

To determine whether the inverted ratio was a feature of Boran cattle only, lymphocytes from cattle belonging to four other breeds (Kenyan East African short-horn Zebu (EASZ), N'Dama, Nelore and Holstein-Friesian) were tested and analyzed (experiment 3). The breeds represent African indicine/taurine admixed, African taurine, Brazilian indicine and European taurine lineages, respectively. The results (Table 2) showed that the percentage of $CD4^+$ cells was greater than that of $CD8^+$ cells in all breeds except Boran cattle and one N'Dama animal (ND230).

The mean $CD4^+/CD8^+$ ratios were 0.54, 1.6, 1.7, 3.28, 1.39 and 2.96 for Boran, EASZ, N'Dama, Nelore, ILRI Holstein-Friesian and UK Holstein-Friesian cattle, respectively, which showed that the inverted $CD4^+/CD8^+$ is most predominant in the Boran cattle (Table 2). The ILRI Holstein -Friesians were all males while the UK Holstein-Friesians were all females which may explain the higher ratio in the latter. The means of the two Holstein-Friesian groups were compared using the unpaired two-samples *t*-test and were significantly different (p value = 6.677e-07).

A generalized linear regression model comparing the $CD4^+/CD8^+$ ratios of the different breeds to that of Boran showed that the Holstein-Friesian cattle (combined ILRI and UK groups) have a significantly higher ratio (p value = < 1.33e-12; Boran n = 52, Holstein-Friesian n = 31). Holstein-Friesian cattle $CD4^+/CD8^+$ ratio is on average 5 times higher than that of Boran. Nelore cattle also had a significantly higher $CD4^+/CD8^+$ ratio than Boran (p value = 0.0002), but only three Nelore were tested. The $CD4^+/CD8^+$ ratios of N'Dama and EASZ were not significantly different from that of Boran cattle, though each breed also had only three animals tested.

When plotted together, Boran cattle are the only breed above the identity line with the highest percentages of $CD8^+$ cells (with the exception of one N'Dama animal). The EASZ and N'Dama fall below the identity line, but are closer to a ratio of 1 than the Holstein-Friesian and Nelore, with one ILRI Holstein-Friesian clustering with the EASZ (Fig. 1).

3.3. Fraction of $CD8^+$ cells prominent in cattle with inverted $CD4^+/CD8^+$ ratio

The $CD8^+$ results presented above were obtained using MAb IL-A51 which reacts with an epitope on the CD8 α chain (MacHugh et al., 1993). MAb IL-A105 also recognizes the CD8 α chain and when tested on PBMCs from one Boran (2124) gave results comparable to those of MAb IL-A51 (31.3 % with IL-A51 and 29 % with ILA-105). This provided confidence that the higher Boran $CD8^+$ percentage was not an artifact of the antibody used.

To determine whether the majority of the Boran $CD8^+$ cells expressed either α/α or α/β dimers, the cells were probed with MAb CC58, which recognizes cells expressing the CD8 α/β heterodimer but not the α/α homodimer (Experiment 4). Boran cattle (2166 and 2227) had 25.7 % and 27.3 % of IL-$A51^+$ cells, and 22.7 % and 25 % of $CC58^+$ cells among circulating PBMCs, respectively. Similar concordance between the two antibodies was seen in the Holstein-Friesians, with animals BP009 and BP012 showing 13.6 % and 13.4 % IL-$A51^+$ cells and $CC58^+$ cells being 13.2 % and 13.7 %, respectively. This showed that the majority of $CD8^+$ T cells in these animals express α/β heterodimers and thus are likely to be actively involved in $CD8^+$ cell effector functions.

In order to determine whether the higher percentage of $CD8^+$ cells in

Table 2
Percentages of $CD4^+$ and $CD8^+$ cells and CD4/CD8 ratio of different cattle breeds (experiment 3).

Breed (no.)	Location	Age	Animal	%CD4 cells	%CD8 cells	CD4/CD8 ratio	Average CD4/CD8 ratio
Boran (52)	ILRI	Adults	2124	17.1	37.3	0.46	0.54
			2166	14.1	40.5	0.35	
			2227	16.9	41.8	0.4	
			2317	24.3	34.2	0.71	
	KAPITI	see Table 1 experiment 2		20.09	25.41	0.79	
N'Dama (3)	ILRI	Adults	ND230	19.1	20.4	0.94	1.69
			ND231	32	12.3	2.6	
			ND234	27.3	17.7	1.54	
EASZ (3)	ILRI	Calves	BP053	42.5	17.9	2.37	1.6
			BP063	34.5	33.6	1.03	
			BP064	31	22.3	1.39	
Nelore	BRAZIL	Adults	A1	37.3	9	4.14	3.28
			A2	25.1	7.26	3.46	
			A3	19.4	8.67	2.24	
Holstein-Friesian (31)	ILRI	Calves	BN085	12.9	7	1.85	1.39
			BN101	23.2	23.2	1	
			BN106	12.7	9.7	1.31	
	ROSLIN	Calves	303,649	19.1	7.6	2.51	2.96
			103,675	30.8	8.9	3.46	
			103,640	27.4	10.3	2.66	
			203,634	23.5	9.8	2.40	
			303,635	18.4	3.1	5.93	
			303,670	22.7	6.9	3.29	
			103,647	26.7	2.6	10.27	
			703,625	24.0	9.3	2.58	
			203,683	22.8	5.7	4.00	
			303,628	20.9	7.1	2.94	
			103,626	26.3	11.4	2.31	
			703,681	18.0	10.1	1.78	
			503,630	20.5	8.7	2.36	
			703,660	23.7	12.1	1.96	
			103,668	15.1	4.7	3.21	
			203,676	25.1	9.0	2.79	
			403,664	16.1	6.7	2.40	
			603,666	16.1	8.4	1.92	
			603,449	22.3	9.0	2.48	
			703,450	19.6	8.4	2.33	
			103,451	30.7	8.6	3.57	
			703,457	28.1	13.4	2.10	
			103,458	22.1	11.8	1.87	
			203,459	25.6	8.8	2.91	
			703,464	18.9	11.2	1.69	
			103,465	20.6	10.7	1.92	
			703,471	20.9	7.4	2.82	
			203,473	28.0	12.1	2.31	

the Boran cattle was due to an increase in the absolute numbers of circulating $CD8^+$ cells or due to a decrease in numbers of $CD4^+$ cells, we quantified lymphocytes and calculated the absolute numbers of circulating $CD4^+$ and $CD8^+$ cells (Experiment 5). The results (Table 3) indicate that the number of $CD8^+$ cells in the Boran animals was approximately twice that of the $CD4^+$ cells, and higher than the numbers of $CD8^+$ cells observed in the Holstein-Friesian cattle. A comparison between the two breeds revealed that the numbers of circulating $CD4^+$ cells were similar in both groups. These results indicate that the inverted $CD4^+/CD8^+$ ratio seen in the Boran cattle is due to an increase in absolute numbers of $CD8^+$ T cells in circulating blood, with maintenance of circulating $CD4^+$ T cell numbers similar to the Holstein-Friesian cattle.

3.4. The inverted $CD4^+/CD8^+$ ratio in Boran is associated with higher percentage of $CD8\alpha\beta^+$ $\gamma\delta^+$ double positive cells and $CD8\alpha\beta^+NK^+$ double positive cells

The CD8 molecule can also be co-expressed with both $\gamma\delta^+$ and $NKp46^+$ molecules in some subsets of bovine PBMCs (Connelley et al., 2014; Park et al., 2015.), whereas the CD4 receptor is not. An experiment was undertaken to investigate if the higher number of circulating $CD8^+$ T cells in the Boran cattle was due to an increase in a specific $CD8^+$ cell fraction (Experiment 6). PBMCs were subjected to multi-colour flow cytometric analysis using a combination of MAbs specific for CD8 α chain (ILA-51/ILA105), CD8 αβ chain (CC58), NKp46 (Gr13) and the γδ TCR (GB21A).

Table 3
Estimated numbers of circulating $CD4^+$ and $CD8^+$ in Boran and Friesian cattle (experiment 5).

	Animal	Lymphocyte counts (10^3/ul)	% $CD3^+$ $CD4^+$ lymphocytes	CD4 counts (10^3/ul)	% $CD3^+$ $CD8^+$ lymphocytes	CD8 counts (10^3/ul)
Boran	2124	6.1	18.70%	1.14	31.80%	1.94
	2166	3.1	14.50%	0.45	31.75%	0.98
	2227	2.5	17.35%	0.43	38%	0.95
	2317	3	23.30%	0.70	34.20%	1.03
Holstein-Friesian	BN085	3.8	14.40%	0.55	12.20%	0.46
	BN101	3.2	26.35%	0.84	21.40%	0.68
	BN106	3.8	11.95%	0.45	16.50%	0.63

The average percentage of CD8α$^+$ T cells in three Boran and three Holstein-Friesian cattle was 33.86 % and 13.28 %, respectively, while the average percentage of CD8αβ$^+$ cells in the same animals was 33.83 % and 13.37 %, respectively. This showed that almost all circulating CD8$^+$ cells in these animals at the time were αβ$^+$. The mean total CD8α$^+$γδ$^+$ cells was also higher in the Boran than Holstein-Friesian cattle with 7.03 % vs 1.22 %, respectively (Supplementary Fig. 1). The majority of these CD8α$^+$γδ$^+$ cells were also CD8αβ$^+$: 6.53 % vs 3.12 % for Boran and Holstein-Friesian, respectively. The CD8$^+$ NK T cell percentages were lower than the other cell percentages with a mean of 1.2 % vs. 0.58 % in Boran and Holstein-Friesian cattle, respectively.

The γδ$^-$CD8αβ$^+$ cell percentages were 26.8 % and 12.15 %, respectively, for Boran and Holstein-Friesian cattle while CD4$^+$ percentages in this experiment were 19.43 % and 16.27 % for Boran and Holstein-Friesian, respectively. All CD8$^+$ cell fractions were higher in the Boran than Holstein-Friesian cattle. These data are represented in supplementary Fig. 1.

PBMCs from 29 Boran cattle were triple stained with monoclonal antibodies against CD8 α chain, NKp46 and TCR δ chain for γδ cells, in order to obtain the percentage of CD8α$^+$ cells that are γδ and NKp46 negative (CD8α$^+$ γδ$^-$ NK$^-$). The CD4$^+$/total CD8$^+$ ratio was then compared with the CD4$^+$/ CD8α$^+$ γδ$^-$ NK$^-$ ratio. In this particular experiment the average percentage of CD4$^+$ cells was 21.1 %, total CD8α$^+$ cells was 24 % and CD8α$^+$γδ- NK$^-$ was 17.7 %. The average CD4$^+$/CD8$^+$(ILA51) ratio was 0.9, while the average CD4$^+$/ CD8$^+$ γδ-NK$^-$ ratio increased to 1.3.

Taking into account that removing the CD8α$^+$γδ$^+$ NK$^+$ resulted in reversal of the CD4/CD8 ratio to >1 and that almost all CD8$^+$ cells are CD8αβ$^+$, we conclude that the inverted ratio is likely to be associated with an increase in the CD8αβ$^+$γδ$^+$ double positive cells, and to a lesser extent by an increase in the CD8$^+$ NK$^+$ double positive T cell populations in Boran cattle.

4. Discussion

The ratio of CD4$^+$/CD8$^+$ cells in peripheral blood is typically ≥1 in both humans and livestock. Studies on Holstein-Friesian cattle have reported a ratio of >1 among healthy animals. Denholm et al. (2017) reported a mean CD4$^+$/CD8$^+$ ratio of 2.38 with a standard deviation of 0.73 in healthy Holstein-Friesian cattle in Scotland. Their study showed that T cell percentages and the CD4$^+$/CD8$^+$ ratio are significantly heritable traits in cattle, hinting at the possibility of improving this ratio via breeding. The same study showed that CD4$^+$/CD8$^+$ ratio can be an effect of the environment on a cow (Denholm et al., 2017). Our study reported a ratio of 1.39 and 2.96 in the ILRI and Roslin Holstein-Friesian cattle, respectively. We suspect that the large variance may be due to a combination of environmental effects and sex differences, since the ILRI Holstein-Friesian were all males while the Roslin animals were all females. Our analysis based on the Boran cattle show that female cattle have higher CD4+/CD8+ ratio than male cattle. A comparison between Nelore cattle and other local zebu breeds, as well as European taurine cattle, in Brazil, showed all breeds to have a CD4$^+$/CD8$^+$ ratio of >1 (Macêdo et al., 2013), with Nelore having a higher ratio than most breeds except the Guzera breed of cattle in the area.

Our study results showed that the inversion in the CD4$^+$/CD8$^+$ ratio in these Boran cattle is as a result of an increase in absolute numbers of circulating CD8$^+$ cells, particularly the CD8αβ$^+$γδ$^+$ T cell fraction, without a reduction in peripheral CD4$^+$ cell count. The CD8$^+$NKp46$^+$ cell fraction was also slightly higher in the Boran than in the Holstein-Friesian comparison group. CD8$^+$ cells that are γδ$^+$ and NKp46$^+$have the potential to act in an MHC unrestricted manner which means these cells have the ability to respond to pathogens and infected cells faster than normal CD8 cells would. It had been thought that human CD8$^+$ T cells do not express γδ and NK receptors (Kalyan and Kabelitz, 2013), however, recent work by Kadivar et al. (2016) showed the presence of both CD8αβ$^+$ and CD8αα$^+$ γδ T cells in circulating blood of humans. Similarly, cattle express the γδ and NK molecules on a proportion of CD8$^+$ T cells (Connelley et al., 2014; Maggioli et al., 2015). However, it should be noted that the proportion of circulating γδ$^+$ T cells is low in humans, representing <5% of circulating lymphocytes, whereas in cattle circulating γδ$^+$ T cells compose 15–60 % of total circulating lymphocytes, making them an important cell type in these animals (Guzman et al., 2014).

An examination of the characteristics of the increased CD8$^+$ T cell population in the Boran cattle showed an increase in CD8αβ$^+$γδ$^+$ T cells in the Boran relative to the Holstein-Friesian cattle used for comparison. The high percentage of CD8$^+$ T cells in Boran can also be attributed to an increase in CD8$^+$γδ$^+$NK$^+$, suggesting that the inversion of the CD4$^+$/CD8$^+$ ratio in these Boran cattle may play a functional role in response to infections. Various research studies have described temporary inversion of the CD4$^+$/CD8$^+$ ratio during some disease states in the bovine (Byoung et al., 2005; Ellis et al., 1990). We did not check these animals for exposure to other diseases except *T. parva* infection, for which we had the history of in the Boran cattle. The animals were kept in an area with strict ectoparasite and helminth control practices. However, Kapiti farm is in an area endemic for a number of cattle diseases such as babesiosis, anaplasmosis, foot and mouth disease as well as malignant catarrhal fever among others, the latter two being viral infections while the former are protozoal and rickettsial infections, respectively.

Existence of higher numbers of activated CD8$^+$ cells in circulation has been linked to a strong response to viral infection through suppression of viral replication in humans (McBrien et al., 2018). CD8$^+$ T cells can also be activated by certain agents such as *Staphylococcus* superantigens, which in turn decrease CD4$^+$ T cell proliferation resulting in a temporary inversion of the CD4$^+$/CD8$^+$ ratio in peripheral circulation of affected bovines (Byoung et al., 2005). Our study indicates that the Boran cattle that were analyzed have higher numbers of CD8$^+$ T cells than CD4$^+$ T cells in circulation due to a general increase in certain CD8$^+$ sub-populations. Most CD8$^+$ cells expressed the α/β chain and thus may play an active role in the immune response. It is possible that the existence of greater numbers of CD8$^+$ T cells in the Boran provides an advantage in their response to infection, although this requires further study.

Although an inverted ratio has been reported to be linked to immunological response to a disease, the few other studies (Flynn et al., 1994; Williams et al., 1991) that have examined the CD4/ CD8 ratio in Boran cattle have reported a normal ratio. It should be noted that, in Flynn et al., the samples measured were from afferent lymph rather than blood, and in Williams et al., the CD4/ CD8 ratio was low – approximately 1. It is unlikely that a particular ongoing or recent infection was the reason for the inversion of the CD4/ CD8 ratio, because the cattle were of different sex and age groups, and were sampled at different times.

Although the Boran cattle were all resident on one farm and we cannot formally rule out that this has contributed to the inverted ratio, given the spread of age, sex and sampling time, the most parsimonious explanation is that the inverted ratio is a feature of the breed. The Holstein-Friesian cattle, the bulk of which were from Edinburgh, UK, and the Nelore from Brazil were the two breeds with significantly higher CD4$^+$ and CD8$^+$ cell percentages. The EASZ and N'Dama from ILRI (notably with similar environment and management system to the Boran cattle tested) had a higher ratio that was within the normal range (>1) but not significantly different from that of Boran cattle (although this lack of significance could be due to the low numbers of EASZ and N'Dama tested).

Studies that have compared the CD4$^+$/CD8$^+$ ratio between male and female cattle have shown that females generally have a higher ratio than males, although they usually fall in the same range. Uppal et al. (2003) undertook a study on healthy adult humans in India and showed that women had a significantly higher CD4$^+$/CD8$^+$ ratio than healthy males in the same population. Bosire et al. (2013) corroborated this finding in a Kenyan population, with CD4$^+$/CD8$^+$ ratios of 1.51 and 1.69 for men

and women, respectively. They attributed this difference to women having on average higher circulating $CD4^+$ cell counts than men, while men have higher $CD8^+$ counts on average. Our statistics suggest that the same is true for cattle. The female Boran cattle had significantly higher $CD4^+/CD8^+$ ratio than the males. We did not find literature that states the same or opposite for cattle.

5. Conclusion

This paper describes a phenomenon observed in Boran cattle and absent in other cattle breeds tested. Boran cattle have a lower $CD4^+/CD8^+$ ratio than Holstein-Friesian, N'Dama, EASZ and Nelore cattle breeds. This inversion is due to an increase in absolute $CD8^+$ cell numbers in the Boran without a decrease in $CD4^+$ cell counts. This paper describes the functional $CD8^+$ cell fractions that are higher in Boran cattle and associates the inversion of the $CD4^+/CD8^+$ with an increased percentage of $CD8\alpha\beta^+$ $\gamma\delta^+$ and $CD8^+$ NK T cells. $CD4^+/CD8^+$ ratio is a heritable immune trait that can be influenced by the environment the cattle are brought up in or by exposure to disease. The Boran cattle used in this study were of different ages, sexes and were sampled at different times thus reducing the likelihood of this inversion being a consequence of an ongoing or recent infection. This said, we did not screen for any endemic diseases in the cattle. It is possible that the inverted ratio could be a consequence of a previous infection that is endemic in the farm where these Boran cattle derived from (Kapiti farm). Alternatively, this could be an inherited adaptation that has evolved to cope with one of the endemic diseases around Kapiti farm, which has been passed down in majority of the cattle from the farm over time. Further studies need to be conducted to narrow down the purpose/ effect of this innate $CD4^+/CD8^+$ ratio inversion. This paper also highlights the higher $CD4^+/CD8^+$ ratio in female cattle compared to male cattle.

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Ethics

The animal procedures were performed in accordance with protocols approved by ILRI's Institutional Animal Care and Use Committee, specifically approvals 2011–11, 2013–03, 2017−01 and 2018−10.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110126.

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