Small Non-Coding RNAs: New Insights in Modulation of Host Immune Response by Intracellular Bacterial Pathogens

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Pathogenic bacteria possess intricate regulatory networks that temporally control the production of virulence factors and enable the bacteria to survive and proliferate within host cell. Small non-coding RNAs (sRNAs) have been identified as important regulators of gene expression in diverse biological contexts. Recent research has shown bacterial sRNAs involved in growth and development, cell proliferation, differentiation, metabolism, cell signaling, and immune response through regulating protein–protein interactions or *via* their ability to base pair with RNA and DNA. In this review, we provide a brief overview of mechanism of action employed by immune-related sRNAs, their known functions in immunity, and how they can be integrated into regulatory circuits that govern virulence, which will facilitate our understanding of pathogenesis and the development of novel, more effective therapeutic approaches to treat infections caused by intracellular bacterial pathogens.

Keywords: small RNAs, *Salmonella*, *Mycobacterium*, *Brucella*, innate immunity, adaptive immunity

INTRODUCTION

Precise control of gene expression is an essential feature of the immune system. The immune system depends on a sophisticated gene expression program equipped with an arsenal of strategies to fight against infections, mainly controlled by well-described transcriptional and posttranscriptional mechanisms [1]. Cells of the immune system are able to undergo dramatic changes in transcription mechanisms to efficiently organize expression of genes critical to defense. Innate and adaptive immune cell differentiation and activation depends largely on these transcription events [2]. Neutrophils, macrophages, and dendritic cells (DCs) exhibit both common and unique sets of toll-like receptors (TLRs), chemokines, and cytokines in innate immune responses. microRNAs (miRNA) and RNA-binding proteins determine the specific region of the gene accessible to transcription factors that ultimately regulate transcription [1].

Small non-coding RNAs (sRNAs) play critical roles in bacterial gene expression and are recognized as key regulators in bacteria. Typically, these RNA regulators range from 50 to 200 nt in length and act on independently expressed targets, often encoded in the intergenic region [3, 4]. sRNA controls bacterial gene expression by employing multiple molecular strategies to regulate the expression of gene targets, including binding directly to complementary sequences present in target mRNA molecules [5, 6]. sRNAs interact with their specific target to exert both positive and negative effects

on gene expression. In positive regulation, sRNAs bind with target mRNA at the 5′-untranslated region (UTR) and alter the secondary structure of the mRNA, to access a ribosome-binding site (RBS) that allows translation (7, 8). In addition, sRNA can bind to the 3′-UTR of target mRNA, ultimately increasing gene expression and stabilizing the transcript (9). Alternatively, sRNAs can also exert inhibitory effects by binding with mRNA 5′-UTR, resulting in decreased stability, degradation of the mRNA, occlusion of the RBS, and inhibition of translation (10). sRNAs are widely identified and their number constantly growing due to their involvement in diverse biological contexts including cell proliferation, development, differentiation, apoptosis, metabolism, stress response and signal transduction (11, 12).

Pathogenic bacteria have to face hostile and changing environments characterized by high concentrations of reactive oxygen and nitrogen species, low pH, and limited nutrient availability that hinder in their replication and infection to succeed (13). Pathogens have evolved a variety of strategies to survive and replicate within eukaryotic cells, establishing mechanisms to manipulate the host-cell machinery for their own benefit (14). After internalization in host cells, pathogenic bacteria modulate their trafficking to avoid lysosomal fusion by occupying a specialized membrane-bound vesicle. Intracellular bacteria are divided in two classes: vacuolar intracellular bacteria, such as *Salmonella*, *Mycobacterium*, *Legionella*, *Brucella*, and *Coxiella*, that survive and replicate either by avoiding vacuole–lysosome fusion or by altering the phagolysosome environment; and cytosolic intracellular bacteria, including *Francisella*, *Shigella*, *Listeria*, *Burkholderia*, and *Rickettsia*, that usually escape to proliferate within the cytosol of host cell (15, 16).

Recent developments in biocomputation have revealed a large number of regulatory sRNAs and have highlighted their potential links to bacterial pathogenesis (17, 18). Bacterial adaptation to intracellular environment niches is efficiently regulated in both time and space. These newly identified sRNAs play an integral part in virulence expression and bacterial stress responses that are ultimately advantageous for pathogens in adaptation and modification of the host-immune response (19). Understanding the mechanisms adopted by sRNAs to control immune cell function and how immune-related cells maintain cell viability and competitiveness in varying environmental niches is critical. However, roles of sRNA regulators in pathogenesis and immune response mechanisms have only begun to be investigated. In this review, we summarize the mechanisms employed by bacterial sRNAs in gene regulation and sRNA-based strategies to counter host immune response mechanisms as well as their implications in the pathogenesis of intracellular bacteria.
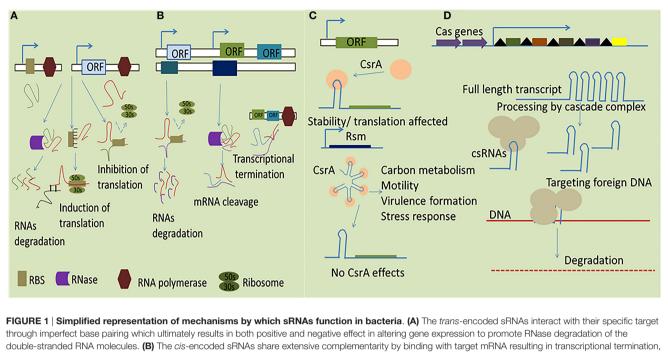
MECHANISMS EMPLOYED BY BACTERIAL sRNAs FOR GENE REGULATION

Bacterial regulatory sRNAs operate at all layers of gene regulation to modulate translation, transcription, DNA maintenance or silencing, and mRNA stability. They use different mechanisms to achieve these outstanding regulatory functions (5). The major

mechanisms employed by bacterial sRNAs for gene regulation are as follows:

(i)   *Trans*-encoded sRNA are usually encoded on the genome in trans location distinct from their targets and share partial complementarity with their target mRNAs. They potentially establish base pairing to the Shine-Dalgarno (SD) sequence of target mRNAs usually 10–25 nt in length to sequester the RBS (4). Additionally, *trans*-acting sRNAs are firmly coupled with the RNases activity to exploit their regulatory functions resulting in RNA turnover through RNA cleavage (20). In many cases, it is thought that *trans*-encoded RNA molecules engage RNA chaperone Hfq to facilitate sRNA-mediated regulation due to limited complementarity between sRNAs and their mRNA targets (21).

(ii) *Cis*-encoded sRNAs are another class of intracellular bacterial sRNAs complementary to their target encoded in the same region of DNA. They have functional ability to interact autonomously as they are transcribed from DNA strand opposite to genes they regulate (22). *Cis*-encoded sRNAs vary greatly in size and usually located in the UTRs of the corresponding gene to establish firm RNA duplex formation which in turn affects ribosome-binding/translation and rearranges the secondary structures to affect mRNA stability or termination events as shown in **Figure 1** (23).

(iii) In addition to RNA–RNA regulation characterized by base pairing, sRNA can also interact with regulatory proteins to directly modify their activities by mimicking and, thus, efficiently compete with DNA or RNA targets. The best suited example for such interaction is CsrA/RsmA family regulators (global carbon storage regulator). All Csr/Rsm-regulatory networks represent a common feature in pathogenic bacteria, the two-component system (TCS) regulate the transcription of the small RNAs to sequester CsrA. The sRNAs directly bind with CsrA/RsmA to sequester it from interacting with mRNA targets usually in close immediate vicinity of the RBS. This phenomena result in the enhancing of the translation of the previously blocked transcripts (24).

(iv) Recent studies indicates that a class of sRNAs participates in an adaptive microbial immune system known as clustered regularly interspaced short palindromic repeats (CRISPR), which provide the bacteria with RNA-based acquired immunity against invading DNA elements, such as from bacteriophages, plasmids, and mobile genetic elements (25). This system is composed of an array of conserved short DNA repeat sequences originating from foreign DNA and interspaced by variable spacer regions. Furthermore, together with the conserved Cas proteins, the crRNA can recognize the complementary DNA target to mediate its degradation (26).

sRNAs AS COORDINATORS OF PATHOGENESIS

After internalization in the host cell, intracellular bacterial pathogens are challenged by diverse changing environmental
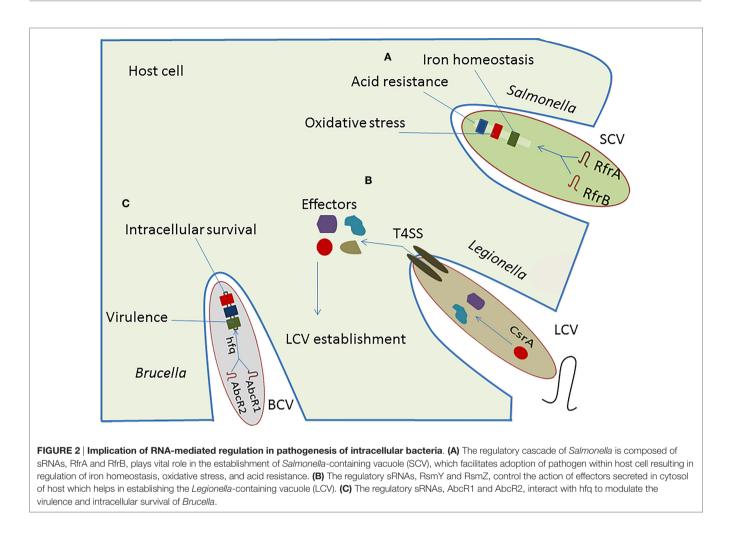
FIGURE 1 | Simplified representation of mechanisms by which sRNAs function in bacteria. **(A)** The *trans*-encoded sRNAs interact with their specific target through imperfect base pairing which ultimately results in both positive and negative effect in altering gene expression to promote RNase degradation of the double-stranded RNA molecules. **(B)** The *cis*-encoded sRNAs share extensive complementarity by binding with target mRNA resulting in transcriptional termination, degradation of the sRNA–target RNA complex, and affecting translation through a putative loop formation and, consequently, the cessation of the RNA polymerase activity. **(C)** The dimeric RNA-binding protein CsrA interacts with target mRNA, typically represented in a hairpin loop structure, transcription termination, leading to an alteration of the accessibility of the translation machinery, and/or the stability of the RNA. **(D)** Mechanism of action of CRISPR arrays for transcription full length RNA which directly target the foreign DNA *via* CAS proteins resulting in subsequent degradation of the exogenous DNA.

conditions. Host cells have established mechanisms to counteract the intracellular bacteria, including degradation of pathogens within the lysosomal compartments. Conversely, as successful pathogens, intracellular bacteria have acquired strategies to avoid lysosomal degradation, such as the arrest or delay of vacuolar maturation in *Salmonella* (late endosome) and *Mycobacterium* (early endosome), control of intracellular trafficking in *Brucella* and *Legionella*, and resistance to lysosome action in *Coxiella* (27, 28). These pathogens have developed strategies to evade host protective mechanisms for adaptation, survival, replication, and persistence within host cells to establish chronic infection that is mainly dictated by the presence of certain structural components and virulence factors (29, 30).

The Csr-type system is the most common posttranscriptional regulatory network in intracellular bacteria and is well-characterized in *Legionella* and *Salmonella*. *Legionella* uses effector proteins to modulate host-cell function and establish a replicative niche by forming a membrane-bound vacuole designated the *Legionella*-containing vacuole (LCV) (31). These effector proteins are under the control of CsrA, which participates in the type IVB secretion system (T4SS) to modulate endoplasmic reticulum (ER)–Golgi vesicular trafficking, with involvement of sRNA-binding protein in survival and replication of intracellular pathogens (32). The VipA, RalF, and YlfA effector proteins have been linked directly to vesicular trafficking to alter the host-cell activity (33). The CsrA controls expression of virulence regulatory genes near the Shine-Dalgarno (SD) sequence of target mRNA located on *Salmonella* pathogenicity islands (SPIs). Also the transition

from a sessile to a motile life form of *Salmonella enterica* serovar Typhimurium is strongly affected by CsrA (34). In *S. enterica* serovar Typhimurium, CsrA seems to act positively on swarming motility, and it is required for accurate flagella expression by stabilizing the *flhDC* and the *fliA* mRNAs, which are the regulators of the flagella operon. This stabilizing effect leads to an increased production of flagellar proteins (34). In *Legionella*, CsrA also affects the flagella sigma factor FliA, but in contrast to *S. enterica* serovar Typhimurium, overexpression of CsrA in *Legionella pneumophila* resulted in lower *fliA* transcription and subsequently to reduced levels of FlaA, the major structural flagellar protein controlled by FliA (35). Although CsrA is apparently a common regulator for flagella expression in different bacteria, the regulatory function differs significantly between them, as it can be either positive or negative (36, 37). sRNAs, such as RsmY and RsmZ, regulate the expression of effector protein RsmA to affect the replication of *Legionella* in macrophages (38), and directly target T4SS regulatory genes to facilitate intracellular survival of pathogen (39, 40).

The RfrA and RfrB are two RybB homologous sRNAs in *Salmonella enterica* that play an essential role in the intracellular replication in macrophages. Additionally, Fur, a well-known repressor of RybB sRNA is also required for phagocytosis and intracellular survival of *Salmonella* in human macrophages (**Figure 2**) (41). These conserved sRNAs regulate expression of virulence genes involved in the oxidative stress, iron homeostasis, and acid resistance within host cells. Furthermore, they have been induced in THP-1 macrophages, fibroblasts, and murine

FIGURE 2 | Implication of RNA-mediated regulation in pathogenesis of intracellular bacteria. (A) The regulatory cascade of *Salmonella* is composed of sRNAs, RfrA and RfrB, plays vital role in the establishment of *Salmonella*-containing vacuole (SCV), which facilitates adoption of pathogen within host cell resulting in regulation of iron homeostasis, oxidative stress, and acid resistance. (B) The regulatory sRNAs, RsmY and RsmZ, control the action of effectors secreted in cytosol of host which helps in establishing the *Legionella*-containing vacuole (LCV). (C) The regulatory sRNAs, AbcR1 and AbcR2, interact with hfq to modulate the virulence and intracellular survival of *Brucella*.

macrophages, suggesting a complementary role of sRNAs in intracellular replication (42). Caswell et al. (43) reported two homologous sRNA in *Brucella*, named AbcR1 and AbcR2, that play significant role in pathogenicity and establishment of chronic infection. Deletion of AbcR1 or AbcR2 alone in *Brucella* did not affect intracellular growth in macrophages, but deletion of both resulted in its significant attenuation in macrophages, as well as in mouse model of chronic *Brucella* infection (43).

Transcriptional regulators are the subject of considerable study at the molecular level, and the number of newly discovered sRNAs is increasing, with more than 100 identified in *Salmonella* (19). In many cases, the ubiquitous RNA-binding protein, Hfq, establishes dynamic interactions with RNA molecules to function in virulence of intracellular pathogens. Due to limited complementarity between sRNA and target mRNA, Hfq is essential to facilitate RNA–RNA interactions. Indeed, deletion of *hfq* has dramatic impact on virulence and intracellular survival in *Brucella abortus* (44), *L. pneumophila* (45), and *Salmonella typhimurium* (46). In addition, Hfq-bound sRNAs are directly involved in regulating metabolic systems and gene expression such as that required for two-component regulatory systems, lipopolysaccharide biosynthesis, host-cell invasion, fatty acid metabolism, central carbon metabolism, and in motility of bacteria (47, 48).

The sRNA GcvBs regulate the ABC transport system through direct binding with extended C/A-rich regions at the mRNA level resulting in lower expression and inhibition of the ABC transport system in *S. typhimurium* (49). Furthermore, IsrJ sRNA-dependent temporal regulation has been reported in SPIs affecting pathogen invasion of intestinal epithelial cells (50).

IsrM is the SPI-encoding sRNA involved in direct regulation of *HilE* and *SopA* virulence genes, which are key regulators of *Salmonella* virulence and essential for bacteria to evade the host immune system. Specifically, *Salmonella* hinders production of *HilE* and *SopA* for invasion of epithelial cells, which facilitates its survival within the host macrophage (51).

AmgR, a *cis*-encoded 1.2 kb long antisense RNA in *S. typhimurium*, which is complementary to *mgtCBR* mRNA, specifies the MgtC protein. The AmgR sRNA plays an essential role in survival of *Salmonella* within macrophages and in its virulence in mice and is required for replication in a low Mg^{2+} environment (52). Surprisingly, transcription of *mgtCBR* mRNA and AmgR sRNA is controlled by the two-component regulatory system PhoP/PhoQ. In detail, when PhoQ senses decrease level of Mg^{2+} in cytoplasm, it phosphorylates PhoP initiating transcription of *mgtCBR* mRNA by direct binding with *mgtC* and *amgR* promoters. As MgtB and MgtC protein levels are decreased by long RNA

regulatory elements, FtsH protease promotes degradation of the MgtR binding to the MgtC proteins. Consequently, AmgR acts as a timing device for sense-encoded MgtC protein, which was shown to diminish virulence in mice (52). Recently, regulatory sRNAs have been identified in *Mycobacterium* that act in pathogenesis by regulating the target gene *Rv0485* that participates in mediation of virulence in mice (53). These studies suggest that intracellular pathogens utilize sRNA-based strategies to establish productive intracellular infection within host cells.

sRNAs IN MODULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSE

Intracellular pathogens have developed well-organized strategies to cope and interfere with host innate immune mechanisms that ultimately facilitate establishment of an environment favorable for an effective, long-lasting, adaptive immune response (54). The mammalian innate immune response mechanism provides first line of defense against invading bacterial pathogens. The recognition of molecules typical of a microbe pathogen-associated molecular pattern is obtained *via* pattern-recognition receptors (PRR) through TLRs that are expressed at high levels on DC and macrophages. The TLRs transmit signals *via* MyD88 to activate the NF-κB, MAPKs, and IRF signaling pathways to initiate microbial clearance (54). Study of a class of sRNAs designated miRNAs, typically 20–22 nt in length, has greatly expanded our understanding of mechanisms involved in gene expression through posttranscription and translation regulation of protein coding genes. miRNAs play pivotal roles in modulation of innate as well as in adaptive immune response mechanisms (55).

Salmonella

At the initial stages of infection, innate immune response mechanisms effectively control the replication and survival of *Salmonella*. *In vitro* studies indicate that *Salmonella* modulates miRNAs in both epithelial cells and macrophages. Key host miRNAs, such as miR-155 and miR-146, are upregulated in immune cells in response to intracellular bacterial pathogens, apparently co-induced during physiological processes stimulated by lipopolysaccharides to repress TLR-mediated recognition of bacterial molecules and NF-κB activity (56). Modulation of miR-146a/b, miR-155, and miR-21 were first reported in *Salmonella* infection, with NF-κB-dependent miRNAs significantly induced upon infection in mouse macrophages (57, 58). miRNAs are usually triggered in response to sense extracellular stimulus. This phenomenon was observed in *Salmonella* mutant strains defective in cell invasion (ΔSPI-1) and replication (ΔSPI-2), as well as in human monocytes (58, 59). It was subsequently found that vaccination of miR-155-null mice with attenuated *Salmonella* vaccine did not confer protection, and mice showed severely defective T-cell cytokine production, indicating the complex role of miR-155 in innate immune responses to *Salmonella* (59, 60).

Schulte et al. (56) reported the importance of miR-155 activation by the sensing bacterial peptidoglycan *via* cytoplasmic NOD2 receptor, suggesting its potential role in innate immune response (56). miR-146 function in zebrafish embryos infected with *S. typhimurium* was found to be disrupted by knockdown of the TRAF6-MyD88 pathway that mediates transduction of TLR signals and cytokine activation (61). miR-146 function characterization has revealed that it acts as a negative regulator of IRAK1 and TRAF6 expression, which, in turn, affects NF-κB signaling pathway (62). miRNA let-7 appears to be a factor in the acute innate immune response that participates in the TLR signaling pathway *via* lipopolysaccharide action. In *Salmonella* infection, let-7 was downregulated in a cell-type dependent manner in HeLa and murine macrophage cells, suggesting that repression of this miRNA family constitutes a common signature of the infection of phagocytic and non-phagocytic cells by *Salmonella* (57). Zhang and colleagues reported miR-128 upregulated expression upon *Salmonella* infection that led to reduced secretion of macrophage colony-stimulating factor-mediated macrophage recruitment, in turn suppressing the host immune response mechanisms (63).

Although innate immunity effectively controls replication and survival of *Salmonella* at the initial stages of infection, a well-organized adaptive immune response is also required at later stages of chronic infection (54). *Salmonella* shows the ability to infect phagocytic and non-phagocytic cells, residing inside phagocytic cells in the so-called *Salmonella*-containing vacuole (SCV). *Salmonella* secretes several virulent SPI-2-encoded proteins into the host cytoplasm to facilitate its intracellular replication and invasion into host cells through two distinct type-III secretion systems (T3SS) (64). Mature DCs may have unique tolerogenic properties for initiation and control of adaptive immune responses. *Salmonella* has developed mechanisms to counteract the function of DCs, such as subversion of cellular trafficking by preventing fusion of the SCV with lysosomes, which ultimately facilitates pathogen entry into the host (65). Additionally, MHC-I and MHC-II molecules expressed on the surface of DCs show elevated numbers of bacterial-derived antigens that facilitate activation of T cells, resulting in enhanced host adaptive immune response (66). The B cell intrinsic requirement of miR-155 is essential for IgG1 antibody production in response to thymus-dependent and -independent antigens, following vaccination with attenuated *Salmonella* (67).

Rodriguez et al. reported bic/miR-155-deficient mice show diminished adaptive immune response against *S. typhimurium* after intravenous immunization that failed to establish strong adaptive immunity, likely due to defective antigen presentation as well as impaired B and T cell functioning by DCs (60). In addition to miR-146 and miR-155, miR-21, miR-23b, miR-27a, miR-24, miR-222, and miR-29 showed upregulation upon *Salmonella* infection in human monocytes. The upregulated miRNA shows that monocytes differentiation is involved in modulation of the TGF-β signaling pathway to counteract host defense mechanisms (58).

The miRNA let-7 family members directly target major immunomodulatory cytokines IL-6 and IL-10, and their downregulation results in increased expression of both cytokines in response to *Salmonella* infection (68). miR-21, miR-146a, and miR-155 show strong induction of NF-κB, leading to decreased regulation of B cell and T cell proliferation in murine macrophages upon

Salmonella infection (69). These findings suggest that *Salmonella* uses miRNA as a strategy to modulate TLR-NF-κB signaling pathways as well as to counteract the function of DCs to subvert its cellular trafficking, and miRNAs play a significant role in the interaction of innate and adaptive immune mechanisms.

Mycobacterium

Recently, several studies have highlighted the role of miRNAs in mycobacterial infection (70). *Mycobacterium* also modulates miRNAs associated with signaling pathways, which enhances its survival in the host. miR-155 was found to be upregulated in *Mycobacterium* infection, resulting in increased apoptosis of infected cells by the involvement of the TLR2 and NF-κB signaling pathways (71). Downregulation of miR-155 result in decreased TNF-α production in response to lipomannan, a component of the bacterial cell wall, was reported in human macrophages, affecting the TLR-MAPK/Akt signaling pathway (72). In addition, MiR-155 is involved in regulation of autophagy-mediated mycobacterial elimination by the repression of the negative regulator Rheb (73). miR-142-3p participates in an effective strategy in mycobacterial infection to control early events of phagolysosome biogenesis *via* targeting the N-Wasp and actin-binding protein (74). On the other hand, down regulation of miR-142-3p negatively regulates the production of NF-κB, TNF-α, and IL-6 in macrophages upon *Mycobacterium bovis* infection, resulting in the activation of the NF-κB pathway *via* the de-repression of the target IRAK1 (75). miR-124 has been found to serve as a potent modulator of the immune response in an *M. bovis* BCG-infected macrophage cell line (Raw 264.7) by targeting components of the TLR signaling pathway including MyD88, TLR6, TRAF6, and TNF-α (76).

miR-146a modulates the inflammatory response upon *Mycobacterium* infection in Raw 264.7 cells by targeting IRAK1 and TRAF6, resulting in remarkably reduced translation of IL-6, IL-1β, and TNF-α (77). Kumar and colleagues reported that *Mycobacterium tuberculosis* down regulates the expression of miRNA let-7f, which targets mRNA of A20, an inhibitor of NF-κB. Significantly, the downregulation of let-7f is accompanied by concomitant upregulation of A20 in mice infected with *M. tuberculosis* (78).

miR-21 was found to induce inhibition of IL-12 production in a NF-κB-dependent manner in DCs and T-cells upon *Mycobacterium* infection, and thus suppress host Th1 responses (79). Interestingly, miR-21 upregulation promotes DCs apoptosis by targeting Bcl-2 in *Mycobacterium*-infected cells (80). miR-99b upregulation has been observed to stimulate the production of proinflammatory cytokines such as IL-6, IL-12, IL-1β, and TNF-α in macrophages and DCs upon *Mycobacterium* infection. Inhibition of TNF-α production is a key strategy of *Mycobacterium* to promote growth within DCs that, in turn, facilitates evasion of host adaptive immune response mechanisms (81). Transfection of T cells with miR-144 precursor has demonstrated that miR-144 possibly regulates antituberculosis immunity by inhibition of IFN-γ and TNF-α production and T cell proliferation (82). Wang et al. (83) reported that miR-223 and miR-424 promotes monocyte differentiation and subsequently downregulates the expression of transcription factor NFI-A78. The downregulation

of miRNAs involved in disorder proportions of T cells and B cells in active tuberculosis patients (83). Collectively, these finding indicates that *Mycobacterium* use miRNA-based strategies for completion of intracellular replication that, in turn, facilitates evasion of the immune response.
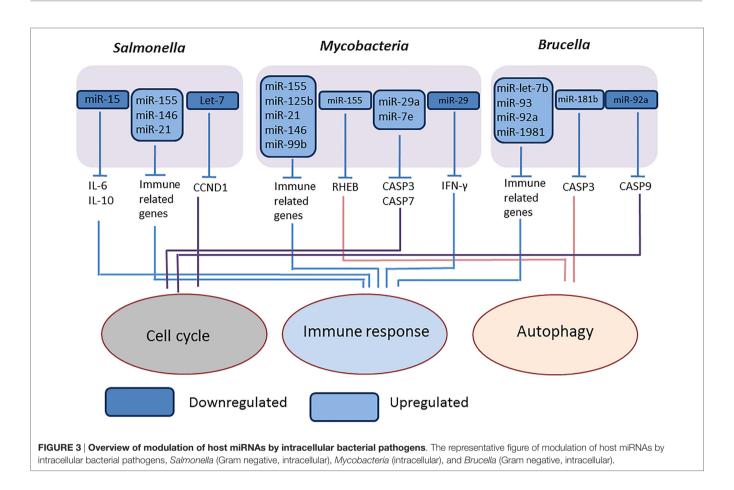
Brucella

We studied the expression of miRNAs expression in *Brucella melitensis*-infected RAW 264 macrophages cells and found several miRNAs such as miR-let-7b, miR-93, miR-92a, miR-181b, and miR-1981, differentially expressed compared to mock-infected cells, and purposed that these miRNAs are involved in regulation of autophagy, apoptosis, innate and adaptive immune response mechanisms (**Figure 3**) (84). Liu et al. (85) reported that the downregulation of miR-125b-5p during *B. abortus* infection enhances the expression of the A20 protein, thereby inhibiting NF-κB activation and facilitating bacterial intracellular survival (85).

APPROACHES TO IDENTIFY BACTERIAL sRNAs

Now that a number of actively transcribed sRNAs have been identified in intracellular bacteria, further research should focus on the determination of their functions and their specific targets. TargetRNA2 was the first webserver specifically designed for identification of bacterial sRNA target which determine sRNA–target interactions through the straight forward hybridization model. It determines the seed region between two putative sRNA targets which is composed of very small short series of consecutive base (86). IntaRNA is also used to identify sRNA targets which work on the principle of hybridization energy between base pairings of sRNA–target interactions to the hybridization energy of interacting regions being unpaired in intramolecular structures. Further, it conform the seed regions more efficiently as compared all other available webtools (87). Target prediction could also be used in conjunction with experimental genome-wide approaches, such as transcriptional profiling (88). Comparison of the transcription profile of a mutant strain or an over-expresser strain to the wild-type strain can highlight putative targets (9). Comparison of the sRNA deletion mutant, the over-expresser strain and the wild-type strain by SDS-PAGE and Coomassie staining may be sufficient to suggest a putative target (89). The proteomic studies could be undertaken or more direct approaches, such as the streptavidin-binding aptamer tag described above could be used (90). Said et al. (91) have performed a systematic analysis for the use of different aptamers and configurations to identify protein targets of sRNA. Further, we need to establish more accurate identification methods for the bacterial sRNA prediction.

SUMMARY AND PERSPECTIVES

Recent advancements in research have revealed diverse functions, wide distribution, and high variability of sRNAs and described their crucial role in biological processes, such

FIGURE 3 | Overview of modulation of host miRNAs by intracellular bacterial pathogens. The representative figure of modulation of host miRNAs by intracellular bacterial pathogens, *Salmonella* (Gram negative, intracellular), *Mycobacteria* (intracellular), and *Brucella* (Gram negative, intracellular).

as infectivity and virulence of intracellular bacteria, stress adaptation, and environmental sensing, as well as in modulation of innate and adaptive immune response mechanisms. Intracellular bacteria are divided in two classes: *vacuolar* intracellular bacteria, such as *Salmonella*, *Mycobacterium*, *Legionella*, *Brucella*, and *Coxiella*, which survive and replicate either by avoiding vacuole–lysosome fusion or by altering the phagolysosome environment; and cytosolic intracellular bacteria, including *Francisella*, *Shigella*, *Listeria*, *Burkholderia*, and *Rickettsia*, which usually escape to proliferate within the cytosol of host cell. Infection is a multidimensional complex event, with host cells having developed immune mechanisms to counteract invading intracellular pathogens *via* lysosomal degradation to maintain a balance between host resistance and bacterial virulence. Intracellular pathogens have evolved several sRNA-based strategies to survive and replicate within phagocytic cells and to manipulate the host-cell machinery for their own benefit. Upon internalization in host cells, the pathogenic bacteria are usually surrounded by a membrane-bound vacuole that protects against proteolytic degradation. Bacterial regulatory sRNAs operate at all levels of gene regulation to modulate translation, transcription, DNA maintenance or silencing, and mRNA stability. They use different mechanisms to perform regulatory functions, including changes in RNA conformation, base pairing with other RNAs, protein binding, and interactions with DNA.

The Csr-type system is the most common posttranscriptional regulator network in intracellular bacteria which participates with type IVB secretion system to modulate the ER–Golgi vesicular trafficking highlighting the involvement of sRNA-binding protein in survival and replication of intracellular pathogens. The effector proteins, VipA, RalF, and YlfA, have been linked directly in vesicular trafficking to affect the host-cell activity. Additionally, sRNA, RsmY and RsmZ, in *Legionella*, RybB sRNA in *Salmonella*, and sRNA AbcR in *Brucella* regulate the expression of effector protein for survival and intracellular replication in macrophages.

At the initial stage of infectious pathogenesis, intracellular pathogens modulate the immune response mechanism of host to quickly translocate through the mucosal immune barrier and are endocytosed by mucosal macrophages and DCs. Intracellular pathogens infect the host-cell machinery by targeting IRAK1 and TRAF6 to limit PRRs which, in turn, affects the TLR/NF-κB signaling cascade. Inhibition of antigen presentation to T cells is a further strategy of intracellular bacteria to dampen innate and adaptive immune mechanisms. They have the ability to hinder activation of DCs to subvert the immune response mechanism by averting function of T-cells and secreting IL-12 to establish a strong Th1 immune response.

Additionally, inhibition of TNF-α production and modulation of MHC-I and MHC-II expression is a key strategy of intracellular pathogen to promote growth within DCs that, in turn,

favors cytokine regulation to facilitate evasion of host adaptive immune response mechanisms. Although numerous strategies employed by bacterial sRNAs have been reported, there are many mysteries that are still veiled, including how are sRNAs involved in modulation of innate immune signaling? What is the role of sRNAs in regulation of apoptosis and autophagy mechanisms? To date, nothing is known about the role of sRNA interfering with innate and adaptive immune mechanism of *Brucella* and *Legionella* and it will be an open question for the next few years. In-depth identification of novel immune evasion strategies employed by bacterial sRNAs will facilitate our understanding of pathogenesis and designing of novel effective therapeutic approaches to combat diseases caused by intracellular pathogens.

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