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Lipopolysaccharide-induced inhibition of transcription of *tlr4 in vitro* is reversed by dexamethasone and correlates with presence of conserved NFκB binding sites

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ABSTRACT

Engagement of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) is a master trigger of the deleterious effects of septic shock. Horses and humans are considered the most sensitive species to septic shock, but the mechanisms explaining these phenomena remain elusive. Analysis of *tlr4* promoters revealed high similarity among LPS-sensitive species (human, chimpanzee, and horse) and low similarity with LPS-resistant species (mouse and rat). Four conserved nuclear factor kappa B (NFκB) binding sites were found in the *tlr4* promoter and two in the *md2* promoter sequences that are likely to be targets for dexamethasone regulation. *In vitro* treatment of equine peripheral blood mononuclear cells (eqPBMC) with LPS decreased transcripts of *tlr4* and increased transcription of *md2* (myeloid differentiation factor 2) and cd14 (cluster of differentiation 14). Treatment with dexamethasone rescued transcription of *tlr4* after LPS inhibition. LPS-induced transcription of *md2* was inhibited in the presence of dexamethasone. Dexamethasone alone did not affect transcription of *tlr4* and *md2*.

1. Introduction

Humans and horses are among the most sensitive species to endotoxemia and septic shock, presenting high mortality rates despite treatment [1–3]. Septic shock is generally associated with infection with lipopolysaccharide (LPS) containing gram-negative bacteria. While mice require as much as 18 mg/kg of LPS to succumb to septic shock [4], humans will develop clinical signs of systemic inflammatory reaction with as low as 2 ng/kg [5] and horses will develop septic shock with 30 ng/kg [6].

Lipopolysaccharides are recognized by Toll-Like receptor 4 (TLR4) in combination with myeloid differentiation factor 2 (MD2) and cluster of differentiation 14 (CD14) [7–12]. The complex formed by TLR4-MD2-CD14 signals through a conserved pathway that results in the phosphorylation of nuclear factor kappa B (NFκB) p65 subunit, which translocates into the nucleus and initiates transcription of several pro-inflammatory cytokines, such as Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) [13–15]. These cytokines are responsible for the initiation of the innate immune response, but also account for the pathogenesis of septic shock and consequently affect mortality.

The alleged higher sensibility of humans and horses to septic shock has generally been supported by anecdotal evidence. Studies of *tlr4* polymorphisms in both humans and horses found substitutions in the TLR4 protein sequences. In the horse, none of them correlated with the inter-individual differences in TNF-α production after LPS challenge *in vitro* [16]. On the other hand, in humans, it was found that one of those substitutions interfere with LPS binding to the receptor ectodomain [17]. Still, to date there is no known mechanism that explains why humans and horses are more sensitive to LPS when compared to rodents.

Sequence similarity of *tlr4*, *md2*, and *cd14* promoters among mammals whose genome was completely sequenced were analyzed, including the conservation of NFκB binding sites. To validate the functionality of these conserved NFκB binding sites, transcription of *tlr4*, *md2*, and *cd14* genes was studied *in vitro* after treatment of equine peripheral blood mononuclear cells (eqPBMC) with LPS and/or dexamethasone. A hypothesis that the anti-inflammatory drug dexamethasone acts by regulating the expression of

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the TLR4-MD2-CD14 complex at the transcriptional level was tested *in vitro*.

2. Materials and methods

2.1. In silico analysis

Nucleotide sequences for the *tlr4*, *md2* and *cd14* genes containing a 5′UTR region of 2 kb upstream the transcription initiation site were retrieved from Ensembl [18] (Table 1). Global pairwise sequence alignments were performed using the rVISTA from the VISTA package [19]. Analysis was performed looking for sequence similarity patterns and conservation of NFκB binding sites across selected sequences, using the horse sequence as reference.

2.2. Equine PBMC

Blood obtained from horses resident at the Veterinary School of the University of São Paulo, Brazil, was used. The cohort included both male and female horses of several breeds in the ages of 10–20 years, all free from clinical signs of disease. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of the University of São Paulo (CEEA 01/2006). Equine PBMC (eqPBMC) were obtained by centrifugation of blood samples on Ficoll–Paque (GE Biosciences) according to the manufacturer's instructions.

2.3. Lipopolysaccharide stimulation and dexamethasone treatment

PBMC were plated at 10^6 cells/mL in DMEM:F12 (1:1, v/v), 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Life Technologies). For experiments using dexamethasone, cells were incubated for 90 min in the presence of different concentrations of dexamethasone (Calbiochem) followed by stimulation with 100 ng/mL ultra-pure LPS from *Escherichia coli* O111:B4 (Invivogen, purified by enzymatic hydrolysis followed by phenol–TEA–DOC extraction) (100 ng/mL equivalent to 10 EU/mL). At established time-points, cells were harvested and processed for qPCR.

Table 1
Complete and annotated genes analyzed.

Species	Genbank entry	Gene
Homo sapiens-human	GRCh37:9:120464610:120466609:1	*tlr4*
	GRCh37:8:74901587:74903586:1	*md2*
	GRCh37:5:140013256:140015255:-1	*cd14*
Equus caballus-horse	EquCab2:25:21964163:21966162:1	*tlr4*
	EquCab2:9:12574784:12576783:-1	*md2*
	EquCab2:14:36267120:36269119:1	*cd14*
Pan troglodytes-chimpanzee	CHIMP2.1:9:117237071:117239070:1	*tlr4*
	CHIMP2.1:8:72435564:72437563:1	*md2*
	CHIMP2.1:5:142392451:142394450:-1	*cd14*
Bos taurus-cow	Btau_4.0:8:112424793:112426792:1	*tlr4*
	Btau_4.0:Un.004.1:1344362:1346361:-1	*md2*
	Btau_4.0:7:51086967:51088966:-1	*cd14*
Mus musculus-mouse	NCBIM37:4:66486645:66488644:1	*tlr4*
	NCBIM37:1:16676514:16678513:1	*md2*
	NCBIM37:18:36886391:36888390:-1	*cd14*
Sus scrofa-pig	Sscrofa9:1:271619935:271621934:1	*tlr4*
	Sscrofa9:4:64133466:64135465:-1	*md2*
	Sscrofa9:2:129357849:129359848:-1	*cd14*
Rattus norvegicus-rat	RGSC3.4:5:83562096:83564095:1	*tlr4*
	RGSC3.4:5:1705241:1707240:-1	*md2*
	RGSC3.4:18:29376329:29378328:-1	*cd14*

2.4. Transcripts quantification

Total RNA was isolated from cells using Trizol. cDNA was generated using 200 ng of total RNA, oligo(dT) primers and Reverse Transcriptase SuperScript III (all from Life Technologies). Expression levels of *tlr4*, *md2*, and *cd14* mRNAs were measured by real-time PCR (qPCR) using Quantifast SYBR green PCR kit (Qiagen) and the primers listed in Table 2. Cycling conditions were one initial cycle of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, plus one final cycle of 95 °C for 60 s followed by 55 °C for 30 s and 95 °C for 30 s. The qPCR products were of predicted sizes and yielded only one conserved peak at melting analysis (data not shown). The amount of specific mRNAs was normalized as a ratio to the amount of equine GAPDH using the $2-\Delta\Delta CT$ method as described by Livak and Schmittgen [20].

2.5. Statistical analysis

Change in transcripts for *tlr4*, *md2*, and *cd14* from baseline were measured at pre-determined time-points. Analysis with one-way ANOVA followed by Bonferroni's test indicated a statistically significant difference between time-points when $p \leqslant 0.05$. IC_{50} was calculated by curve regression (non-linear sigmoid dose–response). All analyses were carried using Prism Graph 5.0 (GraphPad Inc.).

3. Results

3.1. Chimpanzee, horse, human, and pig promoters share sequence similarity

To assess why humans and horses are more sensitive to septic shock, we analyzed the *tlr4*, *md2*, and *cd14* promoters among different mammals for sequence similarity. Sequences upstream the transcription initiation sites of these genes were aligned. Only annotated sequences from fully sequenced mammalian genomes (chimpanzee, cow, horse, human, mouse, pig, and rat) were used (Fig. 1). The similarity profile of *tlr4* promoters (Fig. 1A) revealed that the horse *tlr4* promoter shares extensive regions of similarity with the pig sequence. A medium–high number of regions of similarity were found when compared to the human and chimpanzee promoters. Few regions of significant similarity were found between the horse and cow promoters. Finally, only a small region of sequence similarity was detected among the horse, rat, and mouse promoters. Fig. 1(B) shows the regions of similarity between the horse *md2* promoter and that of different species. As seen before with the *tlr4* promoter, the horse shares regions of similarity within the *md2* promoter with humans, chimpanzees and pigs. However, in this particular case, it also shares similarity with the cow promoter. Again, the regions of similarity between rat and mouse were not significant. Interestingly, when *cd14* promoter was analyzed, only one short region of significant similarity was present in all species (Fig. 1C).

3.2. tlr4 and md2 promoters contain conserved NFκB binding sites

The beneficial effects from use of dexamethasone in human patients with sepsis [21,22] prompted us to focus our analyses on conserved NFκB binding sites since this is the main effector transcription factor resulting from TLR4 engagement. These binding sites could be subject to dexamethasone inhibitory regulation through the blockage of NFκB-p65 translocation caused by this drug. The presence of conserved NFκB binding sites were therefore analyzed, considering only those within the similar regions of all sequences. Four conserved NFκB binding sites were found in the

Table 2
Primer sequences for qPCR.

	Forward primer	Reverse primer
eqTLR4	5′-GGAACTGGACCTGAGCTTTA-3′	5′-CAATTTCACACCTGGACAAA-3′
eqMD2	5′-TAACATGAAGTCCCCGATTT-3′	5′-TCCCCTGAAGGAGAATGATA-3′
eqCD14	5′-GTTGTCAGTCAGTGCAGCTC-3′	5′-GAAGATGCTCCAGGAGAAGA-3′
eqGAPDH	5′-GCCCTCAATGACCACTTTGT-3′	5′-TTACTCCTTGGAGGCCATGT-3′

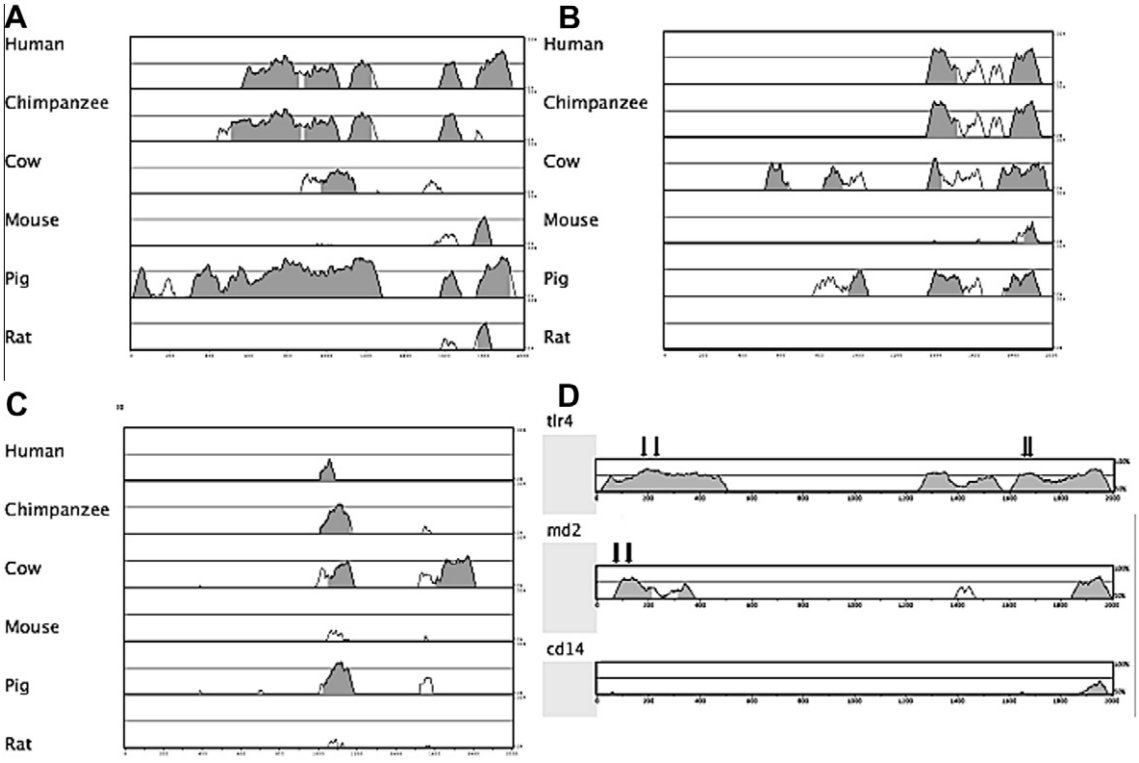

Fig. 1. There are four conserved NFκB binding sites in the promoter region of *tlr4* and two in the *md2* promoter. (A–C) represent the multiple alignment of the promoters of *tlr4* (A), *md2* (B), and *cd14* (C) from analyzed species. The transcription initiation site is located on the left side of each sequence. The gray area represents regions of similarity among sequences. (D) Shows that four conserved NFκB binding sites (indicated by arrows) were found in the promoter region of *tlr4*, two were found in the promoter of *md2* and none was found in the promoter of *cd14*.

promoter region of *tlr4* gene (Fig. 1D), while two were found in the promoter region of *md2* gene. No conserved binding sites for NFκB was found in the promoter region of *cd14* (Fig. 1D).

3.3. LPS inhibits transcription of tlr4 but induces transcription of md2 and cd14 in vitro

To validate our *in silico* analysis, we performed a series of experiments *in vitro* using PBMC from healthy horses (eqPBMC). The horse was chosen because it represents an alternative animal model to sepsis studies and is, along with humans, the most sensitive species to LPS among those whose genome is completely sequenced.

The patterns of transcription of the genes coding for CD14, MD2 and TLR4 found in LPS-stimulated cultures of eqPBMC established from three unrelated healthy horses (Fig. 2) were similar to those patterns found in other mammals. The presence of LPS induced a significant decrease in the levels of *tlr4* transcripts ($p = 0.0035$) (Fig. 2, left panel) and an increase in *md2* and *cd14* transcripts (Fig. 2, middle and right panels). The relative expression of the *tlr4* mRNA dropped to low levels after 8 h of LPS stimulation whereas the relative expression of the *md2* mRNA presented an early peak at 8 h of stimulation and steady transcript levels for 24 h. CD14 transcripts increased earlier peaking at 4 h (Fig. 2, right panel).

3.4. tlr4,md2, and cd14 transcription is affected by dexamethasone in vitro

EqPBMC obtained from three healthy donors were treated for 90 min with different concentrations of dexamethasone followed by stimulation with LPS for 14 h. The basal levels (without LPS) of *in vitro* transcripts of *tlr4*, *md2* and *cd14* were not affected by the presence of different concentrations of dexamethasone (Fig. 3). Dose–response curves for the relative levels of mRNAs induced by LPS in eqPBMC pre-treated with dexamethasone suggested a stimulatory effect of dexamethasone over *tlr4* (Fig. 3, left panel) and *md2* (Fig. 3, middle panel) transcription whereas an inhibitory effect was observed for the transcription of *cd14* ($IC_{50} = 4.1^{-7}$ M) (Fig. 3, right panel). The IC_{50} of dexamethasone for TLR4 and MD2 were 1.3^{-7} M and 1.4^{-6} M, respectively.

3.5. LPS-induced inhibition of tlr4 transcription is reversed in vitro by dexamethasone

The exposure of eqPBMC to LPS caused an initial decrease in *tlr4* transcripts in the absence of dexamethasone (Fig. 2, left panel). EqPBMC were treated with 10^{-7} M of dexamethasone for 90 min before stimulation with 100 ng/mL of LPS. In the presence of 10^{-7} M dexamethasone, the cells regained transcriptional activity for *tlr4* and after 24 h its transcripts level corresponded to more

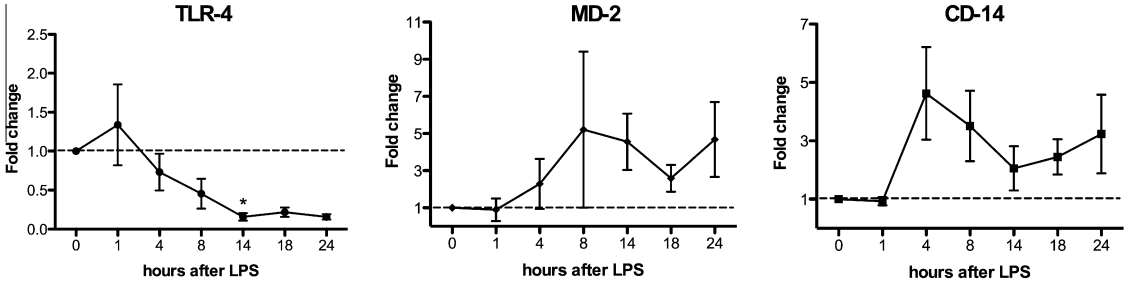

Fig. 2. LPS inhibits transcription of *tlr4* but induces transcription of *md2* and *cd14 in vitro*. PBMC was stimulated *in vitro* with 100 ng/mL of LPS. cDNA was amplified for *tlr4* (round symbols), *md2* (diamond symbols) and *cd14* (square symbols) by qPCR. *gapdh* was amplified for normalization purposes. $\Delta\Delta CT$ was calculated and expressed as fold change in genes expression. The dotted line represents basal levels of transcription (before treatment). Data represents mean ± SD obtained from three unrelated horses for *tlr4* and *md2* genes, and two unrelated horses for *cd14*. All experiments were performed in triplicates.

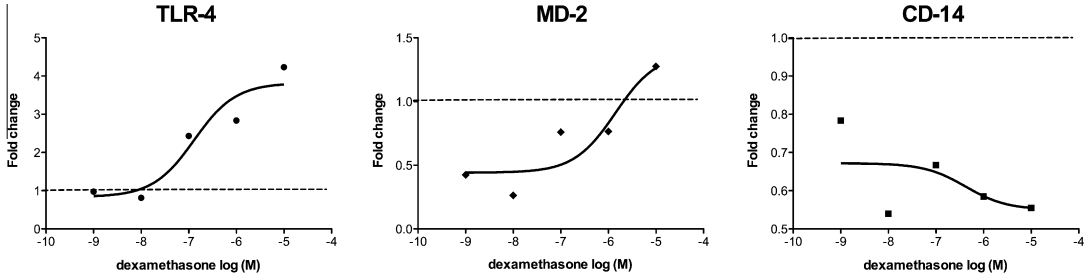

Fig. 3. LPS-induced transcription of *tlr4*, *md2* and *cd14* is affected in a dose–response fashion by dexamethasone *in vitro*. PBMC was cultured in the presence of different concentrations of dexamethasone for 90 min, followed by 14 h of stimulation with 100 ng/mL LPS. cDNA was amplified for *tlr4* (round symbols), *md2* (diamond symbols) and *cd14* (square symbols) by qPCR. *gapdh* was amplified for normalization purposes. $\Delta\Delta CT$ was calculated and expressed as fold change in gene expression. The dotted line represents basal levels of transcription (before treatment). Data represents mean ± SD obtained from three unrelated horses assayed in triplicates.

than 60% of the initial level found before inhibition caused by LPS ($p = 0.0262$) (Fig. 4, left panel). Dexamethasone treatment had an inhibitory effect in the LPS-induced *md2* and *cd14* transcripts ($p < 0.0001$ and $p = 0.0414$, respectively) (Fig. 4, middle and right panels). Compared to LPS stimulation alone (Fig. 2, middle panel), in the presence of dexamethasone the peak of accumulation of transcripts for *md2* occurred sooner, at 1 h, and remained low throughout the experimental period of 24 h (Fig. 4, middle panel). The transcription of *cd14*, that peaked at 6 h after stimulation with LPS alone (Fig. 2, right panel), showed a steady decrease in the presence of dexamethasone (Fig. 3, right panel).

4. Discussion

The engagement of TLRs triggers conserved signaling pathways that result in downstream translocation of NFκB to the nucleus [23–25]. Transcription of several pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, depends on this NFκB translocation [23,26]. Our finding of conserved NFκB binding sites in the mammalian promoters of *tlr4* and *md2*, suggests that translocation of NFκB might also play a role in the transcriptional regulation of these genes. Furthermore, *tlr4* and *md2* promoters showed higher similarity among LPS-sensitive species (chimpanzee, human, and horse) than with LPS-resistant species (rat and mouse).

Two recent reports evaluated trials testing the efficacy of low doses of corticosteroids in the reversal of septic shock in human patients, suggesting that they might reverse shock faster [21,22]. Another study focused on genes that are up- and down-regulated by NFκB, and the effects of corticosteroids over the LPS-induced response. Their results suggest that administration of corticosteroids concomitantly with LPS infusion modifies the initial transcriptional profile and is sufficient to balance the resulting inflammatory response towards a beneficial outcome [27].

By blocking translocation of NFκB-p65 to the nucleus, dexamethasone inhibits transcription of several pro-inflammatory cytokines [23,26]. Our finding that dexamethasone treatment, an inhibitor of NFκB-p65 translocation, reversed the inhibitory effects of LPS over the *tlr4* transcription *in vitro*, suggests that NFκB plays a negative effect over the regulation of this gene. On the other hand, treatment with dexamethasone inhibited *md2* transcription, suggesting a positive effect by NFκB. It is conceivable to hypothesize that by rescuing transcription of one major component of the TLR4-MD2-CD14 complex, dexamethasone might be able to ameliorate, or perhaps reverse, the phenomenon referred to as 'LPS tolerance', when cells become non-responsive to LPS [28]. However, it is important to point out that dexamethasone alone did not affect the basal transcription of *tlr4* and *md2*, suggesting that the basal transcriptional machinery remains unaffected by the presence of this drug. It is also notable that in another study, dexamethasone did not affect the transcription levels of *md2* when this was induced by IL-6 [29].

Our results agree with those recently published showing that NFκB binding sites can be tolerogenic in that they recruit a repressosome containing NcorR-Hdac3-deacetylated-p50 to the promoters of inflammatory genes [30]. The level of transcriptional inhibition found in that study appeared to be dependent on the number of NFκB motifs found in each promoter. Promoters with four NFκB binding sites were the ones most strongly repressed [30]. Our study found four conserved NFκB binding motifs in the mammalian *tlr4* promoter whereas only two were detected in the *md2* promoter. The finding that dexamethasone appeared to recover the transcription of only *tlr4* suggests that the *tlr4* promoter suffers active inhibition by a complex containing LPS-induced NFκB-dependent members, possibly the repressosome

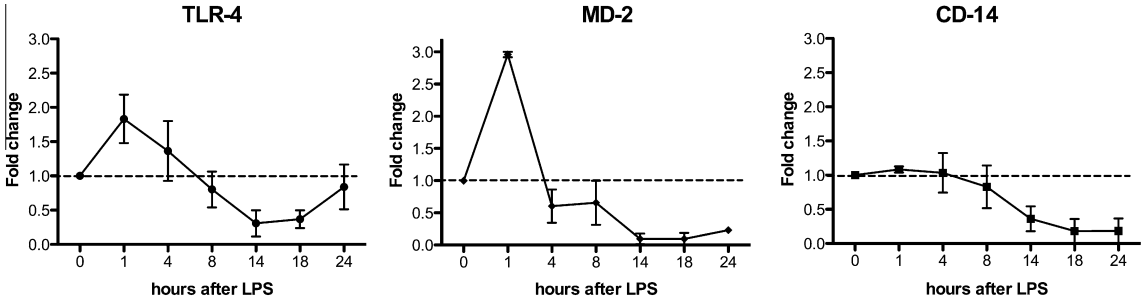

Fig. 4. LPS-induced inhibition of *tlr4* transcription is reversed *in vitro* by dexamethasone. PBMC was cultured in the presence of 10^{-7} M dexamethasone for 90 min, followed by LPS stimulation (100 ng/mL) for different time points. cDNA was amplified for *tlr4* (round symbols), *md2* (diamond symbols) and *cd14* (square symbols) by qPCR. *gapdh* was amplified for normalization purposes. $\Delta\Delta CT$ was calculated and expressed as fold change in gene expression. The dotted line represents basal levels of transcription (before treatment). Data represents mean ± SD obtained from three unrelated horses for *tlr4*, and two unrelated horses for *md2* and *cd14*.

containing NcorR-Hdac3-deacetylated-p50. In contrast, we found that the *cd14* promoter bears no NFκB binding sites, and accordingly, suffered no influence of dexamethasone treatment before/during the LPS challenge.

Finally, our analysis of *tlr4* promoters places the horse in close relationship to LPS-sensitive species such as humans, chimpanzees, and pigs. In contrast, two species that are more resistant to septic shock, rats and mice, presented very few and short regions of similarity at the sequence level. Rodents are the most widely used animal models for studies of sepsis. Still, many promising therapeutic protocols failed at reproducing in humans the same performance they had in rodents, suggesting the existence of a "translational disconnection" between these species [31]. Our results suggest that studies of sepsis pathogenesis in the horse might provide the answers that rodents failed short at. The disease progression between human and horses share more similarities than with rodents. The present study shows that at least some of these similarities can be explained at the gene promoter level, making the horse a better suited model for those studies aiming at unravelling the pathogenesis of human sepsis.

In silico analysis of the *tlr4*, *md2*, and *cd14* promoters from seven mammalian species with completely sequenced and annotated genomes uncovered the existence of four conserved NFκB binding sites in the promoter of *tlr4* and two in the promoter of *md2*. Sequence analysis showed that the horse *tlr4* promoter has more regions of similarity with humans, chimpanzees, and pigs than with the promoter of cows, rats and mice. *In vitro* stimulation of eqPBMC with LPS caused a decrease in the levels of transcripts for *tlr4* and increased transcripts for *md2* and *cd14*. *In vitro* treatment with dexamethasone rescued transcription of *tlr4* after the inhibition provoked by LPS, while inhibiting transcription of *md2*. Dexamethasone treatment alone did not affect transcription of *tlr4* and *md2*. Altogether these data suggest that the NFκB binding sites found in the promoters of *tlr4* and *md2* are functional and respond to TLR4 engagement as a negative (for *tlr4*) and positive (for *md2*) regulator of transcription of the receptor complex genes.

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