



Comparative phenotypic profile of subpopulations of peripheral blood leukocytes in European (*Bos taurus taurus*) and Zebu cattle (*Bos taurus indicus*)

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ABSTRACT. Differences in cellular and humoral immunity in Zebu (*Bos taurus indicus*) and European (*B. taurus taurus*) cattle breeds, which may be related to differences in resistance or susceptibility to infectious or parasitic diseases, are largely unknown. This study aimed to perform a comparative analysis of innate and adaptive immunity of European (including Holstein, Brown Swiss, and Hereford) and Zebu (including Gir, Nelore, and Guzera) breeds, by assessing their peripheral blood leukocyte profiles (i.e., monocytes, eosinophils, and lymphocytes, including CD4⁺ and CD8⁺ T cells, and CD21⁺ B cells).

Higher frequencies of cells involved in innate immunity were observed in Zebu breeds, particularly monocytes and non-T and non-B cells (13.37 ± 0.9058 and 37.67 ± 1.55 , respectively). This finding may contribute to the increased resistance of *B. taurus indicus* to certain infectious and parasitic diseases. Considering other leukocyte populations in the peripheral blood, among-breed variation was greater than differences between the two subspecies. This study will serve as a basis for further investigations regarding comparative immunology and resistance to infectious and parasitic diseases of cattle.

Key words: Bovine; Innate immunity; Adaptive immunity; Leukocyte; Flow cytometry

INTRODUCTION

Zebu cattle often have economic advantages over European breeds under tropical conditions. For example, Brazil has the largest commercial cattle herd in the world with an estimated population of approximately 202 million. In Brazil, as well as in most tropical areas, cattle are extensively raised on pasture, which contrasts with European breeds under temperate climates, where more intensive management conditions are often adopted. Nelore, Guzera, and Gir are the main Zebu breeds (*Bos taurus indicus*), while Holstein, Brown Swiss, and Hereford are the most numerous European breeds (*B. taurus taurus*) reared in Brazil (Serrano et al., 2004).

An effective host immune response requires both innate and acquired immunity. The innate response, which is mediated primarily by macrophages, dendritic cells, and polymorphonuclear cells (such as neutrophils and eosinophils), or by physical barriers (e.g., skin), is the first line of defense, reducing the initial number of pathogens, and generating an environment that is suitable for the development of the acquired immune response (Janeway Jr. and Medzhitov, 2002; Takeda et al., 2003). Conversely, the adaptive response is delayed and primarily mediated by T lymphocytes and their subpopulations, such as CD4⁺ and CD8⁺ T lymphocytes, and B lymphocytes. Importantly, there are particularities regarding the effectors of the innate and adaptive immunity among different animal species or breeds (Takeda et al., 2003; De Visser et al., 2006; Iwasaki and Medzhitov, 2010).

Control of infectious and parasitic diseases is traditionally based on vaccination and drug treatment. However, these strategies are often ineffective and therefore some diseases persist (Cordes and Carter, 1979; Bumstead and Barrow, 1993; Rossetti et al., 2011). Several studies have demonstrated various degrees of resistance or susceptibility of Zebu and European breeds to diseases such as trypanosomiasis, ectoparasitic infestations, and gastrointestinal parasites (Rechav and Kostorzewski, 1991; Wambura et al., 1998; Bock et al., 1999; Naessens et al., 2002). However, little is known about how mechanisms of cellular and humoral immunity differ between Zebu and European breeds, which may be related to differences in resistance or susceptibility to infectious or parasitic diseases. Moreover, studies on innate and adaptive immunity in cattle are generally limited and restricted, rarely focusing on the influence of intraspecific differences.

Therefore, a better understanding of immunological differences between Zebu and European cattle may be informative with respect to differences in resistance against infection. In turn, this knowledge could impact future research leading to improved strategies for

controlling and treating infections of veterinary interest, as well as vaccine development or therapeutically induced immunity. This study aimed to perform a comparative analysis of innate and adaptive immunity of European (including Holstein, Brown Swiss, and Hereford) and Zebu (including Gir, Nelore, and Guzera) cattle breeds, by assessing their peripheral blood leukocyte profiles [i.e., monocytes, eosinophils, and lymphocytes, including CD4⁺ and CD8⁺ T cells, and B cells (CD21⁺)].

MATERIAL AND METHODS

Animals and samples

A total of 70 12-month-old cattle were used in this study (48 + 22 of a preliminary study, described below), including both males and females, belonging to the European breeds Holstein (N = 9), Brown Swiss (N = 3), and Hereford (N = 6), and the Zebu breeds Gir (N = 10), Nelore (N = 10), and Guzera (N = 10). These cattle were maintained under regular management conditions in their respective farms. The European cattle included were predominantly female and were fed mostly hay and corn silage, whereas the Zebu cattle were mostly male and were kept on grass pasture. A preliminary evaluation performed in Guzera cattle indicated that there were no significant differences in any of the parameters between males (N = 10) and females (N = 12) ([Figure S1](#)). These conditions were elected to mimic herd composition and management practices considered routine for these breeds. All animals received mineral supplementation and water *ad libitum*. They were free of ticks and hemoparasites. Blood samples were collected with 5 mL ethylenediaminetetraacetic acid (EDTA) for *ex vivo* cellular response evaluation, and stored at room temperature until further processing.

Immunophenotyping of peripheral lymphocytes

A control test was performed prior to immunophenotyping to evaluate the quality of the cell suspension: 15 μ L cell suspension in 200 μ L Max FaxFix fixative solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.65 g/L sodium chloride, pH 7.2) was analyzed in a flow cytometry tube (FacScan, Becton Dickinson, USA). Immunophenotyping was performed using specific anti-bovine monoclonal cell receptor antibodies (Table 1). These antibodies were titrated, and 1:15, 1:30, 1:60, and 1:120 dilutions were applied to antibodies anti-CD4, anti-CD8, and anti-CD21. With the exception of the monoclonal antibody anti-CD21, all other antibodies were labeled with fluorescein isothiocyanate (FITC). Anti-CD21 was followed by incubation with an anti-mouse secondary antibody labeled with phycoerythrin (PE), which was also titrated (1:200, 1:400, 1:800). A single cell type was analyzed in each tube. Aliquots of 15 μ L blood with EDTA were added to either 15 μ L bovine anti-CD4 (1:60), anti-CD8 (1:60) monoclonal antibodies labeled with FITC or anti-CD21 (1:120) in 5-mL tubes. After a 20-min incubation at room temperature, cells were washed with 2 mL phosphate-buffered solution (PBS), and then centrifuged at 400 g for 7 min. Then, 50 μ L mouse anti-IgG secondary antibody (1:800) labeled with PE was added to the tube incubated with anti-CD21. After incubation, erythrocytes were lysed by incubating with 2 mL lysis solution (FACS™ Lysing Solution) for 8 min at room temperature. After lysis, leukocytes were washed in PBS and fixed with 200 μ L Max FaxFix.

Table 1. Monoclonal antibodies used for immunophenotyping of peripheral lymphocytes from European and Zebu cattle.

Antibody	Fluorochrome	Clone	Manufacturer
Mouse anti-bovine CD4	FITC	CC8	Serotec
Mouse anti-bovine CD8	FITC	CC63	Serotec
Mouse anti-bovine CD21	None	CC21	Serotec
Goat anti-mouse IgG (H+L)	PE	N/A	Southern Biotech

FITC = fluorescein isothiocyanate; PE = phycoerythrin.

Acquisition, storage, and analysis of the data were performed using a FACScalibur flow cytometer (Becton Dickinson) with the CellQuest software.

The leukocyte subpopulations were quantified based on size (laser forward scatter; FSC) and granularity (laser side scatter; SSC) properties commonly used to identify lymphocytes (FSC \approx 200 and SSC < 200), monocytes (FSC \approx 400 and SSC < 200), neutrophils (FSC \approx 500-600 and SSC = 300-600), and eosinophils (FSC \approx 400-500 and SSC = 400-800) (Figure 1A). The lymphocyte subsets were analyzed in cells within the selected lymphocyte region based on the relative fluorescence intensity observed in the FL1 versus FL2 dot plot distributions. Figure 1B-D show representative immunophenotypic profile characteristics of CD4⁺, CD8⁺, and CD21⁺ B cells, respectively. Quadrant statistical analysis was applied to quantify the fluorescent positive lymphocyte subset within the lower right quadrant. The frequency of total T cells was calculated as the sum of CD4⁺ plus CD8⁺ T cell subsets, and the frequency of non-T and non-B (NTNB) cells was determined by the equation:

$$100 - (CD4^+ + CD8^+ + CD21^+) \quad (\text{Equation 1})$$

Calculations of the T/B and CD4⁺/CD8⁺ T cell ratios were also performed.

Statistical analysis

Data were subjected to analysis of variance followed by the Student *t*-test using the Prisma Graphpad 5.0 software. Data were considered to be statistically different when $P \leq 0.05$.

RESULTS

Profile of peripheral blood leukocytes of European and Zebu breeds

The profile of peripheral blood leukocytes was compared among European (i.e., Holstein, Hereford, and Brown Swiss) and Zebu breeds (i.e., Gir, Nelore, and Guzera). When breeds of the different subspecies were grouped together, Zebu cattle had significantly higher percentages of monocytes ($P < 0.001$) and eosinophils ($P < 0.05$) and a lower percentage of total lymphocytes ($P < 0.01$) in the peripheral blood compared to European cattle (Figure 2).

European breeds had similar percentages of lymphocytes, whereas the Zebu breeds Guzera and Nelore had a significantly lower percentage compared to Gir ($P < 0.001$ and $P < 0.0001$, respectively). Furthermore, Nelore had a significantly higher percentage of lymphocytes when compared to Guzera ($P < 0.001$). Intersubspecies comparisons indicated that Holstein and Brown Swiss had significantly higher levels of lymphocytes compared to Nelore ($P < 0.01$) and Guzera ($P < 0.0001$). Hereford had a significantly higher percentage of lymphocytes than Guzera ($P < 0.0001$) (Figure 3A).

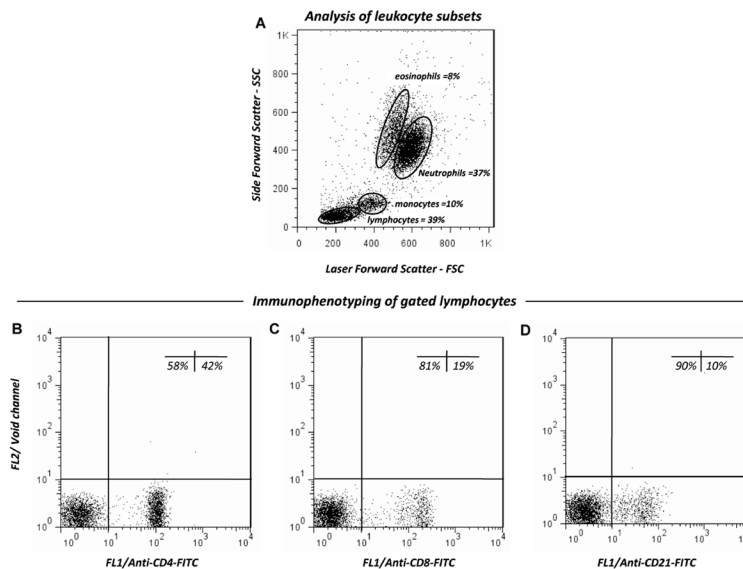


Figure 1. A. Morphometric distribution of peripheral blood leukocytes of cattle based on their size (laser forward scatter - FSC) and granularity (laser side scatter - SSC) properties. Differential profile was used to identify lymphocytes (FSC \approx 200 and SSC $<$ 200), monocytes (FSC \approx 400 and SSC $<$ 200), neutrophils (FSC \approx 500-600 and SSC = 300-600) and eosinophils (FSC \approx 400-500 and SSC = 400-800). B, C, and D. Analysis of CD4⁺ and CD8⁺ T cells as well as CD21⁺ B lymphocytes was performed within the selected lymphocyte region based on the relative fluorescence intensity observed in FL1 versus FL2 dot plot distributions, using quadrant statistics to quantify the percentage of fluorescent cells confined at the lower right quadrant.

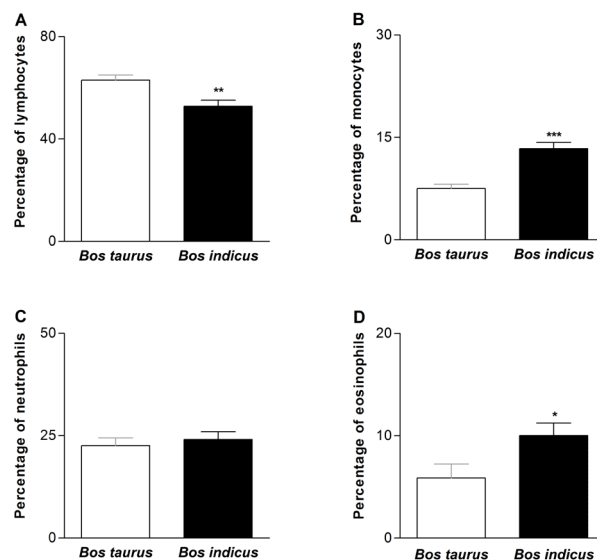


Figure 2. Percentage of leukocytes in peripheral blood from European (*Bos taurus taurus*) and Zebu (*B. taurus indicus*) cattle analyzed by flow cytometry. Data from all breeds from a given subspecies were combined: lymphocytes (A), monocytes (B), neutrophils (C), and eosinophils (D). Data are reported as means and standard error. Statistically significant differences are indicated by asterisks (* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$).

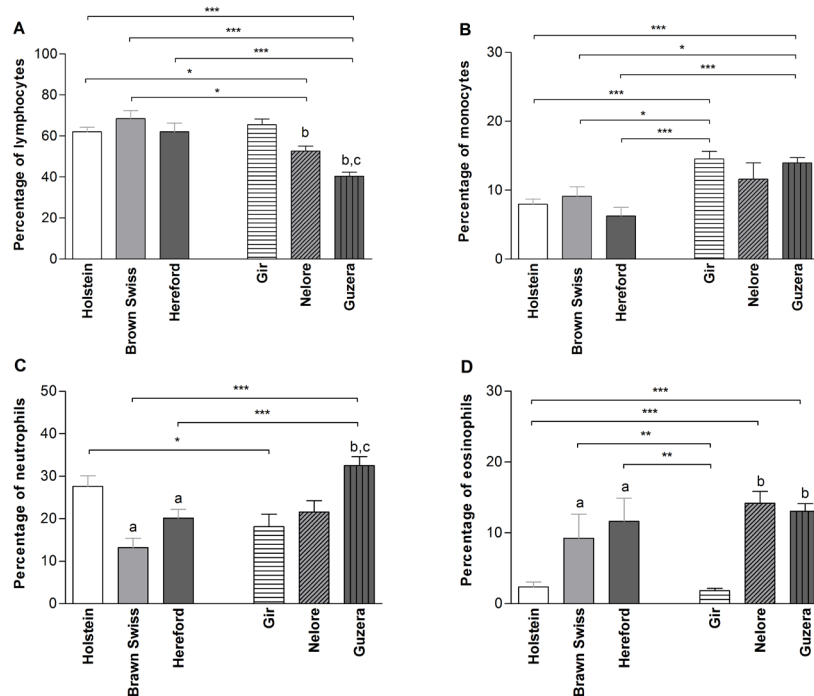


Figure 3. Percentage of leukocytes in peripheral blood from European (*Bos taurus taurus*) and Zebu (*B. taurus indicus*) breeds analyzed by flow cytometry: lymphocytes (A), monocytes (B), neutrophils (C), and eosinophils (D). Data are reported as means and standard error. Statistically significant differences are indicated by asterisks (* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$). “a”, “b”, and “c” indicate statistically significant differences in comparison to Holstein, Gir, and Nelore, respectively ($P < 0.05$).

No significant differences in monocytes were evident among the European or among the Zebu breeds. Intersubspecies analysis demonstrated that the European breeds Holstein, Brown Swiss, and Hereford had significantly lower levels of monocytes compared to the Zebu breeds Gir ($P < 0.0001$, $P < 0.0001$, and $P < 0.01$, respectively) and Guzera ($P < 0.0001$, $P < 0.0001$, and $P < 0.01$, respectively) (Figure 3B).

The percentage of neutrophils was significantly higher in Holstein compared to Brown Swiss ($P < 0.05$) and Hereford ($P < 0.05$) breeds. Among the Zebu breeds, Guzera had a significantly higher percentage of neutrophils compared to Nelore ($P < 0.001$) and Gir ($P < 0.0001$). Intersubspecies comparisons indicated that Holstein had a higher percentage of neutrophils compared to Gir ($P < 0.01$). Interestingly, the European breeds Brown Swiss and Hereford had significantly lower neutrophil levels compared to Guzera ($P < 0.0001$) (Figure 3C).

Holstein had a significantly lower percentage of eosinophils compared to Brown Swiss ($P < 0.001$) and Hereford ($P < 0.001$). Among Zebu breeds, Gir had significantly lower levels of eosinophils than Nelore ($P < 0.0001$) and Guzera ($P < 0.0001$). Intersubspecies comparisons demonstrated that Holstein had a significantly lower percentage of eosinophils compared to Nelore ($P < 0.0001$) and Guzera ($P < 0.0001$), whereas Brown Swiss and Hereford had significantly higher percentages of eosinophils compared to Gir ($P < 0.001$) (Figure 3D).

Phenotypic profile of T, B, and NTNB lymphocytes and the T/B lymphocyte ratio in peripheral blood of European and Zebu breeds

Immunophenotyping of peripheral lymphocytes was performed to evaluate the percentage of T, B, and NTNB lymphocytes, and the ratio between T and B lymphocytes (T/B) in European and Zebu breeds. When breeds of different subspecies were grouped together, Zebu cattle had a significantly lower percentage of T lymphocytes ($P < 0.05$) in the peripheral blood, which was associated with lower levels of $CD8^+$ T lymphocytes, compared to European cattle (Figure 4).

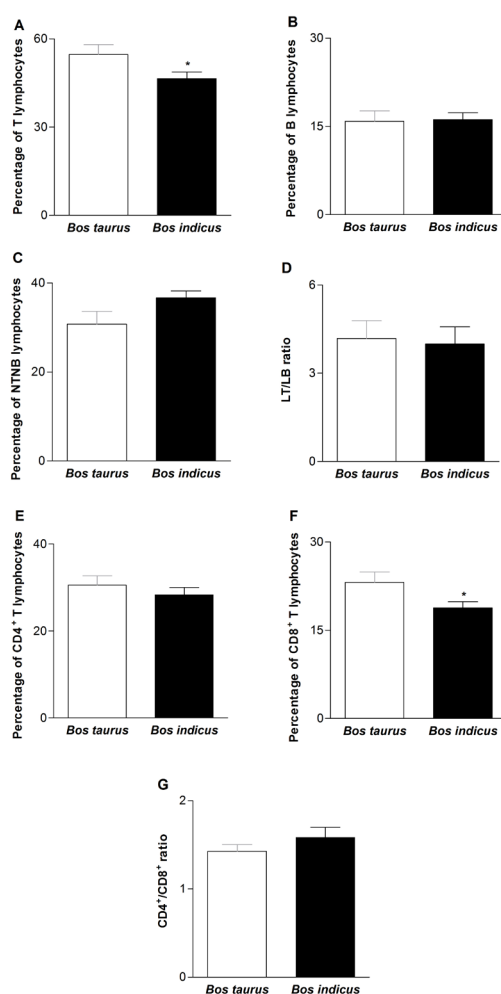


Figure 4. Percentage of lymphocytes in peripheral blood from European (*Bos taurus taurus*) and Zebu (*B. taurus indicus*) breeds analyzed by flow cytometry. Data from all breeds from a given subspecies were combined: T lymphocytes (A), B lymphocytes (B), NTNB lymphocytes (C), and the ratio between T and B lymphocytes (D), $CD4^+$ T cells (E), $CD8^+$ T cells (F), and the ratio of $CD4^+/CD8^+$ (G). Data are reported as means and standard error. Statistically significant differences are indicated by asterisk (* $P < 0.05$).

Holstein had a significantly lower percentage of T lymphocytes compared to Hereford ($P < 0.001$). Among the Zebu breeds, Gir had significantly lower levels of T lymphocytes than Guzera ($P < 0.0001$), whereas Nelore had a significantly higher percentage compared to Gir ($P < 0.0001$) and a significantly lower percentage compared to Guzera ($P < 0.001$). Intersubspecies analysis demonstrated that Holstein had a significantly higher percentage of T lymphocytes than Gir ($P < 0.05$) and a significantly lower percentage compared to Guzera ($P < 0.0001$). Brown Swiss had a significantly higher percentage than Gir ($P < 0.0001$), and Hereford had a significantly higher percentage than Gir ($P < 0.0001$) and Nelore ($P < 0.001$) (Figure 5A).

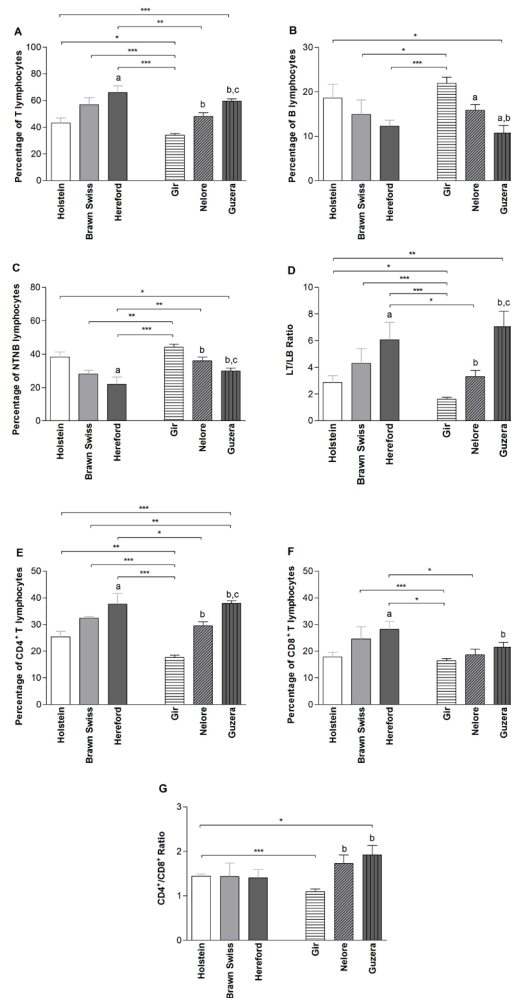


Figure 5. Percentage of lymphocytes in peripheral blood from European (*Bos taurus taurus*) and Zebu (*Bos taurus indicus*) breeds analyzed by flow cytometry: T lymphocytes (A), B lymphocytes (B), NTNB lymphocytes (C), and the ratio between T and B lymphocytes (D), CD4⁺ T cells (E), CD8⁺ T cells (F), and the ratio of CD4⁺/CD8⁺ (G). Data are reported as means and standard error. Statistically significant differences are indicated by asterisks (* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$). “a”, “b”, and “c” indicate statistically significant differences in comparison to Holstein, Gir, and Nelore, respectively ($P < 0.05$).

There were no significant differences in the percentage of B lymphocytes among European breeds. Among the Zebu breeds, Gir had a significantly higher percentage of B lymphocytes compared to Nelore ($P < 0.001$) and Guzera ($P < 0.0001$). Additionally, Guzera had a significantly lower percentage of B lymphocytes compared to Nelore ($P < 0.05$). Intersubspecies evaluation indicated that Holstein had significantly higher levels of B lymphocytes in comparison to Guzera ($P < 0.05$). However, Brown Swiss and Hereford had significantly lower percentages of B lymphocytes compared to Gir ($P < 0.05$ and $P < 0.0001$, respectively) (Figure 5B).

Among European breeds, Holsteins had significantly higher percentages of NTNB lymphocytes compared to Herefords ($P < 0.001$). In Zebu breeds, Nelore had significantly higher levels of NTNB lymphocytes in comparison to Guzera ($P < 0.05$) and a significantly lower percentage in comparison to Gir ($P < 0.05$), and Guzera had a significantly lower percentage than Gir ($P < 0.0001$). Intersubspecies comparisons demonstrated that Holstein had significantly higher NTNB lymphocyte levels than Guzera ($P < 0.05$), while Brown Swiss and Hereford had significantly lower percentages compared to Gir ($P < 0.001$ and $P < 0.0001$, respectively), and Hereford had a lower NTNB lymphocyte percentage than Nelore ($P < 0.001$) (Figure 5C).

Among European breeds, the Holstein T/B ratio was significantly lower than that of Hereford ($P < 0.05$). Among Zebu breeds, Gir had a significantly lower ratio than Nelore ($P < 0.001$) and Guzera ($P < 0.0001$). Intersubspecies analysis demonstrated that Holstein had a significantly higher ratio when compared to Gir ($P < 0.05$) and a significantly lower ratio than Guzera ($P < 0.001$). Additionally, Brown Swiss and Hereford had significantly higher ratios compared to Gir ($P < 0.0001$). Similarly, the Hereford T/B ratio was higher than that of Nelore ($P < 0.05$) (Figure 5D).

Analysis of subpopulations of CD4⁺ and CD8⁺ T lymphocytes

Immunophenotyping results of subsets of T lymphocytes from European and Zebu cattle are shown in Figure 5. Among European breeds, Holstein had a significantly lower percentage when compared to Hereford ($P < 0.05$), whereas among Zebu breeds, Gir had lower levels of CD4⁺ T lymphocytes compared to Nelore ($P < 0.0001$) and Guzera ($P < 0.0001$). Furthermore, Guzera had a significantly lower percentage compared to Nelore ($P < 0.0001$). Intersubspecies analysis indicated that Holstein and Brown Swiss had significantly higher percentages of CD4⁺ T lymphocytes compared to Gir ($P < 0.001$ and $P < 0.0001$, respectively) and significantly lower percentages than Guzera ($P < 0.0001$ and $P < 0.001$, respectively). Additionally, Hereford had a significantly higher percentage of CD4⁺ T cells in comparison to Gir ($P < 0.0001$) and Nelore ($P < 0.05$) (Figure 5E).

Hereford had a significantly higher percentage of CD8⁺ T cells than Holstein ($P < 0.001$). In Zebu breeds, Guzera had a significantly higher percentage of CD8⁺ T lymphocytes than Gir ($P < 0.05$). Intersubspecies comparisons indicated that Brown Swiss and Hereford had significantly higher levels of CD8⁺ T lymphocytes when compared to Gir ($P < 0.05$ and $P < 0.0001$, respectively). Similarly, Hereford had significantly higher levels of CD8⁺ T lymphocytes when compared to Nelore ($P < 0.05$) (Figure 5F). No significant differences in CD4⁺/CD8⁺ ratios were observed among European breeds. Among Zebu breeds, Gir had a significantly lower CD4⁺/CD8⁺ ratio than Nelore ($P < 0.001$) and Guzera ($P < 0.001$). Intersubspecies analysis demonstrated that Holstein had a higher CD4⁺/CD8⁺ ratio compared to Gir ($P < 0.0001$) and Guzera ($P < 0.05$) (Figure 5G).

DISCUSSION

Resistance or susceptibility to disease involves multifactorial mechanisms, but the effect of selection or breed on natural resistance has been clearly demonstrated in farm and laboratory animal species (Adams and Templeton, 1998; Sathiyaseelan et al., 2000; Naessens et al., 2002, Rossetti et al., 2011). Differences between Zebu (*B. taurus indicus*) and European (*B. taurus taurus*) cattle in terms of natural resistance to diseases have also been reported (Martínez et al., 2010; Macêdo, 2012); however, whether these cattle differ in their peripheral blood leukocyte profiles is unclear, although this may account for the more efficient immunity observed in some breeds. Importantly, some evidence suggests that peripheral blood leukocytes from Zebu may respond differently than cells from European breeds when stimulated (Turni et al., 2002). Furthermore, clear differences have been observed in the frequency of alleles of *Nramp1*, a gene that has been linked to natural resistance to intracellular pathogens (Paixão et al., 2006, 2012). Susceptibility to infection is partially determined by genetic or physical characteristics (O'Kelly and Spiers, 1976), but it is also related to the profile and pattern of immune response of the animal. This study is the first to provide a detailed comparative analysis of leukocyte populations in peripheral blood of Zebu and European breeds. Together, our data demonstrated significant differences in the leukocyte profile of peripheral blood between the two subspecies, *B. taurus taurus* (European) and *B. taurus indicus* (Zebu), but also indicated marked variations among breeds within a given subspecies. Zebu had higher levels of monocytes and eosinophils, and lower levels of lymphocytes, particularly CD8⁺ T lymphocytes, when compared to European breeds.

The higher percentages of monocytes in Zebu breeds, and of CD4⁺ T lymphocytes in Hereford and Guzera, may indicate higher resistance of these breeds to infections caused by intracellular pathogens, since T lymphocytes, macrophages, and their cytokines mediate protective immunity against several intracellular pathogens (Brown et al., 1995). Adaptive immunity is highly dependent on CD4⁺ T lymphocytes, which play a fundamental role in releasing proinflammatory cytokines such as IFN- γ and TNF- α , thus contributing to activation of the microbicidal activities of neutrophils and macrophages responsible for phagocytosis and production of intermediate reactive nitrogen and oxygen (Brown et al., 1995; Serafino et al., 2007).

There were also significant differences in the percentage of NTNB lymphocytes among the different breeds. These cells correspond to natural killer (NK) cells and γ T lymphocytes. Among domestic animal species, cattle usually show significant NK activity after infection and/or stress. Bradford et al. (2001) demonstrated that bovine NK cells have high antibody-dependent cytotoxic potential, being able to lyse target cells infected with viruses through a mechanism mediated by Fc receptors. Although γ T lymphocytes constitute a small percentage of the T cells in adult humans and in mice, they represent the majority of intraepithelial lymphocytes in the gut and other mucosal epithelia (Hedges et al., 2003). Furthermore, γ T cells comprise more than 70% of circulating lymphocytes in newborn calves and are an important subset of lymphocytes in adult ruminants (Guzman et al., 2012). Bovine γ cells were found to respond rapidly to *Brucella abortus* infection upon co-culture with autologous macrophages and can inhibit intracellular replication of *B. abortus* in macrophages via IFN- γ , demonstrating that these cells are important for early protection against this pathogen (Skyberg et al., 2011).

Eosinophils are multifunctional leukocytes that play a role in several disease processes, particularly in allergic diseases and parasitic infections (Gleich and Loegering, 1984;

Gleich and Adolphson, 1986; Uhm et al., 2012). Higher numbers of eosinophils in Zebu cattle may be associated with a general increased resistance to parasites compared to European cattle, although a clear cause and effect analysis was beyond the scope of this study.

Several factors may affect the population of leukocytes in the peripheral blood of cattle, including age (Guzman et al., 2012), sex or reproductive stage (Oliveira and Hansen, 2008), and environment (Lejeune et al., 2010). In this study, these factors should not have played any significant role since all cattle studied were prepubertal, within the same age range, and they were raised under standardized, conventional conditions for these breeds.

CONCLUSION

A higher frequency of cells involved in innate immunity, particularly monocytes and NTNB cells, was observed in Zebu breeds. This finding may contribute to the increased resistance of *B. taurus indicus* to certain infectious and parasitic diseases. Considering other leukocyte populations in the peripheral blood, variation among breeds was more evident than differences between the two subspecies. This study will serve as a basis for further investigations regarding comparative immunology and resistance to infectious and parasitic diseases of cattle.

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[Supplementary material](#)

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