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Research paper

Distinct immune response profile during *rhipicephalus (boophilus) microplus* infestations of guzerat dairy herd according to the maternal lineage ancestry (mitochondrial DNA)

Fernanda Fortes de Araújo[a,b], Juliano Bergamo Ronda[a], Eustáquio Resende Bittar[a], Guilherme Costa Venturini[a], Guilherme Caetano Garcia[a], Olindo Assis Martins-Filho[b], Márcio Sobreira Silva Araújo[b,*], Joely Ferreira Figueiredo Bittar[a]

[a] *Universidade de Uberaba (UNIUBE), Medicina Veterinária, Mestrado em Sanidade e Produção Animal nos Trópicos - Avenida Nene Sabino 1697/1698, 38055-500, Uberaba, MG, Brazil*

[b] *Grupo Integrado de Pesquisa em Biomarcadores, Instituto René Rachou - Fundação Oswaldo Cruz. Avenida Augusto de Lima nº 1715, 30190-009, Barro Preto, Belo Horizonte, MG, Brazil*

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ABSTRACT

Rhipicephalus (Boophilus) microplus ticks cause major constraints to public and livestock health, and serious economic losses. It is well known that the immune response to infestations with cattle ticks is influenced by the host genetic background leading to distinct immunological profiles between bovine hosts genetically susceptible and resistant. The influence of *Bos indicus* (Bi) and *Bos taurus* (Bt) maternal lineage ancestry of mitochondrial DNA in the profile of the immune response of Zebu cattle to ticks remains unknown. The present work evaluated the hematological parameters and the immune response profile in the peripheral blood of a Guzerat dairy herd, further categorized into two maternal lineage ancestry subgroups (Bi-mtDNA and Bt-mtDNA) after experimental infestation with larvae of *R. microplus*. Our data demonstrated that although hematological and erythrogram analysis showed a similar profile throughout, some cell populations present a distinct profile between the groups. Especially MON, $CD335^+$ and $CD8^+$ T-cells are predominant in Bi-mtDNA. Moreover, an overall picture of *R. microplus* infestation demonstrated that Bi-mtDNA presented a more efficient and earlier innate immune response. Bi-mtDNA showed a greater number of connections with *R. microplus* counts and also with the $CD25^+$ activation marker of the immune response. Bi-mtDNA showed greater number of connections, with an important participation of the innate immune while Bt-mtDNA showed a delay in the immune response. Elucidating the mechanisms by which resistant animals prevent heavy tick infestation is a crucial step in the development of predictive biomarkers for tick resistance for use in selective breeding programs, and is also potentially useful for the development of anti-tick vaccines.

1. Introduction

Rhipicephalus (Boophilus) microplus ticks cause major constraints to livestock health. The negative impact of ticks on cattle production is due to the direct effects of feeding (weight loss and damage of leather) and indirect effects (transmission of tick-borne pathogens). The costs related to these effects have been estimated around US$ 22–30 billion per annum (Lew-Tabor and Rodriguez Valle, 2016).

The control of ticks is a priority in tropical and subtropical regions (Andreotti et al., 2002; Rodriguez-Valle et al., 2013; Shyma et al., 2015). It is well known that the immune response to infestations with cattle ticks is influenced by the host genetic background leading to a distinct immunological profile between bovine hosts genetically susceptible and resistant (Franzin et al., 2017). Zebu cattle (*Bos indicus*) are considered more adaptive and resistant to *R. microplus*, presenting a cellular immune response more efficient than animals less resistant (Engracia Filho et al., 2017).

Although *Bos indicus* animals are considered well adapted and highly resistant to ticks, a crossbreed with less resistant cattle as *Bos taurus* is common (Marcondes et al., 2007). It is therefore possible to find Zebu animals with two groups of mitochondrial DNA (*Bos indicus* and *Bos taurus* mitochondrial DNA). It has been demonstrated that a

* Corresponding author.
E-mail address: sobreira@cpqrr.fiocruz.br (M.S. Silva Araújo).

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large proportion of American Zebu matrilineages were derived by backcrossing "native" females of *B. taurus* origin to bulls imported from the Indian continent carrying *B. indicus* mitochondrial genotypes (Meirelles et al., 1999). Previous work already demonstrated the influence of mitochondrial DNA (mtDNA) in the productive/reproductive features as well as immunological profiles from bovines (Ribeiro et al., 2009; Macedo et al., 2014). According to Ribeiro et al. (2009) the age at first calving was influenced by the maternal lineage ancestry mtDNA. However, Paneto et al. (2008) showed that mtDNA has no significant effect on lactation milk yield, days in milk, age at first calving, or calving interval. Previous work from our group showed that endogamy and mtDNA influences the immune system of cattle since it is associated with significant changes in the immune cells profile of peripheral blood, mainly with decreased levels of γδ T lymphocytes, an important subpopulation of lymphocytes in cattle (Macedo et al., 2014). In general, the mitochondrial genome of most mammals is assumed to be inherited exclusively from the mother. However, according to Luo et al. (2018), there is novel evidence of biparental inheritance of mtDNA in humans, opening up a new frontier in the study of mtDNA genetics.

The influence of maternal lineage ancestry mtDNA in the profile of the immune response and resistance of Zebu cattle to ticks remains unknown. The present work evaluated the profile of the immune response in the peripheral blood of cattle infested with the *R. microplus* and compared *Bos indicus* and *Bos taurus* animals in response to tick infestation.

2. Materials and methods

2.1. Animals

This work was conducted at the Veterinary Hospital of Uberaba, Minas Gerais, Brazil. Twenty female breed Guzerat dairy cattle aged 18 months, were divided according to their maternal lineage ancestry (Paneto et al., 2008) into (i) *Bos indicus* mtDNA (Bi-mtDNA, n = 10) and (ii) *Bos taurus* mtDNA (Bt-mtDNA, n = 10). The animals of both groups were kept, throughout the experiment, in separate paddocks with green fodder (*Panicum maximum* and hay) and water "*ad libitum*".

This study protocol was approved by the Animal Experimentation Ethical Committee from Universidade de Uberaba, Uberaba-MG, Brazil (CEEA/UNIUBE, protocol number 0014/2013) and followed all the ethical principles of cattle experimentation procedures.

2.2. Experimental design

The animals were kept free of tick infestation for three months through treatment with Dectomax® (Doramectin, Zoetis, Brasil) at 1 ml per 50 Kg of body weight, subcutaneously.

Experimental infestation was performed with *R. microplus* (EMBRAPA SP, Brazil) 45 days after the last doramectin treatment. Each animal received 1 g of larvae (20.000 larvae) at the dorsal midline between the neck and the withers. Following, the cattle were kept immobilized for 30 min to ensure larvae fixation. Tick counting was performed at day 16 (D16) according to Villares (1941). Briefly, for tick counting the animals were taken to the containment trunk for handling. Only engorged females with length of 4 from 8 mm present at one side of the bovine body (left side) were counted (Villares, 1941).

Blood was collected from jugular venipuncture in sterile vacuum tubes containing an anticoagulant (EDTA 7.2 mg) at day 0 (D0 = prior to infestation), day 1 (D1), day 9 (D9) and day 21 (D21) after infestation for hematological and immunophenotypic analysis. The samples were kept at room temperature (RT) and processed within 5 h (Fig. 1).

2.3. Hematological analysis

The hematological measurements including erythrocyte counts, total leucocytes, hemoglobin concentration and hematocrit were carried out in a hematology analyzer (ABC VET - Horiba® ABX Diagnostics). The differential leukocyte count was performed by optical microscopy (Nikon Eclipse E200®), 1000x magnification, of blood smears stained with Fast Panoptic kit (Laborclin®).

2.4. Flow cytometry

Blood cells were collected and evaluated employing the monoclonal antibodies: anti-CD4 FITC (clone CC8), anti-CD8 FITC (clone CC63), anti-CD25 PE (clone IL-A111), anti-CD335 FITC (clone AKS1), anti-WC1 (γδ T cells) FITC (clone CC15) and anti-CD14 FITC (clone CC-G33). All antibodies used are from Serotec (Oxford, UK).

Immunophenotyping analyses were performed according to Bittar et al. (2004), with modifications. Fifty microliters of whole blood cells were placed in polystyrene tubes and incubated in the dark for 30 min, at RT, with each monoclonal antibody fluorochrome-labeled. After this time, the erythrocytes were lysed by adding 3 ml of lysing solution (FACS brand lysing solution; Becton Dickinson San Diego, CA, USA) followed by incubation for 10 min at RT. The samples were centrifuged at room temperature for 7 min at 400 x g. The total leukocytes were washed in with 3 ml of PBS (phosphate buffered saline 0.15 M, pH 7.2) and centrifuged at 400 x g for 10 min at RT. After the washing procedures, labeled cells were then fixed for 30 min at RT, with 200 µl of FACS FIX solution (10.0 *g*/l paraformaldehyde; 10.2 *g*/l sodium cacodylate and 6.65 *g*/l sodium chloride, pH 7.2). The stained cells were stored at 4–8 °C up to 24 h before flow cytometry analysis. The data acquisition (20.000 events) was performed using a FACSCalibur flow cytometer (Becton Dickinson®) and analyzed with FlowJo software. Proper compensation adjustments were performed to guarantee the accuracy and quality of dual color flow cytometric data.

The leukocyte subpopulations were quantified based on size (forward scatter; FSC) and granularity (side scatter; SSC) properties commonly used to identify lymphocytes (FSC^{low} and SSC^{low}). A specific scatter gate using anti-CD14 FITC versus SSC dot plot combination was performed for selecting monocytes, identified as $SSC^{Low}CD14^{high}$ cells amongst the total leukocyte population.

2.5. Statistical analysis

Statistical analyses were performed using the program GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). When the distribution was considered normal and the variance was homogeneous, parametric tests (ANOVA with Tukey post-test) were used. In cases when the distribution was not Gaussian, we used nonparametric tests (Friedmann with post-test of Dunn's). For comparisons between the groups during the experimental period we used Mann-Whitney test and for correlation of the infestation results, the Pearson test. Differences were considered significant when $p < 0.05$.

Biomarker networks were assembled to assess the association between cell subpopulations and hematological parameters for the *Rhipicephalus (Boophilus) microplus* infestation. Significant correlations representing the interaction between biomarkers tested were compiled using the open source software Cytoscape (version 3.7.1), as previously reported (Shannon et al., 2003). The biomarker networks were constructed using circle layouts with each biomarker represented by specific globular nodes: (i) Bi-mtDNA = dark gray nodes and (ii) Bt-mtDNA = light gray nodes. Connecting edges represent correlation scores categorized as positive strong (thick gray line), positive moderate (thin gray line), negative strong (thick gray dotted line), negative moderate (thin gray dashed line) as proposed by Taylor (1990).

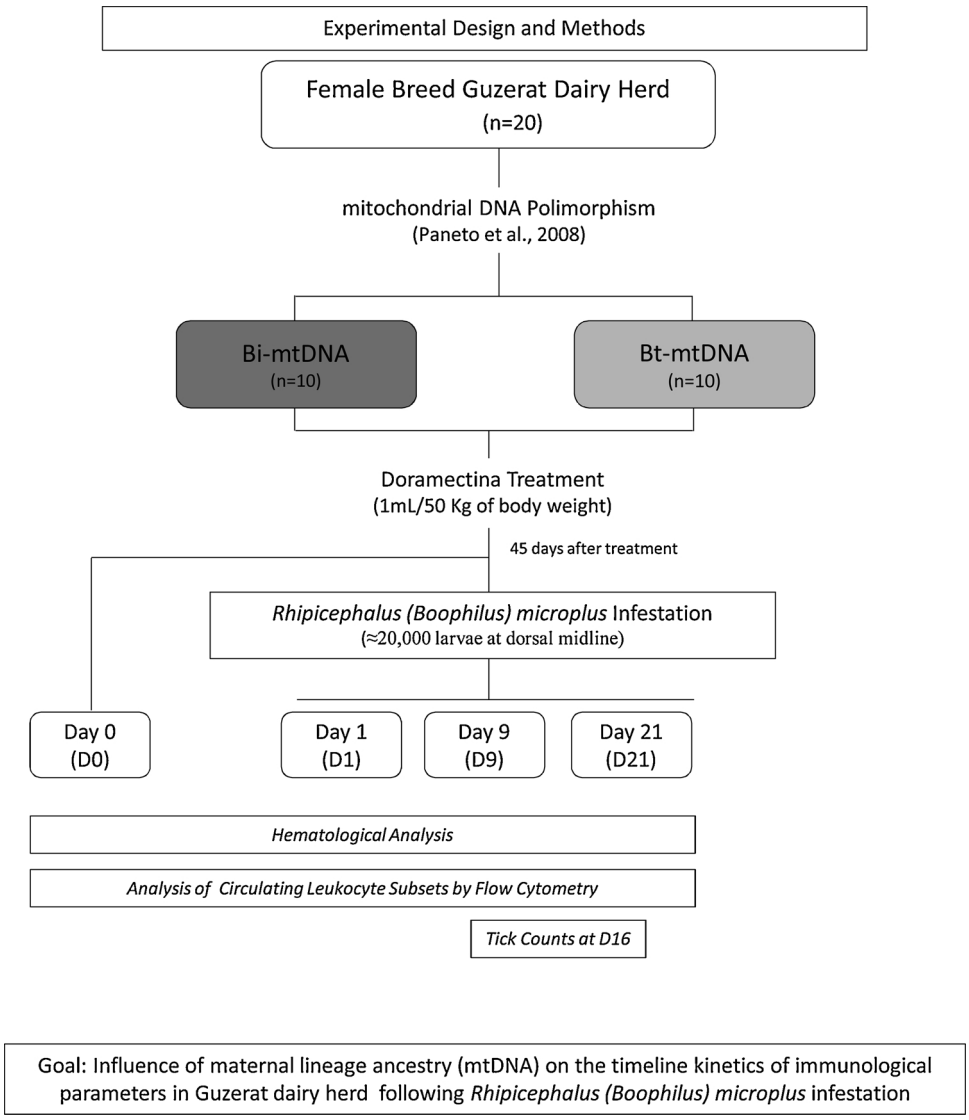

Fig. 1. Experimental Design and Methods.
Schematic timeline of *Rhipicephalus (Boophilus) microplus* infestation of Guzerat dairy herd: *Bos indicus* (Bi-mtDNA; n = 10) and *Bos Taurus* (Bt-mtDNA; n = 10). The groups were treated with doramectin prior infestation. The hematological parameters and the analysis of circulating leukocyte subsets by flow cytometry were evaluated at days 0, 1, 9 and 21 (D0, D1, D9 and D21, respectively).

3. Results

3.1. Influence of mtDNA on hematological parameters in a guzerat dairy herd following tick infestation

Hematological analysis in a Guzerat dairy herd following *R. microplus* infestation demonstrated that in general both groups (Bi-mtDNA and Bt-mtDNA) showed a similar profile of immune cells throughout the days studied, with few exceptions. The Bt-mtDNA group presented an increase in the absolute count of neutrophils (NEU) at D1, but returning to baseline at D21 (Fig. 2). Moreover, a decrease in the absolute count of lymphocytes (LYM) at D9 and D1 was observed for Bi-mtDNA and Bt-mtDNA, respectively, when compared with D0. Regarding to monocytes (MON), a significant opposite profile was observed at D9 between Bi-mtDNA and Bt-mtDNA and for eosinophils at D1, with the counts of these cells becoming comparable at D21 (Fig. 2). Erythrogram, from both groups, presented a related profile with a significant reduction in the absolute count of red blood cells (RBC), hemoglobin (Hb) and hematocrit (Ht) throughout the days analyzed. The results showed an increase in the absolute count of platelets at D9 and D21 when compared with D0 and D1 from both groups. No differences of white blood cells (WBC), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) parameters were observed for the groups (Fig. 2).

3.2. Influence of mtDNA on the immunological parameters in a guzerat dairy herd following tick infestation

The timeline kinetics of the immunological parameters showed a significant increase of $CD14^+$ at D9 and D21 when compared with D0 and D1 while the biomarker $CD25^+$ presented a significantly reduction at D1 when compared with D0 returning to baseline levels at D9 from both Bi-mtDNA and Bt-mtDNA groups. The innate $CD335^+$ cells presented a significant decrease at D0 and D9 from both groups but returning to baseline of each group at D21 (Fig. 3). Significant difference was observed for $CD335^+$ cells between Bi-mtDNA and Bt-mtDNA, suggesting that the two groups present distinct absolute counts of $CD335^+$ throughout infestation. Moreover, our results showed a decrease in the absolute count of T-cells, $CD4^+$ T-cells, $CD8^+$ T-cells and $T/CD21^+$ ratio from both Bi-mtDNA and Bt-mtDNA groups at D1 and

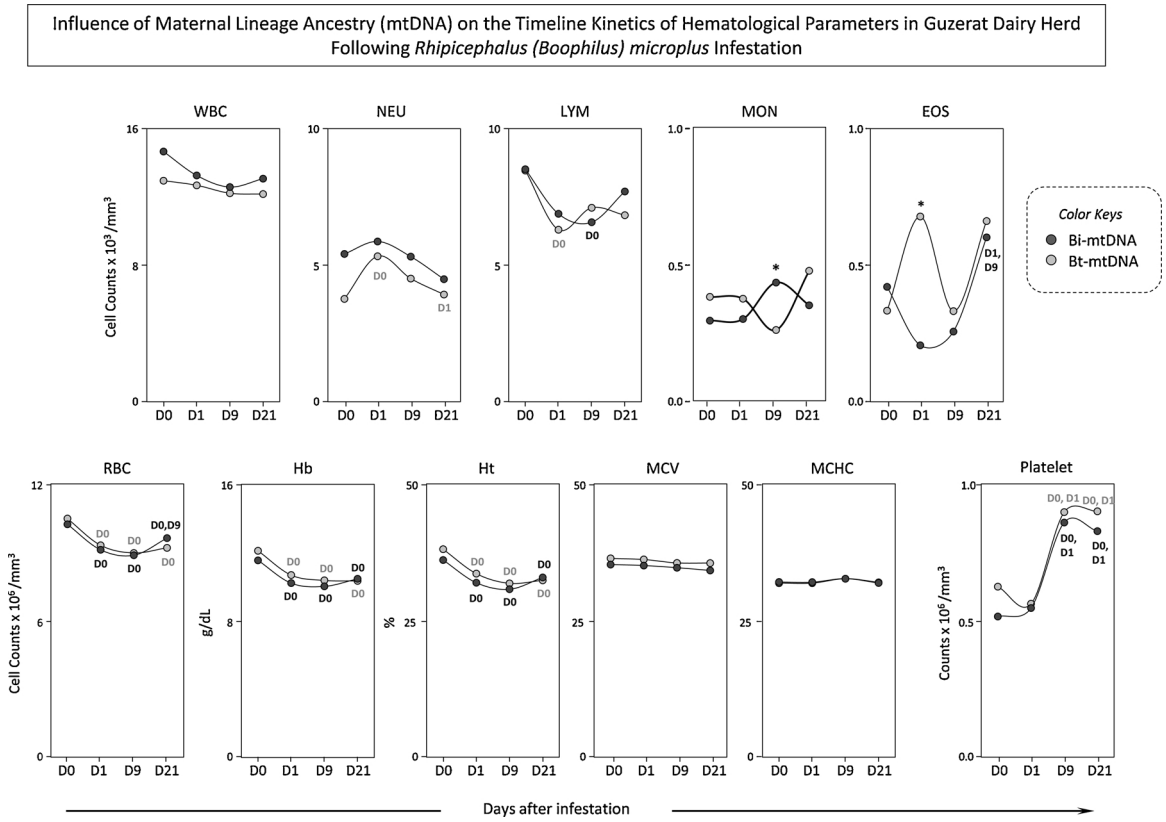

Fig. 2. Hematological parameters in Guzerat dairy herd following *R. microplus* infestation.
Timeline kinetics was built to follow the absolute count of hematological parameters of Bi-mtDNA (dark gray circles) and Bt-mtDNA (light gray circles) groups. The parameters white blood cells (WBC); neutrophils (NEU); lymphocytes (LYM); monocytes (MON); eosinophils (EOS); red blood cells (RBC); hemoglobin (Hb); hematocrit (Ht); mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC) and platelet were evaluated during the course of infestation (D0, D1, D9 and D21). Significant differences amongst the days are represented in the figure by D0, D1 or D9. Significant differences between the groups are represented by *.

D9 when compared to D0 and D21. Significant differences were observed for $CD8^+$ T-cells and $CD4^+/CD8^+$ ratio at D21 between Bi-mtDNA and Bt-mtDNA. No differences of $\gamma\delta^+$ cells and $CD21^+$ cells were observed for the groups (Fig. 3).

3.3. Relation of tick load on hematological and immunological parameters in Guzerat Dairy Herd

Additional analysis was performed to evaluate the relationship between tick load and hematological as well as immunological parameters. Each group (Bi-mtDNA and Bt-mtDNA) was categorized according to median tick load at D16 and referred as Low (< 50 ticks) or High (> 50 ticks) and comprised specifically: Bi-mtDNA(Low), n = 6; Bi-mtDNA(High), n = 4; Bt-mtDNA(Low), n = 5 and Bt-mtDNA(High), n = 5 (Tables 1 and 2). Our data demonstrated that in general Bi-mtDNA with low parasitemia presented a decrease in the absolute count of LYM and an increase of $CD14^+$ and platelets while Bi-mtDNA with high parasitemia presented an increase in the absolute count of MON and EOS. Independent of tick load the absolute count of RBC, Hb and Ht showed a decrease in the Bi-mtDNA group throughout the days of infestation (Table 1 and 2).

The Bt-mtDNA group with low parasitemia presented an increase in the absolute count of MON and $CD14^+$ while the Bt-mtDNA group with high parasitemia presented a decrease in the absolute count of LYM and an increase of $CD4^+$ T-cells. Once more, independent of tick load the absolute count of RBC, Hb and Ht showed a decrease in the Bi-mtDNA group, however the absolute count of platelets increased throughout the days of infestation (Tables 1 and 2).

3.4. Biomarker network overview

A biomarker network analysis from the Bi-mtDNA and Bt-mtDNA groups at the three time points (D1, D9 and D21) demonstrated that at D1, the Bi-mtDNA group presented more connections and more significant negative correlations, with an important participation of the innate immune response (EOS, NEU, MON, $CD14^+$ and $CD335^+$ cells, red circles). In addition, the Bi-mtDNA group showed a greater number of connections with *R. microplus* counts and also with the activation marker of the immune response $CD25^+$ (orange circles). On the other hand, the Bt-mtDNA group did not show connections with innate immune attributes such as EOS, NEU, MON and $CD14^+$ at D1 (Fig. 4, top panel).

At D9, the Bt-mtDNA group had more connections than at D1, however remained with a limited participation of the innate immune response and the activation marker $CD25^+$. The biomarker network analysis demonstrated that the Bi-mtDNA group has a greater number of connections at D9 (Fig. 4, middle panel). The analysis at D21 showed a better number of connections of the Bt-mtDNA group when compared with the previous days, suggesting that this group presents a delay in the immune response, presenting only at D21 a similar immune response to the Bi-mtDNA group at D1. Although improving the immune response, it is clear that the Bt-mtDNA group still had less participation of $CD21^+$ cells (pink circles). In general, Bt-mtDNA group showed more evolvement of platelets (green circles) throughout the days when compared with Bi-mtDNA group (Fig. 4, bottom panel).

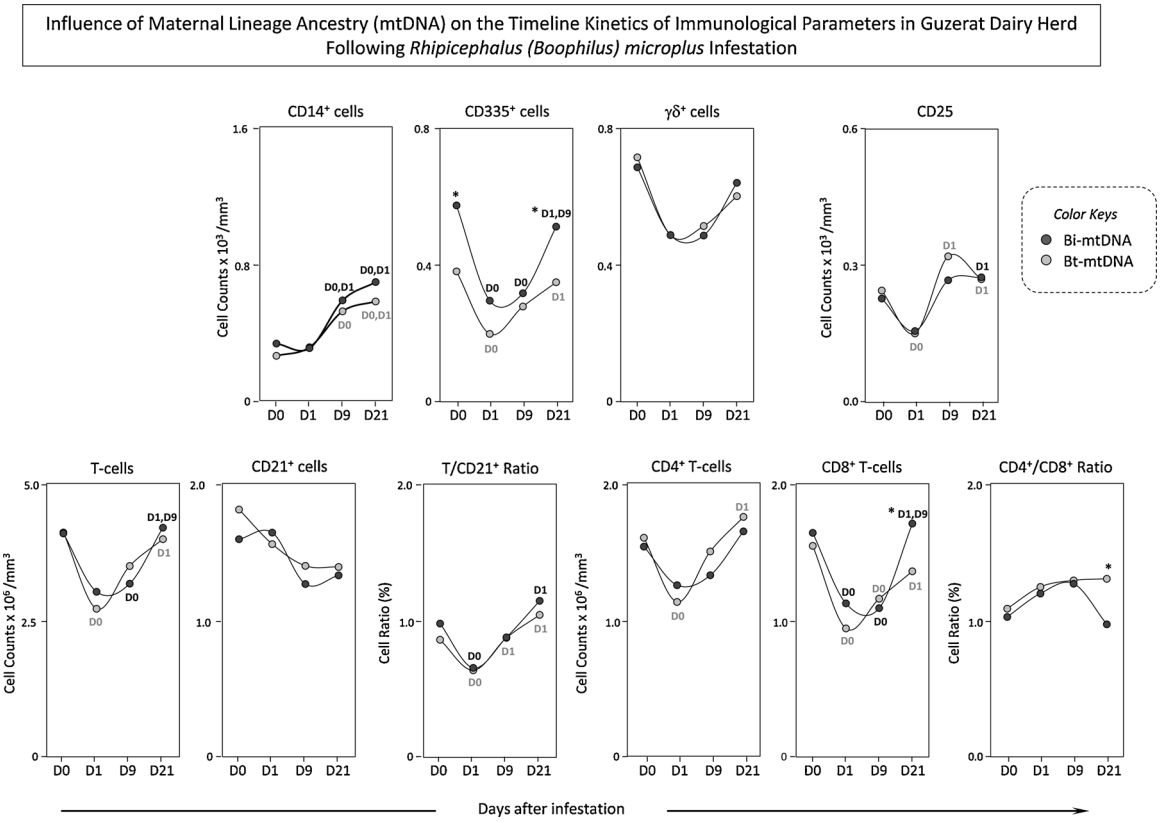

Fig. 3. Immunological parameters in Guzerat dairy herd following *R. microplus* infestation.
Timeline kinetics was built to follow the absolute count of immunological biomarkers of Bi-mtDNA (dark gray circles) and Bt-mtDNA (light gray circles) groups. The markers CD14; CD335, WC1 (γδ T cells); CD25; CD21; CD4; CD8 and the T-cells; the ratios T/CD21 and CD4/CD8 were evaluated during the course of infestation (D0, D1, D9 and D21). Significant differences amongst the days are represented in the figure by D0, D1 or D9. Significant differences between the groups are represented by *.

4. Discussion

To gain an insight into the *Bos indicus* and *Bos taurus* cattle breed against ticks, studies have been conducted comparing the incidence of tick infestation on bovine hosts from divergent genetic backgrounds (Tabor et al., 2017). It is well documented that *Bos indicus* breeds are generally more resistant to tick infestation than *Bos taurus* breeds; however, large variations in resistance can occur within breed (Seifert, 1970). Previous studies have reported the influence of mitochondrial DNA (mtDNA) in the productive/reproductive features as well as immunological profiles in cattle (Ribeiro et al., 2009; Macedo et al., 2014). In general, the mitochondrial genome of most mammals is inherited exclusively from the mother but recently the transmission of paternal mitochondrial DNA (mtDNA) has been convincingly demonstrated in humans (Luo et al., 2018). The pioneer findings of Luo et al. (2018) provide novel insights that alter the widespread belief about mitochondrial inheritance and definitely open novel fields for future investigations in veterinary medicine. The current investigation was focused on the concept of maternal inheritance of mtDNA that still remains valid. Therefore, the results presented in the present work would be interpreted considering the experimental design proposed.

Some studies focused on the physical and mechanical attributes of the skin of indicine and taurine animals. Indeed, Koudstaal et al. (1978) found that animals of high resistance spent significantly more time grooming than animals of low resistance following infestation. According to Jonsson et al. (2014), thickness of skin and the density of the hair coat have both been proposed as characteristics that might confer resistance to tick infestation. Moreover, Kemp et al. (1976) confirmed the findings of proportionally high loss of larvae in resistant cattle within 24 h and also noted that larvae on highly resistant cattle were significantly lighter than those on animals of lower resistance within 24 h of application.

The main difficulty with selecting for host resistance in cattle to ticks is that identifying highly resistant individuals using the standard tick count is not a feasible option in a commercial setting. Consequently, predictive phenotypic markers for tick resistance would be a better approach to develop herds with high levels of tick resistance.

In the complex interaction between tick and host, diverse immune and non-immune mechanisms contribute to variation in host resistance. However, the immunity appears to be important (Jonsson et al., 2014). The immunological parameters have been shown to differ between tick susceptible and tick resistant breeds (Tabor, 2017). Indeed, the identification of the immune cells has become an important tool for both research and diagnosis (Gutierrez et al., 1999).

Our results demonstrated that although hematological and erythrogram analysis in a Guzerat dairy herd following *R. microplus* infestation showed a similar profile throughout the days, some cell populations present differences between the breeds. Especially, MON, $CD335^+$ and $CD8^+$ cells are predominant in Bi-mtDNA group while Bt-mtDNA group presented major counts of EOS. Previous studies concerning immune responses in the skin of tick-infested cattle has associated a strong hypersensitivity reaction with increased tick resistance in *B. taurus* cattle (Schleger et al., 1976, 1981; Kemp et al., 1983), involving the infiltration of eosinophils and concentration of histamine at the tick-attachment site, suggesting that this mechanism can be an alternative to overcome the infestation in situ.

An overall picture of *R. microplus* infestation demonstrated that Bi-mtDNA group presented a more efficient and earlier innate immune response with the participation of cells related to inflammation as NEU, MON, CD335 and EOS. This corroborates the results of Carvalho et al.,

Table 1
Influence of Maternal Lineage Ancestry (mtDNA) and Tick Load Following *Rhipicephalus (Boophilus) microplus* Infestation on the Timeline Kinetics of Hematological Parameters in Guzerat Cattle.

	Parameter	*Rhipicephalus (Boophilus) microplus* Infestation Status							
		Low (< 50 ticks)				High (> 50 ticks)			
		D0	D1	D9	D21	D0	D1	D9	D21
Bi-mtDNA*	WBC	15,100 (±1044)	13,567 (±658)	12,333 (±1102)	13,800 (±761)	14,000 (±2060)	12,800 (±1492)	12,950 (±1638)	11,975 (±1677)
	NEU	5,567 (±718)	5451 (±916)	4562 (±854)	4470 (±754)	5185 (±784)	6498 (±891)	6446 (±1776)	4519 (±790)
	LYM	8,706 (±482)	7485 (±1221)	7,122^{D0} (±409)	8298 (±539)	8228 (±1459)	5983 (±1317)	5755 (±794)	6805 (±906)
	MON	358 (±92)	346 (±107)	328 (±54)	380 (±127)	195 (±77)	227 (±80)	587 ^{D0,D1} (±98)	302 (±138)
	EOS	469 (±135)	283 (±122)	321 (±45)	627 (±166)	348 (±82)	92 (±34)	161 (±35)	280 ^{D1,D9} (±154)
	RBC	9,983 (±0.3)	8,983^{D0} (±0.2)	8700 ^{D0} (±0.2)	9617 ^{D1,D9} (±0.1)	10,650 (±0.2)	9350 ^{D0} (±0.4)	9150 ^{D9} (±0.2)	9650 (±0.5)
	Hb	11.2 (±0.4)	9.9 ^{D0} (±0.2)	9.6 ^{D0} (±0.2)	10.3 ^{D9} (±0.1)	12.1 (±0.2)	10.6 ^{D0} (±0.4)	10.6 ^{D0} (±0.2)	10.6 ^{D0} (±0.4)
	Ht	34.4 (±1.3)	30.9 ^{D0} (±0.7)	29.5 ^{D0} (±0.7)	32.4 ^{D9} (±0.4)	38.6 (±0.8)	33.5 ^{D0} (±1.5)	32.6 ^{D0} (±0.7)	33.6 (±2.1)
	VCM	34.5 (±0.6)	34.5 (±0.6)	33.9 (±0.5)	33.7 (±0.4)	36.4 (±0.5)	35.9 (±0.5)	35.7 (±0.5)	34.9 (±0.5)
	CHCM	32.4 (±0.4)	32.1 (±0.4)	32.8 (±0.2)	31.9 (±0.3)	31.5 (±0.1)	31.8 (±0.3)	32.5 ^{D0} (±0.4)	31.8 (±0.7)
	Platelet	518,333 (±78,570)	563,500 (±55,857)	921,333 ^{D0} (±120,298)	899,000 ^{D0} (±142,779)	509,750 (±81,952)	519,500 (±101,909)	764,750 (±79,182)	719,500 (±179,693)
Bt-mtDNA*	WBC	10,125 (±588)	11,380 (±343)	11,220 (±1243)	11,220 (±823)	14,640 (±937)	13,980 (±920)	13,220 (±739)	13,120 (±1975)
	NEU	3,694 (±550)	5420 (±859)	4801 (±759)	3864 (±532)	3843 (±423)	5237 ^{D0} (±138)	4224 (±952)	3992 (±1113)
	LYM	6,979 (±725)	5265 (±495)	5784 (±383)	5983 (±535)	9957 (±690)	7897 (±854)	8424 (±516)	7681 ^{D0} (±490)
	MON	346 (±69)	305 (±19)	253 (±75)	656 ^{D9} (±154)	413 (±100)	691 (±115)	265 ^{D1} (±59)	610 (±386)
	EOS	240 (±109)	450 (±103)	383 (±144)	716 (±218)	427 (±187)	907 (±300)	281 (±95)	837 (±443)
	RBC	10.2 (±0.5)	9.5 (±0.5)	8.6 ^{D0} (±0.4)	9.4 (±0.4)	10.8 (±0.2)	9.1 ^{D0} (±0.2)	9.3 ^{D0} (±0.2)	8.9 ^{D0} (±1.1)
	Hb	11.9 (±0.4)	11.0 (±0.5)	10.0 ^{D0} (±0.3)	10.7 (±0.4)	12.4 (±0.5)	10.4 ^{D0} (±0.4)	10.7 ^{D0} (±0.3)	10.0 (±1.1)
	Ht	37.6 (±0.8)	34.7 (±1.1)	30.8 ^{D0,D1} (±0.6)	33.3 ^{D0} (±1.2)	38.6 (±1.9)	32.5 ^{D0} (±1.4)	32.9 (±1.3)	31.5 (±3.5)
	VCM	36.9 (±0.9)	36.8 (±0.9)	35.8 (±0.8)	35.2 (±0.8)	35.9 (±1.2)	35.5 (±1.2)	35.2 (±1.2)	35.8 (±1.6)
	CHCM	31.5 (±0.4)	31.6 (±0.5)	32.6 (±0.5)	32.1 (±0.5)	32.1 (±0.7)	32.1 (±0.8)	32.7 (±0.8)	31.9 (±0.7)
	Platelet	672,600 (±70,666)	637,800 (±90,947)	976,400 ^{D0,D1} (±80,846)	1.061,000 ^{D0,D1} (±79,230)	575,600 (±55,855)	485,400 (±33,440)	817,400 ^{D0,D1} (±76,881)	738,800 (±155,814)

* Bi-mtDNA(Low), n = 6; Bi-mtDNA(High), n = 4; Bt-mtDNA(Low), n = 5 and Bt-mtDNA(High), n = 5.

2008, who found that inflammation was associated with high levels of resistance. Moreover, resistant breeds had higher levels of T-cells present in the skin prior to tick infestation and thus seem to respond to ticks more efficiently (Tabor, 2017). These results also align well with the studies of Greer et al. (2018); Hamie et al. (2019) and Greer and Hamie (2016) who have been investigating differences between Romney sheep selected for resistance or resilience to gastrointestinal parasites, suggesting the development of the immunity earlier in resistant line animals.

Additionally, Franzin et al. (2017) showed that there are significant differences between skins from the bovine hosts presenting with different levels of resistance to tick infestations. The authors also demonstrated studying the immune response in skin from Nelore breed (genetically tick-resistant, *Bos taurus indicus*), and Holstein breed (genetically tick-susceptible, *Bos taurus taurus*) that tick-resistant hosts recruit inflammatory responses earlier than susceptible hosts. Our results that clearly showed that *B. indicus* developed an immune response more efficient and earlier than *B. taurus*, corroborate their findings.

On the other hand, Piper et al. (2010) and Tatchell and Moorhouse (1968), showed from both histological and whole genome expression studies of skin sections from these animals that the *B. taurus* host elicits a more vigorous cellular response at the site of tick attachment. Differences between the present data set and the results of others may be attributed to the time post tick exposure that the cells are isolated from the host and also that the majority of the studies were evaluating the tick-attachment site (skin), while the present work evaluated the peripheral blood.

Our additional strategy of analysis evaluating the relation of tick load on hematological and immunological parameters allowed the comparison between the immune profiles of Bt-mtDNA low parasitemia group with the resistant animals (Bi-mtDNA group). Our results demonstrated that Bt-mtDNA group with low parasitemia showed an increase of innate immunity (MON and CD14), suggesting a more resistant profile of these animals.

Interestingly, it is important to notice that Bi-mtDNA group showed a greater number of connections with *R. microplus* counts and also with

Table 2
Influence of Maternal Lineage Ancestry (mtDNA) and Tick Load Following *Rhipicephalus (Boophilus) microplus* Infestation on the Timeline Kinetics of Immunological Parameters in Guzerat Cattle.

	Parameter	*Rhipicephalus (Boophilus) microplus* Infestation Status							
		Low (< 50 ticks)				High (> 50 ticks)			
		D0	D1	D9	D21	D0	D1	D9	D21
Bi-mtDNA*	$CD14^+$ cells	321 (±71)	306 (±63)	475 (±75)	1399 ^{D1} (±682)	351 (±82)	308 (±115)	752 (±173)	634 (±163)
	$CD335^+$ cells	528 (±70)	283 ^{D0} (±46)	390 (±51)	513 ^{D1} (±69)	548 (±145)	261 ^{D0} (±66)	209 ^{D0} (±70)	511 (±142)
	$\gamma\delta^+$ cells	758 (±104)	571 (±131)	599 (±92)	761 (±98)	578 (±158)	362 (±140)	317 (±99)	460 (±115)
	$CD25^+$ cells	256 (±58)	176 (±33)	309 (±62)	318 ^{D1} (±36)	186 (±21)	127 (±20)	206 (±85)	209 ^{D1} (±21)
	T-cells	4,313 (±285)	3433 (±715)	3548 (±338)	4619 ^{D9} (±339)	3749 (±395)	2399 (±494)	2597 (±457)	3550 (±419)
	$CD21^+$ cells	1,590 (±190)	1865 (±242)	1428 (±120)	1547 (±81)	1595 (±491)	1303 (±340)	1017 (±178)	997 (±289)
	$T/CD21^+$ Ratio	3.0 (±0.6)	1.8 (±0.3)	2.5 (±0.3)	3.0 ^{D1} (±0.3)	2.8 (±0.5)	2.1 (±0.4)	2.7 (±0.5)	4.0^{D0,D1,D9} (±0.6)
	$CD4^+$ cells	1,678 (±83)	1509 (±313)	1512 (±188)	1885 (±174)	1317 (±126)	865 (±155)	1042 (±115)	1285 (±225)
	$CD8^+$ cells	1620 (±207)	1177 (±289)	1127 (±121)	1655 ^{D9} (±188)	1669 (±223)	1043 (±268)	1031 (±186)	1595 (±214)
	$CD4^+/CD8^+$ Ratio	1.1 (±0.2)	1.4 (±0.2)	1.4 (±0.2)	1.2 (±0.2)	0.8 (±0.1)	0.9 (±0.2)	1.0 (±0.14)	0.8 (±0.2)
Bt-mtDNA*	$CD14^+$ cells	248 (±52)	352 (±80)	456 ^{D0} (±36)	584 ^{D0} (±72)	275 (±46)	271 (±78)	587 (±203)	575 (±136)
	$CD335^+$ cells	382 (±82)	193 (±31)	295 (±35)	347 ^{D1} (±42)	469 (±123)	204 (±47)	263 (±52)	352 (±81)
	$\gamma\delta^+$ cells	425 (±84)	313 (±82)	303 (±63)	475 (±74)	1006 (±169)	784 (±100)	726 (±163)	728 (±174)
	$CD25^+$ cells	228 (±82)	143 (±7)	293 ^{D1} (±23)	276 ^{D1} (±25)	263 (±37)	159 ^{D0} (±21)	348 ^{D1} (±79)	264 (±46)
	T-cells	3,512 (±286)	2311 ^{D0} (±229)	2864 (±252)	3464 ^{D1} (±175)	4695 (±290)	3466 ^{D0} (±230)	4117 (±420)	4933 (±579)
	$CD21^+$ cells	1,350 (±208)	1263 (±212)	1147 (±122)	1174 (±186)	2268 (±306)	1849 (±344)	1763 (±221)	1603 (±220)
	$T/CD21^+$ Ratio	3.0 (±0.7)	2.0 (±0.4)	2.7 (±0.5)	3.2 (±0.4)	2.2 (±0.2)	1.8 (±0.2)	2.6 (±0.3)	3.0 (±0.6)
	$CD4^+$ cells	1,380 (±119)	950 ^{D0} (±89)	1247 (±164)	1447 ^{D1} (±91)	1817 (±142)	1445 (±107)	1910 ^{D1} (±156)	2278 ^{D1} (±396)
	$CD8^+$ cells	1,479 (±233)	905 (±156)	1022 (±91)	1265 (±101)	1610 (±90)	975 ^{D0} (±88)	1293 ^{D0,D1} (±73)	1451 (±201)
	$CD4^+/CD8^+$ Ratio	1.0 (±0.2)	1.2 (±0.2)	1.2 (±0.1)	1.2 (±0.1)	1.1 (±0.1)	1.3 (±0.1)	1.3 (±0.1)	1.5 (±0.2)

* Bi-mtDNA(Low), n = 6; Bi-mtDNA(High), n = 4; Bt-mtDNA(Low), n = 5 and Bt-mtDNA(High), n = 5.

the $CD25^+$ activation marker of the immune response. The CD25 gene is known to be expressed in activated cells, including T cells, B cells, monocytes, and regulatory T cells (Belkaid, 2007). Our data corroborate those of Piper et al. (2009), who also observed higher expression of CD25 in the blood of resistant Brahman heifers after tick infestation. Furthermore, Constantinoiu et al. (2010) found that after successive tick infestations in Brahman animals, a higher number of $CD25^+$ cells was observed in the skin of resistant animals.

In conclusion, our results pointed out the importance of verifying the evolution of cellular responses through periodic evaluations after tick infestation. The Bi-mtDNA group presents more connections, with an important participation of the innate immune response (NEU, MON, $CD14^+$ and $CD335^+$ cells) while the Bt-mtDNA group showed a delay in the immune response. Elucidating the mechanisms by which resistant animals prevent heavy tick infestation is a crucial step in the development of predictive biomarkers for tick resistance for use in selective breeding programs, and is also potentially useful for the development of anti-tick vaccines. Our findings reinforce that analysis of mtDNA can help define the resistant immune profile of the animals; however additional studies examining cytokines and chemokines can bring a better understanding of the immunological mechanisms.

Authors' contributions

JFFB, MSSA, OAMF, FFA participated in the design of the study, the statistical analysis, and drafting or revising the manuscript. JBR, GCG, GCV, FFA participated in obtaining the samples and conducting the experiments, and performing the immunoassays and parasitological test. ERB participated in the design of the study, in coordination of research, and in drafting the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors confirm that they have no conflicts of interest in this work.

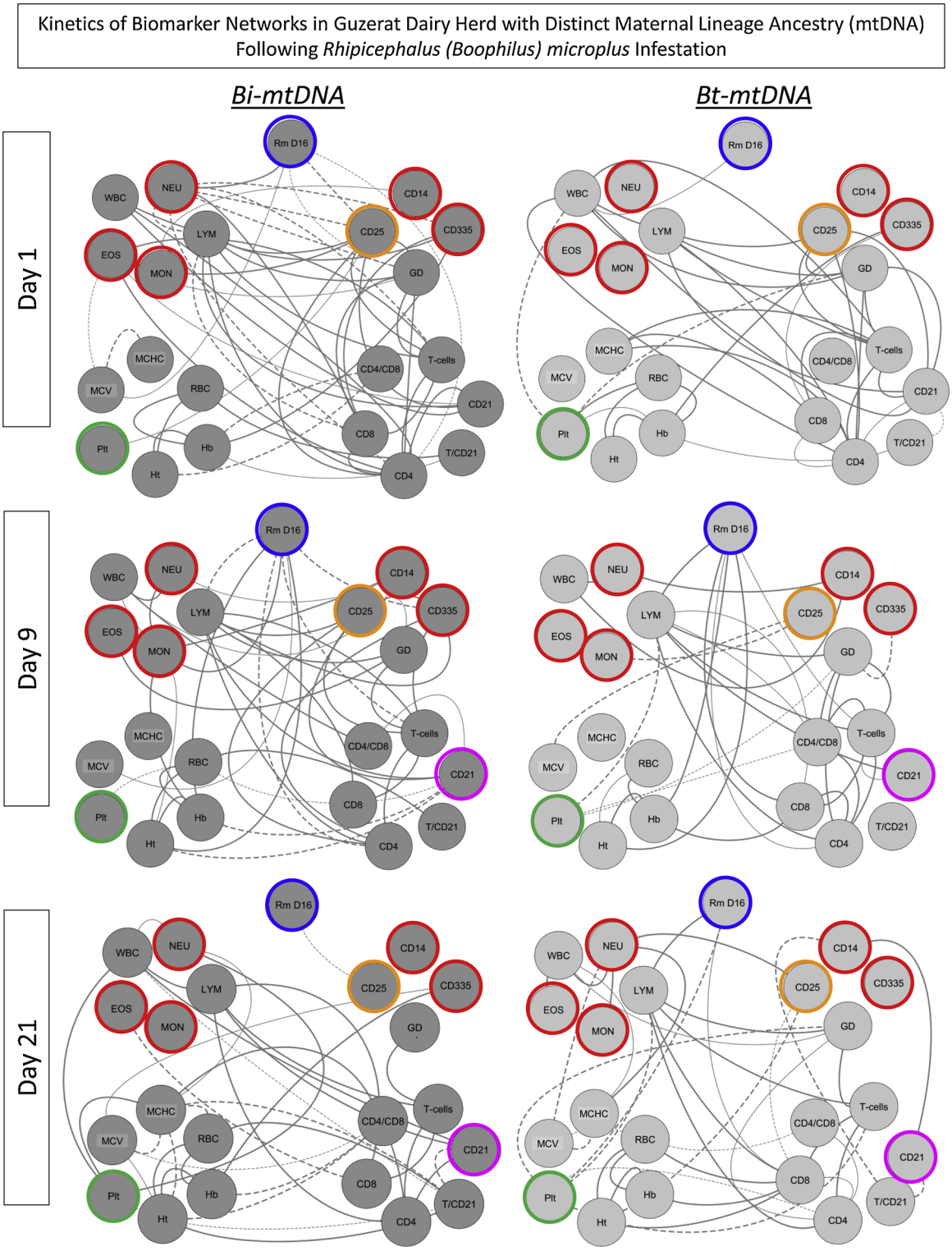

Fig. 4. Biomarker networks in Guzerat dairy herd with distinct maternal lineage ancestry (mtDNA) following *R. microplus* infestation.
Circular layouts were built to underscore the relevant associations between cell subsets using a clustered distribution of nodes for leukogram (left top side); erythrogram (left bottom side); innate immunity cells (right top side) and adaptive immunity cells (right bottom side). The network node neighborhood connections points out the pattern of the immune profile in Bi-mtDNA (dark gray nodes) and Bt-mtDNA (light gray nodes) following *R. microplus* infestation.

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