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Quantification and comparison of TLR2 activity in monocyte-derived macrophages of zebu and crossbred cattle

Baranwal, A.[**]; Sonwane, A.[*, **]; Chauhan, A.; Panigrahi, M. and Sharma, A. K.

Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India

[*]***Correspondence:*** *A. Sonwane, Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, Bareilly, Uttar Pradesh, India. E-mail: arvind.sonwane@icar.gov.in*

[**] These authors contributed equally to this work and should be considered as first authors

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Summary

The present study was conducted to quantify and compare TLR2 (toll-like receptor 2) activity in monocyte-derived macrophages of zebu (Tharparkar) and crossbred (Holstein-Friesian × Jersey × Brown Swiss × Hariana) cattle. The cells were either induced with Pam3CSK4 or kept as control. The TLR2 activity was quantified in terms of IκB-α inhibitory subunit (NFKBIA) messenger RNA (mRNA) copies using real-time, one-step reverse transcription-polymerase chain reaction (RT-PCR). Toll-like receptor 2 activity of induced cells was in the range of 1060421 ± 477937 (n=3) to 3514715 ± 290222 (n=3) copies for Tharparkar cattle (n=7) and in the range of 1365532 ± 47243 (n=3) to 3016510 ± 172340 (n=3) copies for the crossbred cattle (n=7). For uninduced cells, this activity was within the range of 117 ± 51 (n=3) to 293 ± 103 (n=3) copies for the Thraparkar cattle (n=7), and in the range of 182 ± 122 (n=3) to 296 ± 88 (n=3) copies for the crossbred cattle (n=7). The TLR2 activity of induced cells in both groups was found to be significantly higher than that of the respective uninduced cells (P<0.0001). Furthermore, upon comparison, TLR2 activities of induced and uninduced cells of the Tharparkar were not found to be significantly different from those of the crossbred cattle (P=0.8154 and P=0.6670). In the present study, we have quantified and compared, for the first time, TLR2 activity in terms of NFKBIA mRNA copies in monocyte-derived macrophages of Tharparkar and crossbred cattle and found that both have equivalent TLR2 activity.

Key words: Crossbred, Monocyte-derived macrophages, NFKBIA, Tharparkar, TLR2

Introduction

Zebu (*Bos taurus indicus*) cattle have been adapted to the tropical environment of the Indian subcontinent (Chan *et al*., 2010) and are found to be more efficient in coping with infections than taurine (*Bos taurus taurus*) cattle. It has been demonstrated that zebu cattle are more resistant to babesiosis (Parker *et al*., 1985; Bock *et al*., 1999), ticks (Rechav and Kostrzewski, 1991; Wambura *et al*., 1998), and nematodes (Pẽna *et al*., 2000), when compared to taurine cattle. Monocyte derived macrophages are very important cells of the immune system that produce pro-inflammatory cytokines to initiate innate immunity and activate adaptive immunity via pattern recognition receptors (PRRs) when coming into contact with pathogens (Sun *et al*., 2017). Monocyte-derived macrophages from zebu were found to be more efficient to control *Brucella abortus* intracellular survival than macrophages from taurine cattle (Macedo *et al*., 2014). The host's response to a pathogen is a complex phenomenon involving various factors. Delineating varied responses of zebu and taurine cattle or their crossbreeds would be highly useful for breeding and/crossbreeding practices to increase animal disease resistance.

Toll-like receptors (TLRs) are important components of the host's responses to infection. They are a class of highly conserved membrane-bound PRRs that play an integral role in the regulation of the immune system through the recognition of pathogen-associated molecular patterns (PAMPs) and the activation of immune response genes (Beutler, 2004; Creagh *et al*., 2006). Several studies have reported the relationship between TLR gene polymorphisms and disease resistance or susceptibility (Sun *et al*., 2012; Yapan *et al*., 2014).

Toll-like receptor 2 (TLR2), which has been studied in substantial detail, is an important TLR with a wide range of specific agonists such as microbial products representing broad groups of species such as Gram-positive and Gram-negative bacteria, as well as mycobacteria, spirochetes, and mycoplasma (Basith *et al*., 2011). The importance of TLR2 is underscored by the fact that it is considered as a molecular link between microbial products, apoptosis, and host defense mechanisms (Aliprantis *et al*., 1999; Kirschning and Schumann, 2002). Toll-like receptor 2, TLR4 and TLR9 have been implicated in host interactions with *Brucella* (Oliveira *et al*., 2008), and TLR2 was found to be critical for the clearance of pathogenic *Brucella* strains

(Surendran *et al*., 2012).

Activating TLR2 results in the initiation of intracellular signal cascades that involve the adaptor protein encoded by the myeloid differentiation primary response gene 88 (MyD88), which in turn, activates the nuclear factor kappa B (NF-κB) and leads to the production of interferon (Aliprantis *et al*., 2001; Miggin and O'Neill, 2006). As a feedback mechanism, the termination of NF-κB transcription is mediated through the NF-κB-dependent synthesis of the IκB-α inhibitory subunit (NFKBIA). So according to Bottero *et al*. (2003), the transcriptional power of NF-κB can be measured by measuring the NFKBIA messenger RNA (mRNA) level which is a rapid, sensitive, and powerful method for this purpose.

The aim of the present study was to understand the varied host responses of Tharparkar and crossbred cattle at the TLR2 activity level. We quantified and compared, for the first time, the TLR2 activities in terms of NFKBIA copies in monocyte-derived macrophages of these cattle.

Materials and Methods

Animals

Seven animals each from Tharparkar and crossbred cattle of the same age, sex, and health status maintained at the Indian Veterinary Research Institute, Izatnagar, India, were used for the study. The maintenance of these animals and the experiments followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Isolation of peripheral blood mononuclear cells (PBMCs) and derivation of macrophages

Three milliliters of blood were collected aseptically from the jugular vein in a sterile polypropylene vial containing 30 µL of 20% di-Sodium EDTA and mixed properly. It was layered on 3 ml Histopaque-1.083 (Sigma, cat No. 10831-100 ML) in a 15 ml centrifuge tube and centrifuged at 400 × g for 30 min at room temperature. The supernatant was aspirated and discarded without disturbing the buffy coat which was in turn, carefully aspirated and transferred to a clean 15 ml centrifuge tube. It was then diluted with 13 ml sterile calcium, magnesium-free phosphate buffer saline (PBS) (Hyclone cat No. SH3025802) and centrifuged at 800 × g for 10 min after which the supernatant was discarded. RBCs from the cell pellet were lysed by adding five pellet volumes of chilled RBC lysis buffer to the pellet, and incubated for 5 min on ice while being mixed intermittently with a pipette. The PBMCs were then washed three times with PBS and re-suspended in 6 ml of a growth medium containing 90% RPMI-1640 (Hyclone cat No. SH30011.02), 10% fetal bovine serum (FBS), (Gibco cat No. 16000044), 15.6 mM HEPES (Sigma cat No. H3375-100G), 0.3% sodium bicarbonate and 50 µg/ml gentamicin. Two milliliters of cell suspension were then added to each well of a 6-well tissue culture plate. The cells were incubated at 37°C and 5% CO_2 for 24 h. Media were changed to remove non-adherent cells. The adherent monocytes were further incubated for 11 days during which the media was changed at an interval of three days to derive macrophages. Media from the cells were then removed and the cells were washed with 0.5 ml PBS. To dislodge the macrophages, 1 ml PBS-EDTA (PBS with 15 mM EDTA) was added in each well and the cells were incubated on ice for 30 min. This dislodged the macrophages which were then washed with PBS and finally re-suspended in the growth medium.

Induction of monocyte-derived macrophages and their processing for real-time, one step RT-PCR

Monocyte-derived macrophages from each animal were seeded, in triplicates, in a 96-well plate. Each well was seeded with 100 µL cell suspension containing 40,000 cells in the growth medium. After incubation for 48 h, they were induced with 1 µg/ml lipoprotein Pam3CSK4 (Invivogen cat No. tlrl-pms). Cells in control wells were kept uninduced. Six h after induction, cells were processed for real-time, one-step RT-PCR using a FastLane Cell Multiplex Kit (Qiagen cat No. 216513) following the protocol recommended by the manufacturer. Briefly, media from the wells were aspirated and to each well 125 µL of Buffer FCW was added. Immediately after this, Buffer FCW was removed and to each well 50 µL of cell processing mix was added and the plate was incubated at room temperature for 5 min. The FastLane cell lysates containing stabilized RNA obtained in this way from each well were then transferred to 0.6 ml centrifuge tubes and incubated for 5 min at 75°C. The concentration of the RNA was determined using Qubit RNA Assay Kit (Invitrogen cat No. Q32852) and Qubit 2.0 Fluorometer (Invitrogen cat No. Q32866). Finally, the concentration of all the samples was adjusted to 20 ng/µL.

Real-time, one step RT-PCR and determination of NFKBIA mRNA copies

Preparation of standards

Bovine NFKBIA complementary DNA (cDNA) was isolated from LPS-induced bovine monocyte-derived macrophages using RT-PCR. It was then cloned into pTargeT mammalian expression vector (Promega cat No. A1410) to construct pTargeT-NFKBIA. NFKBIA transcripts were produced *in vitro* using RiboprobeSystem-SP6/T7 (Promega cat No. P1460), following the manufacturer's protocol. Briefly, the 100 µL *in vitro* transcription reaction mix consisted of 20 µL Transcription optimized 5x buffer, 10 µL 100 mM dithiothreitol (DTT), 2.5 µL 40 U/µL RNase inhibitor Recombinant RNasin, 5 µL 10 mM adenosine 5'-tri phosphate (rATP), 5 µL 10 mM guanosine 5'-tri phosphate (rGTP), 5 µL 10 mM cytidine 5'-tri phosphate (rCTP), 5 µL 10 mM uridine 5'-tri phosphate (rUTP), 3 µL 2 µg/µL linearized template (pTargeT-NFKBIA)

DNA, 2 µL 20U/µL T7 RNA polymerase and 42.5 µL nuclease-free water. The reaction mix was incubated for 2 h at 37°C for *in vitro* transcription. The DNA was then removed by adding 6U RNase-free deoxyribonuclease (DNase) and incubating the mix further at 37°C for 15 min. The volume of the mixture was finally raised to 250 µL with nuclease-free water and the transcribed RNA was purified using 750 µL Trizol LS reagent (Invitrogen cat No. 10296-010). The concentration of the transcribed RNA was determined as described in the previous section. The NFKBIA transcript copy number was determined using the ENDMEMO DNA/RNA Copy Number Calculator available at www.endmemo.com/bio/dnacopynum.php. Standards comprising of six different dilution points were prepared by 3 fold serial dilution. Every 2 µL of respective standard solution contained 1305467, 3916401, 11749202, 35247611, 105742833, and 317228500 copies of NFKBIA mRNA.

Real-time, one step RT-PCR

Unknown copy numbers of NFKBIA mRNA of the samples were determined by real-time, one-step RT-PCR using a QuantiTect Multiplex RT-PCR Kit (Qiagen cat No. 204643), TaqMan Assay (Life Technologies Assay ID Bt03237837_m1; cat Nos. 4331182 & 4448892), StepOne Plus Real-Time PCR System (Life Technologies) and StepOne Software (Life Technologies) following the manufacturers' protocols recommended. Three replicates for each sample and standard were kept and experiments were repeated thrice. Real-time, one-step RT-PCR was carried out for the samples and standards by adding the following components into 0.1 ml tubes: 2x QuantiTect Multiplex RT-PCR Master Mix, 10 µL; 20x primer-probe mix (TaqMan Assay) 1 µL; QuantiTect Multiplex RT Mix, 0.2 µL; RNase-free water, 6.8 µL and Template RNA (FastLane cell lysates containing 20 ng/µL total RNA or *in vitro* transcribed RNA standards containing respective NFKBIA mRNA copies), 2 µL. The reaction conditions were as follows: 50°C for 20 min (RT); 40 cycles of 95°C for 15 min (PCR initial activation); 94°C for 45 s (denaturation) and 60°C for 45 s (annealing and extension). A standard curve was generated and NFKBIA mRNA copies from the samples were calculated using StepOne software. The results were expressed as NFKBIA mRNA copies per 5×10^5 cells.

Statistical analysis

GraphPad Prism 6.0 was used for the statistical analyses. A two-way ANOVA was performed using the Sidak multiple comparison test (with a 95% confidence interval) to compare the TLR2 activities between the respective groups. All values were considered significant when $P<0.05$. All experiments were performed in triplicates.

Results

TLR2 activity in monocyte-derived macrophages of Tharpakar and crossbred cattle

TLR2 activity was quantified in monocyte-derived macrophages of Tharpakar and crossbred cattle. The cells were induced with lipoprotein Pam3CSK4 and the TLR2 activity was quantified in terms of NFKBIA mRNA copies. For the Tharparkar cattle, the toll-like receptor 2 activity of the induced cells was in the range of 1060421 ± 477937 (n=3) to 3514715 ± 290222 (n=3) copies, while for the uninduced cells (n=7) it was in the range of 117 ± 51 (n=3) to 293 ± 103 (n=3) copies (Table 1). The TLR2 activity of induced cells was found to be significantly higher than that of the uninduced cells ($P<0.0001$) (Fig. 1A). In case of the crossbred cattle (n=7), the toll-like receptor 2 activity of induced cells was in the range of 1365532 ± 47243 (n=3) to 3016510 ± 172341 (n=3) copies, it was in the range of 182 ± 122 (n=3) to 296 ± 88 (n=3) copies for the uninduced cells (n=7) (Table 2). The TLR2 activity of induced cells was found to be significantly higher than that of uninduced cells ($P<0.0001$) (Fig. 1B).

Comparison of TLR2 activities of monocyte-derived macrophages of Tharpakar and crossbred cattle

We compared TLR2 activities in monocyte-derived macrophages of Tharpakar and crossbred cattle using GraphPad Prism 6.0. A two-way ANOVA was performed using Sidak multiple comparison test with a 95% confidence interval. The toll-like receptor 2 activity of induced cells of Tharparkar and crossbred cattle were not found to be significantly different ($P=0.8154$) (Table 3) (Fig. 2A). Similarly, the TLR2 activity of uninduced cells of Tharparkar and crossbred cattle were significantly different ($P=0.6670$) (Table 4) (Fig. 2B).

Table 1: TLR2 activity in terms of NFKBIA mRNA copies in monocyte-derived macrophages of Thraprkar cattle

Animal	NFKBIA mRNA copies per 5×10^5 cells (mean±SEM)		Sidak's multiple comparisons test
	Pam3CSK4$^+$	Pam3CSK4$^-$	
1	1306817 ± 30044 (n=3)	218 ± 100 (n=3)	***
2	2137866 ± 302703 (n=3)	224 ± 99 (n=3)	****
3	1366306 ± 112474 (n=3)	145 ± 65 (n=3)	****
4	1230970 ± 58526 (n=3)	142 ± 43 (n=3)	***
5	1060421 ± 477937 (n=3)	293 ± 103 (n=3)	**
6	3514715 ± 290222 (n=3)	117 ± 51 (n=3)	****
7	3326874 ± 218989 (n=3)	196 ± 106 (n=3)	***

Two-way ANOVA. ** ($P<0.01$), *** ($P<0.001$), and **** ($P<0.0001$)

Table 2: TLR2 activity in terms of NFKBIA mRNA copies in monocyte-derived macrophages of crossbred cattle

Animal	NFKBIA mRNA copies per 5×10^5 cells (mean±SEM)		Sidak's multiple comparisons test
	$Pam3CSK4^+$	$Pam3CSK4^-$	
1	1585380 ± 155347 (n=3)	198 ± 85 (n=3)	****
2	2152301 ± 226099 (n=3)	229 ± 98 (n=3)	****
3	1365532 ± 47243 (n=3)	200 ± 86 (n=3)	****
4	1392479 ± 176759 (n=3)	183 ± 101 (n=3)	****
5	1741054 ± 115253 (n=3)	182 ± 122 (n=3)	****
6	3016510 ± 172341 (n=3)	195 ± 106 (n=3)	****
7	2851933 ± 338464 (n=3)	296 ± 88 (n=3)	****

Two-way ANOVA. **** (P<0.0001)

Table 3: Comparison of TLR2 activity in terms of NFKBIA mRNA copies in induced monocyte-derived macrophages of Tharparkar and crossbred cattle

Animal	NFKBIA mRNA copies per 5×10^5 cells (mean±SEM)		Sidak's multiple comparisons test
	Tharparkar	Crossbred	
1	1306817 ± 30044 (n=3)	1585380 ± 155347 (n=3)	NS
2	2137866 ± 302703 (n=3)	2152301 ± 226099 (n=3)	NS
3	1366306 ± 112474 (n=3)	1365532 ± 47243 (n=3)	NS
4	1230970 ± 58526 (n=3)	1392479 ± 176759 (n=3)	NS
5	1060421 ± 477937 (n=3)	1741054 ± 115253 (n=3)	NS
6	3514715 ± 290222 (n=3)	3016510 ± 172341 (n=3)	NS
7	3326874 ± 218989 (n=3)	2851933 ± 338464 (n=3)	NS

Two-way ANOVA. NS: Not-significant (P=0.8154)

Table 4: Comparison of TLR2 activity in terms of NFKBIA mRNA copies in uninduced monocyte-derived macrophages of Tharparkar and crossbred cattle

Animal	NFKBIA mRNA copies per 5×10^5 cells (mean±SEM)		Sidak's multiple comparisons test
	Tharparkar	Crossbred	
1	218 ± 100 (n=3)	198 ± 85 (n=3)	NS
2	224 ± 99 (n=3)	229 ± 98 (n=3)	NS
3	145 ± 65 (n=3)	200 ± 86 (n=3)	NS
4	142 ± 43 (n=3)	183 ± 101 (n=3)	NS
5	293 ± 103 (n=3)	182 ± 122 (n=3)	NS
6	117 ± 51 (n=3)	195 ± 106 (n=3)	NS
7	196 ± 106 (n=3)	296 ± 88 (n=3)	NS

Two-way ANOVA. NS: Not-significant (P=0.6670)

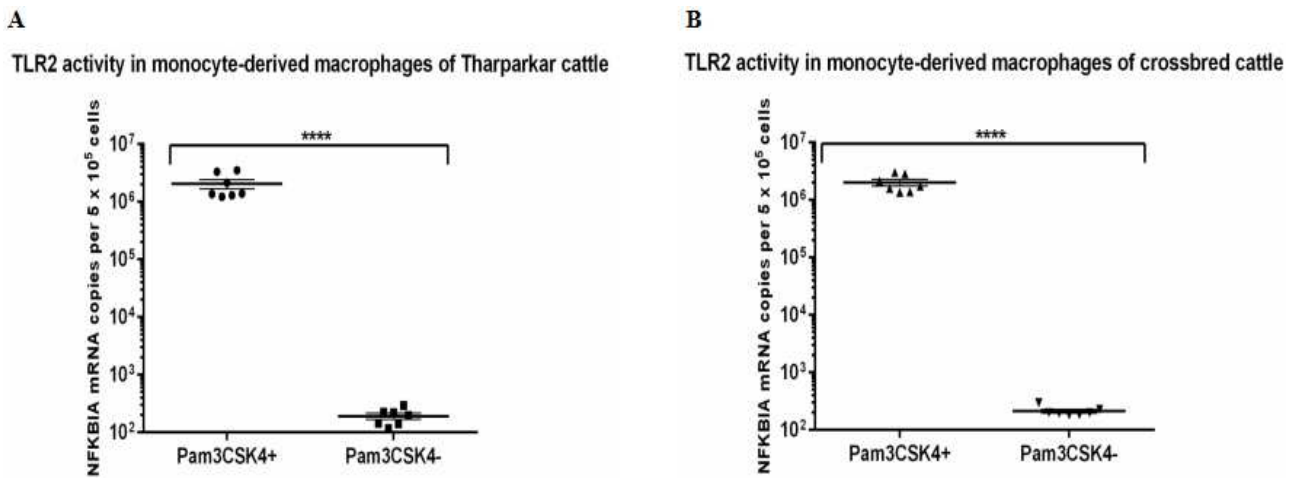

Fig. 1: TLR2 activity in monocyte-derived macrophages of Tharparkar and crossbred cattle. Cells were either induced with TLR2 ligand Pam3CSK4@ 1 µg/ml for 6 h or kept as control. TLR2 activity was determined in terms of NFKBIA mRNA copies using real-time, one step RT-PCR. The experiments were carried out for seven animals for each group and were repeated thrice. TLR2 activities in monocyte-derived macrophages of Tharparkar (**A**) and crossbred (**B**) cattle are presented here. Induction of cells with Pam3CSK4 significantly increased TLR4 activities both in Tharparkar as well as crossbred cattle (P<0.0001)

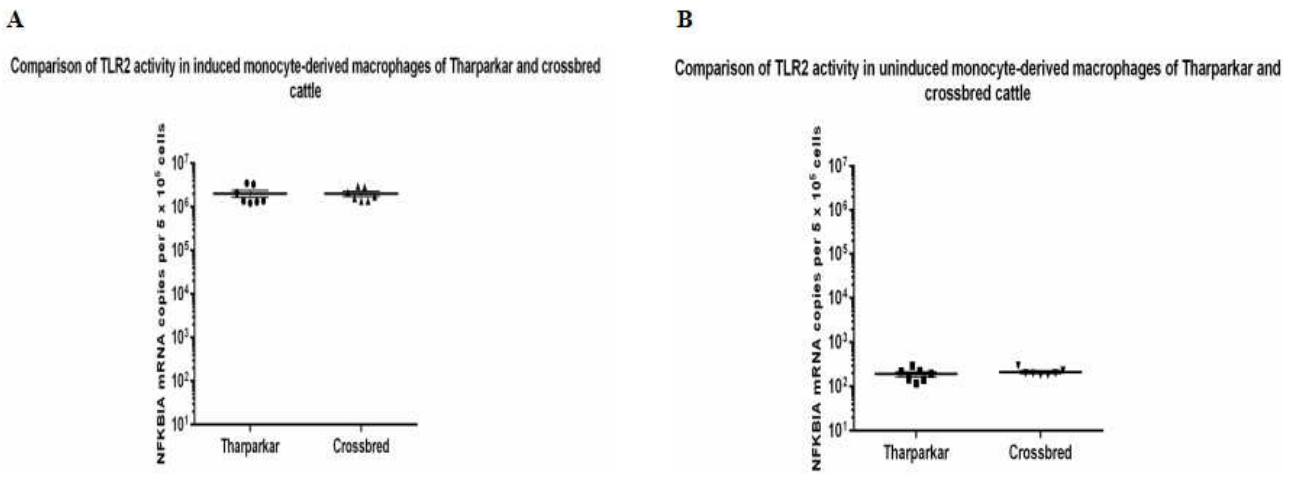

Fig. 2: Comparison of TLR2 activities in monocyte-derived macrophages of Tharparkar and crossbred cattle. Cells were either induced with TLR2 ligand Pam3CSK4@ 1 µg/ml for 6 h or kept as control. TLR2 activity was determined in terms of NFKBIA mRNA copies by using real-time, one step RT-PCR. TLR2 activities in monocyte-derived macrophages of Tharpakar and crossbred cattle were compared using GraphPad Prism 6.0. A two-way ANOVA using Sidak multiple comparison test keeping 95% confidence interval was performed. TLR4 activity of induced (**A**) as well as uninduced (**B**) cells of Tharparkar and crossbred cattle were significantly different (P=0.8154 and P=0.6670)

Discussion

In this study we have quantified and compared, for the first time, TLR2 activity in terms of NFKBIA mRNA copies in monocyte-derived macrophages of Tharparkar and crossbred cattle and found that monocyte-derived macrophages of zebu and crossbred cattle have equivalent TLR2 activity.

It is a widely accepted notion that zebu and taurine cattle cope with infections differently and zebu are more tolerant to the infections (Parker *et al*., 1985; Rechav and Kostrzewski, 1991; Wambura *et al*., 1998; Bock *et al*., 1999; Pĕna *et al*., 2000; Macedo *et al*., 2014). Different host responses to an infection are a function of various components including physical barriers (Potempa and Pike, 2009), body temperature, pH, TLRs and other PRRs (Kawai and Akira, 2010), pathogen-specific receptors, MHC repertoire (Eskra *et al*., 2003; Ko and Splitter, 2003), phagocytosis (Price *et al*., 1990; Qureshi *et al*., 1996; Martínez *et al*., 2010), oxidative radicals (Jiang *et al*., 1993; Macmicking *et al*., 1997; Wang *et al*., 2001; Ko *et al*., 2002; Sun *et al*., 2002), cytokines, interleukins (Zhan *et al*., 1996), essential elements like iron deprivation systems (Gruenheid *et al*., 1995; Adams and Templeton, 1998; Blackwell and Searle, 1999; Barthel *et al*., 2001), interferons (Jiang and Baldwin, 1993; Splitter *et al*., 1996; Oliveira *et al*., 2002) and components of humoral and cellular immune responses along with immunological memory. All these components are influenced by the genetic background of the host, causing it to respond differently to the infection. Several studies have quantified and compared the overall host response to infection vis-à-vis the host's genetic background, but meager information is available on the quantification and comparison of individual components in this context which would be more informative and may lead to novel tools for future diagnosis and/or prognosis.

Toll-like receptor-mediated immunity is an important component of a host's response to infection, and as part of the innate immune system, it eventually activates and aids the adaptive immune system (Adams and Schutta, 2010). Toll-like receptor 2 is an important TLR which recognizes its ligand from a wide range of pathogens and activates the immune system through expression of various pro-inflammatory cytokines (Aliprantis *et al*., 1999; Kirschning and Schumann, 2002). The present study was designed to quantify TLR2 activity in zebu and crossbred cattle and to find differences, if any, in TLR2 activity between these two groups. We used Tharkparkar cattle in our zebu group, while animals of a cross between Holstein-Friesian, Jersey, Brown Swiss and Hariana cattle were used in the crossbred group. The Tharparkar, also known as White Sindh, is an important dual purpose zebu cattle breed used for milk production as well as for draught purpose. It is also known for its heat tolerance ability and considered to be more tolerant to infections compared to taurine cattle or crossbreds produced from crosses with taurine cattle (Cunningham and Syrstad, 1987). The crossbred cattle used in this study carried 50-75% inheritance from taurine cattle (Holstein-Friesian, Jersey and Brown Swiss) and 25-50% inheritance from zebu (Hariana) cattle.

Toll-like receptor 2 activity can be measured by quantifying the mRNA copy number of the NFKBIA gene since TLR2 activation ultimately results in the expression of NF-κB which, in turn, activates NFKBIA gene expression apart from activating expressions of other genes for pro-inflammatory cytokines (Bottero *et al*., 2003). The advantage of this method is its direct quantification of TLR2 activation compared to other rather indirect functional assays. This could shed light on the "inherent potential" of the TLR2 system of a particular individual or group of individuals in question.

We specifically induced the macrophages with TLR2 ligand lipoprotein Pam3CSK4 and quantified the mRNA

copy number of the NFKBIA gene as a function of TLR2 activity. We also quantified the mRNA copy number of the NFKBIA gene in the same cells that were not induced with the ligand in order to find out the basal NFKBIA expression. Here, since cells were activated with pure lipoprotein Pam3CSK4, we can safely assume that whatever mRNA copy of the NFKBIA gene we found above the basal level was a function of the TLR2 activity. We expressed this TLR2 activity in terms of NFKBIA mRNA copy number per 5×10^5 cells. We quantified and recorded the TLR2 activities in lipoprotein Pam3CSK4 induced or uninduced monocyte-derived macrophages of Tharparkar and crossbred cattle. This, we believe, is the first reference of this sort.

We found that the activation of cells with lipoprotein Pam3CSK4 significantly increased TLR2 activity in both Tharparkar and crossbred groups. This may be because lipoprotein Pam3CSK4 activates TLR2 receptors which in-turn, activate the downstream signaling cascade, eventually activating the expression of NF-κB which ultimately up-regulates the expression of the NFKBIA gene.

When we compared the TLR2 activities between Tharparkar and crossbred cattle we found that TLR2 activities in non-activated as well as activated cells did not differ significantly between these groups. This suggests that even though the genetic composition of the animals of these two groups is different and that they may have overall varied responses to the infection, their response at the level of TLR2 activity remained similar. This is highly possible since response to infection is a multi-factorial reaction whose outcome may be different because of the overall activities of several components wherein a certain component(s) may be similar in activity between the groups. For example, Macedo *et al*. (2013) demonstrated that, monocyte-derived macrophages from zebu were able to more efficiently control *B. abortus* intracellular survival than macrophages from taurine cattle. They compared the ability of cells to control the intracellular survival of the pathogen. This phenomenon is the end result of so many individual processes, some of which may be different and others, similar. Toll-like receptor 2 activation may be an example of such a phenomenon which shows similarity between these two groups. Apart from this, the extent of genetic variability will also have an impact on the outcome. Our experiments used animals from zebu and crossbred groups, while Macedo *et al*. (2013) used zebu and taurine cattle in their experiment. Also documented are the differential host responses to infections by different zebu breeds. The zebu breed Nellore is more resistant to *Rhipicephalus* (*Boophilus*) *microplus* and *Babesia bovis* compared to other zebu breeds (Utech *et al*., 1978). So, breeds different from the Tharparkar used here may show different results. A noteworthy observation here is that in our case, crossbreeding did affect TLR2 activity of the crossbred animals in comparison to zebu cattle. This may be important to note since it is commonly believed that upgrading zebu cattle for improved milk production through crossbreeding with taurine cattle makes them less tolerant to infections. Although it is too early to comprehensively claim this without comparing zebu and crossbred animals' overall host responses to infection, we can safely conclude that at least in the above crossbreed, there was no difference with Tharparkar zebu cattle, regarding TLR2 activity levels.

In conclusion, TLR2 activity in Tharparkar zebu and crossbred cattle is equivalent. These results suggests the crossbred cattle were not inferior to Tharparkar zebu cattle as per as their TLR2 activity.

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Conflict of interest

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

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