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MicroRNA-424 regulates the expression of CX3CL1 (fractalkine) in human microvascular endothelial cells during *Rickettsia rickettsii* infection

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ABSTRACT

Cytokines and chemokines trigger complex intracellular signaling through specific receptors to mediate immune cell recruitment and activation at the sites of infection. CX3CL1 (Fractalkine), a membrane-bound chemokine also capable of facilitating intercellular interactions as an adhesion molecule, contributes to host immune responses by virtue of its chemoattractant functions. Published studies have documented increased CX3CL1 expression in target tissues in a murine model of spotted fever rickettsiosis temporally corresponding to infiltration of macrophages and recovery from infection. Because pathogenic rickettsiae primarily target vascular endothelium in the mammalian hosts, we have now determined CX3CL1 mRNA and protein expression in cultured human microvascular endothelial cells (HMECs) infected *in vitro* with *Rickettsia rickettsii*. Our findings reveal 15.5 ± 4.0-fold and 12.3 ± 2.3-fold increase in *Cx3cl1* mRNA expression at 3 h and 24 h post-infection, coinciding with higher steady-state levels of the corresponding protein in comparison to uninfected HMECs. Since CX3CL1 is a validated target of microRNA (miR)-424-5p (miR-424) and our earlier findings demonstrated robust down-regulation of miR-424 in *R. rickettsii*-infected HMECs, we further explored the possibility of regulation of CX3CL1 expression during rickettsial infection by miR-424. As expected, *R. rickettsii* infection resulted in 87 ± 5% reduction in miR-424 expression in host HMECs. Interestingly, a miR-424 mimic downregulated *R. rickettsii*-induced expression of CX3CL1, whereas an inhibitor of miR-424 yielded a converse up-regulatory effect, suggesting miR-424-mediated regulation of CX3CL1 during infection. Together, these findings provide the first evidence for the roles of a host microRNA in the regulation of an important bifunctional chemokine governing innate immune responses to pathogenic rickettsiae.

1. Introduction

Rickettsial diseases caused by established and emerging zoonotic pathogens represent a significant burden to global human health. In the United States, Rocky Mountain spotted fever due to *Rickettsia rickettsii* and other spotted fever rickettsioses are notifiable diseases, the incidence of which as tick-borne infections reflects a consistent upward trend in recent years [1]. As obligate intracellular α-proteobacteria displaying a predilection to target the microvascular endothelial cells lining the blood vessels, infection of humans and susceptible animal hosts with pathogenic rickettsiae are characterized by endothelial activation, vascular inflammation and dysfunction, and dissemination through the vasculature leading to systemic rickettsial vasculitis and compromised vascular permeability [2].

Chemokines are a family of chemotactic cytokines, which signal through seven-transmembrane domain G-protein coupled receptors to regulate a panoply of immune responses primarily through recruitment of inflammatory cells to the sites of injury or infection [3]. Accordingly, chemokines and their receptors represent vital therapeutic targets in inflammatory disorders. Published work on rickettsial pathogenesis has established that endothelial cells express and secrete a variety of pro-inflammatory cytokines and chemokines in response to infection, including T-cell targeting chemokines CXCL9 (Mig), CXCL10 (IP-10), and CX3CL1 (fractalkine) [4,5]. As the only and unique member of CX3C subclass of chemokines, CX3CL1 is synthesized as a transmembrane protein consisting of an extracellular N-terminal domain, a mucin-like stalk, a transmembrane α-helix, and a cytoplasmic tail. Interestingly, membrane-bound CX3CL1 functions as an adhesion molecule, while its soluble form has a chemoattractant function [6]. CX3CR1, the cell surface receptor for CX3CL1, is expressed on NK cells, T cells, and monocytes/macrophages, known to play critical roles in the elimination of pathogens [7]. Despite its importance in the trafficking and/or

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recruitment of inflammatory cells, mechanism(s) underlying CX3CL1 expression during rickettsial infection of host endothelial cells are not understood.

As small non-coding regulatory RNAs of about 20–24 nucleotides, microRNAs (miRNAs) function by virtue of complementarity with their target messenger RNAs (mRNAs) in the 3′UTR region, leading to mRNA degradation or suppression of translation [8]. A single miRNA can interact with a number of different mRNAs and conversely, a single mRNA may be regulated by several miRNAs, suggesting the biological importance of miRNAs in the post-transcriptional regulation of target mRNAs [9]. As such, miRNAs are involved in various cellular processes, including regulation of development, differentiation, and proliferation, cell cycle and metabolism, and pathways of degradation (autophagy and apoptosis) [10]. Regulatory effects of miRNAs have recently been implicated in host-pathogen interactions and differential miRNA expression in response to pathogens is emerging as a novel molecular strategy exploited by bacteria to thwart host defense mechanisms [11]. We have previously demonstrated alterations in the global miRNA profile to reveal that miR-424 and miR-503 are significantly down-regulated during *in vitro* infection of human endothelial cells with *R. rickettsii* and *R. conorii* [12,13]. A follow-up study of this phenomenon further suggests miRNA-mediated regulation of the expression of fibroblast growth factor receptor1 (FGFR1) in facilitating rickettsial entry into host endothelium [13,14], yet the detail of downstream functional roles of miRNA-based regulatory mechanisms in the determination of host cell responses to intracellular rickettsiae remain unexplored. Thus, based on the evidence implicating suppression of miR-424 in the induction of CX3CL1 expression in human epithelial cells subjected to microbial challenge via stimulation with lipopolysaccharide or infection with *Cryptosporidium parvum* [15], the present study was designed with an aim to examine the potential contributory effects of miR-424-5p (miR424) in the regulation of CX3CL1 expression during *R. rickettsii* infection of human microvascular endothelial cells.

2. Materials and methods

2.1. Endothelial cell culture

An immortalized line of human dermal microvascular endothelial cells named CDC/EU.HMEC-1 and displaying all major morphologic, phenotypic, and functional features of microvascular endothelium was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). HMECs were maintained in continuous cultures using MCDB131 growth medium (Caisson Laboratories) supplemented with fetal bovine serum (10% v/v; Aleken Biologicals), epidermal growth factor (10 ng/ml, Thermo Fisher Scientific), L-glutamine (10mM, Thermo Fisher Scientific), and hydrocortisone (1 μg/ml, Sigma) [16]. The cells were routinely monitored microscopically for their growth, propagated by feeding with fresh culture medium every 2–3 days, and passaged at about 90–100% confluence. All experiments were performed at the passage level of ≤35.

2.2. Cell infection and transfection

R. rickettsii (strain Sheila Smith) was grown intracellularly in cultured Vero cells and purified by differential centrifugation as described previously [17]. The infectivity titers of purified stocks were estimated by citrate synthase (*glt*A)-based quantitative PCR and plaque formation assay [17,18]. To avoid repeated freeze-thaw cycles, aliquots (≤500μl) of rickettsial stocks were kept frozen at −80 °C. HMECs were infected with *R. rickettsii* at an MOI (multiplicity of infection) of about 5 intracellular rickettsiae per cell following our standard protocols [14]. The viability of both mock-infected (controls) and *R. rickettsii*-infected HMECs was monitored microscopically. At different times post-infection, culture medium was removed by gentle aspiration and the cells were lysed directly in TRI reagent® (Molecular Research Center) for RNA isolation [14]. The mimics and inhibitors for miR-424-5p (miR-424) and corresponding negative controls (mirVana™ miRNA mimic and inhibitor negative controls) were purchased from Applied Biosystems/Thermo Fisher Scientific and transfected into HMECs for 24–48 h using Lipofectamine RNAimax according to the manufacturer's recommendations.

2.3. RNA preparation

Total cellular RNA was extracted from *R. rickettsii*-infected and control (mock-infected) HMECs using our optimized TRI-reagent® [13]. Resultant RNA preparations were subjected to DNase I treatment to remove any contaminating genomic DNA and quantified using a MultiSkan™ Go Spectrophotometer (Thermo Fisher Scientific).

2.4. Quantitative real-time PCR

TaqMan® two-step RT-PCR assays containing primers for both miRNA-specific reverse transcription and quantitative PCR were obtained from Applied Biosystems. One microgram of total RNA for each sample was reverse transcribed using the TaqMan MicroRNA cDNA synthesis kit (Applied Biosystems) and miRNA-specific primers for miR-424 as well as oligo (dT) primers for concurrent analysis of 18S and Gapdh expression. The miRNA expression was determined by real-time PCR using a TaqMan® assay specific for miR-424 (Applied Biosystems). 18S RNA was employed as an endogenous control to normalize for miRNA expression. The mRNA expression of *Cx3cl1* was measured using gene-specific primers and Gapdh as the housekeeping gene to normalize for mRNA expression among samples [14]. The $^{\Delta}$Ct values for experimental (infected) samples were compared to the mock controls, which were assigned a value of 1 and the relative expression was determined by comparative Ct ($^{\Delta\Delta}$CT method) as described earlier [14].

2.5. Western blot analysis

For the analysis of CX3CL1 protein expression, total protein lysates from HMECs infected with *R. rickettsii* for 3 h and 24 h were prepared. The samples were then subjected to polyacrylamide gel electrophoresis (PAGE) on a sodium dodecyl sulfate (SDS) denaturing gel, followed by wet transfer to a nitrocellulose membrane and immunoblotting using an antibody against CX3CL1 (Thermo Fisher Scientific). The blots were then stripped and re-probed with an α-tubulin antibody to normalize for any variations in the loading of samples.

2.6. Statistical analysis

All experiments were performed at least three times with technical triplicates to calculate the results as the mean ± standard error. Statistical analysis for miRNA-424 and CX3CL1 expression in *R. rickettsii*-infected and uninfected groups was performed by one-way ANOVA with Dunnett's post-test using GraphPad Prism 4.00. The p value for statistical significance among experimental conditions being compared was set at ≤0.05.

3. Results

A unique feature of CX3CL1 is its synthesis as a transmembrane molecule and expression on endothelial cell surface allowing for interactions with its receptor CX3CR1, which is abundantly expressed on monocytic cells, T cell subsets, and NK cells. Thus, CX3CL1 can promote surveillance by adhesion and recruitment of inflammatory cells in the absence of 'endothelial activation'. Proinflammatory stimuli enhance the expression of CX3CL1, promoting the adhesion of monocytic cells and resultant activation of CX3CR1 induces transmigration of adherent monocytes through the endothelial layer. In addition, CX3CL1 acts as a classical chemoattractant for leukocytes after its shedding from the cell

surface by the activity of ADAM10 and ADAM17 [19,20]. We first determined CX3CL1 expression in cultured human microvascular endothelial cells (HMECs) infected with *R. rickettsii* for 3 and 24 h at the levels of transcription and translation (protein) by real-time quantitative PCR and Western blotting, respectively. In comparison to corresponding mock-infected controls, *Cx3cl1* mRNA expression in *R. rickettsii*-infected HMECs was significantly higher as evidenced by 15.5 ± 4.0-fold and 12.2 ± 2.3-fold increase at 3 and 24 h post-infection, respectively (Fig. 1A). Western blot analysis also consistently revealed a pattern of higher steady-state cellular protein levels in response to *R. rickettsii* infection (Fig. 1B). Quantitatively, levels of CX3CL1 protein increased by 8.0 ± 0.5-fold and 6.6 ± 0.5-fold levels at 3 and 24 h, respectively, as compared to mock-infected controls (Fig. 1C). In toto, these results suggest that *in vitro* infection of HMECs with *R. rickettsii* upregulates CX3CL1 expression.

To explore the possibility of CX3CL1 regulation by miRNAs, we next employed targeting algorithms and an online database TargetScan to search for putative miRNAs that might bind to *Cx3cl1* mRNA. Based on the computational screening, we identified miR-424 as a candidate miRNA that could potentially target *Cx3cl1* with a predicted miRNA binding site in the 3′-UTR of *Cx3cl1*. Specifically, the seed region of miR-424 displays complementarity to the 3′-UTR region of *Cx3cl1* mRNA (Fig. 2A). In addition, our previous findings have documented that *R. conorii* infection significantly downregulates the expression of miR-424 in endothelial cells [12], which led us to quantify the levels of miR-424 expression in response to *R. rickettsii* infection. The results revealed a significant reduction of about 83 ± 3% and 85 ± 4% reduction, respectively, in the steady-state expression levels of miR-424 in *R. rickettsii*-infected HMECs at 3 and 24 h post-infection, as compared to its basal level in mock-infected controls (Fig. 2B).

Importantly, published evidence suggests that insertion of 3′-untranslated region of *Cx3cl1* mRNA containing the potential binding site for miR-424 into a pMIR reporter luciferase plasmid and transfection of this construct results in H69 human epithelial cells leads to significant reduction in 3′-associated luciferase activity. In addition, introduction of miR-424 precursor in these cells further decreases the luciferase activity, confirming the validation of CX3CL1 as a target of miR-424 [15]. Thus, to investigate the hypothesis of CX3CL1 regulation by miR-424-5p (miR-424) in host endothelial cells during *R. rickettsii* infection, we next performed a series of experiments based on the gain- and loss-of-function using, respectively, a miR-424 mimic and a

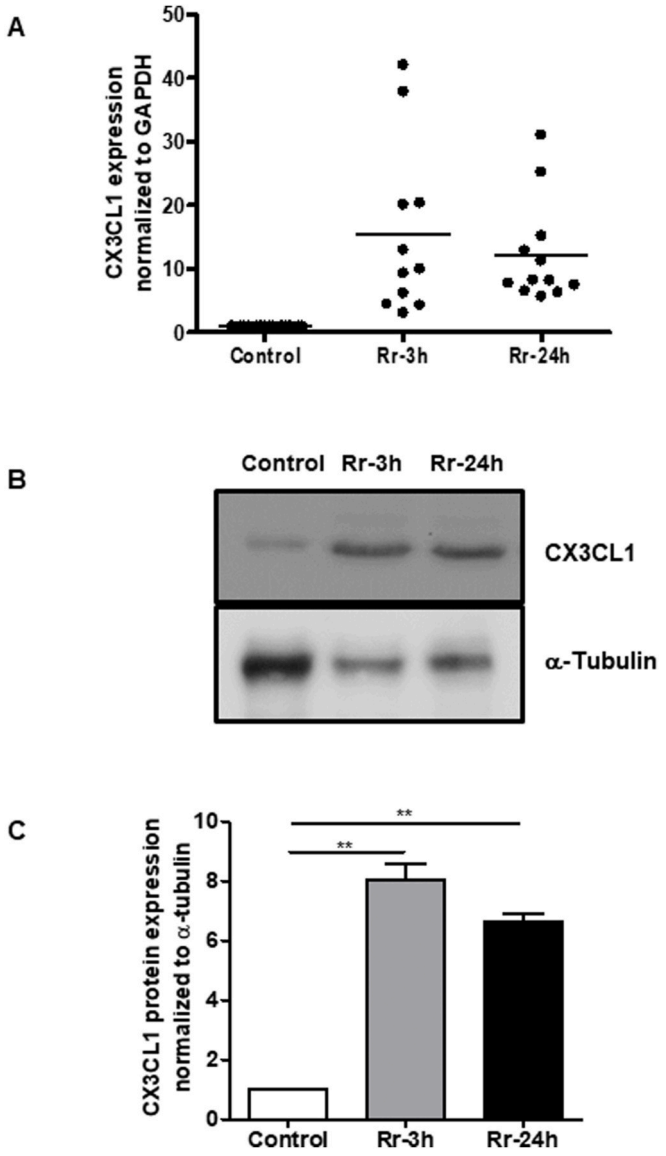

Fig. 1. CX3CL1 expression in *R. rickettsii* infected endothelial cells. (A) Confluent HMECs were infected with *R. rickettsii* for 3 h or 24 h. At each time point post-infection, cells were lysed in Tri-Reagent for the extraction of total RNA, which was subjected to the analysis of *Cx3cl1* mRNA expression by RT-qPCR using a gene-specific primer pair. (B) Total protein lysates from *R. rickettsii*-infected HMECs were prepared in RIPA buffer containing protease inhibitor cocktail and processed for Western blotting using an antibody against CX3CL1 to measure the levels of cellular protein expression. α-tubulin was used as a protein loading control. (C) Quantitation of CX3CL1 protein expression from three independent experiments is presented as the mean ± standard error. The asterisks indicate significant change ($p < 0.01$).

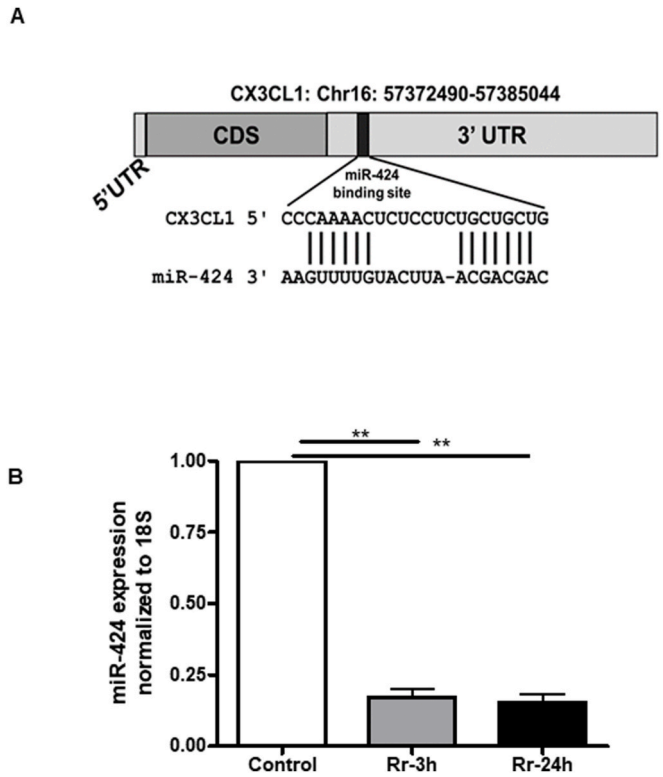

Fig. 2. miR-424-5p (miR-424) expression in *R. rickettsii* infected endothelial cells. (A) Schematic depicting the binding site of miR-424 at the 3′UTR region of *Cx3cl1* mRNA, suggesting *Cx3cl1* as a potential target for miR-424. (B) Confluent HMECs were infected with *R. rickettsii* for 3 h or 24 h and processed using a Tri-Reagent protocol for the preparation of RNA. miR-424 expression was measured by RT-qPCR using a miR-specific Taqman assay. The error bars represent mean ± standard error from three separate experiments. The asterisks indicate significant change ($p < 0.01$).

miR-424specific inhibitor along with corresponding negative controls (mirVana™ miRNA mimic negative control). For this aspect, miR-424 mimic/inhibitor and a negative control were transfected into endothelial cells using Lipofectamine® RNAiMAX and their effects on miR-424, and *Cx3cl1* expression were determined by RT-qPCR. As expected, introduction of the miR-424 mimic resulted in a dramatic increase in miR-424 expression. Infection with *R. rickettsii* was able to counteract the effects of miR-mimic, resulting in reduced miR-424 expression in endothelial cells transfected with the mimic, in comparison to corresponding mock controls (Fig. 3A). Conversely, mRNA expression of *Cx3cl1* was significantly down-regulated in cells transfected with the miR-424 mimic alone and in those infected with *R. rickettsii* after the delivery of miR-424 mimic (Fig. 3B). Similar results were obtained for CX3CL1 protein expression, where introduction of miR-424 mimic in both uninfected and *R. rickettsii*-infected HMECs resulted in significantly reduced levels of CX3CL1 protein (Fig. 3C). Finally, miR-424 inhibitor reduced the cellular levels of miRNA by greater than 75% as expected. *R. rickettsii* infection of ECs further reduced miR-424 expression by about 50% in the presence of miR-424 inhibitor (Fig. 4A). Accordingly, opposite effects on CX3CL1 mRNA levels were also evident in the presence of miR-424 inhibitor (Fig. 4B). Together, these findings suggest potential involvement of miR-424 in the regulation of CX3CL1 expression during rickettsial infection of host endothelial cells.

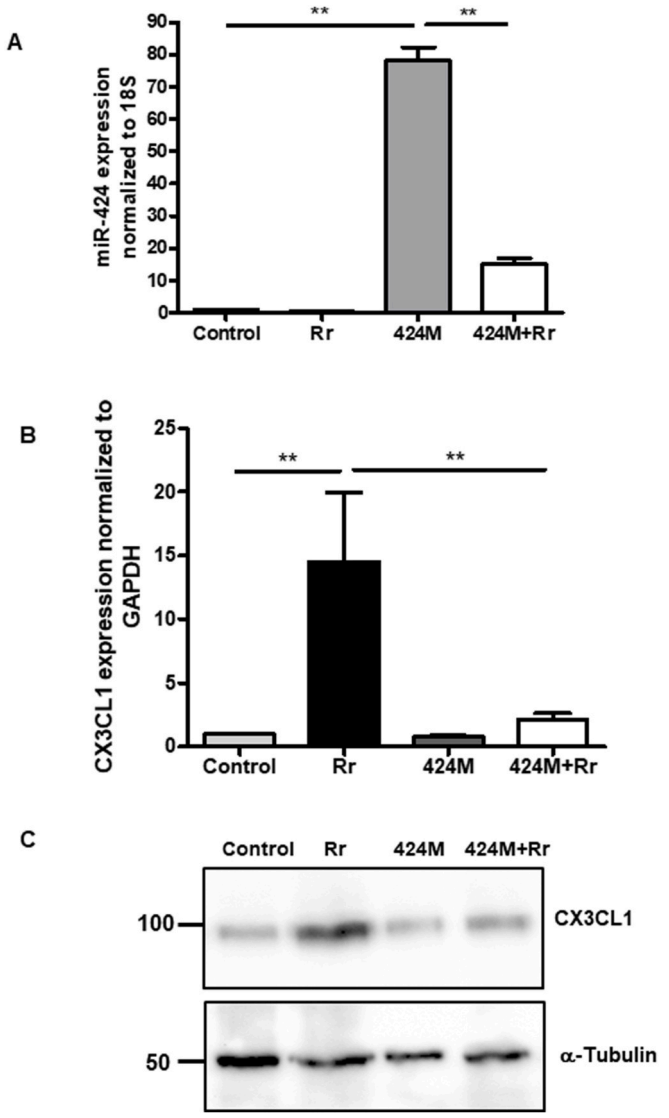

Fig. 3. Regulation of *Cx3cl1* mRNA expression by miR-424-5p (miR-424). (A) Endothelial cells were transfected with miR-424 mimic (1nM for 24 h) prior to infection with *R. rickettsii* for 24 h. Cells were then lysed in Tri-reagent for the isolation of RNA and determination of miR-424 expression by a Taqman assay. (B) *Cx3cl1* mRNA expression was measured by RT-qPCR using a specific primer pair. (C) CX3CL1 protein expression was measured by western blot using an antibody against CX3CL1. The asterisks indicate statistically significant change ($p < 0.01$).

4. Discussion

As small proteins of about 5–20 kDa, cytokines are produced and released by immune cells (macrophages, lymphocytes, and mast cells) as well as other cell types including stromal cells, fibroblasts, and endothelial cells. Based on their cellular origin and functions, cytokines can be further classified into chemokines, interferons, interleukins (ILs), and tumor necrosis factors. Chemokines are a family of mediators of chemotaxis between cells, which include C-X-C (CXC), C–C (CC), X–C (XC), and C-3X–C (CXXXC) motif chemokines depending on the arrangement of first two invariant cysteine residues in the amino acid sequence [21]. Endothelial cells form a monolayer of cells lining the entire lymphatic and vascular systems and functioning as a semi-permeable barrier between the lymph or blood in vessels and surrounding tissues. As conditional immune cells responding to a variety of physiological and pathological stimuli, endothelial cells perform a number of important functions, including participation in the regulation of innate and adaptive immunity [22]. Activation of endothelial cells as a consequence of stimulation by various factors, including infection with

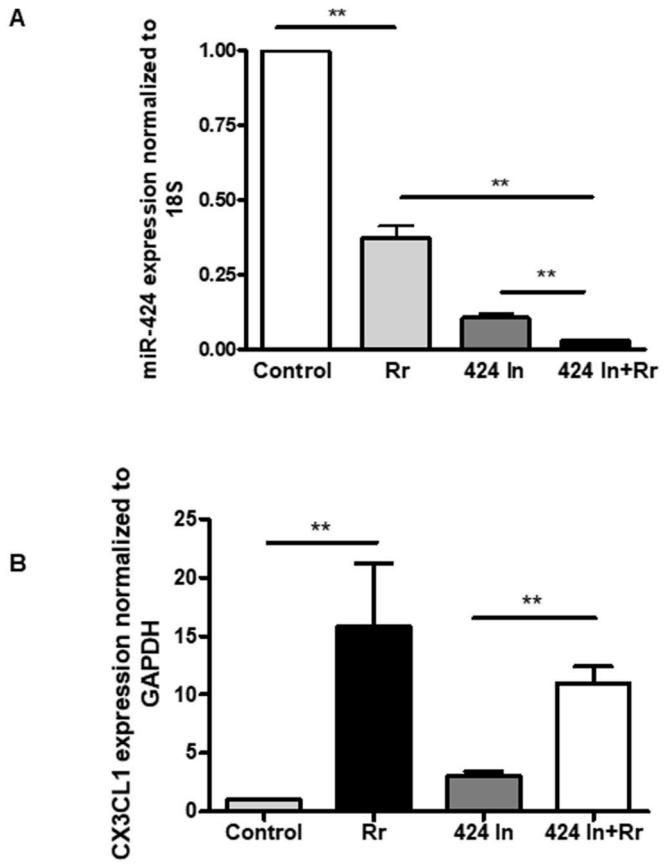

Fig. 4. Regulation of *Cx3cl1* mRNA expression by miR-424-5p (miR-424). (A) Endothelial cells were transfected with miR-424 Inhibitor (200nM) for 48 h prior to infection with *R. rickettsii* for 24 h. RNA was extracted and miR-424 expression was measured by RT-qPCR using a specific Taqman assay. (B) *Cx3cl1* mRNA expression was also measured using a specific primer pair. The asterisks (**) indicate statistically significant change ($p < 0.01$).

pathogenic bacteria or viruses and exposure to pathogen-associated molecular patterns (example, lipopolysaccharide), results in induced expression and secretion of a number of pro-inflammatory cytokines and chemokines [22]. It is now well established that infection of host endothelial cells with *R. rickettsii* and *R. conorii* is characterized by switch from a basal, quiescent phenotype to an activated, pro-inflammatory state defined by enhanced expression and secretion of cytokines/chemokines, including IL-1α, IL-8 (CXCL8), MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), and fractalkine (CX3CL1) [5,23–25].

MicroRNAs (miRs) are small noncoding RNAs responsible for the regulation of a significant proportion (40–60%) of gene expression at the posttranscriptional level. Ample evidence supports the contributory roles of miRs as critically important gene regulators carrying a promise to have tremendous impact on human health and disease. Multiple miRs have been identified for their potential to regulate multiple gene targets involved in different cellular processes controlling innate and adaptive immunity. In the context of infectious diseases, miRs play an important role in the survival and amplification of viruses, bacteria, and other pathogens. Bacterial pathogens exploit miR-directed alterations in protein translation and signal transduction for their own benefit and survival [26]. In addition, pathogens also regulate the production of cellular miRs to downregulate immune activation and cytokine signaling. For example, miR-146 and miR-155 have been strongly implicated in the regulation of inflammation and immunity during bacterial infections [27]. Circulating miRs in biological fluids are also emerging as valuable biomarkers or therapeutic targets for bacterial pathogens as has been described for pulmonary tuberculosis and *Helicobacter pylori*-associated gastritis [28,29]. Previously published reports from our laboratory have identified: (i). significant changes in the expression of a number of miRs in endothelial cells infected with *R. rickettsii*, and (ii). important regulatory roles for miR-424 and miR-503 in potentiating the expression of fibroblast growth factor receptor-1 (FGFR1) as one of the host cell receptors in human dermal, pulmonary, and cerebral microvascular endothelial cells infected with *R. conorii* and in an *in vivo* murine model of *R. conorii* infection [12–14]. The findings of this study corroborate previous reports of increased CX3CL1 expression by host endothelial cells during *R. rickettsii* infection and implicate the involvement of regulatory effects of miR-424 in induced expression of CX3CL1 as a host response mechanism.

CX3CL1 (fractalkine) is an endothelial cell-derived chemoattractant containing a CX3C motif in its N-terminal domain and involved in the regulation of both adhesive and chemotactic functions. It is constitutively expressed in many hematopoietic and non-hemopoietic tissues and exists in two forms as a transmembrane protein with a chemokine domain and as a soluble peptide that cleaves from the cell surface. Membrane-bound CX3CL1 promotes leukocyte binding, adhesion, and activation and the soluble chemokine is chemotactic for T cells and monocytes, which express the CX3CR1 receptor for the fractalkine [30]. Evidence further reveals its chemoattractant properties for natural killer (NK) cells, but not neutrophils [31]. $CD8^+$ T lymphocytes and NK cells have previously been shown to play a critical role in anti-rickettsial immune responses [32]. In an established mouse model based on the sub-lethal dose of *R. conorii*, CX3CL1 expression was found to be significantly increased on almost all segments of the vasculature in target tissues (lungs, kidney, and heart) on day three post-infection, followed by a return to the baseline coinciding with the recovery from disease later on day five or seven post-infection. Importantly, rickettsial burden and infiltration of macrophages also peaked in correlation with CX3CL1 expression in all infected tissues [5]. It is thus likely that increased expression of CX3CL1 recruits immune effector cells expressing its cognate receptor CX3CR1 to the site of infection and activates their effector functions as a host response to facilitate rickettsial clearance.

The immediate early response of host cells to invading pathogens is to trigger the innate immune mechanisms and miRs have been demonstrated to participate in the regulation and fine tuning of such immune responses. Several miRNAs have been implicated in controlling important physiological and pathological responses, including mechanisms underlying regulation of oxidative stress and repair in endothelial cells and vascular inflammation [33]. We and others have determined that both macro-as well as microvascular endothelial cells infected *in vitro* with spotted fever rickettsiae activate different signaling cascades, induce the expression and secretion of cytokines and chemokines, undergo oxidative stress and consequently activate anti-oxidant response mechanisms [25,34,35]. The present study illustrates significant down-regulation of miR-424 in endothelial cells and potential involvement of this host response in inducing CX3CL1 expression, which may constitute one of the pathways for recruitment of immune cells to the site(s) of infection for bacterial clearance. Further *in vivo* studies to determine the precise roles of miR-424 and other regulatory miRNAs in the host immunity to invasive pathogens would be helpful in the design and development of novel therapeutics against human rickettsial diseases and other bacterial infections.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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