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Impact of *Mycobacterium avium* subsp. *paratuberculosis* infection on bovine *IL10RA* knockout mammary epithelial (MAC-T) cells

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Abstract

Mycobacterium avium subsp. *Paratuberculosis* (*MAP*) is an intracellular pathogen that causes Johne's disease (JD) in cattle and other ruminants. *IL10RA* encodes the alpha chain of the IL-10 receptor that binds the cytokine IL-10, and is one of the candidate genes that have been found to be associated with JD infection status. In this study, a previously developed *IL10RA* knockout (*IL10RA*KO) bovine mammary epithelial (MAC-T) cell line and wild-type (WT) MAC-T cells were infected with live MAP for 72 h to identify potential immunoregulatory miRNAs, inflammatory genes, and cytokines/chemokines impacted by MAP infection in the presence/absence of IL10RA. Cytokine and chemokine concentrations in culture supernatants were measured by multiplexing immunoassay. Total RNA was extracted from the MAC-T cells, and qPCR was performed to determine the expression of inflammatory genes and selected bovine miRNAs. Results showed that the levels of TNF-α, IL-6, CXCL8, CXCL10, CCL2, and CCL3 were significantly induced in WT MAC-T cells and IL-10 was significantly inhibited post-MAP infection. However, *IL10RA*KO MAC-T cells had greater secretion of TNF-α, IL-6, IFN-γ, CCL3, CCL4, CXCL8, and CXCL10, and lower secretion of VEGF-α. Moreover, the expression of inflammatory genes (*TNF-α*, *IL-1α*, *IL-6*) was also more significantly induced in *IL10RA*KO cells than in WT MAC-T cells post-MAP-infection, and unlike the WT cells, anti-inflammatory cytokines *IL-10* and *SOCS3* and chemokines CCL2 were not significantly induced. In addition, the expression of miRNAs (miR133b, miR-92a, and miR-184) was increased in WT MAC-T cells post-MAP-infection; however, there was no significant induction of these miRNAs in the *IL10RA*KO cells, which suggests IL10 receptor is somehow involved in regulating the miRNA response to MAP infection. Target gene function analysis further suggests that miR-92a may be involved in interleukin signaling, and miR-133b and miR-184 may be involved in other signaling pathways. These findings support the involvement of IL10RA in the regulation of innate immune response to MAP.

Keywords *IL-10RA* · Bovine mammary epithelial (MAC-T) knockout cells · *Mycobacterium avium* ssp. *paratuberculosis* (MAP) · miRNA · Cytokines

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent for paratuberculosis or Johne's disease (JD) in ruminants, an inflammatory disease that is clinically similar to human Crohn's disease (Rankin 1961; Vary *et al.* 1990; Chacon *et al.* 2004). MAP infection of an animal leads to a reduced ability to absorb nutrients due to inflammation and disruption of the intestinal lining. Clinical JD leads to reduced milk production, and premature culling or death. The cumulative effects of JD are a rising concern to both animal welfare and the dairy industry. MAP infection in dairy operations may have risen by ~ 23% from 2007 to 2013 (68 to 91%) according to the National Animal Health Monitoring System and more recent studies (Lombard *et al.* 2013). This prevalence of MAP infection in the USA may have concurrently resulted in an increased economic loss to the US dairy industry of $1.3 billion from $200 million (Garcia and Shalloo 2015). The cumulative effects of a long subclinical stage of infection, a lack of an effective vaccine, and insensitive diagnostic tools have made it difficult to control JD, and defining protective immune responses to MAP has also been challenging.

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The function of interleukin 10 (IL-10) is well-defined as an immune and anti-inflammatory regulator in terms of both innate and cell-mediated immunity (Moore *et al.* 2001). The IL-10 receptor alpha (IL10RA) plays an essential role in IL10-mediated immunoregulation (Ding *et al.* 2001). The observation that IL10 double knockout (−/−) mice developed spontaneous enterocolitis (Kühn *et al.* 1993), and the implication of *IL10RA* in ileitis mouse models (Kozaiwa *et al.* 2003), has raised the possibility that *IL10RA* mutation(s) play a role in the regulation of the inflammatory response in the human gut. At least 11 single nucleotide polymorphisms (SNPs) have been found on the human *IL10RA* gene of which many are silent mutations (Tanaka *et al.* 1997). Two novel human IL10RA variants have been shown to impact sensitivity to IL-10 (Gasche *et al.* 2003). Also, there is a reduced ability of IL-10 to inhibit lipopolysaccharide (LPS)-induced TNF-α production in human monocytes, indicating that the S138G variant may be a loss of function allele (Gasche *et al.* 2003). In our previous study, the function of IL10RA in the context of JD was interrogated in *IL10RA* knockout (KO) bovine mammary epithelial (MAC-T) cells stimulated with MAP-cell lysate (Mallikarjunappa *et al.* 2020). However, since MAP can evade host immunity, these changes might not truly reflect what occur during live MAP challenge.

MicroRNAs (miRNAs) are 18–25 nucleotides, short non-coding RNAs (Wang *et al.* 2021), which are known to regulate biological processes, and have emerged as key regulators of biological processes in animals (Gebert and MacRae 2019). In farm animal diseases, the potential to use miRNAs as diagnostic biomarkers of disease has been recently reviewed in terms of bovine viral diarrhea, mastitis, and JD (Do *et al.* 2021).

In recent years, a genetic approach to JD control has been investigated. Researchers have calculated heritability estimates of various indicators of JD resistance, and several polymorphic candidate genes have been found to be associated with JD (Koets *et al.* 2000; Mortensen *et al.* 2004; van Hulzen *et al.* 2014), which highlights the potential to breed dairy cattle to specifically reduce the incidence of JD. For example, SNPs in the bovine interleukin-10 receptor α (*IL10RA*) gene were previously found to be associated with MAP infection status (Verschoor *et al.* 2010).

Animal and in vitro cell culture studies have provided insights into the role of JD susceptibility genes and MAP infection. However, the role of *IL10RA* associated with live microbial infection is largely unknown. The objectives of this work therefore were to (1) determine if the IL-10 receptor is involved in the bovine innate immune response to MAP infection, and if so, (2) evaluate candidate miRNAs/cytokines that are affected during MAP infection.

Materials and methods

Bacterial strain and culture conditions The MAP Madonna strain was gifted from the lab of Dr. Lucy Mutharia (University of Guelph, Canada). The MAP was cultured in Middlebrook 7H9 broth (Sigma-Aldrich, St. louis, MO) 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, MO). The bacterial culture was incubated at 37 °C and 5% CO_2.

IL10RA knockout cell line Interleukin-10 receptor α knockout (*IL10RA* -KO) cell line was previously developed in our laboratory using MAC-T cells (Mallikarjunappa *et al.* 2020). The wild-type (WT) and *IL10RAKO* MAC-T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin–streptomycin (100 U/ml; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA), incubated at 37 °C and 5% CO_2 and cultured to a confluency of 80% according to previous study (Huynh *et al.* 1991).

In vitro MAP challenge The *IL10RA*KO and WT MAC-T cells were seeded at 1.2×10^5 cells per well in separate 24-well plates and incubated overnight at 37 °C and 5% CO_2 to reach 80% confluency. Both cell types were either infected with MAP for 72 h, or provided an equivalent volume of MAP-carrier solution media (uninfected control). Bacterial colony-forming units (CFUs) were determined using the pellet wet-weight method, whereby 1 mg of MAP Madonna pellet is equal to 10^7 CFU (Hines *et al.* 2007). MAP was added to each cell type to achieve a 10:1 multiplicity of infection (Lamont *et al.* 2014 ; Shandilya *et al.* 2023), then spun for 2 min at 250 × g to ensure MAP interaction with the cells. This experiment was repeated in quadruplicate at independent times. The culture supernatants were collected and stored at − 80 °C until further analysis.

Cytokines and chemokines multiplex analysis Analysis of culture supernatant cytokine and chemokine concentrations, namely cytokines IFN-γ, IL-1α, and IL-6; vascular endothelial growth factor (VEGF-α), TNF-α, IL-10, and IL-36-α; and chemokines CXCL8 (IL-8), CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL10 (IP-10) from MAP-infected and uninfected *IL10RA*KO and WT MAC-T cells were outsourced to Eve technologies (Calgary, AB, Canada), where they were immunoassay multiplexed using the Luminex 100 system.

RNA extraction Total RNA (mRNA + miRNA) extraction from uninfected (control) and MAP-infected samples of both WT and *IL10RA*KO MAC-T samples was carried out using the RNeasy Mini Kit (Qiagen, Germany), and DNA

traces were removed by DNase I treatment (MBI Fermentas) according to the manufacturer's protocol.

miRNA cDNA synthesis and qPCR The miRNA cDNA synthesis was performed using Qiagen miRCURY LNA RT Kit according to the manufacturer's instructions; cDNA's were diluted to 1:20 with nuclease-free water. The qPCR of previously identified candidate bovine miRNAs, namely miR-92a, miR-184, and miR-133b from the literature (Liang *et al.* 2016; Shaughnessy *et al.* 2020), was performed using a StepOne Plus qPCR machine (Applied Biosystem, Waltham, MA) using Sybr® Green (Bio-Rad Hercules, CA). A master mix of 7 µL containing 5 µL 2×miRCURY SYBR® Green Master Mix, 0.5 µL ROX reference dye, 1 µL resuspended PCR primer mix, and 0.5 µL RNase-free water was added in duplicate to 3 µL of diluted cDNA samples. Pooled samples were used to create a standard curve with a serial dilution of 1:5; this standard curve was used to ensure the efficiency of the reaction for all plates. The qPCR reaction was subjected to denaturation at 95 °C for 2 min then to 40 PCR cycles of 95 °C for 10 s, then 56 °C for 1 min of primer annealing and amplification. The qPCR analysis was performed using the ΔΔCT method with change in miRNA expression being expressed as fold-change (Livak and Schmittgen 2001), using miRNA U6 as the reference gene. All plates analyzed were required to have a standard curve with a reaction efficiency of 91–110%.

mRNA cDNA synthesis and qPCR For mRNA cDNA synthesis, 500 ng of purified RNA was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). For qPCR, the primer sequences for cytokine genes (Table 1) were selected from our previous study (Shandilya *et al.* 2021). Each qPCR reaction was performed in duplicate 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 10 pM 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 "ΔΔCT" method (Livak and Schmittgen 2001) and analyzed with two reference genes (*GAPDH, B2M*) as internal controls.

Target gene prediction and pathway analysis The miRNA target genes were predicted through two database tools: TargetScan (http://www.targetscan.org) and mirDB (http://www.mirdb.org/index.html)] (this URL does not work for some reason). A stringent selection criteria of target genes for both tools was applied; cumulative weighted context + + score < −0.4 for TargetScan, and target score > 70 for miRDb. Only the commonly identified target genes were considered for functional analysis. To facilitate the interpretation of gene targets and aid in the understanding of the potential function of the miRNAs, enrichment analysis for gene ontology (GO) annotation (molecular function, cellular component, and biological process) was performed using WebGestalt (http://www.webgestalt.org/#, version 2019).

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

Gene	Primer sequence (5′-3′)	Ta (°C)	Slope	PCR efficiency (%)
IL1α-F	TTGGTGCACATGGCAAGTG	58.3	−3.260	103
IL1α-R	GCACAGTCAAGGCTATTTTTCCA			
IL1β-F	GCCTTCAATAACTGTGGAACCAAT	58.3	−3.161	107
IL1β-R	GTATATTTCAGGCTTGGTGAAAGGA			
IL6-F	GGCTCCCATGATTGTGGTAGTT	58.3	−3.360	98
IL6-R	GCCCAGTGGACAGGTTTCTG			
TNF-α F	CGGTGGTGGGACTCGTATG	58.3	−3.125	109
TNF-α 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			

Statistical analysis To compare levels of different cytokines/chemokines, and mRNA and miRNA expression in MAP-infected treatments (MAP-infected versus uninfected controls), the values were analyzed using a two-way ANOVA test followed by the Bonferroni test (GraphPad Prism Software, Boston, MA), and a p-value of ≤ 0.05 was considered statistically significant. All data ($n=4$) were presented as the mean ± standard error of the mean (SEM).

Results

Cytokine/chemokine production The effect of MAP infection on cytokine/chemokine production in the culture supernatants by WT and *IL10RA*KO Mac-T cells was determined by multiplex analysis. The levels of TNF-α, IL-6, CCL2, CCL3, CXCL8, and CXCL10 were significantly induced in MAP-infected WT cells compared with uninfected WT cells (Figs. 1 and 2). Conversely, significantly lower amounts of IL-10 and VEGF-α were secreted by MAP-infected WT cells versus the uninfected WT cells. For the *IL10RA*KO cells, the levels of TNF-α, IL-6, IFN-γ, CCL3, CCL4, CXCL8, and CXCL10 were significantly induced ($p < 0.05$) in MAP-infected cells compared to corresponding uninfected *IL10RA*KO cells, whereas VEGF-α was significantly lower. Secretion of IL-1α, IL-10, and CCL2 was not significantly different between the MAP-infected and uninfected *IL10RA*KO cells. When comparing MAP-infected *IL10RA*KO and WT cells, the levels of TNF-α, IFN-γ, IL-6, and CCL3 were significantly higher in the *IL10RA*KO cells than in WT cells; however, CCL2 was lower in the *IL10RA*KO cells than WT cells because it was not induced by MAP challenge.

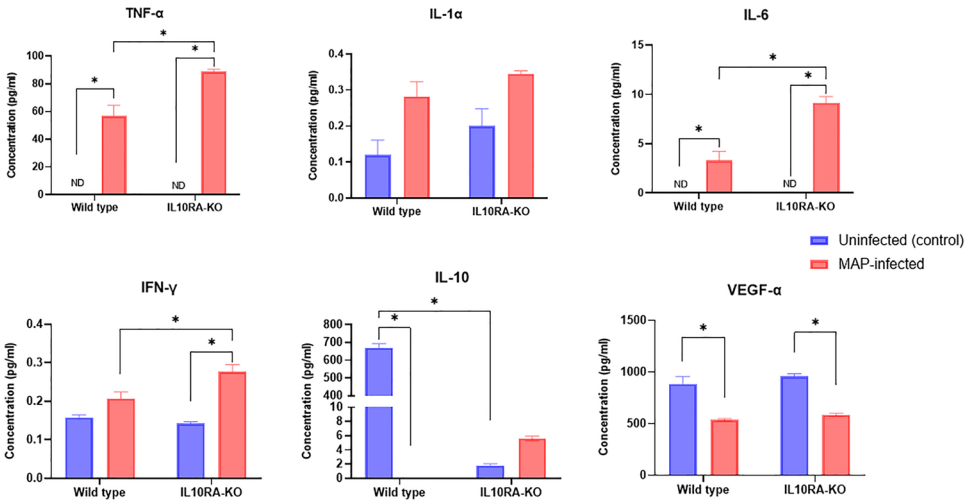

Figure 1. Cytokine concentrations from the culture supernatant of *IL10RA* knockout (*IL10RA*-KO) and wild-type bovine MAC-T cells challenged with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for 72 h. Data are expressed as mean (pg/ml) and SEM, with *asterisks* denoting $p < 0.05$; ND, not detected.

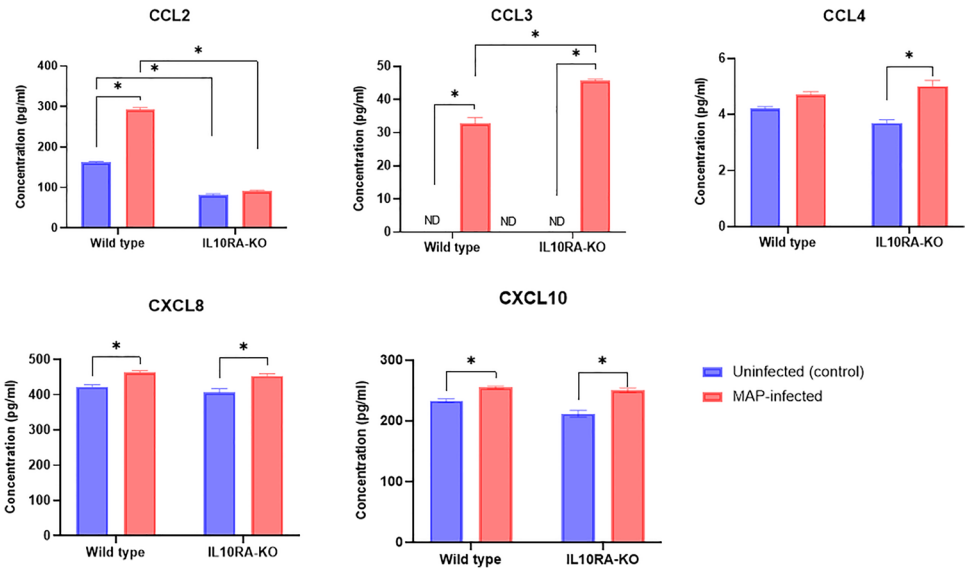

Figure 2. Chemokine concentrations in culture supernatant of *IL10RA* knockout (*IL10RA*-KO) and wild-type bovine MAC-T cells challenged with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for 72 h. Data are expressed as mean (pg/ml) and SEM, with *asterisk* denoting $p < 0.05$; ND, not detected.

Gene expression The expression of pro-inflammatory cytokine genes *TNF-α*, *IL1α*, and *IL6* was significantly increased in *IL10RA*KO cells post MAP infection for 72 h ($P < 0.05$) compared to corresponding uninfected *IL10RA*KO cells, but no significant change in the expression of these genes was observed in WT cells post MAP infection, except IL-6, which overall remained lower in the WT cells (Fig. 3). On the other hand, expression of anti-inflammatory cytokines *IL10* and *SOCS3* was significantly increased ($P < 0.05$) in MAP-infected WT cells compared to uninfected cells. However, there was no significant difference in the expression of *IL-10* and *SOCS3* post MAP infection in *IL10RA*KO cells.

microRNA expression We analyzed the expression of three bovine miRNAs (miR-133b, miR-92a, and miR-184) by qPCR at 72 h post MAP infection (Fig. 4). The WT cells challenged with MAP had significant induction of expression of all 3 miRNAs as compared to the uninfected WT cells ($P < 0.05$). However, the expression of these miRNAs was not altered in *IL10RA*KO MAC-T cells by MAP infection.

Gene enrichment and pathways analysis A total of 911 target genes were predicted for the three bovine miRNAs using two different target gene prediction tools (miRDb and TargetScan). The commonly identified predicted target genes were considered for functional analysis using the WeB-Gestalt webserver, shown in Fig. 5. Bovine miR-133b and miR-92a were found to be involved in the inflammatory signaling pathways; however, miR-184 was found to be involved in the Notch signaling pathway as shown in Table 2. The top three enriched terms in the biological process categories were biological regulation, metabolic process, and response to stimulus. The top three enriched terms in the cellular component categories were membrane, nucleus, and protein-containing complex. The top three enriched terms in the molecular function categories were protein binding, ion binding, and nucleic acid binding.

Discussion

The role of IL-10, a pleomorphic cytokine with diverse phenotypic effects, is well established in paratuberculosis, as it has an important role in the cross-talk between host innate and adaptive immune responses (Hussain *et al.* 2016). IL-10 signals through a tetramer receptor complex (IL-10R) composed of two identical binding subunits IL-10Rα and two homolog signal-transducing IL-10Rβ subunits. Previous studies reported that polymorphisms in bovine *IL10RA* were associated with increased MAP infection in dairy cattle (Sharma *et al.* 2006; Verschoor *et al.* 2010). We also established the role of the IL10RA receptor in MAC-T cell response to immune challenge with MAP cell lysate in vitro. In addition, it has been reported that IL-10 inhibits the anti-mycobacterial activity of macrophages and promotes the intracellular survival of mycobacteria (Nagata *et al.* 2010). Therefore, in this follow-up study, we aimed to investigate the role of the IL10 receptor alpha (IL10RA) in MAP infection using bovine *IL10RA*KO MAC-T cells. We assessed the expression of potential miRNAs, inflammatory genes, and cytokine/chemokine production in culture supernatants of wild-type and *IL10RA*KO MAC-T cell post-MAP infection.

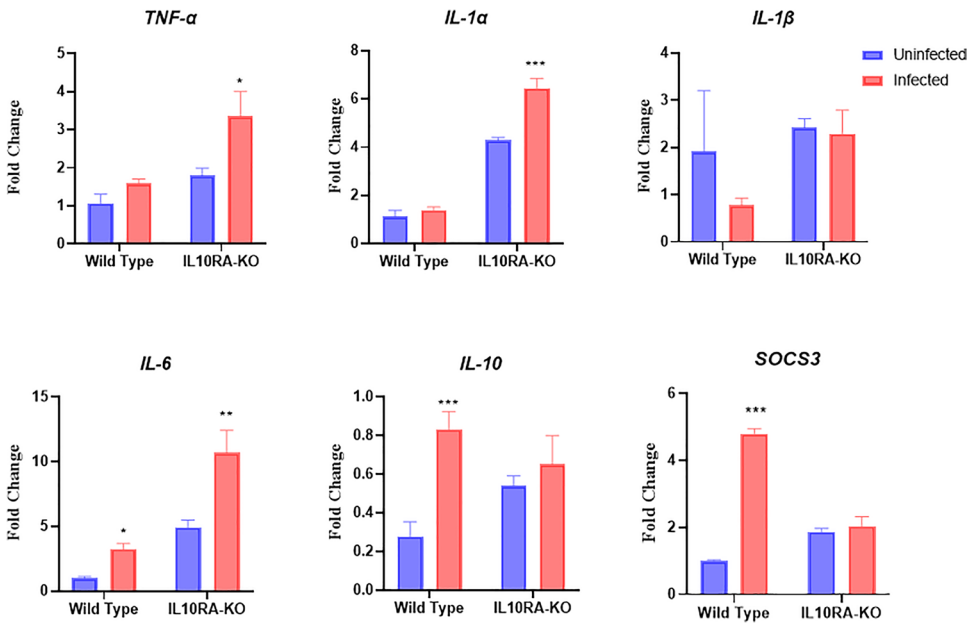

Figure 3. Relative fold change of pro-inflammatory (*TNF-α*, *IL-1α*, *IL-1β*, and *IL-6*) and anti-inflammatory (*SOCS3* and *IL-10*) cytokine gene expression in *IL10RA* knockout (*IL10RA*-KO) and wild-type MAC-T cells challenged with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for 72 h. Data are expressed as mean ± SEM ($n = 4$). Significant differences are denoted by $^{*}p < 0.05$ and $^{***}p < 0.001$.

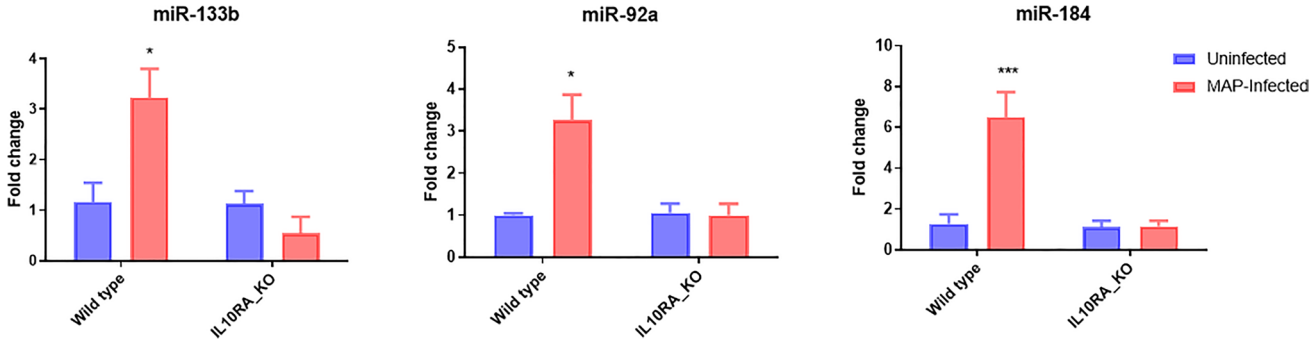

Figure 4. Differential expression of miRNAs in *IL10RA* knockout (*IL10RA*-KO) and wild-type MAC-T cells post MAP-Infection for 72 h. Data are expressed as mean ± SEM ($n = 4$). Significant differences are denoted by $*p < 0.05$ and $***p < 0.001$.

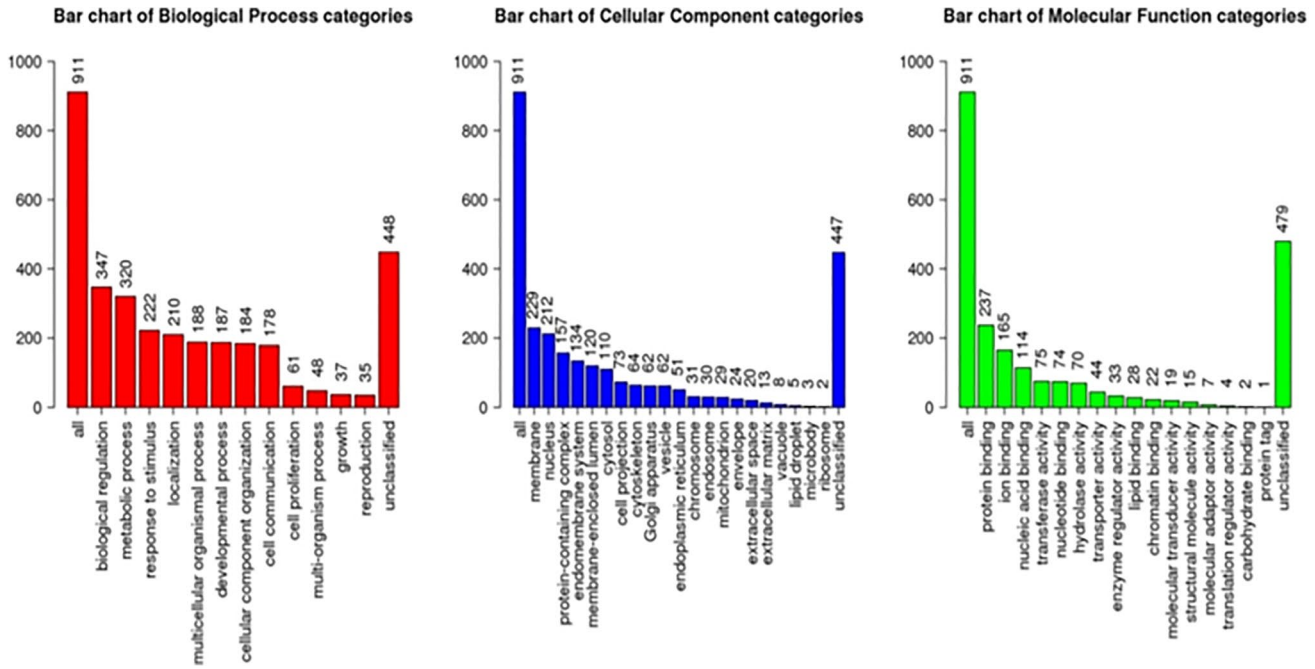

Figure 5. Bar chart of gene ontology (GO) terms predicted by identified target genes of upregulated bovine miRNAs (miR-133b, miR-92a, and miR-184) using Web-GSTALT online tool in three categories: biological processes, cellular components, and molecular functions.

Table 2. Numbers of commonly identified target genes of miR-133b, miR-92a, and miR-184, and selected pathways

miRNA	TargetScan	miRDB	Common targets	Selected pathways
miR-133b	644	654	352	p38 MAPK pathway; Notch signaling pathway; PI3 kinase pathway; Wnt signaling pathway; inflammation mediated by chemokine and cytokine signaling pathway
miR-92a	947	985	612	Interleukin signaling pathway; Toll receptor signaling pathway; inflammation mediated by chemokine and cytokine signaling pathway
miR-184	29	70	11	Notch signaling pathway

There is accumulating evidence that IL-10 may be important in Johne's disease. In vitro, viable MAP stimulates IL-10 secretion from ovine and bovine monocyte-derived antigen presenting cells (Khalifeh and Stabel 2004; Lei and Hostetter 2007). However, we found that IL-10 production decreased in MAP-infected WT cells as compared to uninfected WT cells. It is inconsistent with the findings of other studies. For example, a study analyzed the transcriptome of bovine monocyte-derived macrophages infected with MAP and reported upregulated *IL-10* expression 6 h post-infection compared to uninfected controls (Marino *et al.* 2017), however did not assess IL-10 expression at the protein level. Increased expression of IL-10 has been proposed to be a mechanism of immune evasion by MAP (Magombedze *et al.* 2015). It is possible that differences in cell type, MAP challenge level, and timing of sampling contributed to our contrasting results in terms of IL-10.

MAP is known to upregulate IL-6 and IL-1β expression as early as 1 h post-infection in MAP-infected MDMs (Dudemaine *et al.* 2014), indicating that the development of Th17 cells may be promoted by the local cytokine environment near sites of MAP infection. The levels of IL-6 were greater in the *IL10*RA KO cells compared to the WT for MAP-infected groups. This differs from our previous work that observed greater levels of CCL2 for MAP cell-lysate exposed *IL10*RA KO cells compared to exposed WT cells, as well as IL-10, and CXCL8, for which there were no significant differences in the present study (Mallikarjunappa *et al.* 2020). The higher concentration of IFN-γ in MAP-infected *IL10RA*KO cells is consistent with the findings of another study that found higher concentrations of IFN-γ upon initial MAP infection of macrophage (Stabel and Robbe-Austerman 2011). IL-10, CCL2, and IL-1α were not significantly different between the MAP-infected and uninfected *IL10RA*KO cells. This is inconsistent with findings that attribute activation of anti-inflammatory factors such as IL-10 as a survival tactic of MAP, as well as our previous work done with MAP-lysate (Khalifeh and Stabel 2004; Lucena *et al.* 2021). Both CCL2 and CCL3 play roles in monocyte chemotaxis to sites of inflammation and are known to be markers of severe infection with *Mycobacterium tuberculosis* (Hasan *et al.* 2009; Reichel *et al.* 2009).

In terms of gene expression, we also observed that *IL10RA*KO MAC-T cells displayed a higher pro-inflammatory cytokine gene response (*TNF-α*, *IL-1α*, *IL-6*) when challenged with MAP than challenged WT MAC-T cells. We attribute this higher pro-inflammatory cytokine expression in the KO cells to dysfunctional IL10 signaling. These findings are in support to our previous study that reported that *IL10RA*KO MAC-T cells were hyper-responsive to MAP-cell lysate (Mallikarjunappa *et al.* 2020). This similar response could be attributed to the same mode of interaction of MAP cell lysate and live MAP bacteria to host cells.

IL-10 negatively regulates inflammation by inhibiting pro-inflammatory cytokine production, acting both at the transcriptional and at the post-transcriptional levels (Sabat *et al.* 2010). To gain insights into the post-transcriptional mechanisms whereby IL-10 dampens innate immune cell activation, we investigated the effects of this anti-inflammatory cytokine's receptor on the expression of candidate miRNAs that have been reported to have an important role in MAP infection (Liang *et al.* 2016; Shaughnessy *et al.* 2020), and in fine-tuning the inflammatory response by targeting mRNA translation, processing, and stability (O'Neill *et al.* 2011). Limited studies have clarified the exact role of miRNA involved in host immune-regulation in response to MAP infection in cattle.

In this study, miR-133b and miR-184 were predicted to be involved in the Notch signaling pathway, and their expression was not altered by MAP infection in *IL10RA*KO cells, which implies that miR-184 may regulate the host inflammatory response by altering Notch/TLR4 interaction signaling. Notch signaling was reported to regulate innate immune responses by interaction with toll-like receptor (TLR) signaling, especially for activation of macrophages (López-López *et al.* 2020), and Notch1 enhances inflammation of macrophages in response to TLR4 stimulation by altering NF-κB activation, and excessive interaction between Notch1 and TLR4 signaling may exacerbate the inflammatory response (Li *et al.* 2022). During MAP infection in cattle, miR-133b was reported to be involved in "lymphocyte activation," and miR-184 in "inflammatory response activation" (Liang *et al.* 2016).

In comparison, miR-92a negatively regulates the inflammatory response triggered by TLR signaling by directly targeting mitogen-activated protein kinase kinase 4 (MKK4) in bacterial LPS stimulated macrophages. The MAP infection may regulate TLR signaling pathways and downstream immune responses by altering miRNAs expression, and upregulating miR-146b in infected ileum may be one of the mechanisms by which MAP disrupts TLR signaling pathways after infection (Liang *et al.* 2016). In addition, a previous in vivo study also reported that the TLR signaling pathway was inhibited at 12 h after MAP infection in the ileum (Khare *et al.* 2012). We found the expression of miRNAs increased significantly in WT cells post live MAP challenge, implying that those miRNAs may be involved in the molecular mechanisms regulating the host response to MAP.

Meanwhile, the expression of miRNAs in *IL10RA*KO cells was not affected by MAP infection. Based on these results, we speculate that the lack of IL10/IL10 receptor interactions affects the downstream inflammatory responses by altering miRNA expression. Moreover, Liang *et al.*

(2016) reported that miR-146b is negatively correlated with the predicted target genes interleukin 4-receptor (IL4R) (Liang *et al.* 2016), and spleen tyrosine kinase, which activates the NF-κB-mediated transcription of cytokines (Kashiwada *et al.* 2001; Fleischer *et al.* 2014), suggesting that miR-146b may suppress inflammatory responses triggered via the TLR signaling pathway. A previous study reported that miR-146a negatively regulates the inflammatory response induced by *Porphyromonas gingivialis* through TRAF6/p38 mitogen-activated protein kinases (MAPK) pathway (Tang *et al.* 2019). Overexpression of miR-133b was demonstrated to promote apoptosis of osteosarcoma cells by inhibiting MAPK signaling pathway (Xu *et al.* 2020).

Apart from being an essential immune-regulator in host immunity, IL-10 also accounts for the intracellular survival of mycobacterium due to its inhibitory activity against antimycobacterial functions (Nagata *et al.* 2010). The absence of IL-10 leads to better clearance of some pathogens with no enhanced immunopathology (Brooks *et al.* 2006; Ejrnaes *et al.* 2006). The level of IL-10 production depends on the type and strength of the stimulus, while the molecular mechanisms for the regulation of IL-10 differ according to cell type, although some common mechanisms also exist (Saraiva and O'Garra 2010). The main signaling pathways for the production and regulation of IL-10 in phagocytic cells are MAPK pathways, nuclear factor kappa-B (NF-kB), and signal transducer and activators of transcription-3 (STAT3). By employing target gene function analysis, we found that miR-133b may be involved in the inflammatory response induced by p38 MAPK signaling pathway. PI3 kinase pathway as one of the predicted pathways of both miR-133b and miR-184 may contribute to host inflammation response to MAP challenge. As both pathway prediction tools demonstrated, miR-133b may also be involved in the Wingless/Int1 (Wnt) signaling pathway which is highly interacted with numerous other signaling pathways, such as NF-κB, MAPK, protein kinase B (PKB/AKT), and signal transducer and activator of transcription signaling (Moparthi and Koch 2019).

Conclusion

Taken together, findings reported in the present study indicate that the *IL10RA*KO MAC-T cells show hyper-responsiveness to MAP infection compared to wild-type MAC-T cells, which suggests that interleukin 10 receptor is involved in modulating immune signaling and plays a pivotal role in determining the risk of MAP infection in epithelial cells. Thus, it could be argued that SNPs in *IL10RA* that affect affinity binding of IL-10 receptor alpha to IL-10 may alter the inflammatory response of MAC-T to MAP infection by modulating signaling pathways.

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Data Availability All data generated and/or analyzed during this study are included in the article.

Declarations

Conflict of interest The authors declare no competing interests.

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