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CRISPR-Cas9–mediated knockout of *TLR4* modulates *Mycobacterium avium* ssp. *paratuberculosis* cell lysate–induced inflammation in bovine mammary epithelial cells

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ABSTRACT

Toll-like receptor 4 (TLR4) is a pattern-recognition receptor involved in the recognition of microbial pathogens and host alarmins. Ligation to TLR4 initiates a signaling cascade that leads to inflammation. Polymorphisms in bovine *TLR4* have been associated with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) susceptibility and resistance, the cause of Johne's disease, and milk somatic cell score, a biomarker of mastitis. Although the contribution of TLR4 to recognition of bacterial lipopolysaccharide (LPS) has been well characterized, its role in MAP recognition is less certain. Clustered regularly interspaced short palindromic repeats–Cas9 mediated gene editing was performed to generate *TLR4* knockout (KO) mammary epithelial cells to determine if TLR4 expression is involved in the initiation of the host inflammatory response to MAP cell lysate (5 and 10 μg/mL) and *Escherichia coli* LPS (5 μg/mL). The absence of *TLR4* in KO cells resulted in enhanced expression of key inflammatory genes (*TNFA* and *IL6*), anti-inflammatory genes (*IL10* and *SOCS3*), and supernatant cytokine and chemokine levels (TNF-α, IL-6, IL-10, CCL3) in response to the MAP cell lysate (10 μg/mL). However, in response to LPS, the KO cells showed reduced expression of key inflammatory genes (*TNFA*, *IL1A*, *IL1B*, and *IL6*) and supernatant cytokine levels (TNF-α, IL-6, CCL2, IL-8) as compared with unedited cells. Overall, these results confirm that *TLR4* is essential for eliciting inflammation in response to LPS; however, exacerbated gene and protein expression in *TLR4* KO cells in response to MAP cell lysate suggests a different mechanism of infection and host response for MAP, at least in terms of how it interacts with TLR4. These novel findings show potential divergent roles for TLR4 in mycobacterial infections, and this may have important consequences for the therapeutic control of inflammation in cattle.
Key words: *Mycobacterium avium* ssp. *paratuberculosis*, Johne's disease, mastitis, TLR4, CRISPR-Cas9, gene knockout

INTRODUCTION

Johne's disease (**JD**), or paratuberculosis, is a chronic inflammatory bowel disease of ruminants caused by *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**), a slow-growing, acid-fast, and gram-positive bacterium (Seyyedin et al., 2008). Damage to the intestinal wall during MAP infection compromises nutrient absorption and thus animal production; therefore, JD adversely affects the dairy industry by reducing milk yield, which often leads to early culling. Moreover, MAP might potentially also represent a threat to human health, and it has long been considered to be a potential cause of Crohn's disease (McNees et al., 2015). Animals with subclinical MAP infection can intermittently or persistently shed MAP in their feces, leading to contamination of environment and increased risk of exposure to herd mates (Mitchell et al., 2015). Susceptible animals are typically infected via fecal-oral route or in utero (Whittington and Windsor, 2009), though MAP may also be secreted in milk (Lu et al., 2008).

The innate immune system confers immediate defense against MAP, and this is facilitated by the recognition of MAP-specific pathogen-associated molecular pattern molecules by various host pattern-recognition receptors (**PRR**). The PRR involved in the recognition of

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Mycobacteria spp. may include TLR (Ruiz-Larrañaga et al., 2011), NOD2 (nucleotide binding oligomerization domain containing 2) receptor (Lee et al., 2017; Ruiz-Larrañaga et al., 2010), the C-type lectin receptor CLEC7A (Kumar et al., 2019a), and RIG-I-like receptors (Cheng and Schorey, 2019). Genetic variations in *TLR* genes have been shown to affect pathogen recognition and host innate immune response. Polymorphisms in bovine *TLR1*, *TLR2*, and *TLR4* have been associated with increased susceptibility of cattle to paratuberculosis (Mucha et al., 2009; Ruiz-Larrañaga et al., 2011; Sharma et al., 2015).

Toll-like receptor-ligand interactions are not as clear cut as originally thought. Certain bacteria, spirochetes for example, are recognized by TLR2 as well as TLR4 (Schröder et al., 2000), and HSP was shown to be recognized by both TLR2 and TLR4 (Asea et al., 2002), which can synergize (Beutler et al., 2001). Although TLR4 is mainly involved in LPS recognition, it also recognizes a variety of host alarmins that are released during tissue damage (Yang et al., 2017). In contrast, TLR2 which can heterodimerize with TLR1 and TLR6, has been implicated in the recognition of mycobacterial cell wall lipoproteins (Ruiz-Larrañaga et al., 2010); the role of TLR4 in MAP recognition is less certain and warrants investigation.

Mammary epithelial cells represent a defensive barrier against intramammary pathogens that are capable of pathogen recognition via PRR and initiating an appropriate protective immune response. Mammary epithelial cell culture models have provided the basis for our current understanding of many host-pathogen interactions (Friis et al., 2005; Hemphill et al., 2006), including MAP (Patel et al., 2006; Lamont et al., 2013). Our previous study revealed that IL10RA (interleukin 10 receptor α) knockout (**KO**) mammary epithelial cells (**MAC-T**) cells show higher inflammatory response to MAP cell lysate in vitro that support the immunoregulatory role of IL10RA in eliciting an anti-inflammatory response during MAP infection (Mallikarjunappa et al., 2020). In the present study, the bovine MAC-T cell line was used to generate *TLR4* KO cells to further explore the association of bovine *TLR4* with JD and mastitis. We hypothesized that TLR4 contributes to the modulation of immune response against MAP cell lysate. To test this hypothesis, a *TLR4* KO MAC-T cell line was developed using the clustered regularly interspaced short palindromic repeats (**CRISPR**)-Cas9 technique, and TLR4 involvement in modulating the inflammatory response to MAP cell lysate and *Escherichia coli* LPS (as a positive control) was determined by assessing downstream pro- and anti-inflammatory cytokine and chemokine gene and protein expression.

MATERIALS AND METHODS

Cell Line

The MAC-T cells were cultured according to the previous protocol (Huynh et al., 1991) in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL; Invitrogen; Thermo Fisher Scientific, Inc.) and maintained in an incubator at 37°C with 5% CO_2.

Single Guide RNA Synthesis

Two single guide RNA (**sgRNA**) that target bovine *TLR4* were designed using benching (www.benchling.com/crispr) and Synthego online design tool, and were commercially synthesized by Synthego (https://design.synthego.com/#/). The sequence for sgRNA1 was AUGCACUGGUAACUAAUGUU, and the sequence for sgRNA2 was UUGAUAUGGGGAUGUUGUCG. Both sgRNA targeted exon 2 of *TLR4* gene.

Lipid-Mediated Cell Transfection

The MAC-T cells were transfected with ribonucleoprotein complex of sgRNA and Cas9 using Lipofectamine CRISPRMAX (Invitrogen) reagent as described in our previous study (Mallikarjunappa et al., 2020). Briefly, 24 h before transfection, 80,000 cells were seeded in a 24-well plate (Corning Costar) so that the wells were 70 to 80% confluent at the time of transfection. On the day of transfection, the sgRNA and Cas9 protein complex (1.2:1 ratio) was added to the Lipofectamine CRISPMAX reagent. The sgRNA-Cas9 lipid complex was incubated at room temperature for 15 min, after which it was added to each well to carry out transfection. The cells were then incubated for 72 h at 37°C with 5% CO_2. At 72 h posttransfection, the cells were subcultured in 2 wells; one was used for analysis of the genome cleavage detection (**GCD**) using GeneArt Genomic Cleavage Detection Kit (Invitrogen), and the other was used for single monoclonal cell isolation after confirmation of editing by GCD assay.

Determination of Genomic Modifications

Posttransfection, cells were harvested for subsequent subculture, and modification of target site was detected using Genomic Cleavage Detection Asssay Kit (Invitrogen) as per the manufacturer's instructions. The assay kit used genomic DNA extracted from cells transfected with CRISPR-Cas9. Following cleavage by Cas9, genomic insertions or deletions (indels) were created by the cellular repair nonhomologous

end joining repair mechanisms. Loci (exon 2, *TLR4* gene) where the gene-specific double-strand breaks occur were amplified by PCR using a set of primers (F:5′-TCAGGTGACTTATGATTGGATGGA-3′, R: 5′-CCTGAAGAAGGGAGATAGCTTG-3′) flanking the region of interest in exon 2 of the *TLR4* gene. The PCR product was denatured and reannealed so that mismatches were generated as strands with an indel reannealed to strands with no indel (unedited cells). The mismatches were subsequently detected and cleaved by detection enzyme (T7E1), and then the resultant bands were analyzed by a 2% agarose gel electrophoresis to check induction of indels at the target site.

Isolation of Monoclonal Cell Population

The mixed (edited and unedited) MAC-T cell populations (approximately 3×10^5 cells) in which indels were confirmed by GCD assay were serially diluted so that each well of a 96-well plate received a single cell and was cultured for 4 wk to generate monoclonal cell populations. The progeny cells were subcultured and further subjected to Sanger sequencing to identify indels in the *TLR4* gene.

Western Blot Analysis

Total proteins were isolated from edited and unedited cells using radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc.). Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce). Then, 10 μg/μL of protein was loaded on a 12% SDS-PAGE gel, and protein was then transferred onto a polyvinylidene fluoride membrane (Invitrogen). The membrane was blocked using 5% nonfat milk in PBS, 0.1% Tween 20 detergent for 1.5 h and then incubated with the primary rabbit anti-TLR4 antibody (Antibodies-online Inc.) overnight at 4°C, followed by incubation with secondary antibody horseradish peroxidase–conjugated goat anti-rabbit IgG (Antibodies-online Inc.) at room temperature for 2 h. The enhanced chemiluminescence western blot substrate kit (Bio-Rad) was used for development, and quantified using the Image Lab software (ChemiDoc XRS system, Bio-Rad).

Quantitative PCR of TLR4 Gene

The total RNA was isolated from edited (KO) and unedited (normal) cells using RNeasy mini kit (Qiagen), and complementary DNA (**cDNA**) was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The quantitative PCR (**qPCR**) reactions were performed in a StepOne Plus instrument (ABI) using specific primers of the exon 2 region of *TLR4* genes. Each qPCR reaction was performed in duplicates in a total-reaction mixture of 10 μL comprising 2 μL of cDNA, 5 μL 2× SYBR Green master mix (ABI), 0.4 μL each of 10p*M* forward and reverse primers, and 2 μL of nuclease-free water in PCR tubes.

MAP Cell Lysate Preparation

The MAP cell lysate was prepared according to our previous study (Mallikarjunappa et al., 2020). Briefly, the MAP GC86 strain was cultured into Middlebrook 7H9 broth (Sigma-Aldrich) supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase; Becton–Dickinson Canada), 0.05% Tween 80 (Sigma-Aldrich), and 2 mg/L Mycobactin J (Allied Monitor Inc., Fayette), at 37°C with 5% CO_2 to an optical density of 0.2 to 0.4 at 540 nm, then pelleted by centrifugation at 3,500 × *g* for 20 min at 4°C and washed twice with ice-cold PBS. The pellet was then suspended in PBS and sonicated on ice using a probe sonicator (Model 120, Fisher Scientific) with 60% amplitude for 3 cycles of 10 min bursts, followed by a 10-min chilling period between each sonication. The MAP sonicate was then centrifuged at 12,000 × *g* for 5 min at 4°C, and then the pellet was discarded and the lysate supernatant was saved. The protein concentration of MAP lysate was determined using Bio-Rad protein assay (Richmond).

In Vitro Challenge

We seeded *TLR4* KO (passage 2) and unedited MAC-T cells at 1.2×10^5 cells/well into two 24-well cell culture plates in quadruplicate and incubated overnight at 37°C with 5% CO_2. All experiments were carried out at 4 independent time points (n = 4). Both the cell types were immune challenged for 24 h and 48 h with 2 concentrations of MAP cell lysate (5 μg/mL and 10 μg/mL) or with LPS (5 μg/mL) as a control.

RNA Extraction and Gene Expression

Total RNA was extracted from the edited and unedited MAC-T cells 24 h and 48 h postimmune challenge by using TRIzol (Invitrogen) according to the manufacturer's protocol, and DNA traces were removed by DNAase I (MBI Fermentas) treatment. Subsequently, 500 ng of purified RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

For qPCR, primers for cytokine genes were selected from a previous study (Mallikarjunappa et al., 2020) and are listed in Table 1. Specificity of primers were

checked by sequence alignment using BLASTN at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) genome browser gateway and was further confirmed in a standard PCR reaction, followed by ethidium-bromide staining on 2% agarose gel. The accuracy of primer pairs was also ensured by the presence of a unique peak during the dissociation step at the end of the qPCR reaction. Each qPCR reaction was performed in duplicates in a total-reaction mixture of 10 μL comprising 2 μL of cDNA, 5 μL of 2× SYBR Green master mix (ABI), 0.4 μL each of 10p*M* forward and reverse primers, and 2 μL of nuclease-free water in a 96-well plate (ABI). The reactions were performed in a StepOne Plus instrument (ABI) using the following amplification conditions: 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The data were acquired using the "first derivative" method and subjected for subsequent analysis.

Multiplex Cytokine and Chemokine Analysis

A customized MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel 1 (10-plex) from EMD Millipore Corp. was used for the simultaneous quantification of the following analytes in the culture supernatant obtained from edited and unedited MAC-T cells that had been immune challenged for 48 h: IL-1β, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and TNFα. The assays were performed according to the instructions of the manufacturer, and data were acquired using the Luminex 200 (Luminex Corp.). The median fluorescent intensity of each well was obtained and data were processed using the Milliplex Analyst software; concentrations were determined using the 4PL fitting model. The values are presented as picograms per milliliter. Two-way ANOVA test followed by the Bonferroni test was performed to compare the expression of cytokines and chemokines between the 2 cell types.

Statistical Analysis

To compare differences in the relative expression levels of different genes in different treatments, the cycle threshold (**Ct**) values were analyzed using 2-way ANOVA test followed by Bonferroni test (GraphPad Prism Software), and a P-value ≤ 0.05 was considered significant. All data (n = 4) were presented as the mean ± standard error of the mean.

RESULTS

Genome Cleavage Detection Assay and DNA Sequencing

The sgRNA (sgRNA1 and sgRNA2) were transfected with Cas9 protein as ribonucleoprotein complex into MAC-T cells. To access the performance of sgRNA, genome editing was verified 72 h posttransfection using the mismatch-sensitive endonuclease GCD assay. The assay used genomic DNA extracted from cells transfected with CRISPR-Cas9. Following cleavage, genomic insertions or deletions (indels) were created by the cellular repair mechanisms. Loci where the gene-specific double-strand breaks occurred were amplified by PCR. The PCR product was denatured and reannealed so that mismatches were generated as strands with an indel reannealed to strands with no indel or a different indel. The mismatches were subsequently detected and cleaved by detection enzyme, and then the resultant bands were analyzed by gel electrophoresis. The results

Table 1. Details of primer sequences, annealing temperature (Ta), slope, and PCR efficiency for the target genes used for quantitative PCR

Gene[1]	Primer sequence (5′–3′)	Ta (°C)	Slope	PCR efficiency (%)
IL1A - F	TTGGTGCACATGGCAAGTG	58.3	−3.260	103
IL1A - R	GCACAGTCAAGGCTATTTTTCCA			
IL1B - F	GCCTTCAATAACTGTGGAACCAAT	58.3	−3.161	107
IL1B -R	GTATATTTCAGGCTTGGTGAAAGGA			
IL6 - F	GGCTCCCATGATTGTGGTAGTT	58.3	−3.360	98
IL6 - R	GCCCAGTGGACAGGTTTCTG			
TNFA - F	CGGTGGTGGGACTCGTATG	58.3	−3.125	109
TNFA - R	CTGGTTGTCTTCCAGCTTCACA			
SOCS3 - F	GCCACTCTCCAACATCTCTGT	58.3	−3.382	98
SOCS3 - R	TCCAGGAACTCCCGAATGG			
IL10 - F	AAAGCCATGAGTGAGTTTGACA	58.3	−3.380	98
IL10 - R	TGGATTGGATTTCAGAGGTCTT			
GAPDH - F	TGGAAAGGCCATCACCATCT	60	−3.387	97
GAPDH - R	CCCACTTGATGTTGGCAG			
B2M - F	CTGCTATGTGTATGGGTTCC	60	−3.214	105
B2M - R	GGAGTGAACTCAGCGTG			

[1]F = forward; R = reverse.

showed that indels (insertion and deletions) were induced by sgRNA1 in the target gene as determined by presence of 3 bands (1 parental and 2 cleaved bands) in transfected cells with sgRNA1 (lane S1, Figure 1). However, there was just a single parental band for sgRNA2 (lane S2) and control samples (lane S3) that indicated the normal genome. These results indicated that only sgRNA1 successfully induced the indels in MAC-T cells posttransfection. Indels were further confirmed by DNA sequencing of the targeted areas of the *TLR4* gene. The mixed population of edited and unedited cells from sgRNA1 transfected sample were subjected to single-cell isolation and monoclonal cell proliferation. All monoclonal cells were DNA sequenced, and the *TLR4* KO monoclonal cell populations showed 12 base pair deletions in the targeted region when compared with reference sequences of *TLR4* exon 2 (Figure 2).

Western Blot

The western blot was performed using cell lysate of TLR4 knockout MAC-T cells to compare the expression of TLR4 protein with unedited MAC-T cells. The results demonstrated that there was no TLR4 protein expression in edited cells (lanes S1–S3), whereas the unedited cells (lanes C1 and C2) showed a band approximately 95 KDa in size (Figure 3). Because of the absence of reference protein control, the western blot results may have been entirely conclusive; however, an equal number of cells were used to prepare the cell lysate, and the same protein quantity (10 μg) was loaded in the western blot gel for both KO and unedited samples. The lack of protein in the western blot in the KO sample could be due to misfolding of primary and secondary structures in KO cells. To further confirm the deletion in target region, qPCR was performed using primers of exon 2 of the *TLR4* gene; one of which annealed to the targeted region, over the Cas9 cleavage site, for amplification. The qPCR results showed (data not provided) that there was no amplification (Ct values: undetermined) in KO samples as compared with control unedited cells (Ct values:18–20), which further corroborated the western blot results. No *TLR4* mRNA detection in qPCR could be attributed to the specific primers that were selected for the qPCR; at least one primer was designed to anneal to the targeted region (unedited). Because there was 12 bp deletion in the primer annealing region of KO sample, the forward primer could not anneal during PCR; consequently, the qPCR were undetermined in KO samples.

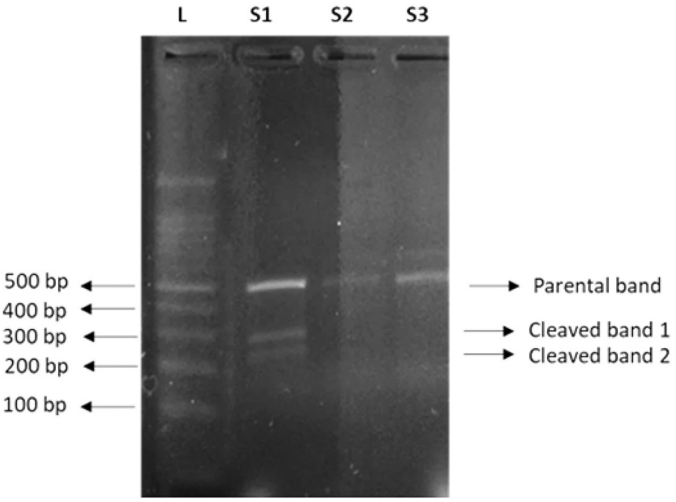

Figure 1. Gel image of Genomic Cleavage Detection Assay of transfected (lane L = DNA ladder, lane S1 = sgRNA1, and lane S2 = sgRNA2) and nontransfected control mammary epithelial cells (lane S3). The samples were assayed for gene editing to assess the performance of sgRNA to induce indels. Lane S1 (sgRNA1) shows expected one parental band (510 bp) and 2 cleaved bands at size 290 and 220 bp after enzymatic cleavage that confirm the gene editing (induction of indels). Lane S2 (sgRNA2) and lane S3 (unedited cells) show just a single parental band that indicates the normal target region with no editing. sgRNA = single guide RNA.

Gene Expression Analysis

Relative mRNA expression of cytokines TNF-α, IL-1α, IL-1β, IL-6, SOCS3, and IL-10 were analyzed at 2 time points (24 and 48 h) postchallenged with MAP cell lysate or LPS in both edited and unedited MAC-T cells by using the geometric mean of 3 house-keeping genes (i.e., *GAPDH*, *ACTB*, and *B2M*) as the internal control reference. The PCR analyses revealed that the expression of all pro-inflammatory genes (*TNFA*, *IL1A*, *IL1B*, and *IL6*) was significantly lower ($P < 0.05$) in TLR 4 KO cells as compared with unedited MAC-T cells after the LPS challenge (Figure 4).

However, for the MAP cell lysate treatment, the expression of *TNFA* was significantly higher ($P \leq 0.05$) in the TLR4 KO cells as compared with unedited cells at 24 h (5 μg) and 48 h (5 and 10 μg). The expression of *IL6* was remarkably higher at both time points in TLR4 KO as compared with unedited cells. In contrast, there were no significant differences in the expression of *IL1A* and *IL1B* between TLR4 KO and unedited cells at both time points. The expression of anti-inflammatory gene *SOCS3* was higher in TLR4 KO cells as compared with unedited cells at both time points, and *IL10* was higher at 24 h and lower at 48 h (Figure 5).

Cytokines and Chemokines Multiplex Analysis

A customized multiplex immune assay was carried out to quantify the concentration of 10 multiple cytokines in the culture supernatant of TLR4 KO and

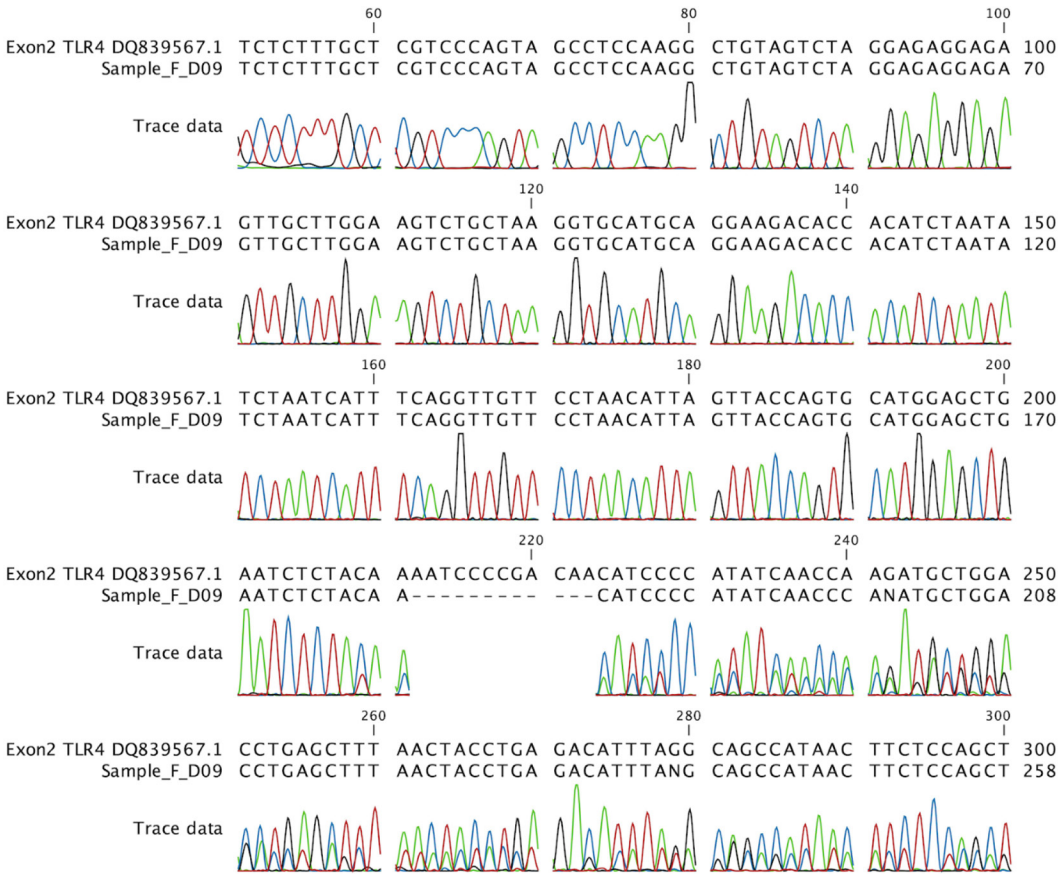

Figure 2. Aligned sequences of exon 2 of TLR4 knockout (KO) mammary epithelial cells with reference sequence using CLC Genomics Workbench 12 software. The edited sample showed a 12-bp deletion as compared with reference sequences (accession no. DQ839567.1) in exon 2 of *TLR4* gene.

unedited cells immune challenged with MAP lysate or LPS (n = 4). The concentration of 4 cytokines (i.e., IL-1β, IL-17A, IP-10 (CXCL10,), and MIP-1β (CCL4) was undetectable, whereas the remaining 6 were detectable (IL-6, IL-8, IL-10, TNF-α, CCL-2, and CCL-3). Protein analysis indicated that TNF-α, IL-6, IL-8, and CCL-2 levels in the culture supernatant of TLR4 KO cells were significantly lower as compared with unedited cells in response to LPS challenge (Figure 6). In contrast, levels of IL-6, TNF-α, CCL-3, and IL-10 were significant higher in TLR4 KO MAC-T cells than unedited cells in response to immune challenge with MAP cell lysate.

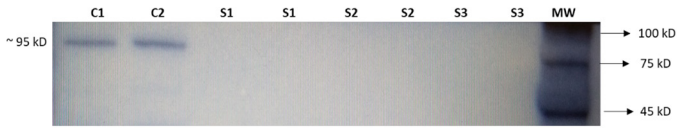

Figure 3. Western blot analysis of TLR4 knockout (KO) and unedited MAC-T cell lysates probed with anti-TLR4 polyclonal antibody, followed by conjugation to secondary antibody. The TLR4 protein band was observed at approximately 95 kD. Protein TLR4 was undetected in lysate from TLR4 KO cells (S1, S2, S3), whereas it was detected in unedited MAC-T cell lysates (C1, C2). MAC-T = mammary epithelial cells; MW = molecular-weight size marker.

DISCUSSION

Several bovine studies have established associations between SNPs in *TLR4* and susceptibility and resistance to both JD and mastitis. Sharma et al. (2015) for example, demonstrated that SNPs in *TLR4* were associated with increased MAP infection and an increase in milk SCS (Sharma et al., 2006). Sharma et al., (2008) later demonstrated that one of these SNP, in the promoter region, was also responsible for altering bovine *TLR4* expression.

The role of TLR4 in the recognition of *E. coli*, which is a common cause of clinical mastitis (Johnzon et al., 2018), has been clearly established. Therefore, the treatment of KO cells with *E. coli* LPS served as positive control in our experiment. Ligation of TLR4 to LPS initiates a first-wave of inflammation via the canonical inflammatory pathway, and endocytosis of TLR4-LPS induces a second-wave of inflammation referred to as

inflammatory endocytosis (Marongiu et al., 2019). More recently, intracellular LPS has also been demonstrated to activate a third noncanonical inflammatory pathway leading to pyroptosis (Huang et al., 2019). In the present study, we also observed that *TLR4* KO MAC-T cells displayed an attenuated cytokine response when

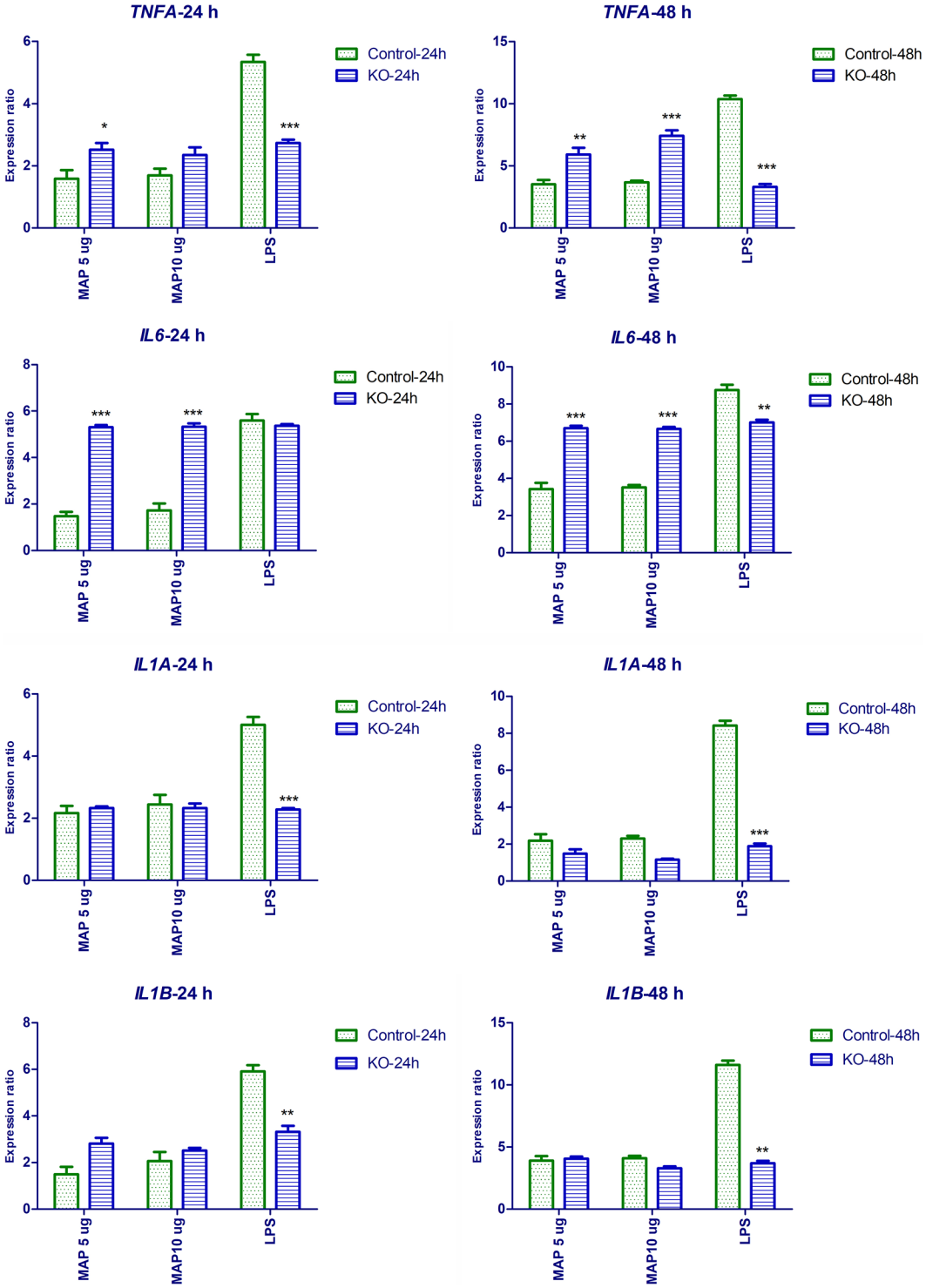

Figure 4. Relative gene expression ratio of pro-inflammatory cytokines *TNFA*, *IL6*, *IL1A*, and *IL1B* in TLR4 knockout (KO) mammary epithelial cells (MAC-T) stimulated with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) lysate with LPS at 24 and 48 h. The values (mean ± SD, n = 4) with asterisks indicate significant differences from the control (unedited) cells. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

stimulated with LPS. As expected, the expressions of all pro-inflammatory cytokine genes (*TNFA*, *IL1A*, *IL1B*, and *IL6*) and pro-inflammatory cytokines and chemokine proteins (TNF-α, IL-6, IL-8, and CCL-2) were significantly decreased in *TLR4* KO MAC-T cells as compared with unedited cells post LPS challenge. We attribute this lower pro-inflammatory cytokine expression in the KO cells to impaired TLR4 signaling. In line with these findings, Lorenz et al., (2002) reported that macrophages and B cells from *TLR4*-deficient mice did not respond to LPS. Collectively, these results support that TLR4 is a major PRR for *E. coli* 0111:B4 LPS and that knocking out *TLR4* in MAC-T cells impaired their responsiveness to LPS.

Although MAC-T cells are also capable of processing of MAP, and this cell line has been previously used to study the course of MAP infection (Patel et al., 2006; Lamont et al., 2013), the role of bovine TLR4 in MAP recognition is less clear. Lee et al., (2014) demonstrated that MAP1305 protein ligation with TLR4 induced murine dendritic cell maturation and pro-inflammatory cytokine production. There are high levels of immune-activating MAP1981c nucleic acid–binding protein in patients with Crohn's disease; this protein is recognized by TLR4 and also induces dendritic cell maturation and pro-inflammatory cytokine production (Kim et al., 2018). There is also evidence that certain microRNA (**miRNA**) may potentially act as TLR4 ligands (Bayraktar et al., 2019). In support of this, miRNA 708–5p was induced in human macrophages during *Mycobacterium tuberculosis* infection, and a miRNA 708–5p mimic reduced pro-inflammatory cytokine production via TLR4 (Li and Zhang, 2019).

Toll-like receptors are known to play a pivotal role in host immune response upon *M. tuberculosis* infection, especially TLR2 and TLR4, which lead to the activation of NF-κB signaling (Sánchez et al., 2010). Accumulating evidence indicates that through targeting TLR, miRNA can modulate innate immune response against *M. tuberculosis* (Zhu et al., 2008; Liu et al., 2014). Niu et al., (2018) reported that miR-125a decreased macrophage inflammatory cytokine production during *M. tuberculosis* infection, and miR-125a levels were dramatically reduced by knockdown of *TLR4* in THP-1 cells during *M. tuberculosis* infection. In contrast, knockdown of *TLR2* had little effect on the expression of miR-125a, indicating that induction of miR-125a during *M. tuberculosis* infection is mainly dependent on TLR4 rather than TLR2 signaling. This could be the one of the possible reasons for the stronger *TLR4* KO cell inflammatory response that was observed in the present study following MAP lysate treatment. Moreover, our results herein agree with recent studies (Kumar et al., 2019b; Gopi et al., 2020), suggesting the

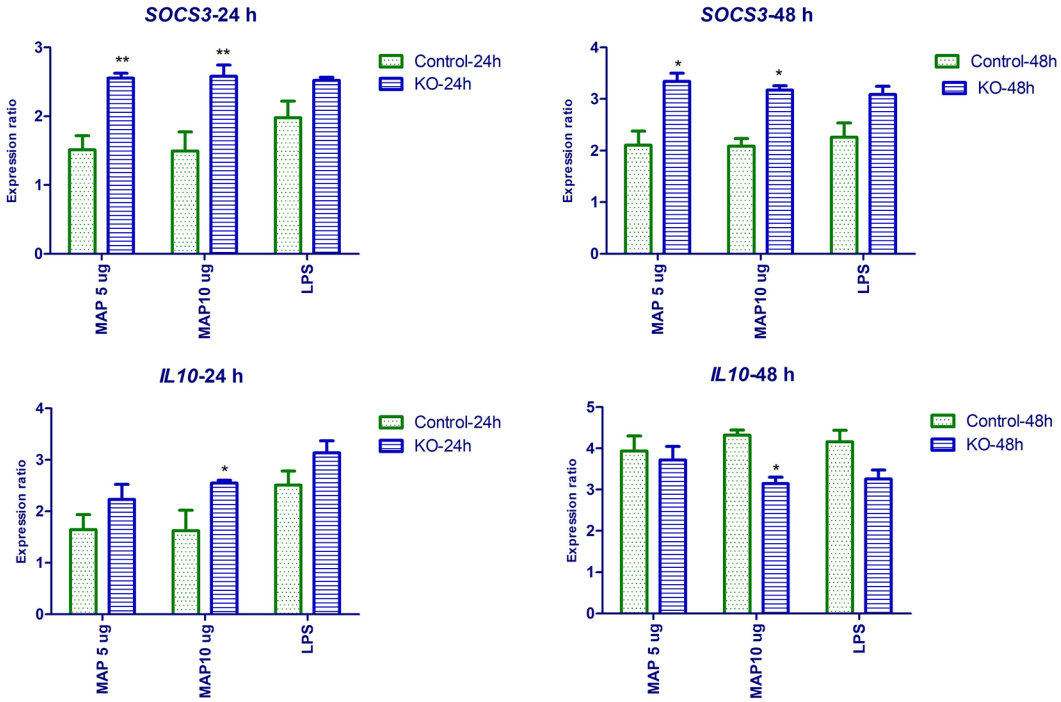

Figure 5. Relative gene expression ratio of anti-inflammatory cytokines *SOCS3* and *IL10* in TLR4 knockout (KO) mammary epithelial cells (MAC-T) and unedited MAC-T cells (control) stimulated with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) lysate with LPS at 24- and 48-h time points. Values are mean ± SD, and significant differences are denoted by $^{**}P < 0.01$ and $^{*}P < 0.05$.

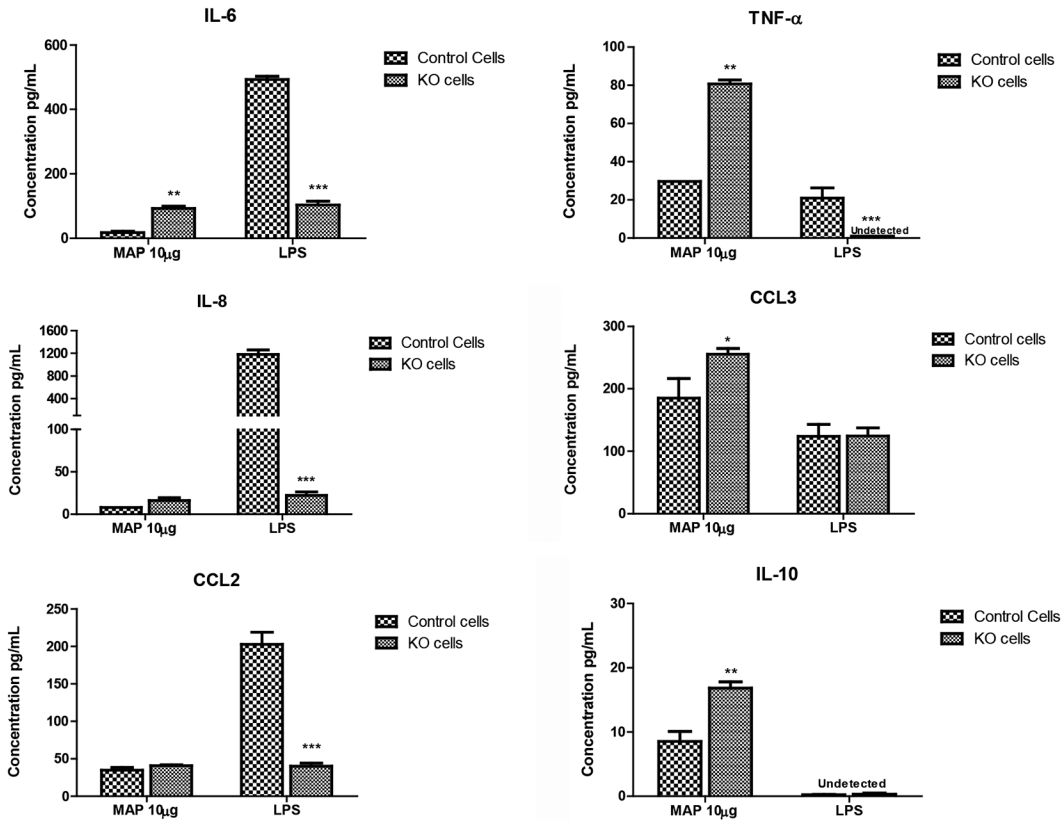

Figure 6. Cytokine and chemokine concentrations in culture supernatant from TLR4 knockout (KO) mammary epithelial cells (MAC-T) and unedited MAC-T cells 48 h postchallenge with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cell lysate or LPS. Values are expressed as mean ± SEM of 4 independent experiments. Asterisks indicate a significant difference (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$) from the control (unedited) cells.

association between SNP in *TLR4* gene and its susceptibility to paratuberculosis (**PTB**) in Indian cattle. Previously, mutations in bovine *TLR4* were reported to have significant association with clinical manifestation of MAP infection (Mucha et al., 2009). In the past, studies highlighted the association of SNPs in bovine *TLR4* with PTB susceptibility in cattle breeds. Haplotypes of 2 SNPs in Canadian Holstein (Sharma et al., 2015) and 3 SNPs in Spanish Holstein cattle (Ruiz-Larrañaga et al., 2011) suggested their involvement in PTB susceptibility and variation in responses to MAP infection. Other *TLR* genes were also reported to be involved in PTB susceptibility. Association between mutations in *TLR1* and MAP infection was reported in Turkish Holstein cattle; however, no association was found with *TLR4* SNPs (Cinar et al., 2018).

The *TLR2* gene has been reported to play role in *M. tuberculosis* infection (Means et al., 1999; Ferwerda et al., 2005) by recognizing its 19-kD lipoprotein (Hertz et al., 2001; Gehring et al., 2003). However, it is currently unknown which MAP ligand(s) engage TLR2, but various immune-active lipoproteins, such as 19-kD lipoprotein (Huntley et al., 2005), 22-kD lipoprotein (Dupont et al., 2005), and 34-kD lipoprotein (Silbaq et al., 1998; Gioffré et al., 2006), have been identified in *M. paratuberculosis*. Therefore, it is tempting to speculate that *M. paratuberculosis* lipoproteins may be recognized by TLR2. Ferwerda et al. (2007) found that human and murine TLR2 recognize sonicated *M. paratuberculosis*

The role of TLR4 in the recognition of mycobacteria is more controversial. The sonicated *M. tuberculosis* failed to stimulate human TLR4 in various experimental models (Ferwerda et al., 2005). On the contrary, some studies reported the beneficial role of TLR4 in tuberculosis-infected mice (Abel et al., 2002; Heldwein et al., 2003), although not all groups could confirm these findings (Shim et al., 2003). Because sonication procedure induces structural changes in some cell wall components that are crucial for TLR4 ligation, TLR4 responds differently to intact versus sonicated mycobacteria (Ferwerda et al., 2005). Our data showed that *TLR4* KO cells responded to MAP lysate, and this led to a greater inflammatory response than unedited cells.

This suggested that TLR4 may not play a direct role in the recognition of MAP cell lysate by bovine MAC-T cells.

Inhibition of TLR4 in human mononuclear cells infected with live MAP reduced the cytokine response, indicating that TLR4 plays significant role in MAP infection. However, there was less production of TNF-α in macrophages of *TLR4* knockout mice infected with sonicated *M. paratuberculosis.* These results suggested that there are differences in interaction of TLR4 with *M. paratuberculosis* in murine and human. There is scant information available on structural components of *M. paratuberculosis* interacting with human TLR4, but some evidence from *M. tuberculosis* interactions indicate that structural components are heat sensitive (Means et al., 1999), which could explain the diverse responses by human TLR4 to sonicated and live *M. paratuberculosis*.

In addition to MAP proteins and possibly miRNA, host alarmins can also act as ligands for TLR4. Defensins, HMGB1, HMGN1, GNLY, S100 proteins, and HSP 60, 70, 90, 96, for example, are all TLR4 ligands (Yang et al., 2017). Interestingly, HMGB1 was detected in bronchial lavage fluid from *M. tuberculosis*-infected mice, and either blocking or administering HMGB1 affected disease outcome depending on its redox state (Hernández-Pando et al., 2015). Hence, it is speculated that the deletion of *TLR4* gene in KO cells not only affected the binding of common ligands such as MAP proteins and miRNA, but also of host alarmins.

In the present study, the *TLR4* KO MAC-T cells expressed an exacerbated cytokine response in comparison to unedited cells when stimulated with MAP cell lysate at the measured time points. These findings imply that TLR4 may be directly or indirectly involved in modulating immune signaling in response to MAP, and the potential contribution of miRNA and alarmins in immune modulation warrants further investigation using this *TLR4* KO cell line. The innate recognition of *M. tuberculosis* has been extensively researched, and many TLR4 ligands have been identified, some involving interactive signaling via TLR2, which is considered the main PRR for *M. tuberculosis* (Kim et al., 2019). Endogenous TLR are also involved in recognition of *M. tuberculosis*, and may be involved in recognition of MAP. In support of this, (Arsenault et al., 2013) reported that bovine monocyte TLR9 signaling was blocked by MAP, possibly as a way to evade monocyte effector function. This raises an important limitation of the present study, in that the *TLR4* KO cells were immune stimulated with MAP cell lysate, rather than being subjected to live MAP challenge. Thus, future MAP pathogen challenge studies will be carried out using these *TLR4* KO cells to further characterize the direct or indirect involvement of TLR4 in bovine immune response to MAP infection.

CONCLUSIONS

In the present study, a *TLR4* KO MAC-T cell line was developed and used to examine the involvement of TLR4 in response to challenge with MAP cell lysate and *E. Coli* LPS. As expected, the *TLR4* KO cells showed hyporesponsiveness to LPS. In contrast, the *TLR4* KO cells were hyperresponsive to MAP cell lysate, which suggests that TLR4 is involved in modulating immune signaling in response to MAP. The results of the present study suggest that *TLR4* genes play a pivotal role in determining the risk to MAP lysate inflammation in epithelial MAC-T cells. These novel findings show divergent roles for TLR4 in *E. coli* and mycobacterial infections. However, further studies with live MAP and accessing miRNA-mediated inflammatory cytokine regulation will be undertaken to further investigate these findings. In addition, the generated knockout cell line of *TLR4* gene could be efficiently used in future studies for a better understanding of the role of TLR4 activation under different pathological conditions.

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