REGULATION OF SOLUBLE IMMUNE MEDIATORS BY NON-CODING RNAS

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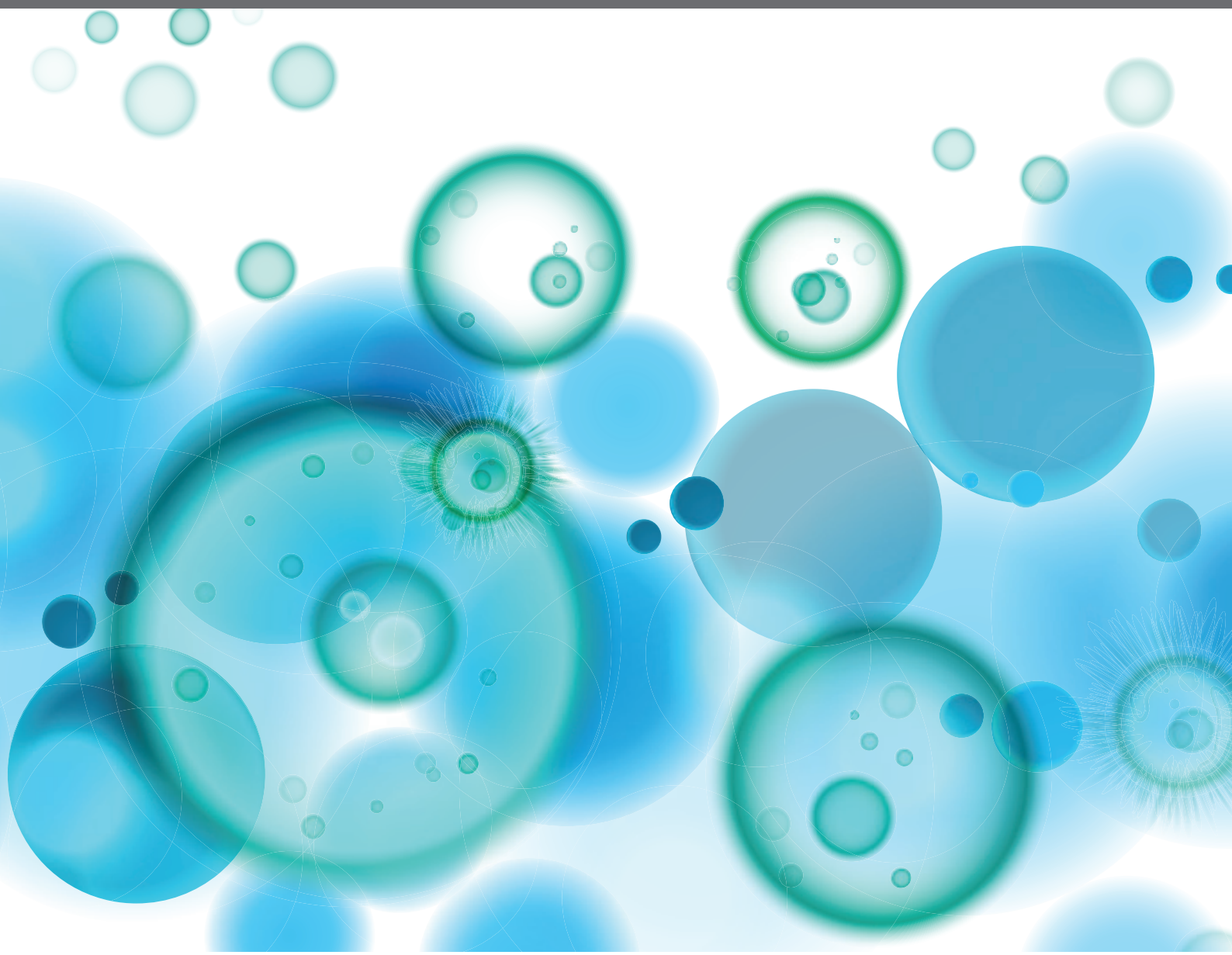

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REGULATION OF SOLUBLE IMMUNE MEDIATORS BY NON-CODING RNAS

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Table of Contents

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EDITORIAL
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Editorial: Regulation of Soluble Immune Mediators by Non-Coding RNAs

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Editorial on the Research Topic

Regulation of Soluble Immune Mediators by Non-Coding RNAs

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Non-coding RNAs (ncRNAs), defined as transcripts that do not encode proteins, are known since long time for their role in translation (i.e. transfer RNAs, ribosomal RNAs) and in splicing events (i.e. small nuclear and small nucleolar RNAs). However, only recently, the revolutionary advances in deep sequencing technology brought to light several new classes of ncRNA, classified according to their length into "short" ncRNAs (<200 nucleotides, that includes piwi-associated RNAs, endogenous short-interfering RNAs, microRNAs, Y-RNAs and others), and "long" ncRNAs (lncRNAs, >200 nucleotides) (1).

Cytokines are crucial soluble messengers of the immune system that regulate and sustain inflammation and immunity. Cytokine expression is tightly regulated, reflecting the need of the immune system to tailor the magnitude and duration of its responses to induce pathogen clearance, but not tissue damage. Thus, understanding cytokine regulation is crucial to gain insight and eventually manipulate undesired immune responses.

In this Research Topic, 53 authors contributed 11 articles touching on many of the combined roles of ncRNAs on the production of cytokines and their consequential effect on cytokine-related functional outputs, as well as inflammatory/autoimmune pathologies.

IMMUNE REGULATION BY INTRACELLULAR ncRNAS

Other than the size limit of 200 nt and a lack of protein-coding potential, the sole other common feature of all ncRNAs consists in being functionally implicated in gene regulatory processes. This is achieved via a multitude of mechanisms, ranging from promoter-specific repression, transcriptional activation, epigenetic remodeling, or post-transcriptional gene regulation such as translational blockade and/or activation (1). Based on these features, the regulatory functions of ncRNAs are recognized to be involved in virtually all homeostatic, developmental and reactive pathways and systems, including the immune response (2, 3).

Among ncRNAs, microRNAs (miRNAs) currently represent the best characterized post-transcriptional regulators of cytokine production. In this Research Topic, Garavelli et al. review the literature concerning regulation of adaptive cytokines by miRNAs, while Salvi et al. concentrate on miRNA-dependent regulation of cytokines that are hallmarks of autoimmune diseases. The emerging picture is quite complicated for several reasons. First, the final effect on cytokine levels may derive by direct regulation of cytokine RNA or by the modulation of cytokine inducers or repressors. In addition, as these authors underline, the coordinated induction and modulation of tens of miRNAs may be required to efficiently affect the components of a genetic network. Thus, it is crucial to rapidly break away from the musty assumption "one miRNA, one cytokine" to boldly embrace the "rheostat" function of miRNAs and to be able to frame the mechanistic miRNA regulation in process-specific contexts. One example of such "integrated view" is beginning to emerge in the multifaceted crosstalk between cell activation, ncRNAs and cytokine expression (**Figure 1**).

Toll-like receptors (TLRs) are key pathogen receptors of the innate immune system. The first miRNA described as induced following TLR activation and controlling TLR signaling through a negative feedback regulation loop dates back to 2006 (4). Since then, our knowledge on the miRNAs induced upon cell activation downstream TLRs and on the mechanisms through which these miRNAs feedback modulate immune cell responses has grown enormously. More recently, a number of lncRNAs have been included among the non-coding transcripts with regulatory functions in the TLR signaling pathway. Three reviews of this Research Topic are focused on the role of ncRNA in immune cell activation downstream TLRs and on the pathologies driven by dysregulation of the TLR-induced responses. Bayraktar et al. summarize the potential role of miRNAs in regulation of gene expression and TLR signaling, with a focus on the ability of miRNAs to act as endogenous ligands of specific TLRs and trigger the downstream immune response (see further). The complexity of this highly regulated network of ncRNAs in macrophage biology is further discussed by Curtale et al., with particular emphasis on the role of miRNAs in macrophage heterogeneity and plasticity in response to environmental cues, bacterial infection, tissue regeneration and endotoxin tolerance. Further studies on TLR-induced miRNAs and lncRNAs in the regulation of endotoxin tolerance are comprehensively reviewed by Vergadi et al., and their impact in the context of innate immune tolerance and of sepsis is discussed. Together with the abovementioned reviews, an original article by Mariotti et al. identifies a role for a lncRNA (namely NRIR) in the expression of type I Interferon Stimulated Genes (ISGs) in monocytes downstream TLR4 activation. Remarkably, this study highlights that aberrant expression on NRIR can be involved in the dysregulation of the innate immune system linked to the development of Systemic Sclerosis.

IMMUNE REGULATION AND BIOMARKER FUNCTION OF EXTRACELLULAR ncRNAs

The picture of immune regulation by ncRNAs is further complicated by their travelling in extracellular spaces, either encapsulated in extracellular vesicles (EVs) or associated to macromolecular structures such as ribonucleoproteins and lipoprotein particles. Despite the function of most extracellular ncRNAs remains largely elusive, they are in the scientific limelight because of a possible role as regulators of intercellular communications as well as a tremendous potential as non-

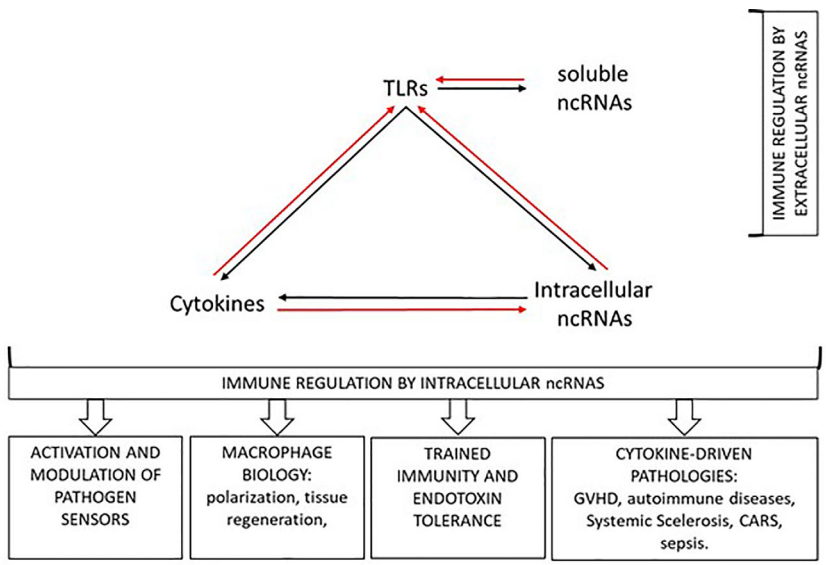

FIGURE 1 | Multifaceted crosstalk between cell activation, ncRNAs, and cytokine expression (see text).

invasive biomarkers for multiple disorders, including pathologies of the immune system (5).

Such burning interest well reflects in our Research Topic, where five contributions deal with different aspects of extracellular ncRNAs biology. Turchinovitch et al. provide a state-of-the-art overview of the transcriptome of EV-associated RNAs, where miRNAs represent the most intensively studied component and, at the same time, the minority of all EV-enclosed RNAs. One prominent class of EV-associated extracellular RNAs involved in a range of immune-mediated processes are the Y-RNAs, discussed here by Driedonks and Nolte-'t Hoen. Both these reviews also address some technical challenges associated with obtaining pure EVs and deep sequencing of the EV-associated RNAs, as well as in assessing whether extracellular ncRNAs are contained in ribonucleoprotein complexes or EVs. These technical aspects are crucial to overcome the frequently observed inconsistency in the identification and quantification of extracellular ncRNAs, which currently impairs our capacity to use them as reliable biomarkers.

Both these reviews, as well as other contributions (Garavelli et al.; Salvi et al.; Bayraktar et al.; Zitzer et al.), also converge in highlighting a role for extracellular ncRNAs as ligands of RNA sensors, TLRs in particular. This function was recently demonstrated for EV-associated miRNAs (6) and may play a role in inducing unwanted inflammation and tissue damage, as reviewed here by Zitzer et al. In this regard, based on the largely sequence-independent impact of nucleic acids on the TLRs (6), Turchinovitch et al. and Driedonks and Nolte-'t Hoen point out the strong possibility that more abundant non-miRNA classes could significantly contribute to such activation.

THERAPEUTING EXPLOITATION OF ncRNAS

It is not surprising to find deregulated ncRNAs as major contributors of cytokine-driven pathologies ranging from acute graft-versus-host disease, autoimmune diseases, Systemic Sclerosis, compensatory Anti-inflammatory Response Syndrome (CARS), endotoxin tolerance and sepsis, as showcased in this Research Topic.

The other side of this same coin would consist of therapeutical exploitation of ncRNAs. Our current lack of a full understanding of their biology and of the intricate network of interactions with the human genome, transcriptome and proteome restrains the translation of such strategies into the clinical use. In addition, a number of specific challenges associated with ncRNA targeting still need to be addressed, such as predicting possible off-target effects and toxicity, improving stability and optimizing the delivery systems (7). In this regard, the original contribution by Macleod et al. focuses on the prevention of paradoxic inflammation following topical delivery of RNA aptamers to treat inflammatory skin diseases.

Despite these knots to be solved, a number of miRNA-based therapeutic tools, mainly for cancer management, entered the clinical trial in the last 5 years (8). Here, one original work by Kim et al. propose miR-135-5p as a target for the development of anti-allergic drugs based on its capability to interact with p62, a selective receptor of autophagy.

CONCLUDING REMARKS

As a result of almost two decades of extensive investigations, nowadays miRNAs can be listed among the soluble mediators of the immune response. In addition, more ncRNAs promise to hold the scene in the near future and for a long time. As we gain more knowledge about the exciting properties of ncRNAs, however, we also get aware of the intricacy of the emerging picture. **Figure 1** schematizes how the bidirectional interplay between cytokine-modulated ncRNAs expression and, in turn, ncRNAs-driven control of cytokine expression and production is further complicated by the recent discovery of the ability of ncRNAs to trigger activation of specific immune receptors. The scrupulous untangling of this intricate web will allow to fully exploit he tremendous potential of ncRNAs as biomarkers and therapeutic tools to safely redirect undesired immune responses.

AUTHOR CONTRIBUTIONS

DB and FB were editors of this Research Topic and wrote this editorial jointly. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Toll-Like Receptor Stimulation by MicroRNAs in Acute Graft-vs.-Host Disease

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Acute graft-vs.-host disease (aGVHD) is a frequent complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT), accounting for substantial morbidity and mortality associated with this treatment modality. The pathogenesis of aGVHD involves a complex cascade of humoral and cellular interactions in which donor T cells target HLA mismatched host tissues, causing tissue injury through secretion of pro-inflammatory cytokines and induction of direct cytotoxicity. Toll-like receptors (TLRs) are key components of the innate immune system that recognize endogenous danger-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs). Patients receiving conditioning chemotherapy and/or whole-body irradiation prior to all-HSCT are prone to gastrointestinal damage and translocation of microbiota across compromised intestinal epithelium, resulting in release of PAMPs and DAMPs. These "danger signals" play critical roles in disease pathogenesis by both initiating and propagating aGVHD through dendritic cell maturation and alloreactive T cell responses. There are 10–15 TLRs identified in mammalian species, a subset of which recognize single-stranded RNA (ssRNA) and serve as a key component of viral immunity. Recently, ssRNAs other than those of viral origin have been investigated as potential ligands of TLRs. MicroRNAs (miRs) are short (19–24 nt) non-coding RNAs that play critical roles in a variety of diseases. While traditionally miRs post-translationally modulate gene expression, non-canonical functions such as regulating TLR stimulation by acting as TLR ligands have been described. Here, we review the role of TLRs in aGVHD pathogenesis, the function of miRs in TLR stimulation, and the recent literature describing miRs as TLR ligands in aGVHD.

Keywords: graft-vs.-host disease, Toll-like receptors, microRNAs, allogeneic stem cell transplantation, innate immunity

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INTRODUCTION

Acute graft-vs.-host disease (aGVHD) is a frequent complication of allogeneic hematopoietic stem cell transplants (allo-HSCTs), with 30–75% of allo-HSCT recipients developing aGVHD (1, 2). Furthermore, aGVHD accounts for ∼10% of all non-relapse mortality in patients that receive allo-HSCT (3, 4), and those with severe aGVHD have a poor prognosis, with an overall 2 years survival of 20–30% (5–7). The morbidity and mortality associated with aGVHD pose a substantial barrier

against the wider and safer application of HSCT as a curative modality.

While current prophylaxis and therapeutics function through systemic immunosuppression (1, 8–14), these treatments increase the risk of systemic infections and leukemia relapse (14–16). Therefore, aGVHD research efforts are being focused on not only the development of novel treatment strategies, but more so on the deeper understanding of aGVHD pathogenesis so that aGVHD may be prevented. The potent activation of Toll-like receptors (TLRs) on antigen presenting cells (APCs) following conditioning regimens is often considered a critical initiating event in the development of aGVHD (2, 17–20). Here, we review the current understanding of the classical role of TLRs and their ligands in aGVHD pathogenesis as well as the recent literature describing microRNAs (miRs) as novel ligands for TLRs both broadly and in the context of aGVHD.

TOLL-LIKE RECEPTORS (TLRs)

Toll-like receptors are a family of evolutionarily-conserved transmembrane pattern recognition receptors (PRRs) that are critical for innate immune responses and the cross-talk between innate and adaptive immune systems. The concept of PRRs is attributed to Dr. Charles Janeway, who, in 1989, proposed the existence of immune receptors on surveillance cells such as APCs. which allow the innate immune system to specifically recognize microbial infections and mount an appropriate immune response (21). The first member of the Toll family was identified in *Drosophila* flies in 1988, although at that time its function was only recognized as being critical for dorsoventral polarity during fly embryo development (22). The connection between *Drosophila* Toll and innate immunity was not recognized until later, when *Drosophila* Toll and human IL-1R were identified as having homologous cytoplasmic domains and the capability of inducing Rel family transcription factor activation (23). Furthermore, it was observed that *Drosophila* flies that carried non-functional *Toll* genes demonstrated significant defects in antifungal responses, although immune responses to bacterial organisms remained intact (24). In 1997, the first human Toll homolog, called hToll (now known as TLR4), was cloned and was shown to signal through the NF-κB signaling pathway, resulting in the production of inflammatory cytokines during the adaptive immune response (25). One year later in 1998, the connection between TLR4 and its ligand LPS was recognized as endotoxin-tolerant mouse strains were shown to have point mutations in the Tlr4 gene (26, 27). To date, there are 13 TLRs identified between mice and humans which allow the innate immune system to recognize not only bacteria but also viruses, fungi, and protozoa (28, 29).

TLRs IN aGVHD

aGVHD is a complex, multistep disease in which immunocompetent donor T cells destroy MHC-mismatched host tissues by secreting inflammatory cytokines and/or direct cytotoxicity (30, 31). However, pathogenesis of aGVHD is a self-perpetuating cycle that often begins even before the graft is transplanted into the patient. Whole body irradiation and/or chemotherapy frequently used as conditioning regimens are very efficient in reducing leukemia burden and clearing any immune or hematopoietic cells prior to transplantation to prevent graft rejection (32). The cytotoxic effects, however, are not specific to only leukocytes or other hematopoietic cells within the body. Instead, the GI tract is one of the most sensitive organs to radiation and chemotherapy induced acute damage (33–36). Following conditioning therapy, there is extensive tissue damage in the GI as well as compromise to the GI epithelium. This allows translocation of GI flora across the mucosal barrier (37, 38) resulting in the release of inflammatory cytokines (39), danger associated molecular patterns (DAMPs), and pathogen associated molecular patterns (PAMPs). These molecules are then recognized by PRRs on APCs, allowing for their activation (40–42).

DAMPs, also called alarmins, are host-derived "danger" signals produced by the body to allow the immune system to recognize times of extreme cellular stress (43). Typically, the release of DAMPs from damaged tissues occurs when the cells undergoes necrosis (as opposed to apoptosis) since the process of necrosis leads to cell swelling and lysis. DAMPs can arise from two sources in the body: intracellular or extracellular (44). Intracellular DAMPs are released from necrotic cells and include shock proteins (45) and purine metabolites such as ATP (46). On the other hand, extracellular DAMPs arise from breakdown products of the extracellular matrix surrounding stressed cells. Examples of extracellular DAMPs include biglycan, heparin sulfate, and hyaluronan (47). PAMPs, in contrast, are molecules found in/on infectious agents that allow the immune system to recognize exogenous organisms. In aGVHD, PAMPs generally arise from translocated GI flora from the lumen of the intestines to tissues or blood. Examples of common PAMPs critical for aGVHD pathogenesis include lipopolysaccharide (LPS), flagellin, peptidoglycans, and microbial CpG-DNA (17, 48–50). Donor and recipient (host) APCs recognize DAMPs and PAMPs through PRRs, the most well-described of which are TLRs (19, 51). For example, microbial PAMPs such as LPS, flagellin, and CpG-motifs, which may be found in or on translocated GI bacteria, are recognized by TLRs 4, 5, and 9, respectively (48, 50). The consequences of TLR activation in aGVHD are upregulation of adhesion molecules, human leukocyte antigen molecules, and pro-inflammatory cytokine production such as IL-1β, IL-6, IL-12, TNFα, and IFNγ. The downstream effects of TLR-induced APC stimulation are the potent donor T cell activation, expansion, differentiation, and trafficking in aGVHD (52).

As TLRs are important for the initiation of aGVHD, researchers have studied the roles of single nucleotide polymorphisms (SNPs) in TLRs of both donor and recipients and their impacts on aGVHD development. The most well-studied TLR polymorphisms in aGVHD are Asp299Gly and Thr399Ile in TLR4. These TLR4 SNPs were first described in allo-HSCT donors and recipients by Lorenz et al. (53). In this report, the authors demonstrated that the presence

of polymorphisms in either the donor or recipient are associated with a lower incidence of aGVHD, although statistical significance was not achieved. Elmaagacli et al. documented slightly conflicting results, with the presence of Thr399Ile SNP in TLR4 in either donor alone or both recipient and donor being associated with more severe aGVHD using univariant analysis; however, statistical significance was lost when using multivariant analysis (54). SNPs in TLR9 have also been shown to impact aGVHD susceptibility, as patients receiving allo-HSCT from donors with either one of two SNPs in TLR9 demonstrated more frequent grade II-IV aGVHD when compared to those receiving allo-HSCT from donors with wildtype TLR9 (55). Sivula et al. demonstrated that many different TLR SNPs found in allo-HSCT donors and/or recipients are associated with aGVHD occurrence when evaluated independently from one another, including one SNP in TLR1, one SNP in TLR4, three SNPs in TLR5, one SNP in TLR6, and one SNP in TLR10, based on multivariant analyses (56). Interestingly, the authors also demonstrate that one SNP in TLR4 found in allo-HSCT recipients was protective from aGVHD.

Yet another focus of research regarding TLRs and aGVHD is how modulation of TLR activation impacts aGVHD development, with the goal of developing novel prophylaxis and therapeutics. Utilizing TLR4 wildtype or deficient mice, Zhao et al. demonstrated that inactivation of TLR4 in either the donor or recipient is protective against aGVHD, with recipient mice having reduced aGVHD symptoms and delayed mortality (57). These finding were also supported by Brennan et al. who showed that administration of heparan sulfate (a TLR4 agonist) promotes aGVHD development while administration of α1-antitrypsin [a serine protease inhibitor which functions as a TLR4 antagonist by disrupting the LPS-TLR4-NF-κB axis (58–60)] reduced aGVHD severity (61). Similar to TLR4, TLR9 inactivation in recipient mice through global deletion, significantly reduces aGVHD severity and mortality (62). Further supporting this data, Taylor et al. demonstrated that repeated administration of CpG oligodeoxynucleotides, a main ligand of TLR9, accelerated aGVHD lethality (63). There are other TLRs such as TLR7 that may accelerate or ameliorate aGVHD depending on time and duration of administration. Chakraverty et al. showed that topical application of R-848, a TLR7/8 agonist, induced severe donor T cell infiltration into the skin of recipient mice (64). Similar results were obtained independently by another research group who repeatedly administered either 3M-011 (a TLR7/8 agonist) or drug vehicle and observed that mice receiving 3M-011 had higher overall mortality when compared to mice receiving vehicle (63). In contrast, from data Jasperson et al. indicated that a single administration of 3M-011 between lethal irradiation conditioning and allo-HSCT induced the tryptophan catabolic pathway in APCs, leading to significantly reduced lethality and colonic pathology scores (65). While the pharmacologic studies described here are all based on well-studied methods of TLR activation or inhibition, researchers are also actively investigating other novel mechanisms by which TLRs are activated and can be pharmacologically manipulated.

MiRs AS TLR LIGANDS

MicroRNAs (miRs) are small, non-coding RNAs that are approximately 19–24 nucleotides long and are found in nearly all plants and animals (66). They function in regulating gene expression for critical cellular processes such as cell development, differentiation, expansion, survival, and function (67). The canonical function of miRs involves the mature miR, loaded into the RISC complex, interacting with target mRNA or proteins in the cell nucleus or cytoplasm, leading to altered gene expression and/or protein function (66, 68, 69). In contrast to canonical biogenesis of miRs, emerging data supports the findings that miRs can also be stably found in a variety of body fluids, such as saliva (70, 71), urine (72), and blood (73, 74) either packaged within exosomes or in complexes with RNA-binding proteins such as Ago or high-density lipoprotein. These miRs, referred to as cell-free miRs (75), may serve as biomarkers of disease (71, 72, 74) and/or facilitators of disease pathogenesis through cell-to-cell communication (75–78).

Viral single-stranded RNA oligonucleotides serve as the primary PAMPs recognized by TLR7 (mice) and TLR8 (humans) located within endosomes (79). Since miRs are short single-stranded RNA molecules, it is conceivable that miRs could function as ligands for these specific TLRs. Indeed, over the past 7 years, a small number miRs have been shown to function as TLR ligands in a variety of diseases, which will be discussed briefly. A summary of the mechanism by which miRs can function as TLR ligands is shown in **Figure 1**.

In 2012, Lehmann et al. demonstrated that secreted miR let-7b functions as a TLR7 ligand in murine microglia and bone marrow-derived, leading to the induction of TNFα release and subsequent neurodegeneration (80). The authors demonstrate that all let-7 family members, not just let-7b, are able to activate TLR7 on murine microglia and propose that this is due to the presence of a conserved 3' GU-rich motif on all let-7 family members that is also present on a known TLR7 ligand, HIV ssRNA40 (79). Interestingly, let-7b was shown to be released from dying neurons *in vitro*, which then functioned in a paracrine manner to accelerate neuronal injury of surrounding neurons. The neuronal damage caused by let-7b release from surrounding degenerative neurons could be ameliorated by pre-treatment with a let-7b inhibitor both *in vitro* and *in vivo* when administered intrathecally. Lastly, the authors also showed elevated let-7b levels in the cerebrospinal fluid of patients with Alzheimer's disease when compared to those who did not have disease. Taken together, these findings demonstrate that let-7b serves as a novel TLR7 ligand on murine microglia, functions as a DAMP to surrounding neurons during times of neuronal injury and could be a novel therapeutic target to reduce neuronal damage.

Kim et al. investigated the role of synovial fluid let-7b in the pathogenesis of an autoimmune disease; rheumatoid arthritis (81). First, the authors identified that let-7b is markedly upregulated in synovial fluid of patients with rheumatoid arthritis. Similar to Lehmann et al., Kim et al. identified that let-7b functions as an endogenous ligand for

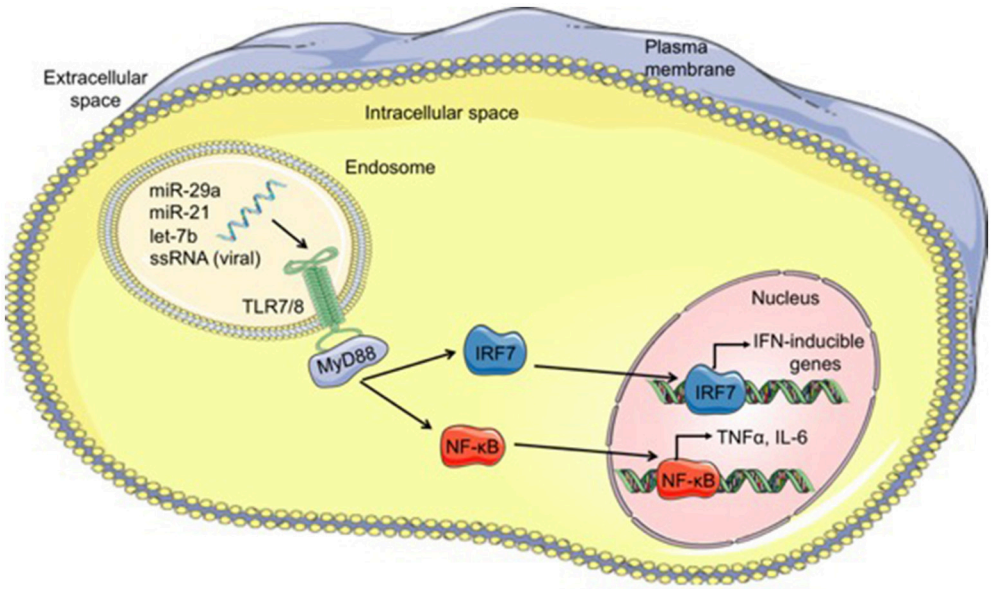

FIGURE 1 | Mechanism of microRNAs functioning as TLR ligands. Cell-derived exosomes containing microRNAs such as let-7b, miR-21, and miR-29a, are taken into cells through endocytosis and fuse with TLR-containing endosomes within cells. The GU-rich microRNA bind to TLR7/8, activating TLR signaling through MyD88 and leading to translocation of IRF7 and NF-κB from the cytoplasm into the nucleus. Once in the nucleus, these transcription factors bind to DNA, resulting in transcription of interferon (IFN)-inducible genes and proinflammatory cytokines such as TNFα and IL-6, respectively. MicroRNA (miR), single-stranded RNA (ssRNA), Toll-like receptor 7/8 (TLR7/8), myeloid differentiation primary response 88 (MyD88), interferon regulatory factor 7 (IRF7), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

both TLR7 and TLR8 within synovial fluid macrophages in patients with rheumatoid arthritis. Additionally, let-7b strongly stimulated TLR7-positive myeloid cells found within synovial fluid, driving their development toward pro-inflammatory M1 macrophages in a murine model of rheumatoid arthritis.

In 2012, Fabbri et al. showed that miR-21 and miR-29a are both secreted from lung cancer cell lines into cell-derived exosomes and interact with TLR-containing endosomes within macrophages at the interface between neoplastic and non-neoplastic tissues (82). Once within macrophage endosomes, miR-21, and miR-29a function as TLR ligands to activate murine TLR7 and human TLR8. In contrast to Lehmann et al. which show indirect interaction between let-7b and murine TLR7, Fabbri et al. utilized co-immunoprecipitation assays to demonstrate the direct binding of miR-21 and miR-29a to TLR7/TLR8. Functionally, the binding of miR-21 and miR-29a to TLR8 on human peripheral blood mononuclear cells induces NF-κB-dependent production of both TNFα and IL-6. Similar to let-7b and TLR7, specific short GU-rich motifs found on miR-21 and miR-29a are critical for modulating their binding to and activation of TLR8. Utilizing the inflammation-induced Lewis lung cancer mouse model, the authors demonstrate that tumor-secreted exosomal miRs, including miR-21 and miR-29a, induce murine TLR7 activation and increase the formation of lung multiplicities. Furthermore, treatment of mice with a locked nucleic acid (LNA) antimiR-21/29a significantly reduced pulmonary tumor multiplicities. Altogether, these findings directly demonstrate that miRs can function as TLR ligands, are important regulators of prometastatic inflammation, and warrant additional investigation as novel cancer therapeutic targets.

MiR29a AS TLR LIGAND IN aGVHD

While there are many studies which describe the importance of miRs in aGVHD pathogenesis, the focus is primarily on intrinsic miR expression in immune cells such as T cells and dendritic cells (83–86). Recent data, however, suggests that miRs in circulation may serve as important modulators of pathogenesis in aGVHD.

Our group demonstrated the a novel role for serum miR-29a in aGVHD pathogenesis as a ligand for TLR7/ TLR8 on dendritic cells following allo-HSCT (84). Using two independent cohorts of patients who received allo-HSCT, we showed that miR-29a is upregulated in the serum of patients who develop aGVHD when compared to those who do not. These findings were also validated in murine models of aGVHD and it was further shown that the miR-29a was localized within serum exosomes. Using liposomal-conjugated miR-29a, we showed that murine bone marrow derived dendritic cells (BMDCs) were potently activated, as indicated by upregulation of maturation markers CD40, CD80, CD86, MHC II, and CCR7 as well as significant secretion of pro-inflammatory TNFα and IL-6. BMDCs activated by miR-29a migrated more efficiently toward CCR7 ligand CCL19 and induced stronger T cell proliferation when compared to BMDCs treated with miR-16 which served as a negative

control. Because TLR signaling results in activation of My-D88-dependent transcription factors including NF-κB and IRF7, we also showed that miR-29a stimulation of murine BMDCs leads to nuclear translocation of both phosphorylated IRF7 and NF-κB-p65. Lastly, utilizing healthy donor human peripheral blood mononuclear cells (PBMCs) and monocyte-derived DCs, we demonstrated that exosomal miR-29a activates human PBMCs and DCs. To conclusively show that these findings were due to direct binding of miR-29a to human TLR8, Flag antibody tagging, and RNA immunoprecipitation were performed and confirmed marked enrichment of miR-29a in TLR8 transfected DCs only. Finally, we showed that administration of LNA antimiR-29a resulted in reduced circulating serum miR-29a, significantly improved survival and decreased clinical aGVHD severity while maintaining beneficial graft-vs.-leukemia effects in murine models of aGVHD. These findings are the first and only to demonstrate miRs as TLR ligands in the context of aGVHD and provide an exciting novel therapeutic target to prevent or treat aGVHD.

FUTURE DIRECTION

Allo-HSCT remains a curative modality a variety of diseases, including hematologic malignancies, myelodysplastic disorders, myeloproliferative neoplasms, and aplastic anemia (3, 87, 88). Despite this, aGVHD remains a frequent and lethal complication of allo-HSCT, underscoring the need for better understanding of aGVHD pathogenesis Furthermore, given the high morbidity and mortality associated with aGVHD, scientists and clinicians are seeking not just novel therapeutics but also novel prophylaxis. TLRs are highly conserved PRRs which are a critical aspect of the innate immune system. Traditionally, TLRs are activated by DAMPs and PAMPs, many of which are involved in the initiation of aGVHD. MiRs, being small ssRNA strands, have recently been documented as serving as ligands for TLR7 and TLR8, propagating such diseases as Alzheimer's disease, rheumatoid arthritis, and aGVHD. With the identification of miR-29a as a soluble mediator of TLR activation in aGVHD, many additional questions arise. Are there other secreted miRs which activate TLRs in the context of aGVHD? While miR-153-3p has recently been identified as a plasma miR which is upregulated and disrupts tryptophan synthesis during aGVHD, the authors do not evaluate the role of miR-153-3p on specific immune cell activation such as T cells or APCs (78). Secondly, do these miRs interact with their associated TLR similarly with specificity dependent on GU-rich motifs and how much specificity does this confer? As miRs are <25 nt long and thousands of miRs are currently recognized, the potential for other miRs to have similar GU-rich motifs which could bind to TLRs seems highly likely. Third, can these miRs serve as novel therapeutic or prophylactic targets in diseases in which TLR7/8 play a pivotal role in pathogenesis, including aGVHD? TLR agonists and antagonists are actively being investigated as novel therapeutics for a broad range of diseases, including cancer, inflammatory disease, allergies, and infectious agents such as HIV and hepatitis C (89–92), although TLR modulation as an aGVHD therapeutic is still very much in its infancy. With this, we have the potential for gaining a better understanding aGVHD pathogenesis and identifying novel prophylactic and therapeutic targets. With all of these questions still needing answers, we have likely only brushed the surface, opening a completely new avenue of study for scientists not only in aGVHD but across many diseases in which TLR7/8 is implicated in their initiation and development.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. NZ and PR wrote the manuscript together. RG edited the manuscript.

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Regulation of Endotoxin Tolerance and Compensatory Anti-inflammatory Response Syndrome by Non-coding RNAs

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The onset and the termination of innate immune response must be tightly regulated to maintain homeostasis and prevent excessive inflammation, which can be detrimental to the organism, particularly in the context of sepsis. Endotoxin tolerance and compensatory anti-inflammatory response syndrome (CARS) describe a state of hypo-responsiveness characterized by reduced capacity of myeloid cells to respond to inflammatory stimuli, particularly those initiated by bacterial lipopolysaccharide (LPS). To achieve endotoxin tolerance, extensive reprogramming otherwise termed as "innate immune training", is required that leads to both modifications of the intracellular components of TLR signaling and also to alterations in extracellular soluble mediators. Non-coding RNAs (ncRNAs) have been recognized as critical regulators of TLR signaling. Specifically, several microRNAs (miR-146, miR-125b, miR-98, miR-579, miR-132, let-7e and others) are induced upon TLR activation and reciprocally promote endotoxin tolerance and/or cross tolerance. Many other miRNAs have been also shown to negatively regulate TLR signaling. The long non-coding (lnc)RNAs (Mirt2, THRIL, MALAT1, lincRNA-21 and others) are also altered upon TLR activation and negatively regulate TLR signaling. Furthermore, the promotion or termination of myeloid cell tolerance is not only regulated by intracellular mediators but is also affected by other TLR-independent soluble signals that often achieve their effect via modulation of intracellular ncRNAs. In this article, we review recent evidence on the role of different ncRNAs in the context of innate immune cell tolerance and trained immunity, and evaluate their impact on immune system homeostasis.

Keywords: endotoxin tolerance, sepsis, immune suppression, microRNAs, non-coding RNAs, soluble mediators, lncRNAs

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INTRODUCTION

The onset and termination of the host immune responses have to be tightly controlled; the initial burst of pro-inflammatory cytokines should be timely blunted to avoid overwhelming inflammatory responses causing tissue damage and secure homeostasis. Endotoxin tolerance is a crucial homeostatic mechanism that prevents from the excessive activation of innate immune responses upon sustained toll-like receptor (TLR) stimulation.

Endotoxin tolerance is defined as the reduced capacity of a cell to respond to gram(-) bacterial lipopolysaccharide (LPS) after an initial exposure to this stimulus (1, 2). Endotoxin tolerance is considered a type of "innate immune memory" (3), a condition describing tolerance to pathogens, characterized by innate immune hypo—responsiveness or "immune-paralysis". It occurs as a result of persistent TLR stimulation not only from LPS but from other TLR agonists and even TLR-independent inflammatory mediators (1). The mechanism by which exposure to a particular TLR ligand or other inflammatory mediators such as cytokines reduces the inflammatory response to different TLR ligands is known as cross–tolerance and cytokine-induced tolerance, respectively (4–7), both being part of the innate immune system training (8, 9).

The phenotype of endotoxin tolerance and cross-tolerance has been extensively studied in monocytes and macrophages, even though the majority of innate immune cells develop tolerance to secondary TLR stimuli. These include dendritic cells, neutrophils, mast cells as well as endothelial and epithelial cells (10–14).

Endotoxin tolerance results to a shift of the cell phenotype from pro-inflammatory to anti-inflammatory (15). Endotoxin tolerant macrophages are reprogrammed to produce less tumor necrosis factor alpha (TNFα), interleukin (IL)-12 and IL-6 upon secondary stimulation and more anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGFβ), compared to the levels produced from naive cells (16, 17). Furthermore, tolerant macrophages and dendritic cells downregulate human leukocyte antigen (HLA-DR) receptors thus have impaired capability for antigen presentation (16, 18). Similar phenotype is also described in cross-tolerance, but to a lesser extent (19). Endotoxin tolerant phenotype is long lasting but reversible in nature.

The clinical manifestation of endotoxin tolerance is recognized as Compensatory Anti-inflammatory Response Syndrome (CARS) (20). CARS represents the phase of immune "exhaustion" otherwise termed "immune paralysis", that is observed in a subset of septic patients usually following the first phase of sepsis, known as Systemic Inflammatory Response Syndrome (SIRS) (21). Endotoxin tolerance explains CARS immunosuppression state in sepsis since blood leukocytes from septic patients exhibit similar phenotype to endotoxin tolerant cells; neutrophils and monocytes from septic patients are refractory to production of inflammatory mediators while they upregulate anti-inflammatory molecules when exposed to secondary TLR stimuli (1, 17, 22, 23). As a result, patients with CARS exhibit increased susceptibility to secondary infections (24).

The mechanism of innate immune cell tolerance and CARS are tightly regulated by complex molecular signatures in macrophages and other innate immune cells. These molecular pathways are controlled not only by modulation of intracellular signaling proteins and histone modifications but also by non-coding (ncRNAs), mostly microRNAs (miRNAs) and long ncRNAs (lncRNAs). In this article, we review recent evidence on the role of ncRNAs, regulated by TLR ligands or other TLR independent soluble signals, in the regulation of endotoxin tolerance and discuss their impact in the context of sepsis.

TLR—DEPENDENT REGULATION OF ENDOTOXIN TOLERANCE VIA ncRNAs

Upon stimulation by pathogen- or danger-associated patterns, TLR mediate signals through two distinct adaptors pathways, myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). The MyD88 pathway employs interleukin-1 receptor-associated kinase (IRAK)1 and 4 kinases and TNF receptor-associated factor (TRAF)6 to activate nuclear factor κB (NFκB) and mitogen activated protein kinase (MAPK)/activator protein 1 (AP-1) signaling, promoting transcription of pro-inflammatory cytokines. Activation of TRIF pathway leads to janus kinase (JAK)/signal transducer and activator of transcription (STAT) and type I interferon activation and increases the expression of interferon-inducible genes (25, 26). In TLR4 tolerance, defects in TLR4 signaling have been observed at all levels, including receptor adaptors, signaling molecules, transcription factors, as well as, chromatin marks as histone modifications (1, 27).

The molecular signature of endotoxin tolerance involves downregulation of TLR4 expression, decreased recruitment of MyD88 or TRIF to TLR4, decreased activation of IRAK1/4 and diminished NFκB signaling via formation of the inactive p50 homodimers (1, 28). Additionally, negative regulatory molecules such as IRAK-M, A20, SH2 domain-containing inositol phosphatase 1 (SHIP1), Pellino-3, suppression of tumorigenicity 2 (ST2), suppression of cytokine signaling (SOCS)3 and SOCS1 are upregulated in endotoxin tolerant cells and inhibit the activation of TLR signaling (1, 28–33). However, during last two decades, an additional level of regulation through non-coding regulatory RNAs has been introduced.

TLR Dependent miRNAs That Regulate Endotoxin Tolerance

MicroRNAs (miRNAs) are a large family of small noncoding RNAs (about 22 nucleotides in length) that regulate gene expression post-transcriptionally, by binding to the 3′-untranslated regions (UTRs) of target mRNAs (34). MiRNAs are recognized as key players in the regulation of endotoxin tolerance since multiple levels of the TLR signaling cascade are controlled by miRNAs (35, 36). At the stage of endotoxin tolerance, two LPS inducible miRNAs, miR-155 and miR-146α have been shown to be coordinately regulated via gene colocalization and transcription factor binding, contributing to the regulation of endotoxin tolerance (37). Indeed, miR-146α was the first miRNA described to promote tolerance (38, 39). MiR-146α is induced upon TLR activation in macrophages and its expression is further upregulated with LPS restimulation (17, 37). MiR-146α then targets IRAK1 and TRAF6, critical components downstream TLR signaling and its prolonged expression has been linked to endotoxin tolerance and cross—tolerance (19, 39–41). On the other hand, miR-155 inhibits the expression of the negative regulators SHIP1 and SOCS1 enhancing TLR signals, promotes TNFα translation and establishes a proinflammatory phenotype in macrophages (42–46). However, other studies show that miR-155 may exert negative regulation of pro-inflammatory

mediators (47) (**Table 1**). Suppression of miR-155 in Akt1$^{-/-}$ macrophages restored sensitivity and tolerance to LPS *in vitro* and *in vivo*, supporting its role in the regulation of endotoxin tolerance (43).

MiR-98 targets IL-10 in macrophages, a key cytokine for development of endotoxin tolerance; miR-98 is decreased by LPS in macrophages, thus failing to suppress IL-10 (68). The miRNAs miR-221, miR-579 and miR-125b are also significantly induced in endotoxin tolerant macrophages and lead to TNFα inhibition; miR-221 promotes TNFα degradation, whereas miR-579 and miR-125b block its translation (52). MiR-132 and miR-212 are also induced upon TLR2 stimulation and their sustained expression promotes cross tolerance (54). In a recent report, miR-221 and miR-222 were identified as regulators of the functional reprogramming of macrophages during LPS tolerization (3). MiR-221 and miR-222 were induced after prolonged LPS stimulation in mice and both promoted transcriptional silencing of a subset of pro-inflammatory genes via regulation of chromatin remodeling mediated by SWI/SNF (switch/sucrose non-fermentable) and STAT transcription factors (3).

However, there is a significant number of other miRNAs that have been shown to negatively regulate TLR signaling (**Table 1**). Among the aforementioned miRNAs, miR-146, miR-155, miR-221 and miR-222 have been extensively studied and appear to have a central role in the regulation of innate immune tolerance. In the context of sepsis, the levels of miR-146, miR-150, miR-221 and miR-222 among other miRNAs, are dysregulated in the peripheral blood leukocytes in sepsis patients and correlate with immunoparalysis and severity of the disease (3, 69–71), thus providing potential prognostic/diagnostic biomarkers.

LncRNAs That Contribute to Endotoxin Tolerance

Long noncoding RNAs (lncRNA) are regulatory RNAs that are over 200 nucleotides in length and do not encode proteins (72–74). LncRNAs are classified based on their site of action into

TABLE 1 | List of the most prominent miRNAs implicated in the regulation of innate immune cell tolerance.

MiRNA	Response to TLR signal	Target	Mechanism of action	References
miR-146α	Induced	TRAF6, IRAK1, TLR2/4, Notch1	Targets TLR and TRAF6, IRAK1 in macrophages critical components downstream TLR signaling	(38, 48)
miR-146b	Induced	TRAF6 IRAK1 TLR4	Targets TLR and TRAF6, IRAK1 critical components downstream TLR signaling	(39, 49)
miR-155	Induced	SHIP, SOCS1 CEBP/β FADD, Ripk1	Inhibits the expression of the negative regulators of TLR signaling, SHIP1 and SOCS1. Promotes TNFα translation. Abrogates expression of anti-inflammatory genes in macrophages	(42–44)
		MyD88 TAB2, IKKe	Negative regulation of inflammatory cytokine production in macrophages and DCs	(50, 51)
miR-221	Induced	TNFα STAT1 STAT2	Promotes TNFα degradation. Induces tolerance via chromatin remodeling mediated by SWI/SNF (switch/sucrose non-fermentable) and STAT1/2 in macrophages	(3, 52)
miR-222	Induced	STAT1, STAT2	Induces tolerance via chromatin remodeling mediated by SWI/SNF (switch/sucrose non-fermentable) and STAT1 and 2 in macrophages	(3)
miR-132	Induced	IRAK4 p300	Responsible for inducing cross tolerance in monocytes/macrophages. Negative effect on the expression of interferon-stimulated genes and antiviral immunity in endothelial cells	(53, 54)
miR-21	Induced	PDCD4 MyD88,IRAK1 IL-12p35	Negative regulation of TLR4 signaling in monocytes. Inhibits the expression of MyD88 and IRAK1 during viral infection.	(55–58)
miR-579	Induced	TNFα	Negative regulation of TNFα translation in monocytes.	(52)
miR-125b	Induced	TNFα MyD88	Negative regulation of TNFα translation. Negatively regulate viral responses by targeting TLR2/MyD88 signaling in monocytes.	(42, 59)
miR-212	Induced	IRAK4	Sustained expression is responsible for inducing cross tolerance in monocytes/macrophages.	(54)
let-7e	Induced	TLR4	Negative regulation of TLR4 signaling in macrophages	(43)
let-7i	Suppressed	TLR4	Post-transcription regulation of TLR4 in epithelial cells	(60)
miR-124	Induced	TLR6, MyD88 TRAF6, TNFα	Negatively regulates TLR signaling in BCG infection in macrophages	(61)
miR-149	Suppressed	MyD88	Represses MyD88 translation in macrophages	(62)
miR-203	Induced	MyD88	Represses MyD88 translation in macrophages	(63)
miR-92a	Suppressed	MAPK4	Inhibits TLR4 —responses in macrophages	(64)
miR-210	Induced	NFκB1	Targets NFκB1 upon stimulation in macrophages	(65)
miR-9	Induced	NFκB1	Negative control of NFκB in monocytes	(66)
miR-718	Induced	PTEN	Down regulates TLR4, IRAK1, and NFκB in a negative feedback loop in macrophages	(67)
miR-98	Suppressed	IL-10	Targets IL-10 in macrophages	(68)

cis-lncRNAs and *trans*-lncRNAs (nearby or remote to genes) and based on their relative position to target mRNAs, being exonic sense, intronic sense, antisense, bidirectional, and intergenic (75, 76). In contrast to miRNAs that have a clear role in promoting post-transcriptional regulation of gene expression, lncRNAs exhibit plethora of actions via transcriptional, post-transcriptional and translational regulation of gene expression as well as via controlling mRNA stability and promoting epigenetic changes (72, 75, 77–79).

LncRNAs have emerged as important regulators of innate immune responses and TLR signaling (74, 79–83). In response to LPS or other TLR stimuli, the lncRNAs expression pattern is altered and lcnRNAs have been shown to either promote or suppress pro-inflammatory responses (80, 84–86).

Several TLR-inducible lncRNAs limit excessive inflammatory responses by negatively regulating TLR signaling. The LPS-responsive lncRNAs Mirt2, THRIL, MALAT1, NKILA, lincRNA-21, and SeT have been shown to suppress expression of pro-inflammatory mediators including TNFα, the central cytokine for tolerance and CARS (**Table 2**). Mirt2 is expressed in macrophages and induced by LPS, negatively regulating TLR4 signaling; Mirt2 inhibits TRAF6 ubiquitination thus blocking NFκB and MARK activation and subsequent TNFα production (87). THRIL is another immuno-regulatory lncRNA that was found to interact with hnRNPL at the promoter region of the TNFα gene inducing TNFα expression (88). However, THRIL is downregulated upon TLR2 triggering indicating that THRIL suppression may be a protective feedback loop controlling TNFα levels and promoting cross–tolerance (88). The lncRNA MALAT1 has been found to negatively regulate TLR response via inhibition of NFκB; MALAT1 is upregulated in LPS-activated macrophages and interacts with NFκB in the nucleus, inhibiting LPS-induced expression of TNFα and IL-6 (89). Importantly, MALAT1 was found to be dysregulated in granulocytes from septic patients indicating its clinical importance in sepsis and CARS (104). Similar to MALAT1, NKILA is another lncRNA that regulates TLR4 signaling and restrains NFκB activation; NKILA is induced by LPS in tumor cells and interacts with the NFκB/IκB complex, preventing its phosphorylation by IKKs and subsequent NFκB activation (90). LincRNA-p21 is induced by LPS in fibroblasts and regulates NFκB activity in monocytes; lincRNA-p21 physically binds to RelA/p65 mRNA blocking translation of p65, resulting in inhibition of NFκB (94, 97, 98). Finally, the lncRNA SeT is expressed in macrophages in response to LPS and its homologous deletion results in biallelic TNFα expression and increase in TNFα levels (91). This finding suggests that lncRNA SeT suppresses expression of one of the two TNFα alleles early upon LPS stimulation (91).

Additional lncRNAs have been shown to suppress pro-inflammatory mediators such as IL-6 but their effect on TNFα expression has not been evaluated (**Table 2**). Lnc-IL-17R is up-regulated significantly in response to TLR2 and TLR4 agonists, promoting H3K27 trimethylation and inhibiting LPS-inducible inflammatory response genes, such as IL-6, adhesion molecules, and chemokines (84). Similarly, the lncRNA IL7-AS (antisense) is induced by LPS in macrophages; knockdown of IL7-AS results in upregulation of IL-6 (92). Finally, lincRNA-EPS is expressed in macrophages and dendritic cells and was downregulated upon microbial infection, while gain-of-function experiments revealed that lincRNA-EPS binds to chromatin, regulates the nuclear ribonucleoprotein L (hnRNPL), thus suppressing LPS-induced pro-inflammatory genes (93). In addition to the lncRNAs outlined, lincRNA-Cox2 is another LPS inducible lncRNA that regulates hundreds of genes, but it appears to act both as an enhancer and as a suppressor of inflammation (80, 94, 95, 105). Finally, in a recent report, TLR4 tolerisation reversed LPS-induced suppression of PCGEM1 and HOTTIP lncRNAs and upregulated snaR lcnRNA, but further investigation is required to define the function of these lncRNAs in the context of tolerance (79).

It appears that the changes in the outlined lncRNAs significantly regulate TLR signaling toward TLR reprogramming. However, the majority of the above lncRNAs were not evaluated in endotoxin tolerant experimental setting per se since their expression and function was not evaluated upon secondary TLR stimulation. Also, their relative contribution to tolerant state in conjunction with several miRNAs, that were mentioned above and have an established central role in endotoxin tolerance, has not been studied yet. Further research is required to address the importance and the level of contribution of these lcnRNAs in endotoxin tolerance and/or cross tolerance.

TLR-INDEPENDENT REGULATION OF ENDOTOXIN TOLERANCE VIA ncRNAs

Establishment of endotoxin tolerance and cross-tolerance is not strictly a result of excessive TLR signaling and subsequent induction of intracellular regulators. The magnitude and duration of the innate cell tolerance is also controlled by a plethora of TLR—independent soluble mediators.

Soluble Mediators in Innate Immune Cell Tolerance and Their Impact in ncRNAs

Cytokines such as IL-1β, IL-10, TGFβ, and TNFα are capable to induce cross-tolerance or cytokine-mediated tolerance initiating intracellular signals similar to those of TLR ligands (17, 106). Indeed, IL-10 and TGFβ are part of a negative feedback loop produced from activated macrophages acting in an autocrine and paracrine manner to promote tolerance and suppress secondary TLR responses. However, LPS priming provokes more sustained tolerance than IL-10 priming, since IL-10-primed monocytes rapidly recover and produce TNFα (107). Also, endogenous hormones, such as adiponectin and glucocorticoids blunt LPS-induced inflammation and promote anti-inflammatory responses (108, 109). In contrast, interferons such as interferon gamma (INF-γ) and α2-interferon, are known to abrogate endotoxin tolerance and restore induction of pro-inflammatory cytokines (110, 111).

The aforementioned soluble mediators have been reported to achieve their effect via modulation of intracellular ncRNAs. The capability of IL-1β priming to induce tolerance and cross-tolerance in monocytes and epithelial cells is mediated via the increase of miR-146α (6). IL-10 has been shown to promote miR-146b upregulation in human monocytes and its transcription

TABLE 2 | LcnRNAs that have been implicated in the regulation of innate immune cell tolerance.

LncRNA	Response to TLR signal	Target	Mechanism of action	References
Mirt2	Induced	TRAF6	Inhibits TRAF6 ubiquitination, NFκB and MARK activation and subsequent TNFα production in macrophages	(87)
THRIL	Suppressed	TNFα	Interacts with hnRNPL at the promoter of TNFα gene inducing TNFα expression in macrophages	(88)
MALAT1	Induced	NFκB	Interacts with nuclear NFκB, inhibits LPS-induced TNFα and IL-6 in macrophages	(89)
NKILA	Induced	NF$\alpha\kappa$B/IκB	Interacts with NFκB/IκB complex in epithelial tumor cells, preventing its phosphorylation by IKKs and subsequent NFκB activation	(90)
SeT	Induced	TNFα	Suppresses expression of one of the two TNFα alleles early upon LPS stimuli in macrophages	(91)
Lnc-IL-17R	Induced	IL-6	Promotes H3K27 trimethylation, inhibits LPS-inducible inflammatory response genes (IL-6, chemokines) in macrophages/endothelial cells	(84)
IL7-AS	Induced	IL-6	IL7-AS suppression induces IL-6 in macrophages	(92)
lincRNA-EPS	Suppressed	NFκB	Binds to chromatin, regulates the nuclear ribonucleoprotein L (hnRNPL), and suppress pro-inflammatory genes in macrophages	(93)
lincRNA-Cox2	Induced	NFκB	Activates the NFκB –regulated late-primary inflammatory genes via interaction with hnRNP-A/B and hnRNP-A2/B1 in macrophages. In epithelial cells it represses TNFα-induced IL-12β transcription via recruitment of Mi-2/NuRD repressor complex to the IL-12β promoter	(80, 94–96)
LincRNA-p21	Induced	RelA/p65	Induced by TLR stimuli in fibroblasts. Physically binds to RelA/p65 mRNA blocking translation of p65 in monocytes	(97, 98)
lnc-DC	Induced	STAT3	Activates STAT3 by preventing SHIP1 mediated STAT3 dephosphorylation, resulting in reduced ability of dendritic cells to activate T cells	(99, 100)
NeST or Tmevpg1	Induced	IFN-γ	Alters H3K4 trimethylation in *IFN-γ* locus, upregulates IFN-γ expression in T cells and indirectly mitigates endotoxin tolerance	(101)
Lethe	Induced	RelA	Binds and inactivates RelA/p65 and decreases p65 binding at NFκB sites to restrict excessive inflammatory response in fibroblasts	(102)
PACER	Induced	p50	Interacts and sequesters excess p50 from COX2 promoter, activates COX2 in macrophages and epithelial cells	(103)
PCGEM1	Suppressed	unknown	TLR4 tolerisation reversed LPS-induced PCGEM1 suppression in macrophages	(79)
HOTTIP	Suppressed	unknown	TLR4 tolerisation reversed LPS-induced suppression of HOTTIP in macrophages	(79)

is driven by STAT3, a transcription factor induced by IL-10 signals (49, 112). Similarly, TGFβ also promotes tolerance in human monocytes via upregulation of miR-146b driven by the transcription factor RUNX3 (112). Glucocorticoids and TGFβ have been shown to downregulate TLR4 signaling via induction of miR-511-5p, which targets TLR4 (113).

Stimulation with TNFα promotes TNFα–induced tolerance via regulation of ncRNAs. The lncRNA implicated in suppression of NFκB inflammatory response in fibroblasts upon TNFα stimulation is Lethe; Lethe binds and inactivates RelA/p65 and decreases p65 binding at NFκB sites (102). Moreover, upon TNFα stimulation, lincRNA-Cox2 is induced and promotes recruitment of the Mi-2/nucleosome remodeling and deacetylase (Mi-2/NuRD) repressor complex to the IL-12β promoter suppressing IL-12β expression (96).

IFN-γ is another mediator that enhances macrophage activation and reverses tolerance via regulation of ncRNAs. IFN-γ is known to inhibit miR-146b expression, a miRNA that contributes to endotoxin tolerance (112). Also, IFN-γ induces phosphatase and tensin homolog (PTEN) via downregulation of miR-3473b; MiR-3473b targets PTEN and promotes Akt/glycogen synthase kinase 3 signaling and IL-10 production (114). Furthermore, NeST, also known as Tmevpg1 or IFNgAS1, is a lncRNA located near the IFN-γ gene in both humans and mice and positively regulates expression of IFN-γ in T cells via histone modifications in IFN-γ locus (101).

Soluble ncRNAs as Modulators of Endotoxin Tolerance

Tissue injury leads to release of extracellular vehicles (EVs) that frequently include miRNAs (115–117). EVs are present in the circulation acting in a paracrine and endocrine manner and can modulate pro-inflammatory cytokine production contributing to a tolerogenic response (116). In addition freely circulating extracellular miRNAs may function as TLR agonists inducing tolerance (55, 118). EVs also promote tolerance in distant cells. For example, Treg derived exosomes deliver miR-150-5p and miR-142-3p to dendritic cells leading to the induction of LPS-induced IL-10 and suppression of LPS-induced IL-6, thus promoting tolerance (119).

CONCLUSIONS

To conclude, it appears that a variety of TLR ligands, cytokines, and soluble mediators control endotoxin tolerance and cross-tolerance via the regulation of ncRNAs. However, there is a significant number of ncRNAs that are implicated in endotoxin tolerance and their relative importance and contribution in this process remains unknown. It is also unclear whether a level of interdependency among these ncRNAs exists and how their function may converge toward common pathways or potentially contradict each other. Further research is required to take into account the levels of contribution of each ncRNA in the context

of innate immune tolerance and to highlight the ones that have the potential to develop into therapeutic tools for CARS, the clinical syndrome associated with innate immune tolerance.

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EV, KV, and CT reviewed the literature and drafted the manuscript.

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The Multifaceted Interface Between Cytokines and microRNAs: An Ancient Mechanism to Regulate the Good and the Bad of Inflammation

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MicroRNAs (miRNAs) are evolutionary conserved small non-coding RNA molecules that affect gene expression by binding to target messenger RNAs and play a role in biological processes like cell growth, differentiation, and death. Different $CD4^+$ T cell subsets such as Th1, Th2, Th17, and T regulatory cells, exert a distinct role in effector and regulatory-type immune responses. miRNAs have been shown to respond to dynamic micro-environmental cues and regulate multiple functions of T cell subsets including their development, survival and activation. Thus, miRNA functions contribute to immune homeostasis, on the one side, and to the control of immune tolerance, on the other. Among the most important proteins whose expression is targeted by miRNAs, there are the cytokines, that act as both key upstream signals and major functional outputs, and that, in turn, can affect miRNA level. Here, we analyze what is known about the regulatory circuit of miRNAs and cytokines in $CD4^+$ T lymphocytes, and how this bidirectional system is dysregulated in conditions of pathological inflammation and autoimmunity. Furthermore, we describe how different T cell subsets release distinct fingerprints of miRNAs that modify the extracellular milieu and the inter-cellular communication between immune cells at the autocrine, paracrine, and endocrine level. In conclusion, a deeper knowledge of the interplay between miRNAs and cytokines in T cells may have pivotal implications for finding novel therapeutic strategies to target inflammation and autoimmune disorders.

Keywords: microRNAs, cytokines, $CD4^+$ Th cell subsets, inflammation, autoimmunity

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INTRODUCTION

MicroRNAs (miRNAs) are small (~22 nucleotides in length), non-coding RNAs, processed from longer transcripts, the pri-miRNAs, first cut to form a stem-loop structure, the pre-miRNAs. These molecules are then further processed to form the mature miRNA duplex by the subsequent action of two type III RNA endonucleases, Drosha (nuclear), and Dicer (cytoplasmic). The miRNA duplex is loaded into the Argonaute (Ago) protein to form a mature RNA interference silencing complex (RISC). The mature single stranded miRNA pairs to sites usually within the 3′ untranslated region of messenger RNAs (mRNAs), causing mRNA decay and block of translation.

A detailed description of miRNA biogenesis goes beyond the scope of the present review but can be found elsewhere (1). miRNA pathway, possibly derived from the ancient RNA interference (RNAi) pathway, is common to all eukaryotes and highly conserved. One of the first miRNAs discovered, *lethal-7* (let-7), a regulator of developmental timing in *Caenorhabditis elegans*, shows a correspondent temporal expression in bilaterian animals and is crucial in regulating mammalian developmental differentiation and glucose metabolism (2–5). In humans, almost two thousand different miRNAs are known and the majority of mRNAs are miRNA conserved targets (6). This broad regulation of the transcriptome expression potential suggests miRNAs may influence all physiological and pathological processes.

A major research effort has investigated the specific impact of miRNAs on the immune system. We will here focus on a population of T lymphocytes, $CD4^+$ T helper (Th) cells, crucial in orchestrating $CD8^+$ T and B cell-dependent adaptive immune response. T cell receptor (TCR) stimulation, the cytokine milieu and co-stimulatory signals together lead to naïve Th cell proliferation and differentiation into effector subtypes, characterized by specific transcription factors, cytokine fingerprints, and pathogenic targets (7). Th1 cells are defined by the master regulator T-bet, produce high levels of Interleukin (IL)-2 and interferon (IFN)-γ and direct immunity toward intracellular bacteria and viruses; Th17 cells, promoted by the expression of the master regulator Rorγt, combat extracellular bacteria, and fungal infections by releasing IL-17; the master regulator Gata3 drives the differentiation of Th2 cells, which produce IL-4, IL-5, and IL-13 and recognize extracellular parasites. Follicular helper T cells (Tfh), characterized by the activity of the master regulator Bcl-6, are located within B cell follicles of secondary lymphoid organs, mostly secrete IL-4 and IL-21 and are responsible for the maintenance of germinal centers and the development of humoral immunity. $CD4^+CD25^{high}FOXP3^+$ regulatory T (Treg) cells represent a functionally distinct lineage committed to exert an anti-inflammatory/immune suppressive control and sustain immunological homeostasis (8). Treg cells act by inhibiting the action of the pro-inflammatory counterpart $CD4^+$ Th1 and Th17 (also referred to as T conventional or Tconv) cell subsets by the production of IL-10, IL-35, and transforming growth factor (TGF)-β. Although the categorization of Th subpopulations is useful, the reported existence of cells with cytokine signatures and functional properties intermediate between the described subsets indicates a certain degree of plasticity (9, 10).

Since the dysregulation of cytokines is associated to deranged inflammation, effector Th cell differentiation/activation must be strictly regulated in order to avoid exaggerated and/or pathological responses (11). Beside epigenetic remodeling and lineage-restricted transcription factors, miRNA-dependent regulation is now recognized to significantly modulate Th gene expression and cytokine-related functional outputs. In this minireview, we will analyze relevant data on miRNA-based networks that regulate the tuned release of specific cytokines by Th subsets, central to mount efficacious immune responses and maintain immune homeostasis.

GLOBAL miRNA MODULATION DURING $CD4^+$ T CELL DEVELOPMENT AND DIFFERENTIATION

During T lymphocyte development, miRNA pool is highly dynamic, ranging from around 30,000 to ∼5,000 copies per cell when comparing the highly proliferative CD4CD8 double negative to the double positive lymphocytes undergoing selection. The miRNAs:total RNA ratio steadily increases during maturation, suggesting that miRNA suppressive potential is also regulated in terms of quantity relative to ribosomal and messenger RNA (12). Furthermore, when Th cells are TCR-stimulated, the RNA yield per cell increases with many housekeeping mRNA transcripts being induced. In parallel, global miRNA expression significantly diminishes, even before any cell division; this down-regulation depends on both pri-miRNA transcription decrease and RISC activity decline secondary to a massive Ago ubiquitination and subsequent proteasome-dependent degradation (13).

Ablation of the machinery for miRNA biogenesis during thymocyte differentiation or Th cell activation has devastating effects, demonstrating the critical role miRNAs play during Th gene expression reprogramming. Dicer or Drosha deletions in murine Th cells result in aberrant development, differentiation and cytokine production. Dicer deficient Th cells are not only unable to engage robust proliferation upon stimulation while actually undergoing increased apoptosis, but also show the preferential expression of IFN-γ, indicating a skewed subset commitment toward the Th1 lineage (14–17). Consistently, when miRNAs are depleted due to Ago deficiency, Th are more prone to differentiate into cytokine producing cells, suggesting that miRNA down-regulation promotes acquisition of effector functions by relaxing the repression of genes that direct Th cell differentiation, like cytokines and/or cytokine regulators (13) (**Figure 1**).

miRNA maturation pathway is also necessary for the development of thymic Treg cells and the induction of FOXP3 by TGF-β. Treg specific deletion of Dicer or Drosha shows a dramatic output, with the development of a lymphoproliferative phenotype resembling the one observed in the absence of FOXP3 itself (18–20).

miRNAs ON THE CUSP OF THE GENE EXPRESSION NETWORKS CONTROLLING $CD4^+$ TH CELL FUNCTION

The most prominent feature of Th cell differentiation is based on cytokine and transcription factor feedback loops that polarize gene expression. Th cell fate is sensitive to subtle changes of these regulatory circuits and therefore particularly responsive to miRNA regulation. Accumulating studies ablating and/or overexpressing single miRNA molecules or miRNA clusters are dissecting miRNA salient action in Th subset differentiation.

miR-17-92 Cluster

A milestone work has been conducted on the mir-17–92 cluster, that encodes miR-17, 18a, 19a, 19b, 20a, and 92

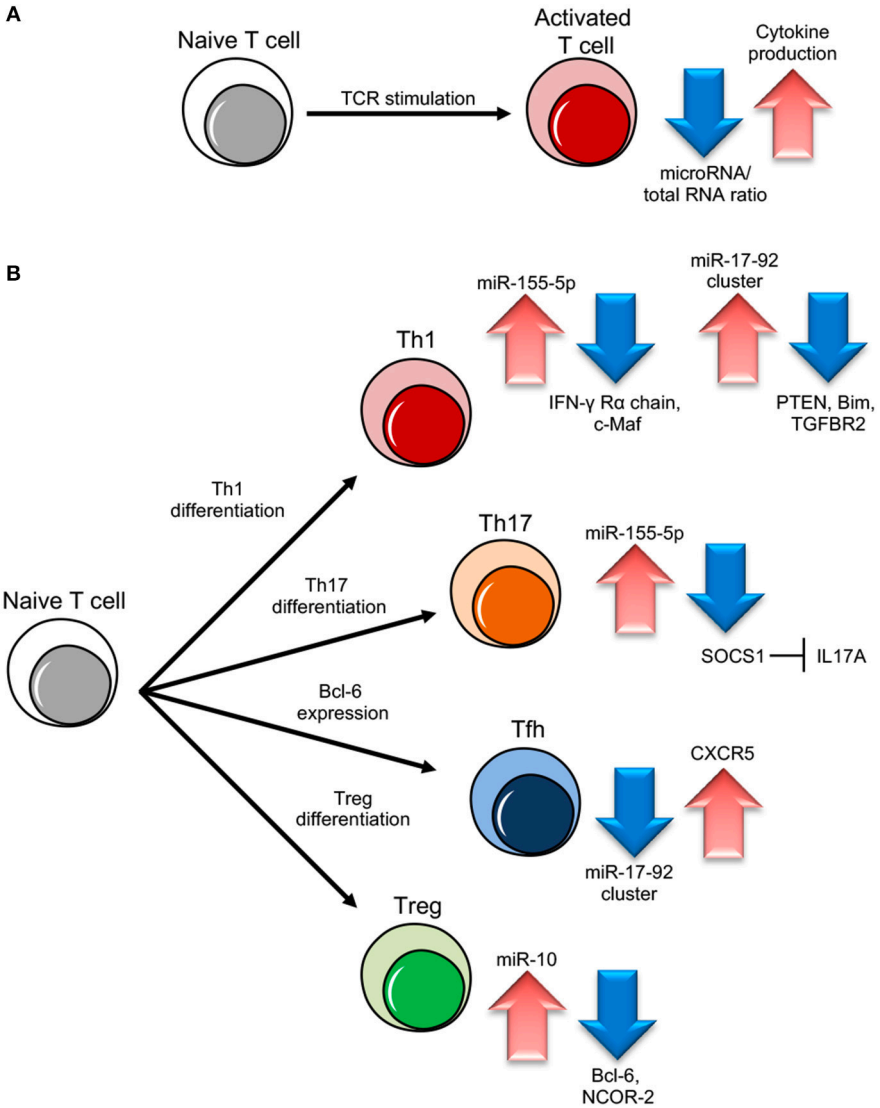

FIGURE 1 | miRNA role in different T cell subset differentiation. **(A)** Upon naive T cell activation, increase in cytokine production is dependent on miRNA/total RNA ratio decrease. **(B)** During Th1 cell differentiation, up-regulation of miR-155-5p and miR-17-92 results in suppression of IFN-γ Rγ chain and c-Maf and PTEN on one side and Bim and TGFBR2 on the other, important to block Th2 differentiation and unlock cell proliferation respectively. During Th17 differentiation, miR-155-5p induction leads to SOCS1 inhibition, which in turn unleashes IL17A production. During follicular helper T cells (Tfh) cell differentiation, CXCR5 up-regulation, important for migration into follicles, is dependent on miR-17-92 cluster downregulation. During Treg cell differentiation, the increase of miR-10 expression blocks the expression of Bcl-6 (Thf differentiation) and NCOR-2 (Th17 maturation).

inside a single polycistronic transcript. This cluster sustains lymphocyte proliferation and inhibits cell death by targeting the tumor suppressor phosphatase and tensin homolog (PTEN) and the proapoptotic protein Bcl-2-like protein 11, commonly named Bim; indeed, lymphocyte-specific transgenic mice over-expressing the cluster die as a consequence of lymphoproliferative disease and autoimmunity (21, 22). In particular, the mir-17–92 cluster pushes toward a more pronounced pro-inflammatory type-1 phenotype, with increased IFN-γ production and, upon viral infection, miR-17–92 expression is required for clonal expansion of virus-specific Th1 and memory formation (23, 24). Two cluster members, miR-17 and miR-19b, are the key players controlling Th1 responses, supporting IFN-γ production and suppressing inducible Treg differentiation, with PTEN and TGFβ receptor 2 (TGFBR2) as the functionally primary targets of miR-19b and miR-17, respectively (25) (**Figure 1**). These two miRNAs are also essential during the induction of graft-vs.-host disease (GVHD) in mice, as the systemic administration of antagomir to block either one of the two significantly inhibits alloreactive T-cell expansion and IFN-γ production, and prolongs survival (26). During Tfh cell differentiation, the master transcription factor Bcl-6 represses

miR-17, miR-18a, and miR-20a and thus releases their repression on C-X-C motif chemokine receptor 5 (CXCR5), required for the migration of cells into follicles (27) (**Figure 1**). On the other hand, in a viral infection model, miR-17–92 acts as a critical regulator of Tfh cell differentiation by restraining the expression of genes "inappropriate" to this cell subset (28); in an airway inflammation model *in vivo*, miR-18a specifically targets three key transcription factors in the Th17 gene-expression program small mother against decapentaplegic 4 (SMAD4), hypoxia inducible factor 1α (HIF1α), and retinoid-related orphan receptor α (RORα), and blocks the differentiation of tissue Th17 cells expressing C-C chemokine receptor 6 (CCR6), RORγt, and IL-17A (29).

miR-155

Another relevant example of a miRNA with dramatic effect on Th subset differentiation is miR-155, which maps within an exon of the non-coding RNA *bic*. This gene is found highly expressed in activated B and T cells and lymphomas and miR-155 transgenic mice develop B cell malignancies (30–33). In conditions of miR-155 deficiency, $CD4^+$ Th cells proliferate normally upon TCR-stimulation but show a significant reduction of Th1 commitment and IFN-γ production and an increase in the number of IL-4 producing cells. miR-155 ability to skew Th differentiation away from the Th2 phenotype and attenuate Th2 cell responses *in vivo* depends on its capacity to directly suppress the transcription factor c-Maf, a potent trans-activator of the IL-4 promoter (34) (**Figure 1**). miR-155 is also able to promote Th1 differentiation and IFN-γ release through the modulation of the IFN-γ signaling by directly targeting IFN-γRα chain (**Figure 1**). Gain and loss-of-function analysis showed that miR-155 also positively regulates Th17 differentiation and induces the release of IL-17A through Janus kinase/signal transducer and activator of transcription (JAK/STAT). The direct target was suggested to be the suppressor of cytokine signaling 1 (SOCS1), which negatively feedbacks cytokine signal transduction (35) (**Figure 1**). Interestingly, in Th2 inducing conditions, miR-155 becomes unable to suppress the IFN-γRα messenger possibly because of preferential binding to high affinity Th2 specific mRNA targets, such as c-Maf, or because this suppression requires additional factors, including other miRNAs, exclusively expressed in Th1 cells (36).

THE BIDIRECTIONALITY OF CYTOKINE-miRNA RELATIONSHIP

A pivotal study has described the pleiotropic effect of TGF-β on the miRNome. SMADs, signal transducers of TGF-β, promote the expression of a plethora of miRNAs by facilitating the cleavage by Drosha, through the recognition of a consensus sequence within the stem region of miRNA primary transcripts, illustrating that TGF-β gene regulation also relies on miRNA modulation (37). Another example of cytokine-dependent miRNA regulation is recordable during the switch from a resting state to clonal expansion of antigen-activated Th lymphocytes, when the suppressor of proliferation Forkhead box protein O1 (FOXO1) is initially inactivated by post-translational modifications, and then post-transcriptionally inhibited by IL-2-induced miR-182 (38).

An intriguing case of miRNA-cytokine tango is that of miR-29a and IFN-γ. A wide screen for miRNA function in primary Th cells identified miR-29 as able to correct the aberrant IFN-γ expression associated with global miRNA deficiency. This miRNA targets both T-bet and EOMES, two transcription factors known to induce IFN-γ production, but it also suppresses IFN-γ production by directly targeting its mRNA (39).

The transgenic expression of a "sponge" target to compete with endogenous miR-29 targets in *Listeria monocytogenes* infected mice increased IFN-γ serum concentrations and decreased infection burdens, further suggesting that miR-29 suppresses immune responses to intracellular pathogens by targeting IFN-γ (40). The direct involvement of miR-29 in IFN-γ regulation remains controversial, as no correlation between miR-29a and IFN-γ expression of Th cells was observed in patients during active tuberculosis in more recent works (41, 42).

miRNA REGULATION OF TREG CELL IDENTITY AND THE CONTROL OF IMMUNE HOMEOSTASIS

In 2010, it was demonstrated that a single miRNA can control immune homeostasis. Treg specific deletion of miR-146a-5p resulted in a breakdown of immunological tolerance manifested in fatal IFN-γ dependent lesions in a variety of organs, associated with the augmented expression and activation of the direct target Signal transducer and activator of transcription 1 (STAT1) (43). Another study confirmed that miR-146a null mice lose peripheral T cell tolerance and die prematurely of a spontaneous autoimmune disorder, characterized by splenomegaly, lymphadenopathy, and multiorgan inflammation (44). miR-146a is part of a regulatory negative feedback loop that controls TCR signaling to NF-κB and the resolution of Th responses: mice Th cells lacking miR-146a are hyperactive in both acute antigenic and chronic inflammatory autoimmune responses because in physiological conditions TCR-driven NF-κB activation up-regulates the expression of miR-146a, which in turn down-regulates NF-κB activity, at least partly through repressing the NF-κB signaling transducers TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) (45). Upon Treg induction, TGF-β is able to specifically induce miR-10a. By simultaneously targeting the transcriptional repressor Bcl-6 and the corepressor nuclear receptor corepressor 2 (NCOR2), miR-10a hampers the phenotypic conversion of Treg into Tfh cells and at the same time blocks differentiation into the Th17 subset. In other words, TGF-β can fine-tune the plasticity and fate of Th cells also through the specific induction of a single miRNA (46) (**Figure 1**). Notably, although under basic conditions miR-17–92-deficient Treg cells are able to maintain immune homeostasis, the expression of miR-17–92 cluster (above described as central for Th1 differentiation) reveals to be also critical for the accumulation of activated antigen-specific Treg, the differentiation into IL-10-producing effector cells and clinical remission from experimental autoimmune

encephalomyelitis (EAE, a model of human multiple sclerosis) (47). Furthermore, while Treg cells do not seem to need miR-155 to exert their suppressive function, FOXP3 positively regulates miR-155 expression and this miRNA deficiency impairs Treg development by increased levels of SOCS1 and reduced responsiveness to IL-2 (48, 49).

GENETIC BASIS OF miRNA REGULATION

A remarkable work of miRNA expression quantitative trait loci (miR-eQTL) analysis discovered that most of these loci are located upstream of their associated intergenic miRNAs by mapping more than five thousand individuals. Cis-miR-eQTLs miRNAs display differential expression in relation to the corresponding trait, and distal regulatory elements may also affect interindividual variability associated with a variety of complex traits (50). A single-miRNA based meta-analysis has extensively reviewed studies suggestive of an association between the miR-146a single nucleotide polymorphisms (SNPs) and susceptibility to autoimmune diseases confirming that specific miR-146a SNPs are associated with susceptibility to multiple sclerosis (MS) and systemic lupus erythematosus (SLE) (51). miRNA regulation can also change in response to genetic variants in the 3′ untranslated region (UTR) of mRNA targets that may affect mRNA stability, translation and miRNA binding. An SNP inside the IKAROS Family Zinc Finger 3 (IKZF3) gene is predicted to create a new recognition site for miR-326 and lead to significantly lower levels of IKZF3 in subjects carrying the allele. IKZF3 is a transcription factor important for B-cell activation, and the lack of this gene causes a lupus like syndrome in mice, suggesting a role for the regulatory loop of IKZF3 and miR-326 in autoimmunity (52). On the other hand, Steri et al. described a genetic variant located in the 3′UTR of the TNF Superfamily Member 13 (TNFSF13B) gene which shortens the untranslated region and deletes a miR-15a binding site. As a consequence, the protein encoded by this mRNA, BAFF, a soluble cytokine important for B cell development, and differentiation, increases in the blood of variant individuals, leading to augmented circulating B cells and immunoglobulins and an increased susceptibility to MS and SLE (53). A significant effort of data integration has more recently linked the prediction of SNPs affecting miRNA binding sites, statistics from 12 studies on different autoimmune diseases, public expression quantitative trait locus (eQTL) data and mRNA/small RNA-seq data and succeeded to reveal new autoimmune disease non-coding risk SNPs that might be involved in the miRNA-dependent causal mechanisms, providing valuable information for further functional studies (54).

miRNAs AS POTENTIAL THERAPEUTIC TARGETS IN AUTOIMMUNITY

The capability of miRNAs to skew Th subset differentiation candidates them as therapeutic targets in autoimmune conditions. T cell-specific miR-17–92 deficiency reduces Th17

TABLE 1 | A list of bibliographic references for the reported functional links between miRNAs and cytokines (either direct or indirect), ranked according to miRNA nomenclature.

	Direct or Indirect Cytokine Target	System	Cellular Type	PMID
miR-7	IL-6 [↑]	Human	PBMCs	27749601
miR-9	IL-2 [↑] IFN-γ [↑]	Human	$CD4^+$ T cells	22585398
miR-10a	IL-12 [↓] IL-23 [↓]	Human	Dendritic cells $CD4^+$ T cell	25281418
	IFN-γ [↑]	Human	Treg cells	23825948
miR-10b	IL-17A [↓]	Human	$CD4^+$ T cells Th17 cells	28039186
miR-15a/16-1	IL-22 [↓]	Mouse	$CD4^+$ T cells	29023933
miR-17, miR19b (miR-17∼92)	IFN-γ [↑]	Mouse	$CD4^+$ T cells	26138686
[-10pt]	IFN-γ [↑]	Mouse	Th1 cells	21972292
miR-18 (miR-106∼363)	IL-17A [↓]	Mouse	$CD4^+$ T cells	28617945
miR-19 (miR-17∼92)	IL-4 [↑] IL-5 [↑] IL-13 [↑]	Human	$CD4^+$ T cells	25362490
miR-20a-5p (miR-17∼92)	IL-17 [↓]	Human	$CD4^+$ T cells	28972028
miR-20a (miR-17∼92)	IL-2 [↓] IL-6 [↓] IL-8 [↓] IL-10 [↓]	Human	$CD4^+$ T cells	25884400
miR-20b	IL-17 [↓]	Mouse	$CD4^+$ T cells	24842756
miR-21	IL-4 [↑] IL-5 [↑] IL-12-p35 [↓] IL-13 [↑]	Mouse	$CD4^+$ T cells $CD8^+$ T cells	28379062
	TGF-β [↓]	Human	Plasma Treg cells	26383248
	TGF-β [↓]	Mouse	Bone marrow MSC	26086742
	TNF-α [↑] IFN-γ [↑] IL-17A [↑]	Mouse	T cells	23395552
	IL-12 [↓] IL-4 [↑] IFN-γ [↓]	Mouse	Dendritic cells $CD4^+$ T cells	21849676
miR-23a cluster	IFN-γ [↓]	Human	$CD8^+$ T cells	25030422
miR-24	IFN-γ [↓]	Human	$CD4^+$ T cells	24704866

(Continued)

TABLE 1 | Continued

	Direct or Indirect Cytokine Target	System	Cellular Type	PMID
miR-25 (miR-106b∼25)	TGF-β [↓]	Human	Treg cells	20637509
miR-26a	IL-6 [↓]	Mouse	$CD4^+$ T cells	25728641
miR-27	IL-4 [↓] IL-5 [↓]	Human, mouse	$CD4^+$ T cells	22088562
miR-29	IFN-γ [↓]	Human	$CD4^+$ T cells	22772450
	IL-32nonα [↓]	Human	PBMCs $CD4^+$ T cells $CD14^+$ monocytes	25808800
	IFN-γ [↓]	Mouse	$CD4^+$ T cells, $CD8^+$ T cells	21706005
	IFN-γ [↓]	Mouse	$CD4^+$ T cells	21820330
miR-30a	IL-17A [↓]	Human, Mouse	$CD4^+$ T cells	27581464
	IL-17A [↓] IL-17F [↓]	Human, Mouse	$CD4^+$ T cells	27006279
miR-31	IFN-γ [↑] IL-2 [↓] IL-4 [↓]	Human	$CD4^+$ T cells	26978146
	IL-2 [↑]	Human	T cells	23303246
miR-101	IL-2 [↓]	Human	$CD4^+$ T cells	27898347
miR-106a (miR-106∼363)	IL-17A [↓]	Mouse	$CD4^+$ T cells	28617945
miR-106b (miR-106b∼25)	TGF-β [↓]	Human	Treg cells	20637509
miR-125b	CCL4 [↓]	Human	Monocytes $CD8^+$ T cells	25620312
	IFN-γ [↓] IL-2 [↓]	Human	$CD4^+$ T cells	21706005
miR-126	IFN-γ [↓]	Mouse	$CD4^+$ T cells	28987000
miR-128	IL-4 [↓] IL-5 [↓]	Human, mouse	$CD4^+$ T cells	22088562
miR-146a	IL-6 [↓] IL-21 [↓]	Mouse	$CD4^+$ T cells	28872459
	TGF-β [↑]	Mouse	Dendritic cells	26700406
	IL-10 [↑]	Mouse	Monocytes	26526003
	IFN-γ [↓] IL-2 [↓] IL-17 [↓]	Mouse	T cells	22891274
miR-150	IL-10 [↑]	Human	$CD4^+$ T cells	26746193
	IL-2 [↓] TNF-α [↓]	Human	$CD4^+$ T cells	26549736
miR-155	IL-17 [↑]	Human	$CD4^+$ T cells	28471953
	IL-6 [↑] IL-23 [↑] IL-1β [↑] TNF-α [↑] IL-17A [↑]	Mouse	Dendritic cells $CD4^+$ T cells	27052830
	IFN-γ [↑] IL-17 [↑]	Rat	$CD4^+$ T cells	26349986
	IL-21 [↑]	Human	$CD4^+$ T cells	26055806
	IL-17 [↑]	Human	$CD4^+$ T cells	25761610
	IL-17 [↑]	Mouse	Dendritic cells $CD4^+$ T cells	25651871
	IFN-γ [↑]	Mouse	$CD4^+$ T cells	24891206
	IL-13 [↑]	Mouse	$CD4^+$ T cells	25024218
	IL-9 [↑] IL-10 [↑] IL-22 [↑]	Mouse	$CD4^+$ T cells	24856900
	IL-2 [↓]	Human	$CD4^+$ T cells	22785227
	IL-17 [↑]	Mouse	Th17 cells	23686497
	IL-17 [↑]	Mouse	Th17 cells	23091595
	IFN-γ [↑]	Mouse	T cells	23200854
	IL-17A [↑] IL-6 [↑] IL-12 [↑] IL-23 [↑] TNF-α [↑]	Mouse	$CD4^+$ T cells Dendritic cells	20888269
	IL-4 [↓] IL-5 [↓] IL-10 [↑]	Mouse	$CD4^+$ T cells	17463290
miR-181	IFN-γ [↓]	Human	$CD4^+$ T cells	24704866
miR-181c	IL-2 [↓]	Human	$CD4^+$ T cells	21112091
miR-182	IL-2 [↓]	Human	Treg cells	23825948
miR-200a	IL-17 [↑] IL-23 [↑]	Human	$CD4^+$ T cells	28738533
	IL-2 [↑]	Mouse	$CD4^+$ T cells	28438897
miR-210	TNF-α [↑]	Human	$CD8^+$ T cells	27749601
	IL-17 [↓]	Mouse	T cells	24608041

(Continued)

TABLE 1 | Continued

	Direct or Indirect Cytokine Target	System	Cellular Type	PMID
miR-212/132	IL-10 [↓]	Mouse	$CD4^+$ T cells	25862525
	IL-17 [↑]	Mouse	$CD4^+$ T cells	23818645
miR-301a	TNF-α [↑] IL-17 [↑]	Human Mouse	$CD4^+$ T cells Th17 cells	26338824
	IL-17 [↑]	Mouse	$CD4^+$ T cells	22517757
miR-326	IL-17 [↓]	Human	$CD4^+$ T cells	27454344
	IL-17 [↑]	Human	Th17 cells	19838199
miR-340	IL-4 [↓] IL-5 [↓]	Human, mouse	$CD4^+$ T cells	22088562
miR-363-3p (miR-106∼363)	IL-17A [↓]	Mouse	$CD4^+$ T cells	28617945
miR-425	IL-2 [↓] IFN-γ [↓]	Human	$CD4^+$ T cells	28192189
Let-7 family	IL-10 [↓]	Human	$CD4^+$ T cells	22586040
	IL-13 [↓]	Human	T cells	21616524
Let-7a	IL-13 [↓]	Mouse	$CD4^+$ T cells	20630862
Let-7e	IL-4 [↓] IL-10 [↓] IL-17 [↑] IFN-γ [↑]	Mouse	$CD4^+$ T cells, CNS-mononuclear cells	23079871
Let-7f	IL-17 [↓]	Human	$CD4^+$ memory T cells	21508257
Let-7i	IL-2 [↑]	Human	$CD4^+$ T cells	27145859
	IL-10 [↓]	Rat	Dendritic cells	26755202

The species in which the observation was made and the cell type are also registered.

differentiation and ameliorates EAE symptoms, identifying this miRNA cluster as a potential target for the clinical intervention of MS (55). miR-155 expression is found highly elevated in heart tissue in an inflammatory cardiac disease driven by autoantigen-specific $CD4^+$ Th cells (experimental autoimmune myocarditis, EAM) and miR-155 inhibition results in attenuated severity of disease and cardiac injury, reduced Th17 immune response, and decreased dendritic cell function of secreting Th17-polarizing cytokines. Th cells from miR-155-inhibited EAM mice exhibit reduced proliferation and IL-17A secretion in response to autoantigens. These findings demonstrate that miR-155 adversely promotes inflammation by driving a Th17/Treg imbalance in favor of Th17 cells, and anti-miR-155 treatment can significantly reduce the autoimmune response (56). miR-155 was also proposed as a therapeutic target in a model of Th1/Th17-related inflammation during chronic cardiac rejection (57). Furthermore, *in vivo* silencing of let-7e, found up-regulated in Th cells of EAE mice, is able to inhibit encephalitogenic Th1 and Th17 cells and attenuate the disease, with reciprocal promotion of Th2 cell maturation (58). miR-340 is increased in memory Th cells from patients with MS, and favors pro-inflammatory Th1 responses while inhibiting Th2 cell development. These effects are mediated by IL-4 direct suppression, resulting in decreased GATA3 levels, and a Th2 to Th1 cytokine shift; treatment of Th cells from MS patients with miRNA inhibitors leads to the restoration of Th2 responses (59). Finally, miR-146a-deficient mice develop more severe EAE, with Th cells being more prone to differentiate into Th17 cells. In these animals, an enhancement of IL-6- and IL-21-induced Th17 differentiation pathway suggests miR-146a functions as a molecular stop signal for this autocrine pathway in autoreactive cells, and highlights miR-146a potential as a therapeutic target for treating autoimmune diseases (60).

THE EXTRACELLULAR VESICLE-ASSOCIATED miRNAs AS NOVEL MEDIATORS OF INFLAMMATION

Most cells in the body release membrane bound vesicles of nanometric size (from 50 nm to 1 micron), either formed by the inward budding of multi-vesicular endosomes and subsequent fusion to the plasma membrane (exosomes), or directly budding from the plasma membrane (61, 62). Vesicle lumen contains miRNAs and other non-coding RNAs, not randomly but instead preferentially exported (63–67). Th subsets also release miRNAs not passively mirroring specific signatures at the intracellular level (68, 69). miRNA expression in Treg-cell-derived exosomes are distinct from that of pro-inflammatory Th1/Th17 subsets, suggesting a regulatory mechanism enforcing subset-specific vesicular diversity (69, 70). Extracellular vesicles (EVs) play an important role in T cell-to-cell communication, intervening in antigen presentation, cell stimulation, differentiation, cell killing, cytokine transport and stability, tolerance induction and allograft rejection (71–84). In both human and mouse, gene silencing mediated by miRNA-containing EVs was shown to participate into Treg-dependent immune suppression (69, 70).

The hypothesis that miRNA release into the microenvironment adds a further mechanism of plasticity to fine-tune specific Th responses at the paracrine level *in vivo*, is strengthened by the finding of miRNA-containing EVs in all tested biological fluids [blood, urine, saliva, breast milk, among others (85–92)], that suggests also an endocrine role. Very recently, systemic extracellular miRNA dysregulation in MS was implicated in the reduced frequency and dysfunctional suppression of Treg cells in disease. Kimura et al. showed that induction of human IFN-γ^-IL-17A$^-$FOXP3$^+$CD4$^+$ T cells is inhibited in the presence of patient (compared with healthy) blood exosomes, and that the exosomal miRNA profile of patients is characterized by significantly higher level of let-7i, able to target insulin like growth factor 1 receptor (IGF1R) and TGFBR1 in naïve Th cells (upon up-take of let-7i containing

exosomes) and suppress induction of Treg cells, thus fueling MS pathogenesis (93).

Therefore, extracellular miRNAs may represent novel pathogenic mediators in the onset of autoimmune reactions and potential therapeutic targets in these diseases.

CONCLUSIONS

miRNAs are "rheostats," capable to fine-tune mammalian gene expression. Single miRNAs may only marginally regulate target genes but, when the cell responds to environmental changes, the coordinated modulation of tens of miRNAs altogether is a powerful strategy to efficiently affect many components of a genetic network. We have described the most relevant examples, but a more exhaustive list of miRNA-dependent cytokine modulation is reported in **Table 1**.

Studies in different Th subsets concur to show that miRNAs are able to direct differentiation by restraining the expression of genes "*inappropriate*" to that specific cell subset, including cytokines characterizing the function of other subsets. Furthermore, master regulatory transcription factors positively induce Th differentiation also through "repression of miRNA-based repression" of genes "*appropriate*" to that specific cell subset. In most cases, a single miRNA targets different sets of mRNAs depending on cell context and the co-expression of other miRNAs and/or higher affinity gene targets, resulting in different functional outputs. Finally, the contiguity of different Th subsets, or better their (not yet completely revealed) plasticity, is also evident when considering that the same miRNAs are crucial in the differentiation of functionally divergent subsets such as Th1/17 and Treg. Hence, we need to not only identify *which* miRNAs regulate *which* cytokines but also frame the mechanistic miRNA regulation in a *subset-specific context*. The picture is further complicated by EV-associated miRNAs traveling in the extracellular space and becoming regulatory signals in cell-to-cell communication likewise cytokines themselves.

In conclusion, if we want to take advantage of the powerful regulatory action of miRNAs for therapeutic purposes, in the next years we will have to fully untangle the intricate web of miRNA-target genes to safely re-direct the differentiation and function of $CD4^+$ Th cell subsets in pathological conditions such as autoimmunity.

AUTHOR CONTRIBUTIONS

PdC conceived the article. PdC and SG collected and assembled data and wrote the manuscript. VD substantially contributed to draft writing and critical revision of the article. All authors approved the final version of the manuscript.

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REVIEW
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Circulating Y-RNAs in Extracellular Vesicles and Ribonucleoprotein Complexes; Implications for the Immune System

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The exchange of extracellular vesicles (EV) between immune cells plays a role in various immune regulatory processes. EV are nano-sized lipid bilayer-enclosed structures that contain a multitude of proteins and small non-coding RNA molecules. Of the various RNA classes present in EV, miRNAs have been most intensively studied because of their known gene-regulatory functions. These miRNAs constitute only a minor part of all EV-enclosed RNA, whereas other 20–200 nt sized non-coding RNAs were shown to be abundantly present in EV. Several of these mid-sized RNAs perform basic functions in cells, but their function in EV remains elusive. One prominent class of mid-sized extracellular RNAs associated with EV are the Y-RNAs. This family of highly conserved non-coding RNAs was initially discovered as RNA component of circulating ribonucleoprotein autoantigens in serum from Systemic Lupus Erythematosus and Sjögren's Syndrome patients. Y-RNA has been implicated in cellular processes such as DNA replication and RNA quality control. In recent years, Y-RNA has been abundantly detected in EV from multiple different cell lines and biofluids, and also in murine and human retroviruses. Accumulating evidence suggests that EV-associated Y-RNA may be involved in a range of immune-related processes, including inflammation, immune suppression, and establishment of the tumor microenvironment. Moreover, changes in plasma levels of extracellular Y-RNA have been associated with various diseases. Recent studies have aimed to address the mechanisms underlying their release and function. We for example showed that the levels of EV-associated Y-RNA released by immune cells can be regulated by Toll-like receptor (TLR) signaling. Combined, these data have triggered increased interest in extracellular Y-RNAs. In this review, we provide an overview of studies reporting the occurrence of extracellular Y-RNAs, as well as signaling properties and immune-related functions attributed to these RNAs. We list RNA-binding proteins currently known to interact with Y-RNAs and evaluate their occurrence in EV. In parallel, we discuss technical challenges in assessing whether extracellular Y-RNAs are contained in ribonucleoprotein complexes or EV. By integrating the current knowledge on extracellular Y-RNA we further reflect on the biomarker potential of Y-RNA and their role in immune cell communication and immunopathology.

Keywords: Y-RNA, extracellular vesicles, exosomes, ribonucleoprotein complexes, immune signaling, biomarker

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INTRODUCTION

Extracellular vesicles (EV) are 50–300 nm sized lipid bilayer-enclosed vesicles containing proteins and nucleic acids (1), which are released by virtually all cells. All living cells, including archaea, bacteria, and eukaryotes release EV, which suggests that the release of EV is a conserved mechanism of cellular communication (2, 3). EV have been found in many body fluids and have been implicated in several diseases, including immune-related disorders, cancer, neurological disorders and cardiovascular diseases (4–7). Characterizing the protein, lipid, and RNA content of EV is an active area of research. One of the major topics in the field is to delineate how differences in EV composition relate to differences in their function, and to determine whether differences in the protein/RNA content of EV can be used as biomarkers for disease.

It has been shown that EV-enclosed RNAs can be functionally transferred to target cells (8–11). Many studies have focused on elucidating the miRNA composition of EVs because of their known effects on gene regulation. However, miRNAs only constitute a minor percentage of EV-enclosed RNA. In contrast, the majority of EV-RNA consists of other types of small- to mid-sized non-coding RNAs [20–200 nt] (12–16). Of these RNAs, Y-RNA attracted attention because this conserved RNA has been detected in EV from many different cell types and in various vertebrate species (12–14, 16–18). Moreover, Y-RNAs are highly abundant in body fluids, such as blood and seminal fluid (19, 20). Recent data indicate that as much as 67% of sequencing reads in plasma samples of healthy donors map to Y-RNA (19). There are also indications that the levels of Y-RNA in body fluids could correlate with disease (21, 22). Research on the regulation of Y-RNA sorting into EV and the function of EV-associated Y-RNA is in its early days. Our laboratory recently showed that incorporation of Y-RNA in EV released by dendritic cells is regulated by immunogenic and tolerogenic stimuli imposed on these cells (16). Initial studies on the function of EV-enclosed Y-RNA reported pro- and anti-inflammatory effects (23–25). Given the increasing interest in and number of publications on extracellular Y-RNA we took the initiative to compile an inventory of data and assess the inter-study comparability of discoveries in this field. In this review, we provide an overview of reports describing the occurrence of extracellular Y-RNA in EVs from various cell types and biofluids, as well as its signaling properties and potential immune-related functions. After introducing general aspects of EV-associated RNA and the role of Y-RNA inside cells, we summarize current knowledge on Y-RNA association with EV and with extracellular ribonucleoprotein complexes. In addition, we provide an overview of protein partners of Y-RNA that have also been detected in EV and may therefore be involved in sorting these RNAs into EV. Finally, we provide an overview of the proposed functions of extracellular Y-RNA and reflect on its biomarker potential. Key steps in the Y-RNA life cycle, putative pathways for Y-RNA release into the extracellular space, and ideas on the function of Y-RNA transferred to target cells are illustrated in **Figure 1**.

INTERCELLULAR COMMUNICATION VIA EV-ASSOCIATED RNA

EV constitute a unique way in which molecular messages are exchanged between cells. Upon transfer, the lipids, proteins, and RNA associated to EV can modify the function of recipient cells (1, 3, 26–28). EV are either formed by inward budding into multivesicular bodies, which upon fusion with the plasma membrane are released as exosomes, or by direct budding off the plasma membrane (microvesicles) (1, 3). Exosomes and microvesicles cannot be separated using currently available methods, and are therefore collectively referred to as EV. EV are heterogeneous in size and molecular composition, but unique molecular markers to distinguish biologically distinct EV subtypes are yet to be discovered. Various classes of RNA have been detected in EV, including mRNA, lncRNA, circRNA, and small non-coding RNA. Most of the EV-RNA consists of small non-coding RNA types, such as miRNA, tRNA, rRNA, snoRNA, Y-RNA, SRP-RNA (7SL), and Vault RNA (12, 13).

It is important to realize that not all extracellular RNA is associated with EV. Other macromolecular structures in the extracellular milieu, such as ribonucleoproteins (RNPs) and lipoprotein particles, also contain RNA (16, 29–31). These other structures overlap in size and/or density with EV and are therefore frequent contaminants in EV preparations (32, 33). The degree to which some of these contaminants co-isolate with EV depends on the fluid used as source of EV and the applied EV isolation method. The most widely used techniques are ultracentrifugation, size exclusion chromatography (SEC), and density gradient centrifugation, in which particles are separated based on mass, size, or buoyant density, respectively. Ultrafiltration-based methods concentrate particles by molecular weight. Precipitation-based methods, on the contrary, concentrate all macromolecules in solution. The advantages and disadvantages of available methods have been extensively reviewed (32, 34). Sequential application of methods that separate particles based on size and on density likely yields the purest EV preparations.

It has been demonstrated that both the protein- and miRNA composition of EV can change upon exogenous stimuli imposed on EV-producing cells (9, 13, 16). These changes in the "molecular message" that is conveyed via EV can lead to alterations in the function of EV-recipient cells. We have previously shown that, in addition to miRNA, the levels of Y-RNA and snoRNA in EV are regulated by exogenous stimuli imposed on the EV-producing immune cells (16). Importantly, the activation-induced changes in EV-RNA composition we observed only partly reflected changes in cellular RNA, which suggests that the cell stimuli triggered mechanisms for sorting of specific RNA types into EV (16).

Several *in vitro* and *in vivo* studies have demonstrated that intercellular transfer of EV-associated miRNA and mRNA leads to changes in recipient cell function (8, 10, 32, 35–37). For example, EV-mediated transfer of miR-155 and miR-146a from wildtype dendritic cells to recipient cells deficient for these miRNAs modulated the response of these recipient cells to

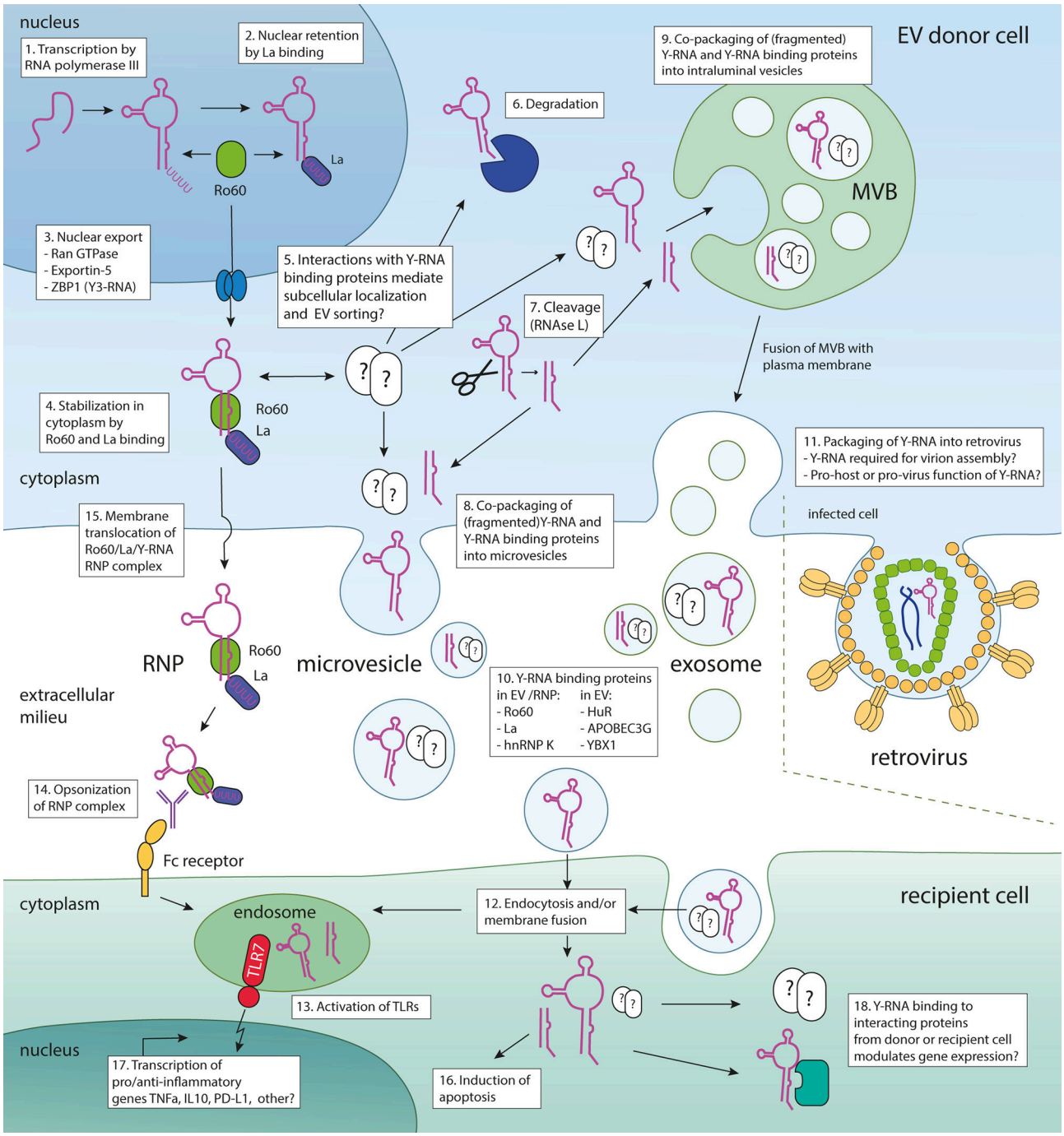

FIGURE 1 | Model illustrating the Y-RNA life cycle, putative pathways for Y-RNA release into the extracellular space, and ideas on the function of Y-RNA transferred to target cells. Various steps in the process of Y-RNA transport within cells and between cells are indicated numerically. Upon transcription (1), newly generated Y-RNA may remain in the nucleus through binding of La (2). Alternatively, upon binding of Ro60, it can be transported into the cytoplasm by Ran GTPase, Exportin-5 and/or ZBP1 (3). In the cytoplasm, Ro60 binding stabilizes Y-RNA (4). Y-RNA can also bind to various other RNA-binding proteins (for instance the Y-RNA binding proteins summarized in **Table 3**) that may influence its subcellular localization and/or fate (5). Y-RNA may be degraded (6), or be cleaved into fragments by RNAse L (7). Both full-length and fragmented Y-RNA are packaged into EV, either via passive engulfment of Y-RNA by budding membranes, or through protein-mediated shuttling toward sites of EV biogenesis [such as the plasma membrane (8) or late endosomes/multivesicular bodies (9)]. Certain proteins known to bind Y-RNA are co-packaged into EV, but others may only serve to shuttle Y-RNA to the sites of EV biogenesis. The Y-RNA binding proteins from **Table 3** that have been found in extracellular space, associated with EV and/or RNP are listed in (10). In the case of retrovirus infected cells, Y-RNA may be additionally released from cells by incorporation into virions (11). EV can be taken up by recipient cells by endocytosis and/or membrane fusion (12). Y-RNA may be delivered to the endosome, where it

(Continued)

FIGURE 1 | may activate TLRs (13). TLR triggering also occurs after uptake of opsonized Y-RNA/Ro60/La RNP complexes (14) which may be released from cells after translocation across the cellular membrane (15). Naked Y-RNA has been shown to induce apoptosis (16). TLR triggering of Y-RNA drives the transcription of various pro- and anti-inflammatory cytokines (17). On a more speculative note, transferred Y-RNA could affect the function of recipient cells through the action of Y-RNA binding proteins present in recipient cells or co-transferred by the EV (18). For example, binding to translation enhancer proteins, such as HuR and HuD, may alter mRNA stability and translation efficiency.

lipopolysaccharide (LPS). Transfer of miR-155 into miR-155 negative recipient cells increased IL6 release via repression of SHIP1 and BACH1, while transfer of miR-146a dampened this LPS response by repression of TRAF6 and IRAK1 (10). Functional transfer of mRNA was evidenced by demonstrating that EV-associated mRNA derived from *in vitro* cultured mast cells could be translated in recipient cells (8). *In vivo* evidence for EV-mediated transfer of mRNA was provided by the use of Cre-Lox mouse models. Hematopoietic cells or tumor cells expressing Cre-recombinase were shown to release EV containing Cre-mRNA, which induced recombination-mediated expression of floxed fluorescent reporter genes in recipient cells at local or distant sites (36, 38). The functional effects of other RNA classes, which compose the major part of all EV-RNA, are beginning to be unveiled. The experimental approaches used to study miRNA transfer may serve as a basis to gain understanding of how other EV-associated RNA classes affect recipient cell behavior, but these RNAs likely exert their functions via mechanisms other than base-pairing with RNA targets. Although many questions remain to be answered, EV-mediated transfer of RNA appears to be a common, frequent, and adaptable process that cells employ to communicate with other cells.

INTRACELLULAR LOCATION AND FUNCTION OF Y-RNAs

In order to unravel the role of Y-RNA in EV, it is important to understand the function of Y-RNA inside cells. Y-RNAs have been studied for many years and multiple comprehensive reviews are available on this topic (39–44). Y-RNAs were initially discovered as RNA components of circulating ribonucleoprotein (RNP) autoantigens Ro60 and La in serum from lupus patients (45). These RNP are major targets for autoimmune responses in rheumatic diseases such as Systemic Lupus Erythematosus (SLE) and Sjögren's Syndome (SS) (46, 47). Y-RNAs are well-conserved through evolution and have been found in all vertebrate species (48, 49), and related ncRNAs have been found in some bacteria (44) and in nematodes (50, 51). Although the nematode ncRNAs called "stem-bulge RNAs" resemble Y-RNA because of their stem-loop structure (51), they differ from bona fide Y RNAs in that they have not been found complexed with Ro60 in cells (50). The human genome encodes four different Y-RNAs (hY1, hY3, hY4, and hY5) while only two different Y-RNAs exist in rodents (mY1 and mY3) (52). All Y-RNAs contain a long stem, formed by basepairing the 5′ and 3′ ends, that contains the Ro60 binding site, but individual Y-RNAs differ slightly in their primary and secondary structures (53).

Y-RNAs are transcribed in the nucleus by RNA polymerase III (54) (**Figure 1**, box 1). Binding of La to the 3′ oligo-uridine tail of Y-RNA mediates its nuclear retention and protects Y-RNA from 3′ to 5′ exonucleolytic degradation (55, 56) (**Figure 1**, box 2). Binding of Ro60 to the stem region of Y-RNA enhances nuclear export (55), which is mediated by Ran GTPase and exportin-5 (57) (**Figure 1**, box 3). Y3-RNA can also be exported via an alternative pathway through binding of Ro60/Y3-RNA to zipcode binding protein (ZBP1), enabling export via exportin-1 (alternatively named CRM1) (58). It is not fully understood whether Y-RNA is transported from the nucleus in complex to La, or whether La reassociates to Y-RNA after nuclear export. Binding of Ro60 stabilizes Y-RNA in the cytoplasm (**Figure 1**, box 4), as knockout of Ro60 was shown to drastically reduce Y-RNA levels (59). The loop of Y-RNA is known to interact with various other proteins including nucleolin (60), polypyrimidine tract-binding protein (PTB/hnRNP I) (61), and zipcode-binding protein 1 (ZBP1) (62). It has been proposed that interactions with these proteins could affect the localization and/or function of Y-RNA (43) (**Figure 1**, box 5). Conversely, Y-RNA can influence the localization of Y-RNA binding proteins, since siRNA-mediated knockdown of Y-RNA leads to nuclear accumulation of Ro60 (63).

Various housekeeping functions of Y-RNA have been described, such as involvement in DNA replication (43) and quality control of non-coding RNA (64, 65). The effects of Y-RNA on DNA replication were first observed in cell-free reactions, in which addition of purified Y-RNA subtypes increased the percentage of dividing nuclei (66). siRNA-mediated knockdown of Y1-RNA in cells was sufficient to reduce the percentage of cells in S-phase, during which DNA replication takes place (66). In a later study, association of Y-RNA with chromatin was shown to increase 2–4 fold during S-phase and to decrease during G1 phase and mitosis, which suggests an association with the origin replication complex (ORC) (67). It has been shown that a specific sequence in the Y-RNA stem was sufficient to increase DNA replication in cell-free reactions (68). However, Ro60 knockout cells that contain ∼30-fold lower Y-RNA levels did not show reduced growth rates (59). The exact molecular mechanisms by which Y-RNA affects DNA replication therefore remain unresolved.

Y-RNAs are involved in regulating the degradation of misfolded RNAs through its interaction with Ro60 (47, 64, 69). Misfolded RNAs that contain a 3′ single-stranded end and adjacent helices can bind Ro60 (70, 71). This has been shown for 5S rRNA in *Xenopus* (72) and for U2 snRNA in mouse embryonic stem cells (59), and suggested for a wider variety of structured RNAs (70). Structural analyses revealed that the single-stranded tail of the misfolded RNAs extend through the Ro60 cavity, while helices bind on its outer surface (70). Y-RNAs sterically

blocked binding of misfolded RNAs to Ro60, thereby regulating the RNA quality control function of Ro60 (64). A similar mechanism has also been demonstrated for the bacterial ortholog of Ro (*r*o-*s*ixty *r*elated, Rsr) (71). In the bacterium *Deinococcus radiodurans* Y-RNA tethers Rsr to the exonuclease PNPase, thereby forming a RNA-degrading RNP complex resembling the eukaryotic exosome (71). It was proposed that tethering to Rsr potentiates PNPase to specifically degrade structured RNAs. Although mammalian PNPases localize inside mitochondria, it has been proposed that Y-RNAs could potentially tether Ro60 to other proteins involved in RNA metabolism, including exoribonucleases, helicases or RNA chaperones (40).

Interestingly, it was recently discovered in neuronal cells that Y3-RNA can act as a molecular sponge for the enhancer protein HuD (ELAVL4) (73). HuD can enhance gene expression by binding and stabilizing the 3′ untranslated regions (UTRs) of specific mRNAs involved in motor neuron differentiation and axonogenesis. This activity is counteracted by Y3-RNA binding to HuD, which leads to changes in HuD localization and reduced expression of the involved mRNAs (73). Moreover, dysregulation of Y-RNA binding to HuD has been found to cause alternative splicing in neurons of Alzheimer patients (74).

In cells, Y-RNA does not only occur in its full length form, but has also been shown to be cleaved into specific fragments of 25–35 nt. This cleavage, which is carried out by the enzyme RNase L (75) (**Figure 1**, box 7) occurs in response to UV irradiation or by polyI:C-mediated activation of the innate immune system (76, 77). Because Y-RNA fragments arise from conserved ends of the Y-RNA hairpin and have comparable sizes to miRNAs, it was proposed that Y-RNA fragments function similar to miRNAs (78). Although interactions of Y-RNA fragments with Argonaute have been demonstrated, mRNA reporter constructs could not be repressed by Y-RNA/Argonaute complexes (79).

Taken together, the highly conserved family of Y-RNAs interacts with, and regulates the localization and activity of various RNA-binding proteins involved in basic cell functions.

Y-RNAs ARE ABUNDANTLY PRESENT IN THE EXTRACELLULAR MILIEU

RNA sequencing studies aiming to characterize the small transcriptome of EV have indicated that cells release Y-RNAs into the extracellular milieu (12–18, 20, 24, 25, 31, 80). There is now strong evidence that Y-RNAs are abundantly present both in supernatants of multiple *in vitro* cultured primary and immortalized cell types, as well as in various biofluids (see **Table 1**). In fact, Y-RNA was found to be the most abundant non-coding RNA species in plasma from healthy individuals (19). Multiple studies reported a strong enrichment of Y-RNA in EV relative to intracellular levels, which suggests that the shuttling of Y-RNA into EV is highly efficient (12–14, 25, 31).

The frequent detection of Y-RNAs in the external milieu of cells suggests that release of Y-RNAs from cells is a common and ubiquitous process. We compared the abundance of Y-RNA subtypes reported in each of the RNA sequencing studies and ranked these from 1 (highest) to 4 in **Table 1**. Although differences exist between studies that used different cell types or EV-purification methods, Y4 is most abundantly detected in the

TABLE 1 | Overview of RNA sequencing studies reporting the presence of extracellular Y-RNA in *in vitro* cell cultures or in body fluids.

References	Sample type	EV-enrichment	RNA size selection?	Y1	Y3	Y4	Y5
Cambier et al. (24)	Cardiosphere derived cells (CDC)	EV precipitation	No	2	3	1	4
Haderk et al. (25)	Chronic leukemic lymphocytes	UC pellet (100,000 g)	No	2	4	1	3
Kaudewitz et al. (81)	Platelet rich and platelet poor plasma	No	No	3	4	1	2
Dhahbi et al. (82)	Plasma	No	No	n.d.	n.d.	1	n.d.
Vojtech et al. (20)	Seminal fluid	UC pellet (100,000 g)	No	4	3	1	2
Tosar et al. (14)	MCF7 and MCF-10A breast cancer cell lines	UC pellet (100,000 g)	<60 nt	3	2	1	4
van Balkom et al. (17)	Human endothelial cells	Density gradient	No	2	4	2	1
Chakrabortty et al. (80)	K562 myelogenous leukemia and BJ primary fibroblast	EV precipitation	<200 nt	-	-	-	1
Repetto et al. (22)	Primary macrophages	No	No	3	4	2	1
Shurtleff et al. (15)	HEK293T cell line	Density gradient	No	3	1	2	4
Wei et al. (31)	Glioblastoma cell line	Ultrafiltration	<65 nt	1	4	2	3
Driedonks et al. (16)	Primary bone-marrow derived dendritic cells (mouse)	Density gradient	<275 nt	2	1	Not in mouse	Not in mouse
Lunavat et al. (18)	Melanoma cell lines	UC pellet (100,000 g)	<175	Not specified			
Nolte-'t Hoen et al. (12)	DC - T cell co-cultures (mouse)	UC pellet (100,000 g)	<70 nt	Not specified			
Bellingham et al. (13)	Neuronal cells (mouse)	UC pellet (100,000 g)	<150 (incl adapters)	Not specified			
Yeri et al. (19)	Plasma, saliva, urine	No	n.s.	Not specified			

Different Y-RNA subtypes are ranked based on their relative abundance reported by each study. The Y-RNA subtype with the highest RPM value in a study is ranked with 1, the second highest RPM value as 2, etc. The column 'EV-enrichment' indicates the method that was used to concentrate EVs from supernatant/biofluid. n.d., not determined, UC, ultracentrifugation.

extracellular milieu. A number of studies indicate that differences exist between the relative abundance of Y-RNA subtypes inside cells and those released by these cells into the extracellular milieu (14, 25, 31), which supports subtype-specific differences in Y-RNA release.

The data in **Tables 1**, **2** indicate that different size selections were applied during sequencing library generation for extracellular Y-RNA detection. Several of the studies primarily focused on miRNA detection and therefore applied a narrow size selection (<65 nt). This hampers detection of longer transcripts such as full-length Y-RNA, which are 83–110 nt in size. The sequencing approach in these studies may therefore bias toward detection of Y-RNA fragments (12, 14, 19, 31). However, Y-RNA fragments have also been detected in sequencing studies where no size selection was applied (**Table 2**) (17, 19, 20, 25, 80–82). Most studies show that the extracellular Y-RNA fragments derive from both the 5′ and 3′ arms of the Y-RNA hairpin and that they can be categorized in defined lengths of ∼21 nt, ∼30 nt and ∼40 nt (**Table 2**). Fragments of the 5′ arm of the Y-RNA hairpin were generally found to be more abundant than the 3′ fragments. Although these data suggest that Y-RNA fragments are frequently released from cells, reliable detection of full-length Y-RNA in these studies may have been hampered by technical limitations. Y-RNA forms complex RNA structures that are known to negatively influence cDNA synthesis efficacy and to introduce bias in deep sequencing. Reverse transcriptases may not efficiently read through these complex RNA structures, leading to overestimation of fragmented non-coding RNA in sequencing data (83, 84). This is corroborated by recent sequencing studies deploying reverse transcriptases that are insensitive to secondary structures, which detected mostly full-length Y-RNAs (and other structured ncRNA such as tRNA) (15, 85). By using Northern blot analysis, we also recently confirmed that EV contain mostly full-length Y-RNA and only a small amount of 19–35 nt fragments (16). This urges caution in drawing conclusions on the presence of Y-RNA fragments in EV based on RNA sequencing data (16). Taken together, both full-length and fragmented forms of extracellular Y-RNA are abundantly detected in body fluids and in culture supernatant of various cell lines.

Y-RNA BINDING PROTEINS IDENTIFIED IN EV

Several different proteins are known to interact with Y-RNA inside cells and determine its function or localization (see chapter 2). Additionally, protein binding may shield motifs in Y-RNA which may trigger cellular RNA sensors. For instance, the La-protein potentially shields the triphosphate moiety (56), which may prevent activation of RIG-I (86). Ro60 covers the stem-motif (87), which may prevent activation of dsRNA sensor TLR-3. In the context of EV release, protein partners of Y-RNA may be involved in shuttling of the RNA into EV and in functional effects of transferred Y-RNA in target cells. It is largely unknown which protein partners are associated with Y-RNA in EV and whether this differs between EV of different cellular origin (**Figure 1**, box 8 and 9). We therefore composed a list of known Y-RNA protein partners and evaluated whether these proteins have been detected in EV by searching public databases of mass spectrometry data of EV-associated proteins (Vesiclepedia) (88). The list of known Y-RNA protein partners can be found in **Table 3**. Most of these proteins have been identified by immunoprecipitation with antibodies against RNA binding proteins followed by Y-RNA detection, or by using tagged Y-RNA molecules to pull down proteins from cell lysates that directly interact with this RNA (RNA affinity purification). Studies that initially discovered the interaction between an RNA-binding protein and Y-RNA subtypes, as well as later studies further validating this interaction have been listed in **Table 3**. Twenty-three proteins have been reported to directly interact with Y-RNA. Ro60 and La, which are the best characterized protein partners of Y-RNA, have been discussed in chapter 2. Many of the other Y-RNA-binding proteins (hnRNP I, hnRNP K, RoBPI, ZBP1, YBX1, YBX3, ELAVL1 (HuR), CPSF1, CPSF2, FIPL1 SYMPK, and HuD) function in processing or splicing of mRNA transcripts. Several of these proteins mediate 3′ end processing of human histone-H3 mRNA in conjunction with a truncated form of Y3-RNA called Y3** (89). As mentioned earlier, the protein HuD is specifically expressed in neuronal cells where it enhances translation efficiency by stabilizing the mRNAs of mTORC1-responsive genes, which is counteracted by Y3-RNA binding to HuD (73). Similarly, the related protein HuR, also known to bind Y3-RNA, can bind AU-rich elements in mRNA transcripts. Via this mechanism, HuR was for example shown to influence cytokine production, evidenced by increased interferon-β expression in synoviocytes of arthritis patients, and reduced production of inflammatory cytokines including TNFα and TGFβ in LPS-treated macrophages (95, 96). Two other proteins, MOV10 and Argonaute, are important players in miRNA-mediated gene silencing. Additionally, a number of Y-RNA interacting proteins are involved in virus infection or innate immunity, such as MOV10, APOBEC, IFIT5, SYMPK, YBX1. Interestingly, not all proteins were found to interact with all four human Y-RNA subtypes. This suggests specialized functions for different Y-RNA subtypes, dependent on their associated proteins.

Next, we searched Vesiclepedia (www.microvesicles.org), a repository for extracellular vesicle proteomics data (88), to investigate which of the known Y-RNA binding proteins have been detected in EV. Interestingly, 18 out of 23 known Y-RNA binding proteins were reportedly present in EV from various cell types (**Table 3**). In addition, 10 of these proteins have been detected in EV from biofluids such as blood and urine. Of note, most entries in Vesiclepedia are based on mass-spectrometry, which may be prone to false-positive identification of proteins due to its high sensitivity. Therefore, we additionally searched the literature to determine whether the presence of these proteins in EV has been validated by Western blot detection. This was the case for 6 proteins: Ro60 (20, 31), La (31), hnRNP K (97), YBX1 (98), APOBEC3G (99), and ELAV1 (HuR) (100, 101). In studies reporting the presence of Ro60 (20, 31),

TABLE 2 | Overview of studies reporting the presence of Y-RNA fragments by RNA sequencing analysis of extracellular RNA.

References	Sample type	Sequencing method	Size selection (nt)	Y-RNA fragments in sequencing	5′ length (nt)	3′ length (nt)	Fragment detected by Northern blot
van Balkom et al. (17)	HMEC	Illumina smallRNA	No	Y1, Y4, Y5	30–39	19 and 33	No
Cambier et al. (24)	Cardiosphere derived cells (CDC)	Ion Total RNA seq	No	Y1, Y3, Y4, Y5	n.s.	n.s.	No
Chakrabortty et al. (80)	K562 myelogenous leukemia and BJ primary fibroblast	Illumina TruSeq SmallRNA	20–200	Y5	23, 29, 31	31	Y5 5p
Dhahbi et al. (82)	Plasma	Illumina TruSeq smallRNA	No	Y4	27, 30–33	-	Y4 5p
Dhahbi et al. (21)	Plasma (healthy vs. cancer)	Illumina TruSeq smallRNA	No	Y4	30-33	25 - 29	Y4 5p
Driedonks et al. (16)	Primary bone-marrow derived dendritic cells (mouse)	NebNext smallRNA	15–275	Y1, Y3	30	21	Y1 5p and 3p
Haderk et al. (25)	Chronic leukemic lymphocytes	NebNext smallRNA	No	Y4	30-32	-	Y4 5p
Kaudewitz et al. (81)	Platelet rich and platelet poor plasma	Illumina smallRNA	No	Y1, Y3, Y4, Y5	-	-	No
Nolte-'t Hoen et al. (12)	DC - T cell co-cultures (mouse)	SOLiD Small RNA Expression Kit	20–70	Yes, but not specified which subtypes	-	-	No
Repetto et al. (22)	Primary macrophages	NebNext Small RNA	25–40	Y4	-	-	Y1 5p
Tosar et al. (14)	MCF7 and MCF-10A cell lines	NebNext smallRNA	<60 nt	Y4	30–33	30−33	No
Vojtech et al. (20)	Human seminal fluid	ScriptMiner smallRNA seq	No	Y1, Y3, Y4, Y5	30–33	-	No
Wei et al. (31)	Glioblastoma	NEBnext smallRNA	15–65 nt	Y1, Y4, Y5	32 nt	-	No
Yeri et al. (19)	Plasma, saliva, urine	Illumina TruSeq	Not specified	Yes, but not specified which subtypes	Not specified	-	No

Indicated are the Y-RNA subtypes from which the fragments derived, the reported fragment length, and whether the presence of Y-RNA fragments was confirmed by Northern blot analysis.

La (31), and hnRNP K (97), EV had only been enriched by ultracentrifugation/ultrafiltration. These proteins may therefore be associated to EV or RNP or both. Association of HuR and APOBEC3G to EV was convincingly demonstrated using EV purification by density gradient centrifugation (99, 101). Additionally, it was shown that lipid membrane-enclosed YBX1 was protected from protease degradation, indicating that this protein is found inside EV (98).

We noticed that several of the Y-RNA-binding proteins detected in EV have previously been implicated in sorting of miRNAs into EV. ELAV1 (HuR), for example, dissociates miRNA-122 from AGO2/mRNA complexes in hepatocytes and drives subsequent expulsion of miR-122 from the cell via EV (101). Furthermore, YBX1 was shown to package miR-223 into EV from HEK293T cells (102). Initial evidence suggests that YBX1 also plays a role in sorting Y-RNA into EV (15). Knockout of this protein in HEK293T cells resulted in a reduced packaging of Y-RNA into EV. However, disruption of YBX1 did not completely abolish Y-RNA packaging, suggesting the involvement of additional proteins in this process. Moreover, YBX1 knockout also affected the packaging of other small non-coding RNAs such as tRNAs and Vault RNA, which suggests a more general function in EV-RNA packaging. Delineating the mechanisms underlying sorting of RNAs into EV is an area of intense research. Identification of proteins that specifically interact with EV-RNAs of interest, as performed above for Y-RNA, is a starting point to investigate potential involvement in sorting these RNAs into EV. Besides the assumed involvement of RBP in this process, sorting of RNAs into EV may also be influenced by the presence of specific motifs, modifications, or structures in RNA, post-translational modifications in RBP, and local enrichment of RNA close to membrane compartments [reviewed in (32)]. RNA sorting into EV may additionally be modulated by signaling processes triggered in the parental cells. There is strong evidence that miRNA sorting is influenced by cell stimulation (9, 16). Our own laboratory recently showed that EV-mediated release of Y-RNA is influenced by immune-related stimuli imposed on EV-producing cells (16). The EV-associated changes in Y-RNA were not reflected in cellular Y-RNA levels, which suggests that the Y-RNA shuttling rate, rather than the transcriptional level of Y-RNA, is modulated by these stimuli. Condition-dependent changes in the levels of extracellular Y-RNA have also been observed *in vivo*. Physical exercise was shown to increase the levels of circulating Y4-RNA, while Y1, Y3, and Y5 were decreased relative to resting conditions (103). Further research is needed to evaluate whether regulation of Y-RNA shuttling to the extracellular space is driven by differential expression or localization of Y-RNA binding proteins.

TABLE 3 | Overview of proteins known to interact with Y-RNA, as identified by immunoprecipitation or RNA affinity purifications.

References	Protein name	Protein function in cells	Method to identify Y-RNA binding proteins	Y1	Y3	Y4	Y5	Entry in vesicle pedia	Source of EV
Hendrick et al. (45); Köhn et al.(89)	Ro60	Binds misfolded RNA	Immunoprecipitation (anti-Ro60), RNA affinity purification	++	++	++	++	Yes	Blood/cell lines
Hendrick et al. (45); Köhn et al.(89)	La (SSB)	Binds 3′ poly-(U) tail of RNA pol III transcripts	Immunoprecipitation (anti-La), RNA affinity purification	++	++	++	++	Yes	Cell lines
Fouraux et al. (60)	Nucleolin	Associates with intranucleolar chromatin	Immunoprecipitation (anti-Ro60 and anti-La)	++	++	–	–	Yes	Blood/urine/cell lines
Fabini et al. (61)	hnRNP I (PTBP1)	Pre-mRNA splicing	RNA affinity purification	++	++	–	–	No	–
Fabini et al. (61); Köhn et al.(89)	hnRNP K	Pre-mRNA binding	RNA affinity purification	++	++	–	–	Yes	urine/cell lines
Thomson et al. (79)	Ago	RNAi mediated gene-silencing	Immunoprecipitation (anti-Ago)	n.d.				No	–
Cheng et al. (90)	Calreticulin	Calcium-binding chaperone	Electrophoretic mobility shift assay (EMSA)	++	++	++	++	Yes	Blood/pleural effusions/saliva/ urine/cell lines
Hogg and Collins (69)	L5 (RPL5)	Component of ribosome	RNA affinity purification	–	–	–	++	Yes	Saliva/urine/cell lines
Bouffard et al. (91); Hogg and Collins (69)	RoBPI (PUF60)	Pre-mRNA splicing, apoptosis and transcription regulation	Yeast three-hybrid assay, immunoprecipitation, RNA-immunoprecipitation (Ro60)	++	++	–	++	Yes	Cell lines
Hogg and Collins (69)	IFIT5	Interferon-induced RNA binding protein, senses viral 5′triphosphorylated RNA	RNA affinity purification	n.d	n.d.	n.d.	++	Yes	Urine/cell lines
Köhn et al. (58); Sim et al.(62); Kohn et al. (89)	ZBP1 (IFGB2P1, IMP1)	Recruits mRNAs to protein-RNA complexes, allowing mRNA transport and transient storage	Immunoprecipitation (anti-Ro60-FLAG), RNA affinity purification	+	++	n.d.	n.d	Yes	Cell lines
Sim et al. (58); Köhn et al. (89)	YBX1	Regulation of mRNA transcription, splicing, translation and stability	Immunoprecipitation (anti-Ro60-FLAG), RNA affinity purification	++	++	++	+	Yes	Cell lines
Sim et al. (58)	YBX3	Binds to GM-CSF promoter. Also binds full-length mRNA and short RNA	Immunoprecipitation (anti-Ro60-FLAG)	n.d				No	–
Sim et al. (58)	MOV10	Required for miRNA-mediated gene silencing. Involved in human hepatitis delta virus transcription	Immunoprecipitation (anti-Ro60-FLAG)	n.d.				Yes	Urine/cell lines
Yamazaki et al. (92); Köhn et al. (89)	Matrin-3	Nuclear matrix protein, nuclear retention of RNA, involved in antiviral response	RNA affinity purification	++	++	–	–	Yes	Urine/cell lines
Köhn et al. (89)	ELAVL1 (HuR)	Stabilizes mRNA and regulates translation	RNA affinity purification	–	++	–	–	Yes	Cell lines
Köhn et al. (89)	CPSF1	Involved in mRNA poly-adenylation	RNA affinity purification	++	++	–	–	Yes	Cell lines
Köhn et al. (89)	CPSF2	Involved in mRNA poly-adenylation	RNA-immunoprecipitation	++	++	–	–	Yes	Cell lines
Köhn et al. (89)	FIP1L1	Involved in mRNA poly-adenylation	RNA-immunoprecipitation	++	++	–	–	No	–
Köhn et al. (89)	SYMPK	Histone mRNA 3′-end processing	RNA-immunoprecipitation	++	++	+	+	Yes	Urine/cell lines
Bogerd et al. (93); Apolonia et al. (94)	APOBEC3G	Inhibitor of retrovirus replication, broad antiviral activity	RNA-immunoprecipitation	n.d.				Yes	Cell lines
Bogerd et al. (93); Apolonia et al. (94)	APOBEC3F	Inhibitor of retrovirus replication, broad antiviral activity	RNA-immunoprecipitation	n.d.				Yes	Cell lines
Tebaldi et al. (73)	HuD (ELAVL4)	Translational enhancer of mTORC1-responsive genes, regulation of mRNA abundance and alternative splicing	RNA-immunoprecipitation	–	++	–	n.d.	No	–

Indicated are known functions of the human variants of these proteins. Binding to the different Y-RNA subtypes is indicated with +, – and ++, or not-determined (n.d.). Furthermore, identification of these proteins in EV is indicated based on entry in the Vesiclepedia database of EV-associated proteins, as well as the source of EV in which these proteins were detected.

In conclusion, a large number of proteins known to interact with Y-RNA have been detected in EV. Some of these proteins may be involved in sorting of Y-RNA into EV, but the underlying mechanisms should be further explored (**Figure 1**, box 10). The co-presence of Y-RNA and Y-RNA binding proteins in EV also highlights the need to study the functional effects of EV-associated Y-RNA in the context of its protein partners.

Y-RNA AND VIRUSES

Extracellular Y-RNA has not only been detected in EV and RNP, but also in various retroviruses such as murine leukemia virus (MLV) and human immunodeficiency virus (HIV) (104–108) (**Figure 1**, box 11). These viruses incorporate not only Y-RNA, but also various other host-derived non-coding RNAs, such as tRNA and 7SL. The presence of extracellular Y-RNA in both retroviruses and EV is interesting because both structures are formed via overlapping biogenesis routes (109, 110). In addition, several Y-RNA binding proteins that have been identified in EV also interact with retroviral RNA. In the case of HIV, these proteins included YBX1, hnRNP K, PTBP1, Nucleolin, and Matrin-3 (111). This raises the interesting question whether there is overlap in mechanisms underlying the sorting of RNAs into EV and retroviruses.

Retroviruses use specific host tRNAs to prime reverse transcription, which is a key step in the retroviral life cycle (112). In addition, encapsidated host non-coding RNAs may mediate packaging of antiviral proteins, such as the antiviral cytidine deaminase APOBEC3G into virions (113). It has been hypothesized that newly synthesized host RNAs, including Y-RNAs that have not been bound by Ro60, may act as a scaffold for virion assembly (105, 106, 114). Moreover, it has been suggested that Y-RNA may benefit the host via potential triggering of TLR7 in newly infected cells, thereby initiating an antiviral immune response (107). Additionally, it was reported that many packaged RNAs, including Y-RNA, can mediate APOBEC packaging which leads to mutations in the viral genome or restricts retrotransposition (94). It remains to be investigated whether these Y-RNA-driven processes only occur during virus infection and whether we could learn from retroviruses to further delineate the function of Y-RNA in EV.

THE ROLE OF EXTRACELLULAR Y-RNA IN IMMUNE REGULATION

The high abundance of Y-RNA in EVs and RNPs raises the question whether extracellular Y-RNAs have signaling functions when transferred to target cells (**Figure 1**, box 12). In general, it is technically challenging to assess the role of individual RNAs in EV-mediated effects because EV mediate simultaneous transfer of multiple proteins, lipids and RNAs to target cells. Until now, functional transfer of EV-associated miRNAs have been addressed either by using target cells with luciferase reporter constructs containing the 3′UTR of the mRNA target, or by investigating EV released by miRNA knockout cells, or by assessing the effects of transfecting synthetic analogs of the RNA of interest into target cells [reviewed in (32)]. It is unlikely that, upon transfer to target cells, Y-RNA functions similar to miRNA, as it has been shown that Y-RNA fragments bound to Ago2 were unable to repress reporter mRNAs (79). In addition, the effects of Y-RNA in EV may differ from those elicited by Y-RNA-containing RNP. Although the number of studies addressing the effects of extracellular Y-RNA are limited, the majority of these studies described effects of Y-RNA on immune regulation. Interestingly, both pro- and anti-inflammatory effects have been described, which will be discussed in more detail below.

Table 4 summarizes the immune-related effects that have been reported for extracellular Y-RNA subtypes in various experimental settings. Some of these studies specifically addressed the function of Y-RNA containing RNP (23, 115, 116), whereas others focused on the function of EV-associated RNA (24, 25). In the recent study by Haderk et al. it was shown that Y4 and 5′-fragments of Y4 were abundantly present in EV released by B cell leukemia cells. These EV not only induced inflammatory effects, such as the release of CCL2, CCL4 and IL6 by monocytes, but also induced PD-L1 expression on these cells, which inhibits T-cell activation (117). To investigate whether the EV-induced effects were mediated by Y4, monocytes were transfected with synthetic homologs of full-length Y4 or fragments thereof. Full length Y4, but not Y4 fragments, induced similar pro- and anti-inflammatory effects in monocytes as those observed after incubation with EV. Based on these data it was suggested that Y-RNA in tumor EV could contribute to establishing a favorable tumor microenvironment via suppressing the immune system (25).

Y4-RNA containing EV have also been implicated in myocardial infarctions (24). Cardiosphere-derived cells (CDC) can reduce damage during myocardial infarction by modulating inflammatory responses via an unknown mechanism. It was found that CDC-EVs contain a relatively large percentage of Y-RNAs, and that one specific 5′ fragment of Y4-RNA was particularly abundant in CDC-EV compared to normal human dermal fibroblasts. Evidence was provided that EV could transfer Y4-fragments to bone-marrow derived macrophages, and that transfection of this Y4-fragment into macrophages resulted in strong and prolonged upregulation of IL10, and to a lesser extent TNFα. Additionally, administration of this Y4-fragment *in vivo* induced IL10 release and reduced damage in a myocardial infarction model in rats (24). Thus, the abundance of Y4-fragments in CDC-EVs correlated with the potency of these RNA fragments to mitigate damage after myocardial infarction.

The function of EV-associated Y-RNA has until now been addressed by transfecting Y-RNA (fragments) into target cells as a model for EV-mediated transfer of these RNAs. Although this may currently be the most feasible approach, several limitations can be identified. The naked, synthetic RNAs employed in these studies are not complexed to proteins, whereas RBP may play a role in the function of truly EV-associated Y-RNAs. Additionally, the route of uptake of lipofected RNA complexes may be different from EV-enclosed RNAs, resulting in delivery of the Y-RNA to subcellular locations other than those reached after EV-mediated delivery.

TABLE 4 | Overview of immune-related effects of extracellular Y-RNA.

References	Source of Y-RNA	Y-RNA subtype	Approach	Recipient cell type	Immune-related effect	Conclusion
Clancy et al. (115)	*In vitro* transcribed RNA and Ro60/Y-RNA complexes assembled *in vitro*	Y3-RNA	DOTAP transfection of *in vitro* transcribed RNA, and addition of Ro60/Y-RNA complexes to medium	Human macrophages; Fetal cardiac cells	macrophages: TNFa release, cardiac fibroblasts: collagen secretion	Increased TNFa release in macrophages; Increased collagen secretion by cardiac fibroblasts
Greidinger et al. (116)	*In vitro* transcribed RNA	All Y-RNAs	Addition to medium	RL-95 epithelial cells and HEK293 transiently transfected with TLR reporter constructs	RL-95: release of IL6, TLR-reporters: increased luciferase release,	Y-RNAs differ in their capacity to stimulate various RNA-sensing TLRs; Y1 stimulates TLR7 whereas Y3 stimulates TLR3
Hizir et al. (23)	Affinity purification from lysates from 293T cells treated with and without staurosporine (induces cleavage of Y-RNA)	Not specified	Addition to medium	Mouse and human monocytes/ macrophages	Apoptosis (caspase-3 cleavage, IkBa)	Cleaved Y-RNA associated with Ro60 induces inflammation and apoptosis, while naked Y-RNA does not. TLR7 triggering is involved
Cambier et al. (24)	Synthetic Y-RNA	Y4-RNA fragment	Transfection (Dharmafect 4 reagent)	Bone-marrow derived macrophages	mRNA expression (Arg1, IL4RA, Nos2, IL10, NFkB, TNF, TGFb, Vegfa) and increased IL10 release	Transfection of Y4-fragment in BMDM leads to prolonged induction of IL10
Haderk et al. (25)	EV isolated from MEC1 cell line by ultracentrifugation; Synthetic Y-RNA	Y4-RNA	Transfection (Effectene)	Monocytes	Cytokine release (CCL2, CCL4, IL-6), increased levels of surface markers (PD-L1, CCR2)	Transfected Y4-RNA or Y4-RNA enclosed in EV induces anti-inflammatory PD-L1

A few other studies provide indirect support for a role of EV-associated Y-RNA in immune-modulatory processes. For instance, Y-RNAs and tRNAs are particularly abundant in seminal plasma EV (prostasomes) (20), which are known to confer immune-suppressive effects leading to reduced rejection of sperm cells (118). Similarly, EV released by the parasite *Heligmosomoides polygyrus* contain high levels of nematode stem-bulge RNAs (which are related in sequence to Y RNAs) and suppress cytokine release in mice (119). Furthermore, in our latest study, we demonstrate that EV released from dendritic cells with an immune-suppressive function are more enriched in Y-RNA than EV released by dendritic cells with an immune-activating phenotype (16).

While EV-associated Y-RNAs seem to induce a range of different immune-related effects, circulating RNP containing Y-RNA predominantly induce immune activation. Many of these effects reportedly depend on TLR-mediated triggering (**Figure 1**, box 13). However, Y-RNA subtypes differ in their capacity to trigger different TLRs. Y3-RNA predominantly triggers TLR3, while Y1, Y3, and Y4 trigger TLR7 (116). Whereas, unbound Y-RNA may trigger TLR signaling, Y-RNA bound to protein partners such as Ro60 and La has a reduced stimulatory potential, likely because these proteins shield dsRNA hairpin structure and 5′ triphosphate group that are ligands for TLR and other pattern recognition receptors. In support of this idea, Clancy et al. showed that Ro60-associated Y3-RNA, in contrast to naked Y3-RNA, does not induce TNFα release in macrophages (115). The pro-inflammatory effects of Y-RNA-Ro60 complexes in autoimmune diseases such as SLE and SS are likely explained by binding of auto-antibodies to these RNP. Opsonization of Ro60-associated Y3-RNA by anti-Ro60 IgG was shown to be required for stimulation of TNFα release by macrophages, supporting a role for FcγR in this process (115) (**Figure 1**, box 14). However, not only FcγR-mediated triggering, but also RNA-mediated triggering of TLR7 contributed to the inflammatory effects elicited by the RNP (115). It is not known whether exposed Y-RNA-Ro60/La complexes only occur as RNP or whether these complexes are also present on the surface of EV. During apoptosis of fibroblasts, Y3-RNA was shown to drive the translocation of Ro60 to the outer leaflet of the plasma membrane (120) (**Figure 1**, box 15). Upon opsonization with anti-Ro60 antibodies, these apoptotic fibroblasts induced TNFα release in macrophages in a TLR7 dependent manner (120). Since apoptotic cells release various types of EV as well as apoptotic bodies (121), it is possible that some of these EV display surface-exposed Y-RNA-Ro60 complexes. In fact, it is known that EV associate with autoantibodies in several autoimmune diseases, thereby forming proinflammatory complexes that contribute to disease [reviewed in (122)].

Besides triggering inflammation in SLE and SS, extracellular Y-RNA complexes have also been demonstrated to induce apoptosis in atherosclerosis (23) and cancer (80). In atherosclerosis, lipoproteins accumulating in arteries can lead to activation of macrophages and subsequent apoptosis induction in these cells. *In vitro* cultured macrophages treated with lipids release increased levels of fragmented Y-RNA into the medium (22). Hizir et al. showed that affinity purified Y-RNA fragments/Ro60 RNPs from apoptotic HEK293T cells induced cell death in macrophages (**Figure 1**, box 16). Y-RNA fragment-containing RNP released by macrophages could therefore contribute to a negative feed-back loop in which more and more macrophages in the lipid-rich environment die by apoptosis. In the context of cancer, it was shown that EV released by myelogenous leukemia cell lines contain high levels of fragmented Y5-RNA (80). Not only these EV, but also deproteinized total RNA from these EV and synthetic Y5 fragments were shown to induce apoptosis in healthy cells, but not in cancer cells. Via this mechanism, Y5-fragments in EV could favor cancer cell proliferation and invasion of tissues.

The studies described above suggest that the functional effects of extracellular Y-RNAs depend on both the macromolecular structure to which it is associated and the conditions under which the Y-RNA is released. In addition to the TLR-mediated effects of Y-RNA that have been reported to date (**Figure 1**, box 17), Y-RNA may also mediate functional effects via their interacting proteins (**Figure 1**, box 18). This highlights the importance of separating EV from RNP in studies addressing the function of EV-associated RNA (32, 123).

BIOMARKER POTENTIAL OF EXTRACELLULAR Y-RNA

The abundance of circulating Y-RNA in body fluids has triggered interest in the potential use of Y-RNA as biomarker for disease. Increased levels of Y-RNA have been observed in the circulation of cancer patients (14, 21, 25, 31). In breast cancer patients, the abundance of 3′ Y-RNA fragments was higher than in healthy controls (21). A more recent study on chronic lymphocytic leukemia (CLL) reported the increased abundance of Y4-RNA in serum from CLL patients compared to healthy controls (25). However, these studies were performed with small groups of patients and the data currently lack power to confirm the suitability of Y-RNA (fragments) as biomarkers for cancer. Whereas there is no evidence that EV from tumor cells are more enriched in Y-RNA than their non-tumorigenic counterparts, most tumor cells release relatively high numbers of EV (14, 25). The cancer-related increase in circulating Y-RNA may therefore be explained by increased numbers of tumor cell-derived EV in the circulation. Alternatively, other cell types may react to the presence of the tumor by increasing cellular export of Y-RNA.

Increased levels of circulating extracellular Y-RNA have also been observed in the context of atherosclerosis and coronary artery disease. Repetto and colleagues observed a higher number of 5′ Y1-fragments in the blood of ApoE$^{-/-}$mice used as a model for atherosclerosis (22). Likewise, increased levels of circulating 5′-Y1 were observed in a cohort of 43 men with stable coronary artery disease (CAD), as compared to 106 age-matched healthy men. These data were validated in an independent sub-cohort including 220 patients vs. 408 controls. In 45 CAD patients an increased abundance of Y4-RNA 5′-fragments was observed (22). This raises the question of which cells are the main producers of extracellular Y-RNA fragments present in the circulation. The suggested candidates include macrophages (23) and platelets (81). However, it is important to note that pre-analytical variables can strongly affect characterization of extracellular RNA in plasma. Plasma samples are commonly contaminated by platelets, which may disintegrate during freezing (34), thereby releasing their internal (RNA) content. Indeed, plasma miRNA levels were shown to correlate with platelet counts prior to freezing (124). Thus, there is an urgent need for standardization of sample collection, storage conditions and sample processing for reliable assessment of Y-RNA and other extracellular RNAs present in body fluids.

In conclusion, differences in circulating Y-RNA may be further explored as biomarkers for disease, but it is critically important to evaluate and standardize the various methods used to isolate different carriers of Y-RNA in body fluids. Additionally, acquisition of knowledge on how disease-associated changes in cells affect the release of Y-RNAs will help to better understand their biomarker potential.

CONCLUDING REMARKS

Current data suggest that the family of Y-RNAs does not only play a role in intracellular processes to maintain cell function, but also acts as versatile intercellular messengers. Various studies have indicated that extracellular transport of Y-RNA is a highly efficient process employed by many different cell types. Additionally, Y-RNA is one of the most abundant extracellular non-coding RNAs in human plasma. Such extracellular RNA can occur in RNP or in EV. One potential trigger that regulates extracellular release of Y-RNA is TLR signaling. Moreover, currently available data suggest functional involvement of extracellular Y-RNA in various immune-related processes. Y-RNAs can bind to several different proteins. We here provided an overview of Y-RNA binding proteins that occur inside cells and in Y-RNA-containing RNP or EV released by cells into the extracellular milieu. We propose that binding to these proteins not only determines how Y-RNA regulates cellular processes, but may also drive their sorting into EV and could be essential for functional effects of Y-RNA transferred to recipient cells (**Figure 1**). Partly based on currently available data, we envision that Y-RNA may affect the function of recipient cells via different mechanisms. These include direct effects of Y-RNA, such as activation of RNA sensors (e.g., TLRs), leading to the release of pro- and anti-inflammatory cytokines. Additional effects may be mediated by Y-RNA binding proteins, many of which function in regulation of transcription and

translation. Initial data suggest that levels of extracellular Y-RNA may correlate with disease. However, more research is needed as to how Y-RNA release is altered in diseased cells and how this affects other cells in order to delineate the contribution of extracellular Y-RNA in (immune-related) diseases and to correctly interpret its applicability as a disease biomarker.

AUTHOR CONTRIBUTIONS

EN performed literature research, drafted the manuscript, and edited the text. TD performed literature research, made the inventory of Y-RNA binding proteins in EV by searching data repositories, and wrote the manuscript.

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Cytokine Targeting by miRNAs in Autoimmune Diseases

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Persistent and excessive cytokine production is a hallmark of autoimmune diseases and may play a role in disease pathogenesis and amplification. Therefore, cytokine neutralization is a useful therapeutic strategy to treat immune-mediated conditions. MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression in diverse biological processes. Altered miRNA levels are observed in most autoimmune diseases and are recognized to influence autoimmunity through different mechanisms. Here, we review the impact of altered miRNA levels on the expression of cytokines that play a relevant pathogenic role in autoimmunity, namely primary pro-inflammatory cytokines, the IL-17/IL-23 axis, type I interferons and IL-10. Regulation can be either "direct" on the target cytokine, or "indirect," meaning that one given miRNA post-transcriptionally regulates the expression of a protein that in turn influences the level of the cytokine. In addition, miRNAs associated with extracellular vesicles can regulate cytokine production in neighboring cells, either post-transcriptionally or via the stimulation of innate immune RNA-sensors, such as Toll-like receptors. Because of their tremendous potential as physiological and pathological regulators, miRNAs are in the limelight as promising future biopharmaceuticals. Thus, these studies may lead in the near future to the design and testing of therapeutic miRNAs as next generation drugs to target pathogenic cytokines in autoimmunity.

Keywords: TNF-α, IL-6, IL-17/IL-23, IFN, SLE, RA, psoriasis, MS

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INTRODUCTION

Autoimmune diseases are chronic and often life threatening conditions characterized by an undesired activation of the immune system against self-antigens, whose incidence and prevalence has markedly increased over the second half of the twentieth century (1). The pathogenesis of these diseases is complex and largely remains to be investigated, but it is now widely accepted that environment, genetic background and immunity all contribute to the development of autoimmunity.

The ability of the immune system to avoid activation toward self-antigens is called tolerance. "Central" tolerance in the thymus and bone marrow plays a key role in shaping immune system homeostasis by inactivating or deleting autoreactive T and B lymphocytes. However, even under strict vigilance of "central" tolerance, small numbers of potentially self-reacting lymphocytes can still "leak out" into the periphery. This phenomenon does not necessarily lead to pathology because additional mechanisms of "peripheral" tolerance restrain the activation of these cells, including permanent inactivation of potentially autoreactive lymphocytes that recognize antigens in the absence of innate immune activation and inflammation (2). Any defect or failure in tolerance

mechanisms can lead to breakdown of tolerance and to the development of autoimmunity (3). For example, some autoimmune diseases, such as the so called "interferonopathies," are triggered by the recognition of self or foreign molecules by innate sensors (4, 5) which, in turn, trigger inflammation and engagement of previously quiescent autoreactive T and B cells (3).

Cytokines are crucial immune mediators that activate and polarize the immune response to grant host defense and recovery of homeostasis. On the other hand, excessive or persistent cytokine production results in deregulated immune activation and plays a role in both the initiation and the amplification phases of immunopathologies (6, 7). The key role of deregulated cytokine production in autoimmunity represents the rationale for therapeutic cytokine targeting with biologicals, an approach that has led to major successes in the treatment of diseases such as rheumatoid arthritis (RA) and psoriasis (8).

MicroRNAs (miRNAs) are a large family of short, non-coding, single stranded RNAs that regulate the expression of one third of human genes (9). As such, they play crucial roles in most physiological and pathological processes, including cell growth and differentiation, metabolism, immunity, cancer, and autoimmune disorders (10–12). Within the cell cytoplasm, miRNAs regulate gene expression post-transcriptionally by binding to complementary sequences in the coding, 5′- or 3′-untranslated region (UTR) of target mRNAs that is either silenced or degraded (9). In addition, miRNAs are now known to master cell-to-cell signaling via the association with extracellular vesicles that protect them from degradation and allow efficient entry into neighboring cells, where they regulate the expression of target mRNAs (13, 14). Interestingly, extracellular miRNAs were also shown to exert cell-to-cell regulation via a non-conventional mechanism consisting on the interaction with innate immune RNA sensors, such as Toll-like receptors 7 and 8 (TLR7 and TLR8) (15–17). Because of their tremendous potential as physiological and pathological regulators, miRNAs are in the limelight as promising future biopharmaceuticals (18).

In this review, we will summarize the literature describing miRNAs that influence the pathogenesis and course of autoimmune diseases by deregulating key pathogenic cytokines. In addition to shedding pathogenetic insights, our work may contribute to the identification of attractive candidate targets for the development of miRNA-based next generation drugs for immune-mediated pathologies.

SEARCH METHODS

We searched the related articles indexed in PubMed database from inception to August 2018 using the following search details: ("micrornas"[MeSH Terms] OR "micrornas"[All Fields] OR "mirna"[All Fields]) AND ("cytokines"[MeSH Terms] OR "cytokines"[All Fields] OR "cytokine"[All Fields]) AND ("disease name"[MeSH Terms] OR "disease name"[All Fields]). We restricted our search to the best characterized autoimmune diseases, namely RA, systemic lupus erythematosus (SLE), psoriasis, Sjogren's syndrome (SS), type 1 diabetes, and multiple sclerosis (MS). Search results were screened for the source of analyzed miRNAs and cytokines. Works performed in cell lines stimulated to reproduce pathological tissue conditions were deliberately excluded. Original research papers clearly referring to basal miRNA and cytokine levels in pathology, either in the circulation/tissues or in cells from patients and murine models were selected to be discussed in paragraph 4 and summarized in **Table 1**. Additional literature was added, concerning cytokine biology and modulation in autoimmune diseases and miRNA biology, function and candidate therapeutic targets/tools.

MECHANISMS OF CYTOKINE TARGETING BY miRNAs

Figure 1 summarizes the four main mechanisms through which miRNAs regulate cytokine levels. Regulation can be either "direct" on the target cytokine, or "indirect," meaning that one given miRNA post-transcriptionally regulates the expression of a protein that in turn influences the level of the cytokine. "Direct" regulation comprises both the targeting of cytokine mRNA, reflecting in decreased cytokine levels, and the stimulation of TLR7/8, reflecting in cytokine increase. In "indirect" regulation, if one miRNA targets a cytokine activator, the cytokine level is expected to be decreased. By contrast, if a repressor is targeted the cytokine level increases. However, as discussed in specific paragraphs and summarized in **Table 1**, the neat result in terms of cytokine production also depends on the level of the analyzed miRNA in the specific pathology (increased or decreased in respect to healthy individuals). Please note that, in the present review, the terms "repressor" and "activator" are intended in their wider meaning and one single protein may be considered repressor or activator depending on the cytokine under consideration [e.g., FOXP3 is considered "activator" for IL-17 production and "repressor" of IL-10, based on its role of Th17-promoting transcription factor (19), see **Table 1**].

miRNA-MEDIATED CYTOKINE TARGETING IN AUTOIMMUNE DISEASES

Primary Pro-Inflammatory Cytokines (TNF-α, IL-1β, IL-6)

Primary pro-inflammatory cytokines are increased in RA patients and play a vital role in the pathogenesis of this disease, characterized by chronic inflammation of the synovial tissue, joint dysfunction, and tissue damage in the joints. Collectively, these cytokines facilitate the recruitment of leukocytes into the joints to maintain chronic inflammation, induce the proliferation of synovial fibroblasts that leads to pannus formation and contribute to angiogenesis and cartilage and bone destruction in the course of arthritis (7, 8). However, pro-inflammatory cytokines also display non-overlapping pathogenic functions that are not fully understood in autoimmunity. Indeed, while TNF-α and IL-β inhibition turned out to be effective approaches in the treatment of RA and of other chronic arthritis, the therapeutic effect of IL-1 inhibition proved unexpectedly modest (8, 20).

TABLE 1 | miRNAs involved in cytokine modulation in autoimmune diseases.

Cytokine	miRNAs	Disease	Expression in disease	Source	Target	Effect on cytokine	References (PMID)	Mechanism
TNF-α	GU-rich miRNAs	SLE	⇧	Plasma exosomes	TLR7	⇧	29769437	Direct effect (TLR activation)
		RA	⇧	Synovial fluid of Macrophages	TLR7	⇧	26662519	
	miR-10a	RA	⇩	Synovium	TBX5	⇧	28782180	Indirect effect (activators)
	miR-23b	RA-SLE-MS	⇩	Synovia, renal biopsies, spinal cords	TAB3, TAB2, IKK-α	⇧	22660635	
	miR-155	RA	⇧	PBMC, monocytes, macrophages, synovial fluid	SOCS1; SHIP-1	⇧	24351865; 27411480; 21690378	Indirect effect (repressors)
	miR-522	RA	⇧	RASFs	SOCS3	⇧	29394098	
	let-7a/e	SLE	⇧	Kidney	TNFAIP3	⇧	26110642	
	miR-21	PSO	⇧	Lesional skin	TIMP3	⇧	24574341	
	miR-106b	RA	⇧	Ankle tissues from CIA mice	N.A.	⇧	28957555	N.A.
	miR-146a	RA	⇧	PBMC	N.A.	⇧	21810022	
	miR-155, miR132, miR-26a	MS	⇧	PBMC	N.A.	⇧	27310932	
	miR-125b	RA	⇧	Serum, synovial tissues	N.A.	⇧	28738524	
IL-1β	miR-10a	RA	⇩	Synovium	TBX5	⇧	28782180	Indirect effect (activators)
	miR-23b	RA-SLE-MS	⇩	Synovia, renal biopsies, spinal cords	TAB3, TAB2, IKK-α	⇧	22660635	
	miR-155	RA	⇧	PBMC, monocytes, macrophages	SOCS1; SHIP-1	⇧	24351865; 21690378	Indirect effect (repressors)
	miR-522	RA	⇧	RASFs	SOCS3	⇧	29394098	
	miR-31	PSO	⇧	Lesional skin	STK40	⇧	23233723	
	miR-448	MS	⇧	PBMC, cerebrospinal fluid (CSF)	PTPN2	⇧	28342869	
	miR-106b	RA	⇧	Ankle tissues from CIA mice	N.A	⇧	28957555	N.A.
	miR-125b	RA	⇧	Serum, synovial tissues	N.A.	⇧	28738524	
IL-6	miR-410	SLE	⇩	Kidney (SLE mouse model)	IL-6	⇧	27028192	Direct effect
	GU rich miRNAs	SLE	⇧	Plasma exosomes	TLR7	⇧	29769437	Direct effect (TLR activation)
		RA	⇧	Synovial fluid of Macrophages	TLR7	⇧	26662519	
	miR-10a	RA	⇩	Synovium	TBX5	⇧	28782180	Indirect effect (activators)
	miR-140	RA	⇩	Synovial tissue and RASFs	TLR4	⇧	28987944	
	miR-22	RA	⇩	Synovial tissue	Cyr61	⇧	24449575	
	miR-23b	RA-SLE-MS	⇩	Synovia, renal biopsies, spinal cords	TAB3, TAB2, IKK-α	⇧	22660635	
	miR-155	RA	⇧	Monocytes, macrophages	SHIP-1	⇧	21690378	Indirect effect (repressors)
	miR-203	RA	⇧	RASFs	NF-κB repressors and SOCS	⇧	21279994	
	miR-106b	RA	⇧	Ankle tissues from CIA mice	N.A.	⇧	28957555	N.A.
IL-23	miR-21	PSO	⇧	Lesional skin	TIMP-3	⇧	24574341	Indirect effect (repressors)
	miR-200a	PSO	⇧	CD4+ T cells	N.A.	⇧	28738533	N.A.
IL-17	miR-340	PSO	⇩	T cells (Imiquimod model)	IL-17A	⇧	30012847	Direct effect
	miR-20b	MS	⇩	Th17 cells (EAE mice)	RORgt, STAT3	⇧	24842756	Indirect effect (activators)

(Continued)

TABLE 1 | Continued

Cytokine	miRNAs	Disease	Expression in disease	Source	Target	Effect on cytokine	References (PMID)	Mechanism
	miR-30a	MS	⇩	CD4+ T cells (EAE mice)	IRF4	⇧	27581464	
	miR-210	PSO	⇧	CD4+ T cells	FOXP3	⇧	24316592	
	miR-451a	SLE	⇧	Spleen and thymus (mouse model)	IRF8	⇧	28120198	
	miR-326	MS	⇧	CD4+ T cells, EAE mice	Ets-1	⇧	19838199	
	miR-26a	MS	⇩	PBL of MS patients; brain of EAE mice	IL-6	⇧	25362566	
	let-7e	MS	⇧	CD4+ T cells in EAE model	IL-10	⇧	23079871	Indirect effect (repressors)
	miR-21	PSO	⇧	Lesional skin	TIMP3	⇧	24574341	
	miR-448	MS	⇧	PBMC, CSF	PTPN2	⇧	28342869	
	miR-155, miR132	MS	⇧	PBMC	N.A.	⇧	27310932	N.A.
	miR-200a	PSO-MS	⇧	CD4+ T cells	N.A.	⇧	28738533; 25938517	
	miR-146a	PSO-RA	⇧	Lesional skin, PBMC, synovium	N.A.	⇧	23018031; 20840794	
IFN-α	GU-rich miRNAs	SLE	⇧	Plasma exosomes	TLR7	⇧	29769437	Direct effect (TLR activation)
	miR-146a	SLE	⇩	PBMC	IRF5, STAT1	⇧	19333922	Indirect effect (activators)
	miR-302d	SLE	⇩	Monocytes	IRF9	⇧	28318807	
	miR-155, miR-17 and miR-181b	SLE	⇩	PBMC	N.A.	⇧	25775145	N.A.
IL-10	let-7e	MS	⇧	CD4+ T cells in EAE model	IL-10	⇩	23079871	Direct effect
	miR-410	SLE	⇩	CD3+ T cells	STAT3	⇧	27351906	Indirect effect (activators)
	miR-223	RA	⇧	T cells	IGF-1R	⇩	24816316	
	miR-210	PSO	⇧	CD4+ T cells	FOXP3	⇩	24316592	
	miR-21	SLE	⇧	PBMC	PDCD4	⇧	21602271	Indirect effect (repressors)

SLE, Systemic Lupus Erythematosus; RA, Reumathoid Arthritis; RASF, RA Synovial Fluid; MS, Multiple Sclerosis; CSF, Cerebrospinal Fluid; PSO, Psoriasis; EAE, Experimental Autoimmune Encephalomyelitis; PBL, Peripheral Blood Lymphocyte; PBMC, Peripheral Blood Mononuclear Cell; CIA, Collagen-Induced Arthritis; N.A., Not Addressed.

miRNAs were described to play a role in the pathogenic increase of pro-inflammatory cytokines in RA, but also in SLE, psoriasis and MS. A few reports describe miRNA-mediated "direct" regulation, while many more demonstrate "indirect" modulation of pro-inflammatory cytokine production, either via the targeting of activators or of repressors (**Figure 1** and **Table 1**).

Direct mRNA targeting was shown in kidneys of MRL/lpr SLE mouse model, where increased IL-6 levels depended on a decrease in miR-410, which targeted the 3′-UTR region of IL-6 mRNA (21).

Two groups reported a role for TLR7 stimulation in increased pro-inflammatory cytokine secretion in autoimmune conditions. Let-7b was markedly upregulated in synovial fluid of patients with RA and capable of inducing TNF-α and IL-6 production by macrophages via TLR7 ligation (22). Our own group recently demonstrated that TNF-α and IL-6 are produced by human primary plasmacytoid dendritic cells (pDCs) stimulated with exosomes isolated from plasma of SLE patients. This effect depends on the triggering of TLR7 by exosome-associated miRNAs (17). Both groups found that TLR7 triggering can be mediated by several miRNAs rich in guanosine and uridine. This is in line with recent structural studies demonstrating that TLR7 works as a dual sensor for guanosine and uridine-containing ssRNAs by associating with degradation products of RNA instead of recognizing specific RNA sequences [reviewed in (23)].

In the synovium of RA patients, the down-modulation of miR-10a promoted the expression of TBX5, a member of T-box transcription factor family. TBX5 is an important regulator of synovial fibroblast that in turn increased the expression of TNF-α, IL-6, and IL-1β (24).

miR-23b was found down-regulated in human lesions and in murine models of SLE and RA, as well as in a model of MS. This suppression depended on IL-17 and contributed to autoimmune inflammation by promoting the expression of pro-inflammatory cytokines. Indeed, miR-23b suppresses NF-κB activation and inflammatory cytokine expression by targeting TGF-β-activated kinase 1/MAP3K7 binding protein 2 (TAB2), TAB3 and inhibitor of nuclear factor κ-B kinase subunit α (IKK-α). As expected, these second messengers that are essential in the pathway leading

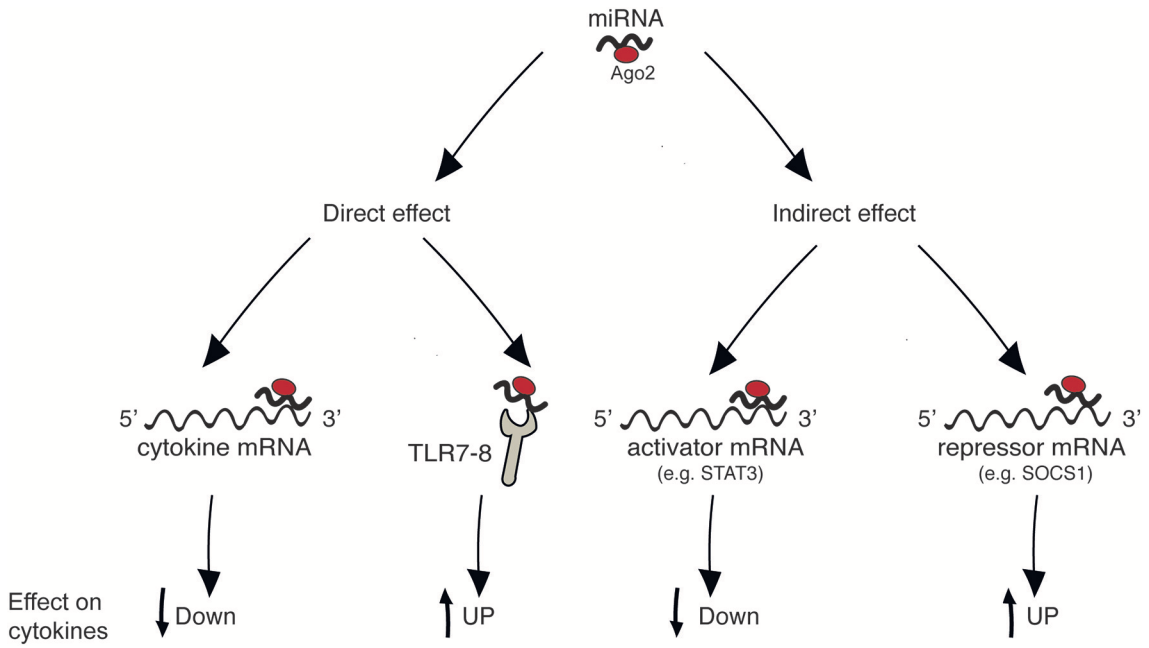

FIGURE 1 | Mechanisms of cytokine regulation by miRNAs. "Direct" regulation comprises targeting of cytokine mRNA and triggering of innate immune receptors leading to cytokine production. "Indirect" regulation comprises targeting of molecules that act as inducers or inhibitors of a given cytokine.

to inflammatory NF-κB activation were upregulated both in RA patients and in murine models (25).

miR-155 was increased in peripheral blood mononuclear cells (PBMCs) (26), peripheral blood monocytes (27) and synovial macrophages and monocytes (28) isolated from RA patients as compared with healthy controls. Increased miR-155 could increase the expression of pro-inflammatory cytokines by targeting Suppressor of cytokine signaling 1 (SOCS1) (26) and Src homology 2-containing inositol phosphatase-1 (SHIP-1) (28) in the respective cell type. Similarly, miR-522 and miR203, which are up-regulated in synovial fibroblasts of RA patients, respectively, increased the expression of TNF-α and IL-1β via targeting SOCS3 (29) and of IL-6 by targeting inhibitors of the NF-κB pathway, although these could not be further identified (30).

IL-6 production was also stimulated by two other miRNAs, miR-140 and miR-22, both down-regulated in synovial tissue samples from RA patients. In the case of miR-140, IL-6 upregulation was induced by a significant increase of TLR4, its direct target (31). Indeed, it is well-established that the slightest increase in the expression of TLRs may translate in overt autoimmune phenotypes [reviewed in (32)]. miR-22 expression was found to negatively correlate with that of Cyr61, a secreted extracellular matrix protein that promotes fibroblast-like synoviocyte proliferation. This increased IL-6 production and consequent Th17 differentiation (33).

Let-7 deregulation was reported to influence SLE pathogenesis. In particular, let-7a and let-7e were up-regulated in kidney biopsies of SLE patients independent of lupus nephritis and increased the production of TNF-α by suppressing TNF-α Induced Protein 3 (TNFAIP3), an ubiquitin-editing enzyme that negatively regulates the activation of NF-κB (34).

miR-21 and miR31 were involved in increased expression of pro-inflammatory cytokines in psoriasis. Increased miR-21 levels in epidermal lesions of psoriatic patients correlates with increased expression of TNF-α, because of reduced expression of epidermal Tissue Inhibitor of Metalloproteinase 3 (TIMP3) and consequent activation of TNF-α Converting Enzyme (TACE), responsible for the shedding of the functional ectodomain of TNF-α from cell membranes (35). miR-31, markedly over-expressed in psoriatic keratinocytes, was responsible for IL-1β over-expression, as demonstrated by the block obtained with an anti-miR31. The authors found that increased miR31 suppressed Serine/Threonine Kinase 40 (STK40), a suppressor of NF-κB activation (36).

miR-448 is significantly increased in both PBMCs and cerebrospinal fluid of patients with MS and enhances the production of pro-inflammatory cytokines, including IL-1β and IL-17, through targeting protein tyrosine phosphatase non-receptor type 2 (PTPN2) thus promoting Th17 differentiation (37).

In addition to the evidence discussed above, the levels of additional miRNA were found to correlate with pro-inflammatory cytokine expression, although the mechanisms remained not addressed. In collagen induced arthritis, mice displayed increased expression of miR-106b, an important miRNA involved in bone remodeling (38). miR-106 inhibition led to decreases arthritis severity and reduced levels of serum pro-inflammatory cytokines (39). In PBMCs of MS patients, the upregulation of miR-155, miR-132, and miR-26a associated to increased expression of TNF-α and IL-17 (40). Finally, in patients

with RA the expression of miR-146a and 125b was increased as compared to healthy controls and positively correlated with levels of pro-inflammatory cytokines (41, 42).

All in all, these studies indicate that TNF-α, IL-1β, and IL-6 are relevant targets of miRNAs that are deregulated in autoimmune diseases. Because these cytokines share most of the inducing stimuli and pathways, miRNAs acting via indirect mechanism are often found to regulate all of them. Thus, miRNAs could represent relevant deregulators of pro-inflammatory cytokines and, as such, interesting therapeutic targets for controlling their aberrant production in autoimmune diseases. However, our survey also shows that, at present, it is not possible to identify one or a small group of miRNAs representing the miRNA signature of the disease, i.e., the miRNAs mainly responsible for pro-inflammatory cytokine deregulation and possible therapeutic candidate/s. Indeed, single reports investigate different aspects or cell types within the different diseases making it difficult to gain an integrated view of cytokine deregulation by miRNAs.

The IL-23/IL-17 Axis

IL-23 is a crucial player in T-cell-mediated responses and a key promoter of immune-mediated pathological conditions. With the requisite assistance of other cytokines such as IL-6 and TGF-β, IL-23 masters the polarization of naïve $CD4^+$ T cells into Th17 effector cells (43). Many other innate immune cells characterized by the expression of the transcription factor RORγt and γδ T cells are also responsive to IL-23 (44). Collectively, these cells are responsible for the production of inflammatory cytokines including IL-17, IL-22, and TNF-α, inciting local tissue inflammation and immune-mediated inflammatory conditions. Aberrant IL-17 production has been identified in many autoimmune diseases including psoriasis, inflammatory bowel disease, RA, and MS (45). Consistently, IL23/IL-17 axis blockade is a successful therapy for psoriasis and psoriatic arthritis.

A direct regulatory effect of this axis was described for miR-340, which controls the expression of endogenous IL-17A by specifically binding to its 3′ UTR. miR-340 was decreased in T cells from the Imiquimod psoriasis mouse model, thus increasing the release of IL-17A. Furthermore, treatment with miR-340 alleviated the clinical severity of Imiquimod-induced psoriasis (46).

Many other miRNAs were found to regulate the IL-23/IL-17 axis in autoimmune diseases by indirect mechanisms.

In experimental autoimmune encephalomyelitis (EAE) miR-20b, miR-30a, and miR-26a were reduced. Decreased miR-30a and miR-26a was confirmed also in peripheral blood $CD4^+$ T cells of MS patients (47, 48). miR-20b was shown to suppress Th17 differentiation *in vitro* and *in vivo* by targeting RORγt and STAT3, thus acting as a negative regulator of EAE (49). Similarly, over-expression of miR-30a inhibited Th17 differentiation and prevented the full development of EAE, whereas interference of miR-30a promoted Th17 differentiation. miR-30a was shown to reduce IRF4 expression by specifically binding its 3′-UTR (47). miR-26a was shown to be a IL-6-associated miRNA and therefore an indirect regulator of the Th17/Treg cells balance, which inhibition substantially aggravated EAE severity (48). miR-326 and Let-7e were significantly up-regulated EAE. miR-326 expression also correlated with disease severity in MS patients. It was shown to promote the generation of Th17 cells by targeting Ets-1, a negative regulator of Th17 cell differentiation (50). Let-7e indirectly enhanced IL-17 production by targeting the 3′UTR of IL-10 mRNA (51).

$CD4^+$T cells from patients with psoriasis vulgaris showed miR-200a and miR-210 over-expression. miR-200a expression positively correlated with that of RORγT, IL-17, IL-23 (52, 53). miR-210 deregulation led to decreased IL-10 and increased IL-17 production, thus impairing the immunosuppressive functions of Treg cells, via the inhibition of FOXP3 expression (54).

In lesional skin from psoriatic patients miR-21 was up-regulated. Anti-miR-21 treatment of mice receiving patient-derived xenotransplants resulted in IL-17 and IL-23 down-regulation (35). Similarly, miR-146a was up-regulated in lesional skin and PBMCs of psoriatic patients (55), but also in RA synovium (56), and positively correlated with IL-17 expression and disease severity (55, 56).

miR-451a expression was increased in spleen and thymus of a SLE mouse and its blockade decreases serum level of IL-17. *In vitro* and *in vivo* studies identified IRF8 as a target of miR-451a (57).

Type I IFNs

Type I IFNs are a family of cytokines produced by innate immune cells (pDCs in particular) and by tissue cells upon sensing of viral nucleic acids via RIG-Like Receptors (RLRs) and TLRs. By binding to a common, ubiquitously expressed receptor, these cytokines induce viral resistance in tissues and exert important immunostimulatory functions (58). Increased levels of type I IFNs are the hallmark and a pathogenic mechanism of a class of autoimmune diseases known as "interferonopathies" comprising SLE, psoriasis, SS, and others (5, 6, 59). Indeed, several inhibitors of type I IFN are currently under clinical trial for the treatment of SLE and psoriasis (6).

A direct regulation of type I IFN production by miRNAs was described by our own group. Indeed, together with pro-inflammatory cytokines, we found that exosome-associated miRNAs from the plasma of inactive SLE patients induced also the release of type I IFNs by human primary pDCs via TLR7 triggering (17).

A decreased expression of several miRNAs was implicated in the over-expression of type I IFNs in SLE patients. Under-expression of miR-146a, a negative regulator of innate immunity, in both active and inactive patients negatively correlated with clinical disease activity and with IFN scores. However, in active patients the levels were significantly lower than in inactive individuals. In healthy PBMCs, inhibition of endogenous miR-146a increased the induction of type I IFNs, while over-expression repressed type I IFN production by targeting IRF5 and STAT1. Importantly, introduction of miR-146a into the patients' PBMCs alleviated the coordinate activation of the type I IFN pathway (60).

miR-302d is an estrogen-regulated miRNA that was found decreased in SLE monocytes, where it inversely correlated with

the IFN-dependent genes MX1 and OAS1. It also inversely correlated with the levels of its predicted target, IRF9, a critical component of the transcriptional complex that regulates expression of genes induced by type I IFNs. Furthermore, significantly reduced miR-302d levels and increased IRF9 levels were identified in SLE patients with active disease as compared to inactive individuals (61).

Another study found a strong inverse correlation between type I IFNs expression and the levels of miR-155, miR-17, and miR-181b in PBMCs of active SLE patients, but the molecular mechanism was not elucidated (62).

IL-10

IL-10 is a pleiotropic cytokine produced by multiple cell types including innate immune cells, B cells, Th1, and Th2 cells, $CD4^+CD25^+FOXP3^+$ Treg cells, and keratinocytes (63). It exerts anti-inflammatory and immunomodulatory effects mainly acting on innate myeloid cells. Indeed, IL-10 directly inhibits the production of primary pro-inflammatory cytokines, thus representing a key anti-inflammatory mediator. In addition, it indirectly inhibits the activation of adaptive immunity also by blocking the production of IL-12 and the expression of MHC and costimulatory molecules. Ultimately, IL-10 is thought to play a crucial role in terminating excessive T-cell responses to prevent chronic inflammation and tissue damage, especially at the mucosal level (64), as demonstrated by the observation that IL-10-deficient mice develop spontaneous enterocolitis and other Crohn's disease-like symptoms as well as exaggerated asthmatic and allergic responses (65).

let-7e is significantly up-regulated in EAE and directly decreases IL-10 production by targeting its 3′UTR (51).

In activated T cells from RA patients, increased levels of miR-223 were implicated in decreased production of IL-10. This effect depended on IGF-1R targeting by miR-223. Indeed, IL-10 secretion was shown to depend on IGF in these cells (66).

$CD4^+$ T cells from patients with psoriasis vulgaris showed miR-210 over-expression. This study showed that miR-210 inhibits FOXP3 expression, thus impairing the immunosuppressive functions of Treg cells and decreasing the levels of IL-10 (54).

Elevated IL-10 levels were shown to correlate with disease activity in SLE (67) miR-410. was down-regulated in $CD3^+$T cells of SLE patients as compared to healthy controls and was shown to target the 3′ UTR of STAT3 mRNA. This would result in increased STAT3 levels, which is a positive regulator of IL-10 production in $CD3^+$T cells (68). Also, miR-21 upregulation strongly correlated with SLE disease activity. Its silencing decreased IL-10 production by T cells. Investigation of putative gene-targets showed PDCD4 (a selective protein translation inhibitor) to be effectively suppressed by miR-21. Accordingly, PDCD4 expression was confirmed to be decreased in active SLE (69).

miRNAs AS FUTURE THERAPEUTICS

Cytokine targeting with monoclonal antibodies or recombinant peptides is nowadays a powerful therapeutic option for autoimmune diseases that is dramatically improving patient outcomes (70). However, it does not work for everyone: in the case of RA, for example, improvement is usually seen in about two thirds of patients and it remains impossible to predict which patients will benefit of the treatment. In addition, high costs and lack of oral absorption have often represented major barriers for the success of biologicals (71).

The discovery of miRNAs as important regulatory agents for gene expression and their widespread deregulation in several pathological settings boosted the idea to exploit them as therapeutic targets and tools [reviewed in (18, 72–74)]. Available literature confirms that cytokines are relevant targets of miRNAs that are deregulated in autoimmune diseases. Thus, miRNAs could represent interesting therapeutic targets for controlling aberrant cytokine production involved in the onset and amplification of autoimmunity. However, at present, it is not possible to identify signature miRNAs, i.e., the miRNAs mainly responsible for cytokine deregulation in specific autoimmune diseases to be addressed as therapeutic candidate/s.

miRNAs possess unique characteristics that render them very attractive in terms of drug development (72). First, they are small, with known sequences and are often conserved among species. Second, it is possible both to supplement downregulated miRNAs by using synthetic oligonucleotides and to block the effects of increased miRNAs through artificial antagonists, either oligonucleotides or small molecules. In this regard, miRNA-based therapies can also take advantage from decades of research on other therapeutic oligonucleotides. Third, the ease of administration through local or parenteral injection routes and sufficient uptake in tissues gives miRNA therapeutics an extra edge. Last, but not least, one single miRNA can regulate different targets and potentially influence entire cellular pathways or processes. However, our current lack of a full understanding of miRNA biology and of the intricate network of interactions between miRNAs and the human genome, transcriptome and proteome restrains the translation of miRNA-based therapy into the clinical use. Also, as above anticipated, the identification and validation of signature miRNAs has yet to come for most diseases. In addition, a number of specific challenges associated with miRNA targeting still need to be faced, such as predicting possible off-target effects and toxicity, improving miRNA stability and optimizing the delivery systems.

In the last 5 years, a number of miRNA-based therapeutic tools entered in clinical trials, mainly for cancer management (73–75). Thus, an increasing amount of preclinical and clinical data for miRNA replacements and antagonists is expected to become soon available. This, together with progresses in characterizing disease-signature miRNAs, will determine the therapeutic future of this potentially powerful technology.

CONCLUDING REMARKS

Altered miRNA levels are observed in most autoimmune diseases and are recognized to influence autoimmunity through different mechanisms, among which deregulation of pathogenic cytokines may be of crucial importance. Literature describing novel deregulated miRNAs and putative targets is tumultuously

growing. Although much work has still to be performed to gain an integrated overview of the relevant miRNAs and molecular mechanisms of cytokine modulation in specific autoimmune diseases, these studies will hopefully lead to the identification of disease-specific signature miRNAs. These, in turn, will represent interesting candidates for next generation drugs aimed at controlling the production of pathogenic cytokines in autoimmune conditions.

AUTHOR CONTRIBUTIONS

DB conceived the article and wrote the manuscript. VS substantially contributed to draft writing and prepared the table. VG contributed to draft writing and table editing and provided artwork. LT contributed to draft writing. SS conceived the work and contributed critical revision of the manuscript. All authors approved the final version of the manuscript.

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The Long Non-coding RNA NRIR Drives IFN-Response in Monocytes: Implication for Systemic Sclerosis

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TLR4 activation initiates a signaling cascade leading to the production of type I IFNs and of the downstream IFN-stimulated genes (ISGs). Recently, a number of IFN-induced long non-coding RNAs (lncRNAs) that feed-back regulate the IFN response have been identified. Dysregulation of this process, collectively known as the "Interferon (IFN) Response," represents a common molecular basis in the development of autoimmune and autoinflammatory disorders. Concurrently, alteration of lncRNA profile has been described in several type I IFN-driven autoimmune diseases. In particular, both TLR activation and the upregulation of ISGs in peripheral blood mononuclear cells have been identified as possible contributors to the pathogenesis of systemic sclerosis (SSc), a connective tissue disease characterized by vascular abnormalities, immune activation, and fibrosis. However, hitherto, a potential link between specific lncRNA and the presence of a type I IFN signature remains unclear in SSc. In this study, we identified, by RNA sequencing, a group of lncRNAs related to the IFN and anti-viral response consistently modulated in a type I IFN-dependent manner in human monocytes in response to TLR4 activation by LPS. Remarkably, these lncRNAs were concurrently upregulated in a total of 46 SSc patients in different stages of their disease as compared to 18 healthy controls enrolled in this study. Among these lncRNAs, Negative Regulator of the IFN Response (NRIR) was found significantly upregulated *in vivo* in SSc monocytes, strongly correlating with the IFN score of SSc patients. Weighted Gene Co-expression Network Analysis showed that NRIR-specific modules, identified in the two datasets, were enriched in "type I IFN" and "viral response" biological processes. Protein coding genes common to the two distinct NRIR modules were selected as putative NRIR target genes. Fifteen *in silico*-predicted NRIR target genes were experimentally validated in NRIR-silenced monocytes. Remarkably, induction of CXCL10 and CXCL11, two IFN-related chemokines associated with SSc pathogenesis, was reduced in NRIR-knockdown monocytes, while their plasmatic level was increased

in SSc patients. Collectively, our data show that NRIR affects the expression of ISGs and that dysregulation of NRIR in SSc monocytes may account, at least in part, for the type I IFN signature present in SSc patients.

Keywords: long non-coding RNAs, monocytes, systemic sclerosis, interferon, NRIR

INTRODUCTION

Toll-like receptor 4 (TLR4) is a member of the pattern recognition receptors (PRR) family, which detects conserved structures found in a broad range of pathogens and triggers innate immune responses. TLR4 signals through two major pathways: (i) the MyD88-dependent pathway, that elicits the release of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-12p40; (ii) the TRIF (Toll/IL-1R domain-containing adaptor-inducing IFN-beta)-dependent pathway, that contributes to pro-inflammatory cytokine responses and, most importantly, induces type I IFN responses, particularly IFN-β (1). IFNs confer their activity by regulating networks of interferon-stimulated genes (ISGs), a process that requires *de novo* transcription and translation of both IFN and downstream ISGs (2). Other than being activated by different exogenous pathogen-associated molecular patterns (PAMPs), the IFN pathway is activated also by TLR4 ligation of endogenous danger-associated molecular patters (DAMPs) released upon cell damage or stress (3, 4). Thus, TLR4-mediated activation of innate immunity plays a key role not only in host defense against pathogens but also in numerous autoimmune diseases, including systemic sclerosis (SSc) (5). Indeed, endogenous ligand-induced TLR4 activation has been recognized as a key player driving the persistent fibrotic response in SSc (5–7). Different endogenous TLR4 ligands, including fibronectin extra domain A (FnEDA) and S100A8/A9, are indeed increased in the circulation of SSc patients and have been correlated with fibrotic-related clinical complications (8, 9). Moreover, activation of TLR4 response leads to transforming growth factor-β production, a crucial mediator for fibrosis development in SSc (10).

Likewise, production of type I interferon is closely linked to TLR4-mediated innate immune signaling in SSc (11–13). In fact, several lines of evidence suggest that both the IFN network and monocytes are implicated in SSc immune-pathogenesis. First, the development of SSc has been reported in patients undergoing IFN treatment (14) and IFN-α injections worsen SSc-related clinical features (15). Most importantly, increased expression of type I IFN-regulated genes, known as "type I IFN signature," is a hallmark of SSc, and type I IFN signature is present both in the fibrotic skin and in peripheral blood cells (11, 13), as well as in monocytes of SSc patients from the earliest phases of the disease, even before the skin fibrosis is evident (16). Moreover, in the fibrotic subsets of SSc patients we identified an increase in non-classical monocytes spontaneously producing the IFN-responsive CXCL10 (17), a chemokine associated with faster progression rate from pre-fibrotic SSc to worse disease stages (18).

The IFN pathway downstream TLR4 activation has been focus of intense investigation and a number of known protein-mediated mechanisms that mediate the complex signaling pathways and gene expression programs involved in the interferon response have been identified (2). Recent studies point at long non-coding RNAs (lncRNAs) as a novel class of IFN pathway regulatory molecules (19). LncRNAs are RNA transcripts longer than 200 nucleotides, characterized by lacking protein coding capability, but able to regulate gene expression both at the transcriptional and post-transcriptional levels (20). Existing data indicate that lncRNAs are critically involved in various biological and immunological processes (21), including several pathways related to innate immunity (22–29). However, with respect to the IFN response, while IFN-induced changes in the expression of protein-coding RNAs and their functional outcome have been well-documented, our knowledge of the impact of IFNs on lncRNA genes is highly incomplete. Moreover, the involvement of lncRNAs in diseases such as SSc, where both TLR4 and type I IFN concur to disease pathogenesis, is still unexplored.

This study aims to investigate the profile and the role of lncRNAs in the IFN response initiated by TLR4 activation of primary human monocytes and their implication in the immune dysregulation present in SSc patients.

MATERIALS AND METHODS

Patients

Patients affected by systemic sclerosis (SSc) and sex- and age-matched healthy controls (HC) were obtained from the University Medical Center Utrecht (UMCU), The Netherlands, and the Scleroderma Unit of Fondazione IRCCS Policlinico of Milan, Italy. Patients fulfilling the ACR/EULAR 2013 criteria (30) were classified in relation to the extent of skin fibrosis as limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) (31); patients satisfying the classification criteria without skin fibrosis were referred to as non-cutaneous SSc (ncSSc). Additionally, early SSc (eaSSc) subjects were defined as patients presenting with Raynaud's phenomenon and SSc-specific autoantibodies and/or typical nailfold videocapillaroscopy abnormalities (32). Three separate cohorts, herein named "definite SSc" cohort, "non-fibrotic SSc" cohort, "SSc cohort 3," were recruited for the current study. Demographics and clinical characteristics of the three cohorts are reported in **Tables 1–3**. All patients and healthy donors signed an informed consent form approved by the local institutional review boards prior to participation in the study. Samples and clinical information were made de-identified immediately after collection.

Cell Purification and Culture

Human CD14+ monocytes and neutrophils (PMNs) were purified from heparinised whole blood of SSc patients and matched HC or from buffy coats of healthy donors after

TABLE 1 | Demographics and clinical characteristics of the donors included in the definite SSc cohort.

Patient group (*n*)	HC (9)	ncSSC (7)	lcSSc (11)	dcSSc (7)
Age (yr.)	52 (30–64)	45 (26–63)	59 (45–70)	58 (34–72)
Female (*n*, %)	5 (56%)	6 (86%)	8 (73%)	3 (43%)
ANA (*n* pos, %)	–	6 (86%)	10 (91%)	7 (100%)
ACA (*n* pos, %)	–	3 (43%)	6* (55%)	1 (14%)
Scl70 (*n* pos, %)	–	2 (29%)	2* (18%)	4 (57%)
mRSS	–	0	6 (0–12)	14* (5–36)
ILD	–	1 (14%)	2 (18%)	5 (71%)
Disease Duration (yr.)		4 (1–12)	9 (1–19)	10 (2–27)

Values reported indicate the number (n) of patients and the median for each parameter [Interquartile Range (IQR)], if not otherwise indicated. ACA, anticentromere antibodies; ANA, antinuclear antibodies; dcSSc, diffuse cutaneous SSc; HC, healthy controls; ILD, Interstitial Lung disease; lcSSc, limited cutaneous SSc; mRSS, modified Rodman Skin score; ncSSc, non-cutaneous SSc; pos, positivity; Scl70, anti-topoisomerase antibodies; Yr., years.

**1 patient unknown.*

TABLE 2 | Demographics and clinical characteristics of the donors included in the non-fibrotic SSc cohort.

Patient group (*n*)	HC (9)	eaSSC (11)	ncSSc (10)
Age (yr.)	38 (28–49)	57 (40–77)	52 (25–70)
Female (*n*, %)	9 (100%)	11 (100%)	10 (100%)
ANA (*n* pos, %)	–	10 (91%)	10 (100%)
ACA (*n* pos, %)	–	7 (64%)	8 (80%)
Scl70 (*n* pos, %)	–	2 (18%)	1 (10%)
mRSS	–	0	0
ILD	–	0	0
Disease Duration (yr.)	–	–	Unknown

Values reported indicate the number (n) of patients and the median for each parameter [Interquartile Range (IQR)], if not otherwise indicated. ACA, anticentromere antibodies; ANA, antinuclear antibodies; eaSSc, early SSc; HC, healthy controls; ILD, Interstitial Lung disease; mRSS, modified Rodman Skin score; ncSSc, non-cutaneous SSc; pos, positivity; Scl70, anti-topoisomerase antibodies; Yr., years.

centrifugation over Ficoll-Paque gradient. Briefly, CD14+ monocytes were purified from PBMCs using the anti-CD14 microbeads (Miltenyi Biotec), on the autoMACs Pro Separator (Miltenyi Biotec) according to manufacturer's protocol. Purity of monocyte preparations was usually >98%. PMNs were recovered after dextran sedimentation and hypotonic lysis of erythrocytes followed by EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, Canada) (33). Purity of neutrophils preparations was usually 99.7 ± 0.2%.

Monocytes (3×10^6 cells/ml) and PMNs (5×10^6 cells/ml) were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS (<0.5 EU/ml; Sigma-Aldrich) and 2 mM Glu in the presence or absence of 100 ng/ml ultra-pure lipopolysaccharide (LPS, from E. coli strain O111:B4, InvivoGen, San Diego, CA, USA), 5 μM R848 (Invivogen), 1,000 U/ml IFNα CRI003B, Cell Sciences), 100 ng/ml palmitoyl-3-cysteine-serine-lysine-4 ($Pam3CSK_4$, Invivogen), 50 μg/ml polynosinic:polycytidylic acids [poly(I:C), Invivogen], as indicated. In selected experiments, CD14+ monocytes were incubated for 30 min with 5 μg/ml Brefeldin A (BFA, Sigma-Aldrich) or 5 μg/ml αIFNAR (PBL InterferonSource, Piscataway, NJ, USA) or its isotype control antibody (mouse IgG2a), before cell stimulation.

Human Monocyte Transfection

Freshly purified monocytes (8×10^6) were transfected with 200 pmol NRIR-specific Silencer Select siRNA or Silencer Select negative control #2 (both from Ambion, Thermo Scientific), using the Human Monocyte Nucleofector Kit and the AMAXA Nucleofector II device (both from Lonza), according to the manufacturer's protocol. Once transfected, cells were plated in recovery medium [50% RPMI 1640 + 10% FCS + 2 mM Glu, and 50% IMDM (Lonza) + 10% FCS + 2 mM Glu], at 3×10^6 cells/ml overnight. The next day, medium was changed to RPMI 1640 + 10% FCS + 2 mM Glu, and cells were stimulated as indicated. NRIR specific Silencer Select siRNA sequence (34) is reported in **Table S1**.

Extraction of Total RNA

Total RNA was purified with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. DNAse treatment (RNAse Free DNase I set, Qiagen) on column was performed. RNA quantification, purity and integrity were assessed at the Nanodrop 2000 spectrophotometer (Thermo Scientific) and by capillary electrophoresis on an Agilent Bioanalyzer (Agilent Technologies), respectively. Purified RNA was used for sequencing analysis or RT-qPCR, as described below.

RNA Sequencing Analysis

RNA sequencing data of peripheral blood monocytes purified from SSc, together with sex- and age-matched healthy controls (HC) enrolled in the "definite SSc" cohort, were obtained from the University Medical Center Utrecht (UMCU), The Netherlands (35).

RNA sequencing libraries were generated from total RNA extracted from CD14+ monocytes of SSc patients and matched HC enrolled in the "definite SSc" and "non-fibrotic SSc" cohorts, or from RNA pools of three different donors of freshly isolated and LPS-treated monocytes. RNA-seq library preparation was accomplished using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on a HiSeq 2000 system (Illumina) using pair-end sequencing reads (2×90 bp for SSc and matched HC libraries and 2×51 bp for resting and LPS-treated monocytes libraries); a minimum of 20 million fragments per sample were analyzed. After quality filtering according to the Illumina pipeline, reads were firstly aligned to the human transcriptome annotated in Ensembl 77 (*Homo sapiens* gene model annotation) and secondly converted to genomic mapping using as reference the human reference genome GrCh38 (Genome Reference Consortium Human build 38) by means of TopHat (v 2.0.14) (36). On average, 23,969,150 (concordant pair alignment rate: 91.84%), 24,404,133 (concordant pair alignment rate: 89.90%), and 43,071,006 (concordant pair alignment rate: 92.67%) paired-reads of the "definite SSc," "non-fibrotic SSc"

and LPS-treated-monocytes dataset, respectively, mapped to the reference genome.

Differential expression analysis was performed using the generalized linear model (GLM) implemented in DESeq2 (v 1.6.3) on the summed exon reads count per gene estimated using HTSeq-count (v 0.6.1p1) (37, 38). Differentially expressed genes were identified from the comparison of each single SSc group and matched HC. Significance was tested using the Wald test. Genes with a $\log_2$(FC) value $\geq$0.58 or $\leq$-0.58 and a $p \leq 0.05$, were considered significantly modulated. Differentially expressed genes in LPS-treated monocytes were identified using the Likelihood Ratio Test (LRT). Raw *p*-values from differential expression analyses were adjusted to control the false discovery rate (FDR) using the Benjamini–Hochberg method. Genes with adjusted $p < 0.05$ were considered significantly modulated by LPS. Gene expression levels were expressed as variance stabilized data (vsd) or FPKM, calculated according to DESeq2 instructions. Gene type were associated according to the Ensembl 77 annotation. All genes not belonging to the gene type protein coding and pseudogene and with a transcript length of at least 200 bp were considered as lncRNAs. Raw and processed sequencing data are available from Gene Expression Omnibus under the following accession numbers: GSE123532 and GSE124075.

Gene Expression Data of PBMC From SLE Patients and Relative Healthy Controls

Gene expression profiles of PBMC purified from systemic lupus erythematosus (SLE) and relative healthy donors (HC) were downloaded from Gene Expression Omnibus Database (GEO number: GSE122459). Gene expression levels and differential expression analysis were retrieved from the dataset present in the GEO database.

GO-Term and Pathway Enrichment Analysis

Protein coding genes (PCGs) were subjected to Gene Ontology (GO) and pathway enrichment analysis using ToppFun[1] (39). *p*-value was calculated according to the probability density function and corrected for the False Discovery Rate (FDR) according to Benjamini-Hochberg method. Pathways and GO-terms associated to biological processes (BP) with a FDR$\leq$0.05 were considered significantly enriched.

Weighted Gene Co-expression Network Analysis

Co-expression networks were generated using WGCNA R-package (40). Signed weighted adjacency matrix of connection strengths was constructed using the soft-threshold approach with a scale-independent topological power $\beta = 18$ for LPS-treated and freshly isolated monocytes and $\beta = 13$ for the definite SSc data. Genes were aggregated into modules by hierarchical clustering and refined by the dynamic tree cut algorithm. Biological function of each module was evaluated by pathway and BP GO-terms enrichment analysis using ToppFun (39). All terms enriched with a FDR < 0.05, were considered. Redundancy of significantly enriched BP GO-terms was solved by means of REVIGO (41) using the simRel score to assess similarity between two GO-terms (42). NRIR-specific modules were visualized using Cytoscape v3.2.1 (43).

Gene Expression Analysis by Real-Time PCR

RNA samples were reverse transcribed using 5 ng/μl random primers, 1 U/μl RNase inhibitor (RNAse Out, Invitrogen) and 5 U/μl reverse transcriptase (SuperScript III, Invitrogen), according to manufacturer's instruction. NRIR expression was quantified in duplicates by RT-qPCR from 9 ng RNA-equivalent cDNA in the presence of SYBR Select Master Mix (ThermoFisher Scientific, Applied Biosystems) and 400 nM specific primers (**Table S1**), on the ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific, Applied Biosystems) using the standard protocol. PCG expression was quantified in duplicates by RT-qPCR from 9 ng RNA-equivalent cDNA in the presence of Fast SYBR Green Master mix (ThermoFisher Scientific, Applied Biosystems) and 200 nM of specific primer pairs (**Table S1**), on the ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific, Applied Biosystems). Primers were designed using the Oligo Explorer software[2], for only fifty-six out seventy-nine NRIR putative target genes was possible to design specific primer pairs. Data were analyzed with LinReg PCR 7.0[3] and Q-Gene software[4] Gene expression was calculated as mean normalized expression [MNE (44)] units after normalization over the stably expressed RPL32 or ACTIN B.

Multiplex Immunoassay

CXCL10, CXCL11, and CCL8 concentrations in cell-free supernatants and/or plasma from SSc patients and matched HC enrolled in the "SSc cohort 3" were measured using an in-house developed and validated (ISO9001 certified) multiplex immunoassay (Laboratory of Translational Immunology, University Medical Center Utrecht) based on Luminex technology (xMAP, Luminex Austin TX USA). The assay was performed as previously described (45). Aspecific heterophilic immunoglobulins were pre-absorbed from all plasma samples with heteroblock (Omega Biologicals, Bozeman MT, USA). All samples were measured with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with the xPONENT software (v 4.2, Luminex). Data were analyzed by a 5-parametric curve fitting using the Bio-Plex Manager software (v 6.1.1, Biorad).

Statistical Analysis

Data are expressed as mean ± SEM unless otherwise indicated. Statistical evaluation was determined using the Mann Whitney test or the two-way analysis of variance (ANOVA), followed by Bonferroni post-test, with α set to 0.05. Correlation analysis

[1]https://toppgene.cchmc.org/enrichment.jsp

[2]http://www.genelink.com/tools/gl-downloads.asp

[3]http:/LinRegPCR.nl

[4]http://www.gene-quantification.de/download.html.

TABLE 3 | Demographics and clinical characteristics of the donors included in the SSc cohort 3.

Patient group (n)	HC (21)	eaSSc (15)	ncSSc (27)	lcSSc (23)	dcSSc (19)
Age (yr.)	52 (35–82)	62 (25–81)	59 (29–80)	60 (41–80)	52 (27–80)
Female (*n*, %)	19 (90%)	15 (100%)	27 (100%)	22 (96%)	15 (79%)
ANA (*n* pos, %)	–	15 (100%)	26 (96%)	22 (96%)	16 (84%)
ACA (*n* pos, %)	–	12 (80%)	20 (74%)	12 (52%)	0 (0%)
Scl70 (*n* pos, %)	–	2 (13%)	1 (4%)	9 (39%)	11 (58%)
mRSS	–	0	0	4 (0–8)	12 (2–29)
ILD	–	0	2 (7%)	7 (30%)	14 (74%)
Disease Duration (yr.)	–	N.A.	10* (0–29)	16** (1–38)	10 (1–25)

Values reported indicate the number (n) of patients and the median for each parameter [Interquartile Range (IQR)], if not otherwise indicated. ACA, anticentromere antibodies; ANA, antinuclear antibodies; dcSSc, diffuse cutaneous SSc; eaSSc, early SSc; HC, healthy controls; ILD, Interstitial Lung disease; lcSSc, limited cutaneous SSc; mRSS, modified Rodman Skin score; N.A., not assessed; ncSSc, non-cutaneous SSc; pos, positivity; Scl70, anti-topoisomerase antibodies; Yr., years.

**2 patients unknown.*

***3 patients unknown.*

were performed using the *rcorr()* function in R using the non-parametric Spearman method. Correlation with $p < 0.05$ were considered significant.

RESULTS

Identification of LPS-Modulated lncRNAs in Primary Human Monocytes

To identify lncRNAs potentially involved in the responses of peripheral human monocytes downstream TLR4 activation, CD14+ monocytes purified from buffy coats of healthy donors were cultured in the presence or absence of LPS (100 ng/ml) for 1.5 h or 4 h, and subsequently subjected to RNA sequencing. 1,812 transcripts annotated as lncRNAs in Ensemble (**Figure 1A**) were identified as significantly (p-adj < 0.05) modulated in response to LPS. Specifically, 1278 lncRNAs (i.e., 70.53%) were up-regulated, while 534 lncRNAs (i.e., 29.47%) were down-regulated (**Figure 1B**). Moreover, K-means clustering arranged the LPS-modulated lncRNAs in three main groups according to their kinetic of expression (**Figure 1C**): (i) lncRNAs rapidly and consistently modulated by LPS within 1.5 h, representing the majority (52.32%) of LPS-modulated lncRNAs (early group, **Figure 1D**); (ii) lncRNAs modulated by LPS within 1.5 h in a transient manner (22.57%) (early and transient group, **Figure 1E**); (iii) lncRNAs modulated by LPS at 4 h (25.11%) (late group, **Figure 1F**).

Identification of Type I IFN Signature-Associated lncRNAs

LncRNAs possibly involved in the regulation of type I IFN pathway activated downstream TLR4 were identified using the strategy depicted in **Figure 2**. Specifically, 3,248 PCGs up-regulated in response to LPS (FPKM > 2) were retrieved and subjected to GO term enrichment analysis. 469 LPS-induced PCGs associated to significantly enriched IFN-response and anti-viral response-related GO-terms were then subjected to correlation analysis with the 1,812 LPS-modulated lncRNAs. Finally, based on the knowledge that lncRNAs can regulate the transcription of PCGs located *in cis* (46), only the lncRNAs localized *in cis* ($\pm$ 150 Kb) to correlated PCGs were retrieved ($n = 99$) (**Figure 2** and **Table S2**). This group of lncRNAs ($n = 99$) will be referred from now on as the "IFN/viral" lncRNAs.

To verify whether the selected "IFN/viral" lncRNAs were effectively related to the IFN signature in an *in vivo* setting where the IFN pathway is known to play a pathogenetic role, the expression level of the 99 selected lncRNAs was then retrieved and analyzed from the transcriptomic profile of monocytes purified from the "definite SSc" (35) and "non-fibrotic SSc" cohorts of patients and matched healthy donors (**Tables 1**, **2**). The patient cohorts included individuals presenting with different SSc phenotypes according to clinical features and the extent of skin fibrosis, i.e., early SSc (eaSSc, $n = 11$), non-cutaneous SSc (ncSSc, $n = 17$), limited cutaneous SSc (lcSSc, $n = 11$), diffuse cutaneous SSc (dcSSc, $n = 7$).

Four out of ninety-nine lncRNAs, namely NRIR, PSMB8-AS1, RP5-1091N2.9, and RP11-24F11.2, were expressed at significantly higher levels in at least two groups of SSc patients as compared to their respective healthy donors in the "definite SSc" cohort (**Figure 3A**), whereas only NRIR was significantly up-regulated in ncSSc and showed a trend in eaSSc (FC $= 1.30$, $p = 0.104$) in the "non-fibrotic" cohort (**Figure 3B**). Remarkably, only the expression of NRIR significantly correlated in both cohorts with the patients' IFN score (**Figures 3C,D**), calculated on the basis of the expression of IFI27, IFI44L, IFIT1, IFIT2, IFIT3, and SERPING1 (16).

IFNα was demonstrated to be central to the pathogenesis also of other systemic autoimmune diseases, with Systemic lupus erythematosus (SLE) being the prototype one. To verify whether NRIR is effectively related to the IFN signature in an *in vivo* setting in IFN-related diseases other than SSc, we retrieved from the Gene Expression Omnibus database RNA-seq data from PBMCs of SLE patients and matched healthy controls (GSE122459). Seventeen out of ninety-nine lncRNAs were commonly modulated in LPS-treated CD14+ transcriptome and SLE PBMCs compared to healthy controls (**Figure S1A**), and only three lncRNAs, namely NRIR, PSMB8-AS1 and RP5-1091N2.9, were modulated in all the three datasets

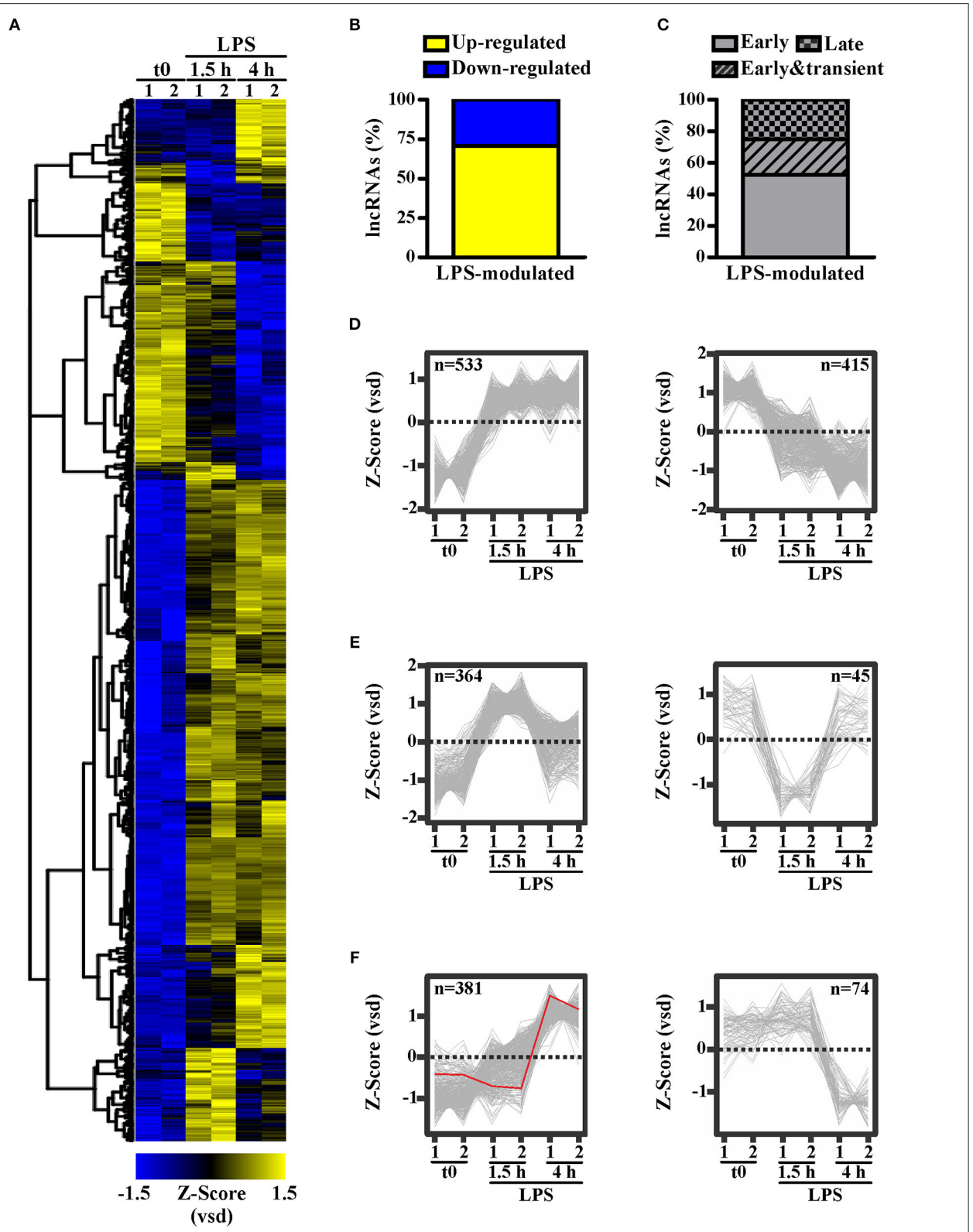

FIGURE 1 | LPS modulates the expression of long noncoding transcripts in human monocytes. CD14+ monocytes were cultured for 1.5 or 4 h with LPS (100 ng/ml) or left untreated (t0). Two pools of three donors for each condition were used to create polyA library for RNA-seq. Sequencing data were analyzed as described in Materials and Methods. The expression levels of the LPS-modulated (adjusted $p < 0.05$) lncRNAs **(A)** are shown as row mean-centered *z*-Score of the variance

(Continued)

FIGURE 1 | stabilized data (vsd). **(B)** The percentage of up- and down-regulated lncRNAs modulated by LPS. **(C)** The percentage of early, early & transient and late lncRNAs modulated by LPS. K-means clustering analysis was applied on the significantly modulated lncRNAs. Early modulated **(D)**, early and transiently modulated **(E)** as well as late modulated **(F)** lncRNAs are shown. The expression of each lncRNA belonging to the three groups is shown. LncRNAs up regulated and down regulated by LPS are shown separately. NRIR expression is highlighted in red. LncRNA expression is depicted as row mean-centered *z*-Score of the variance stabilized data (vsd), number of lncRNA belonging to each KMC group is shown.

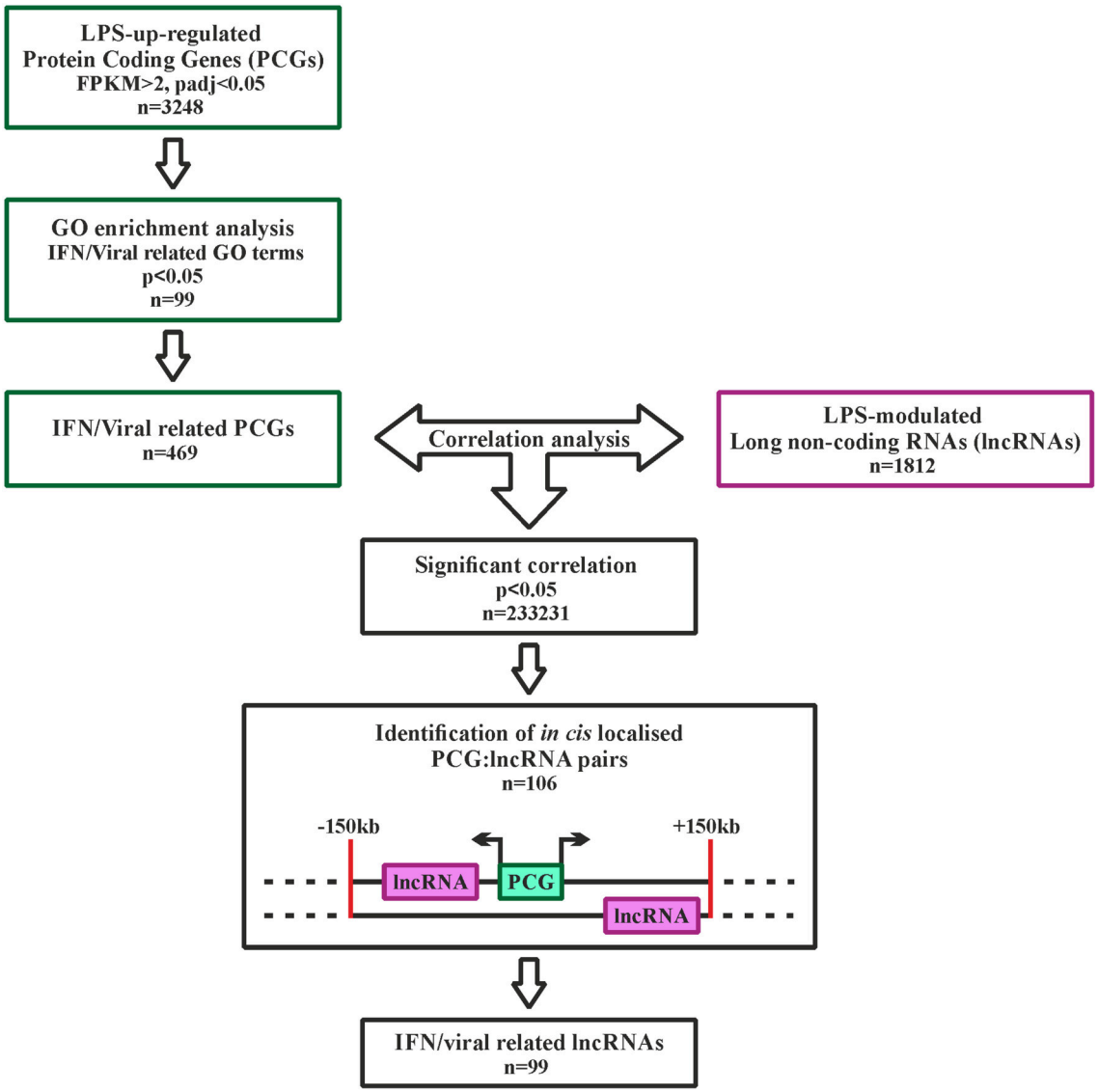

FIGURE 2 | Analysis pipeline to identify IFN/viral-related lncRNAs, modulated by LPS in monocytes. Green squares represent the selection of IFN/viral related protein coding genes, while the purple square represents the selected lncRNAs modulated by LPS. Black squares represent the workflow for integration of protein coding genes and lncRNAs by correlation analysis.

(i.e., LPS-treated CD14+ monocytes, SSc CD14+ monocytes and PBMC from SLE patients) (**Figure S1B**). Remarkably, NRIR was the only one lncRNA significantly up-regulated in all the three datasets and the lncRNA most differentially expressed ($\log_2$FC = 1.90, $p = 3.83 \times 10^{-8}$) in PBMC from SLE patients as compared to healthy controls (**Figure S1B**).

Collectively, data from three different biological datasets (i.e., transcriptome of monocyte activated *in vitro* by LPS, transcriptome of circulating monocytes from SSc patients and transcriptome of PBMC from SLE patients) converged in identifying NRIR as belonging to the IFN signature. Therefore, we focused our study on the pathways underlying NRIR

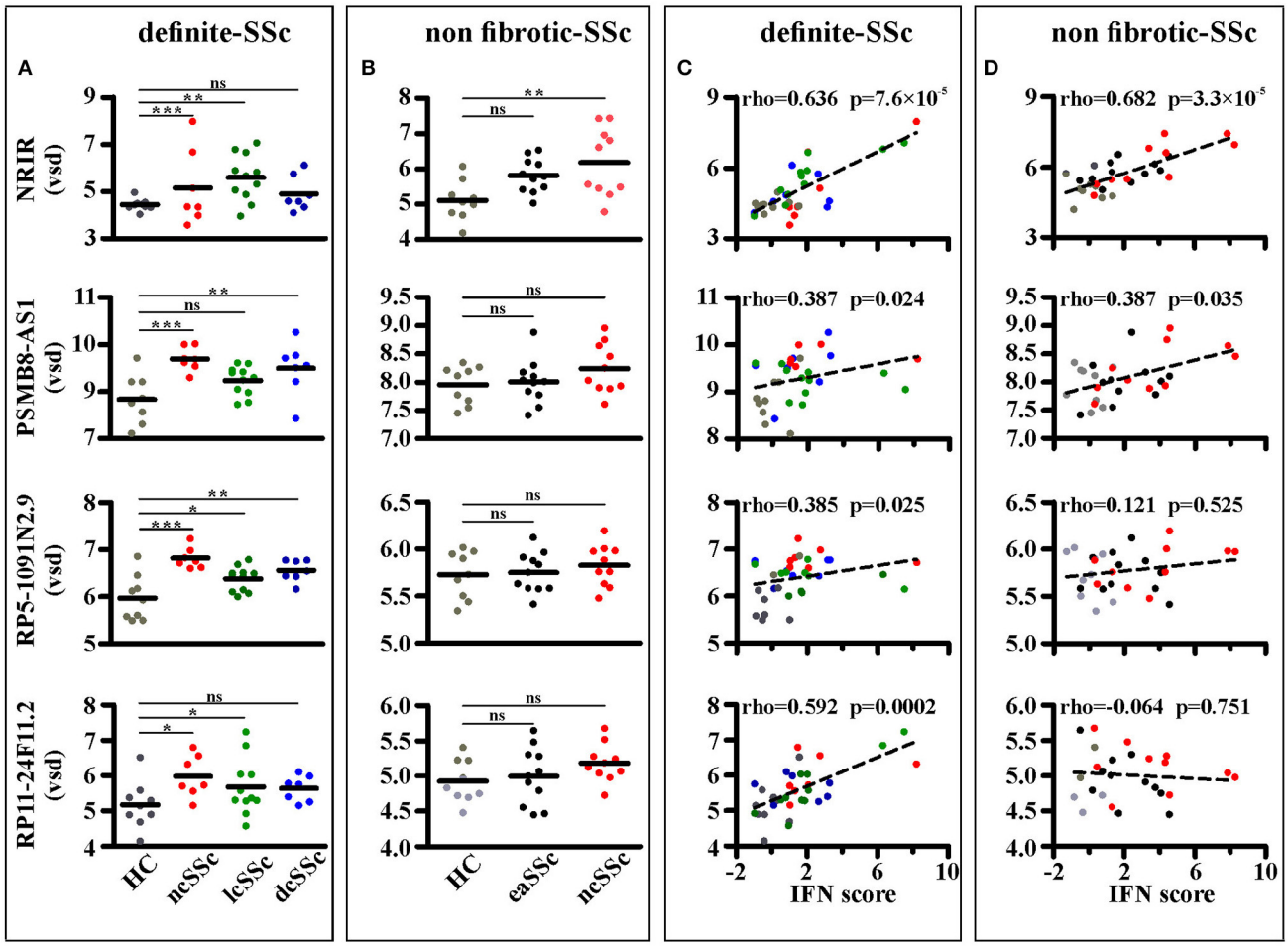

FIGURE 3 | NRIR expression is increased in monocytes from SSc patients and correlates with the IFN-score. RNA sequencing data of CD14+ monocytes from SSc patients and matched healthy controls (HC) from both the definite SSc and non-fibrotic SSc cohorts were analyzed as described in Meterials and Methods. NRIR, PSMB8-AS1, RP5-1091N2.9, and RP11-24F11.2 expression were considered. LncRNAs expression in HC and patients with established Systemic Sclerosis (ncSSc, lcSSc, and dcSSc, definite-SSc cohort) **(A)** and in patients with early stages of SSc (eaSSc and ncSSc, non-fibrotic SSc cohort) **(B)** is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant, by Wald test **(C)** Correlation of NRIR expression with the IFN-score of HC (gray), ncSSc (red), lcSSc (green), and dcSSc (blue) patients is depicted. **(D)** Correlation of NRIR expression with the IFN-score of HC (gray), eaSSc (black) and ncSSc (red) patients is shown. Spearman's Rho and *p*-value are reported. NRIR expression levels are expressed as vsd, IFN Score was calculated according to Brkic et al. (16).

upregulation as well as on the role of this lncRNA in the type I IFN signature.

NRIR Is a Type I IFN Dependent lncRNA

Consistent with KMC analysis of RNA-seq data that classified NRIR as a "late" transcript (**Figure 1F**, red line), kinetic analysis confirmed that NRIR expression is slowly induced by LPS stimulation in monocytes, being detectable after 4 h and steadily increasing over 16 h (**Figure 4A**). In addition, monocyte activation with agonists of TLR3 [polyinosinic:polycytidylic acid, poly(I:C)] and TLR7/8 (Resiquimod, R848), both known to promote type I IFN production, resulted in up-regulation of NRIR (**Figure 4B**). Conversely, a synthetic lipoprotein agonist of TLR2 (Pam3CSK4), unable to induce type I IFN transcription and secretion (47), was ineffective (**Figure 4B**). Consistent with this observation, treatment of monocytes with brefeldin A or with IFNα receptor (αIFNAR) blocking antibodies before LPS stimulation completely abolished NRIR induction by LPS (**Figure 4C**), indicating that endogenously produced type I IFNs is responsible for the upregulation of NRIR. Additionally, NRIR expression is significantly induced by IFNα but not by LPS, in human polymorphonuclear neutrophils (PMNs), that do not activate the IFN pathway downstream TLR4 (**Figure 4D**) (48). Taken together, these data demonstrate that type I IFN production is necessary and sufficient to increase NRIR expression in response to LPS.

The Type I IFN-Dependent NRIR Plays a Role in the Expression of Several ISGs

Identification of pathways likely associated to NRIR function was conducted by weighted gene co-expression analysis (WGCNA). Two specific co-expression networks were created, one composed

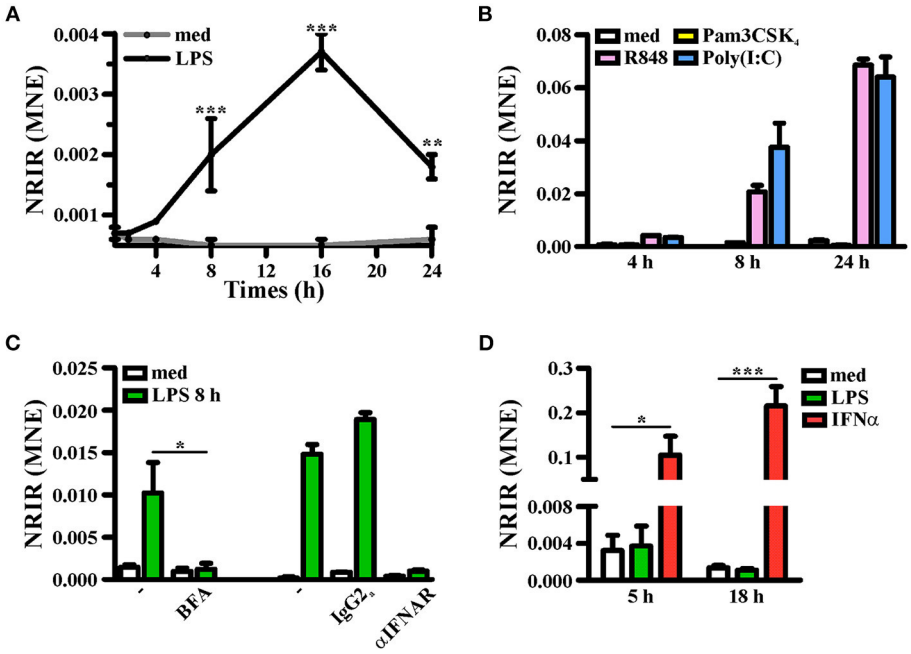

FIGURE 4 | Induction of NRIR expression is IFN-dependent. **(A)** CD14+ monocytes were cultured for the indicated time point in presence of LPS (100 ng/ml, black line) or left untreated (gray line). NRIR expression levels were analyzed by RT-qPCR and expressed as mean normalized expression (MNE). Results are shown as mean ± SEM of three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA. **(B)** CD14+ monocytes were stimulated with $Pam3CSK_4$ (100 ng/ml), poly(I:C) (50 μg/ml), R848 (5 μM) or left untreated for the indicated time points. NRIR expression levels were analyzed by RT-qPCR and expressed as MNE. One experiment representative of two performed is shown. **(C)** CD14+ monocytes were stimulated with LPS or left untreated for 8 h in presence or absence of brefeldin A (BFA, left) or αIFNAR or the control IgG2a antibody (right). NRIR expression levels were analyzed by RT-qPCR and expressed as MNE. For BFA experiments results are shown as mean ± SEM of three experiments, $^{*}p < 0.05$ by two-way ANOVA, while for αIFNAR experiments one experiment representative of two performed is shown. **(D)** Human neutrophils were stimulated with LPS (100 ng/ml), IFNα (1,000 U/ml) or left untreated for 5 and 18 h. NRIR expression levels were analyzed by RT-qPCR and expressed as MNE. Results are shown as mean ± SEM of three experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ by two-way ANOVA.

of 13 modules in the transcriptome of LPS-treated monocytes and the second one composed of 26 modules in the "definite SSc" cohort. The NRIR-related module was identified in both LPS-treated monocytes (blue module) and SSc monocytes (cyan module) co-expression networks. The blue module contained 2060 PCGs and 548 ncRNAs (**Figure S2**), while the cyan module was composed of 116 PCGs and 8 ncRNAs (**Figure S3**).

GO-term and pathway enrichment analysis of the PCGs of each module underlined that biological processes related to "response to type I IFN," "response to virus," and "immune system process" (**Figure 5**) and related pathways (**Tables S3**, **S4**) were significantly enriched in both modules. Comparative analysis of the two modules identified 83 common transcripts: specifically, 79 PCGs and 4 ncRNAs (**Figure 6A**), the majority (63.3%) of which were associated to IFN, antiviral and immune response (**Figure 6B**). The 79 common PCGs were selected as putative NRIR target genes.

To investigate the role of NRIR in the regulation of IFN and anti-viral response secondary to TLR4 activation, we analyzed the expression of 56 PCGs, that were co-expressed with NRIR and common to the both blue and cyan modules (**Figure 6**), in NRIR-silenced monocytes. Monocyte transfection with NRIR siRNA led to an average reduction of 60.83 ± 4.81 and 55.47 ± 4.83% of the constitutive and LPS induced NRIR expression, respectively (**Figure 7A**). Under these conditions, the induction of fifteen PCGs by LPS was significantly impaired as compared to cells transfected with a scramble siRNA (**Figures 7B–P**). Precisely, decreased induction of CXCL10, CXCL11, APOBEC3A, MX1, USP18 mRNA was observed 4 h after LPS stimulation and remained reduced at 8 h as well; decreased induction of CCL8, EPSTI1, DDX58, IFI44, IFIH1, IFIT2, and OAS2 was observed at shorter time point (4 h); whereas the ability of LPS to upregulate the expression of IFITM3, ISG15 and OAS3 could be detected only at later time point (8 h) (**Figures 7B–P**). The induction of the remaining forty-one PCGs was unaffected by NRIR knock-down (**Figures S4**, **S5**), Strikingly, all genes modulated by NRIR silencing were also significantly upregulated in at least one group of SSc monocytes as compared to cells isolated from healthy donors (**Figure S3**).

Among the IFN-responsive genes, CXCL10, CXCL11 and CCL8 have been shown to be implicated in SSc pathogenesis and/or to correlate with the degree of skin fibrosis (18, 49–51). Analysis of CXCL10, CXCL11 and CCL8 protein level in cell-free supernatants of LPS-stimulated monocytes showed a significant reduction of CXCL10 (mean reduction: 62.48 ± 8.94%, $n = 7$) and CCL8 (mean reduction: 56.13 ± 7.37%, $n = 7$) production in response to LPS (**Figures 8A,B**), while CXCL11 was below the detection levels (not shown). Noticeably, plasma level of CXCL10 and CXCL11 in the SSc subjects enrolled in this study was

significantly higher as compared to their healthy counterparts (**Figures 8C,D**).

Collectively, these data substantiate the role of NRIR in the expression of several interferon-responsive genes upregulated by LPS *in vitro* or constitutively increased in circulating monocytes from SSc patients.

DISCUSSION

The aim of this study was to investigate the potential role of lncRNAs in the type I IFN pathway elicited in human monocytes by TLR4 activation and to explore their functional role *in vivo*, in the IFN signature displayed by SSc monocytes. Several studies have shown that lncRNAs are involved in numerous aspects of the innate and adaptive immune responses (22), and, more recently, a critical role for a small group of lncRNAs in the regulation of the IFN response has been reported (19). Likewise, evidence clearly supports the involvement of lncRNAs in the pathogenesis of autoimmune and inflammatory diseases (25, 31), where the physiologic response of immune cells is dysregulated. However, no lncRNA has been associated to the immune dysregulation present in SSc yet. Characterization of the role of lncRNAs in the regulation of monocytes IFN response to TLR4 activating agents is an important aspect to understand both the physiologic response and the disease biology of SSc arising from alteration of physiologic pathways. In fact, the link between monocytes, TLR4 activation and the downstream IFN response with SSc pathogenesis is supported by several observations: (i) circulating monocytes have been indicated as one prominent leukocyte subset playing a role in the pathogenesis of SSc (52–55); (ii) circulating SSc monocytes are characterized by an increased type I IFN signature (11, 12, 16) (iii) TLR activation may represent the connection between immune activation in SSc and tissue fibrosis (7, 10, 52, 56).

The lncRNA landscape of LPS-activated human monocytes, characterized by RNA sequencing, identified 1,278 annotated lncRNAs as upregulated and 534 as downregulated. Modulated lncRNAs were further clustered according to their kinetic of expression into early, early and transient and late. Correlation with the expression of PCGs enriched in the IFN- and anti-viral response related GO-terms allowed us to retrieve lncRNAs likely comprised into the type I IFN pathway. Moreover, as some lncRNAs have been described to regulate the expression of neighboring genes (46), lncRNAs that may have functional relevance in the expression of LPS-induced mRNAs related to the IFN/anti- viral response were retrieved on the basis of their localization *in cis* to their respective correlated PCGs.

To validate the relevance of these "IFN/viral" lncRNAs in an *in vivo* setting where the IFN response constitute a major hallmark, we examined the expression level of each of the 99 lncRNAs in monocytes from two distinct cohorts of SSc patients as compared to the relative healthy control groups. The cohorts comprised patients with the full spectrum of SSc phenotypes, from pre-clinical eaSSc, to definite groups either presenting with (lcSSc and dcSSc) or without (ncSSc) skin fibrosis. Most importantly in both cohorts a remarkable IFN signature had been identified in previous studies (16, 35). Remarkably, monocytes from lcSSc and ncSSc patients showed consistently higher levels of NRIR expression, that correlated significantly with the IFN signature in both cohorts analyzed, strikingly confirming the implication of NRIR in the IFN response also in a pathological condition. Consistently, it must be noted that NRIR had the highest expression levels in patients with ncSSc, that is the SSc subset presenting with the strongest IFN-signature (16). In addition, it is intriguing to observe that NRIR shows a trend of

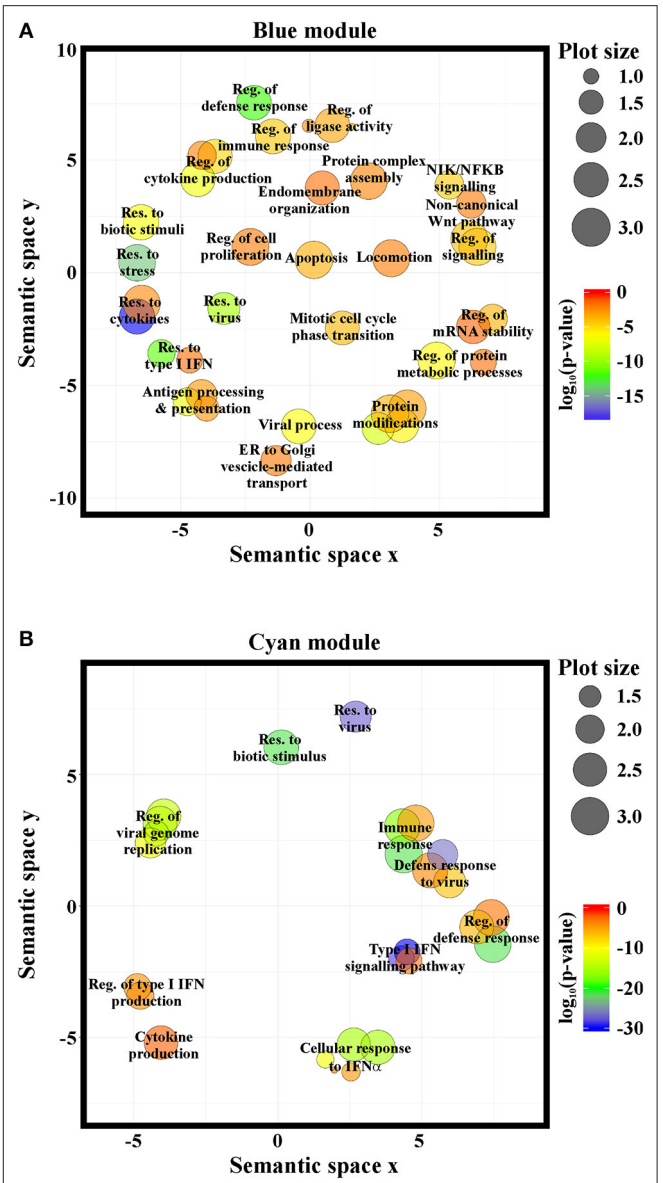

FIGURE 5 | NRIR is implicated in biological processes related to immune response and the IFN/antiviral response. GO-term enrichment analysis was performed to identify biological processes enriched in the blue- **(A)** or the cyan-module **(B)**. Significantly enriched GO terms are represented as circles according to their semantic similarities. Circle size represents term specificity (bigger, general terms; smaller, specific terms), while circle color represents the $\log_{10}$ (*p*-value FDR B&H) of the enrichment.

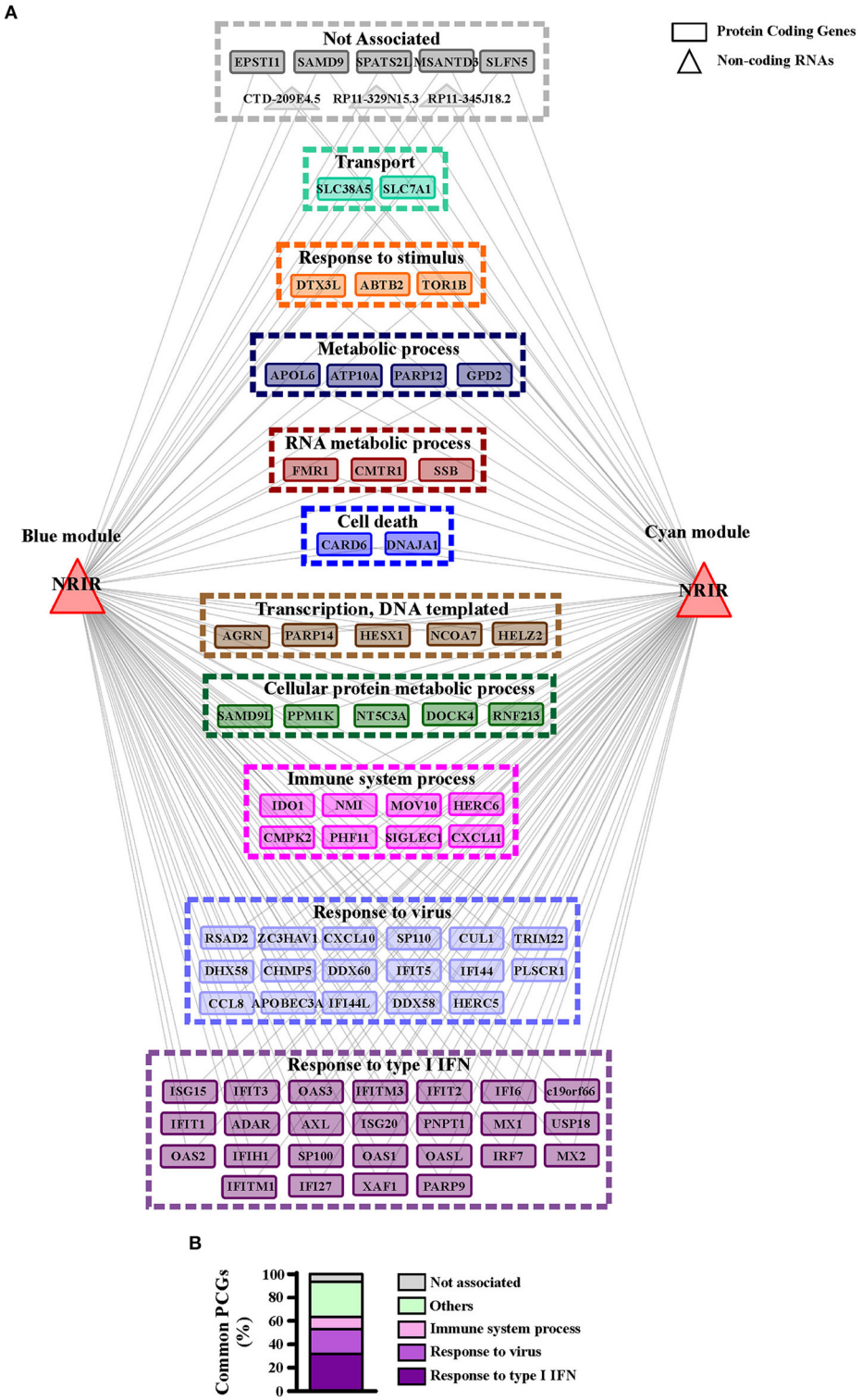

FIGURE 6 | PCGs common to the blue and cyan modules are mainly involved in the immune and IFN/antiviral response. **(A)** Representation of the transcripts common to blue and cyan-module. The seventy-nine protein coding genes and the four ncRNAs are represented as rectangles and triangles, respectively. Transcripts are grouped according to their associated biological process related GO-terms. Different colors highlight different group of GO-terms, the most general GO term, summarizing each group, is reported. Genes associated to any GO-term are signed as not associated and depicted in gray. **(B)** Protein coding genes found in both modules are associated to their GO terms. Percent of common protein coding genes associated to different GO terms is shown.

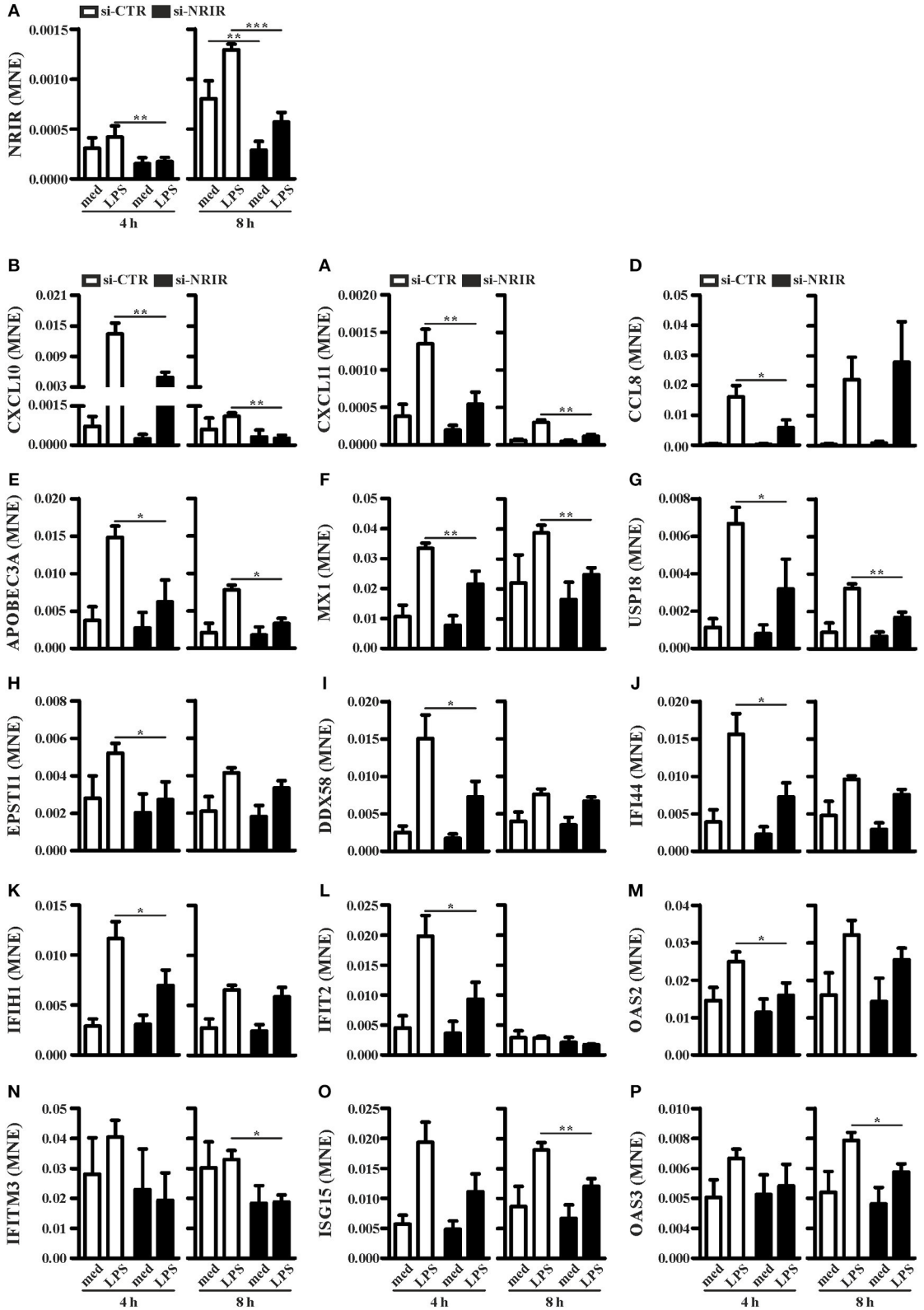

FIGURE 7 | NRIR regulates fifteen of its co-expressed genes. CD14+ monocytes were transfected with si-NRIR or si-CTR and 18 h later were stimulated with LPS for 4 or 8 h or left untreated. The expression of NRIR **(A)** and its co-expressed genes **(B–P)** was analyzed by RT-qPCR and expressed as MNE. Results are shown as mean ± SEM of at least three different experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA.

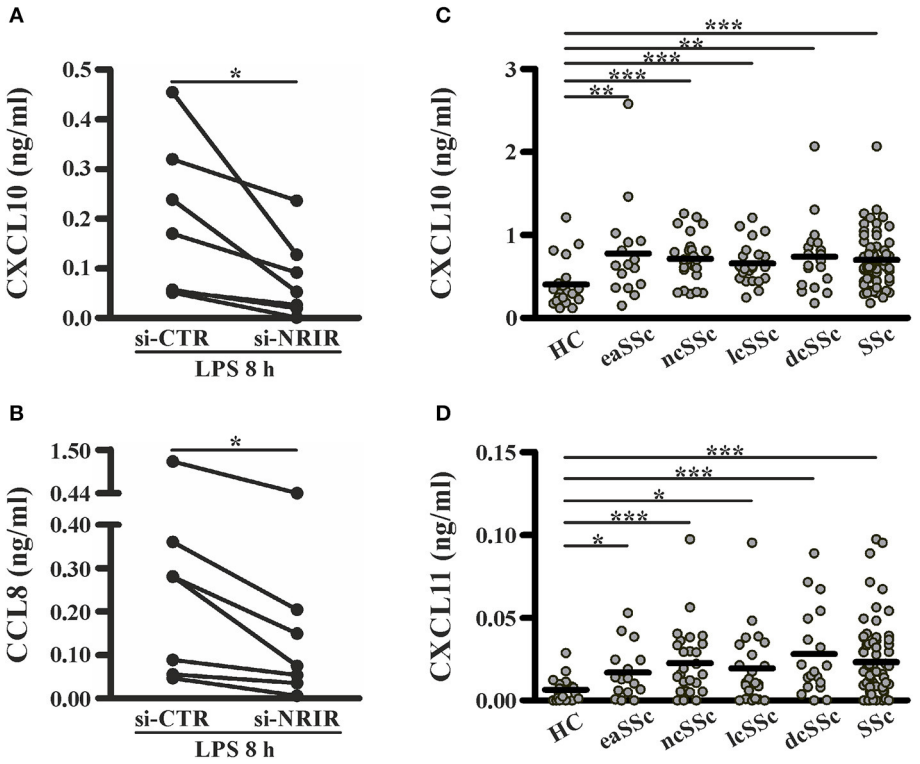

FIGURE 8 | NRIR regulated proteins CXCL10 and CXCL11 are elevated in plasma of SSc patients. CD14+ monocytes from seven different donors were transfected with si-NRIR or si-CTR and 18 h later were stimulated with LPS for 8 h. Cell-free supernatants were collected, and the release of CXCL10 **(A)** and CCL8 **(B)** was measured by the multiplex immunoassay. *$p < 0.05$ by Wilcoxon matched-pairs signed rank test. CXCL10 **(C)** and CXCL11 **(D)** level in plasma from SSc patients and matched HC was measured by the multiplex immunoassay. eaSSc, early SSc; ncSSc, non-cutaneous SSc; lcSSc, limited-cutaneous SSc; dcSSc, diffuse-cutaneous SSc. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Mann Whitney test.

upregulation also in the eaSSc group, characterized by higher levels of ISGs as well. Considering that most patients with eaSSc are prompt to progress toward definite SSc (57, 58), one could speculate a potential implication of NRIR in the IFN signature intertwined with SSc progression. Remarkably, NRIR was the lncRNA most differentially expressed in PBMC from SLE patients as compared to healthy controls, thus further supporting that dysregulation of the IFN-dependent NRIR lncRNA represents a hallmark of different IFN-driven pathologies.

Identification of NRIR-related pathways was conducted according to the "guilt-by-association" method (59), that remains the only approach allowing to characterize lncRNAs based on the function of their co-expressed PCGs. NRIR was found in two distinct co-expression modules, retrieved from WGCNA analysis of the transcriptome of monocyte activated *in vitro* by LPS or isolated from SSc patients. The majority (63%) of the PCGs common to both modules was included in "response to type I IFN," "response to virus," and "immune system process" biological processes, thus strengthening the likelihood that NRIR plays a role in these processes. Experimental validation of the *in silico* analysis demonstrated that NRIR is a type I IFN-responsive gene, induced in monocytes upon activation of only those TLRs that can trigger type I IFN production (i.e., TLR4, TLR3 and TLR7/8). This is further supported by the demonstration that inhibition of LPS-induced release of soluble mediators, and specifically blockade of type I IFN receptor abolished the ability of LPS to upregulate NRIR. Moreover, monocyte activation with agonists of TLR2 (unable to induce type I IFN transcription and secretion) or neutrophil activation of TLR4 (that does not mobilize the TRIF-IFN pathway) (48) failed to upregulate NRIR expression.

Consistently with the NRIR role suggested by the WGCNA approach, data shows that NRIR-silencing mainly reduces the LPS-induced expression of type I IFN target genes, including, among the others, CXCL10, MX1, IFITM3, and ISG15. Moreover, measurements of CXCL10 and CCL8 secretion further endorsed the role of NRIR as a positive regulator of a subset of LPS-induced IFN-dependent genes.

The inhibition of ISGs upon NRIR-silencing is in sharp contrast with recent reports showing that NRIR acts as a negative regulator of specific ISGs (CMPK2, CXCL10, IFIT3, IFITM1, ISG15, Viperin, and IFITM3) in hepatocytes (34) or epithelial cells (60). Overall, our findings strengthen the role of NRIR as a regulator of the IFN response, but they strongly point out that NRIR function is highly cell-type or stimulus specific. Such behavior is not uncommon among lncRNAs implicated in the regulation of immune response; one example is IL7-AS, that was described either as a positive regulator of IL-6 expression in

IL-1β-activate epithelial cells (61) or as negative regulator in LPS-stimulated monocytes/macrophages as well as in IL-1β activated chondrocytes (62).

It must be underlined that all the ISGs inhibited by NRIR silencing are also upregulated in SSc monocytes, that display concomitantly a prominent IFN signature as well as NRIR upregulation. These observations strengthen the relevance of the NRIR-ISGs axis in both physiological as well as pathological conditions. Among the ISGs inhibited upon NRIR silencing, numerous genes have been frequently linked to SSc. Increased levels of CXCL10, CXCL11, IFI44, and MX1 correlate with the severity of different clinical features in SSc patients (63, 64). Higher MX1 expression was associated with ischemic ulcers and reduced forced vital capacity (64, 65). The extent of skin fibrosis measured by the modified Rodman Skin Score (mRSS) correlates with the expression of IFI44 (63). Most importantly, increased levels of circulating CXCL10 and CXCL11, both NRIR targets, highly correlate with the type I IFN signature as well as with a more severe clinical phenotype, with lung and kidney involvement (11, 63, 66). In fact, serum level of CXCL10 and CXCL11 has been recently proposed as biomarker for the identification of early and non-fibrotic subset of SSc (18). Conversely, inhibition of type I IFN signature in SSc patients with anifrolumab, that blocks IFN receptor signaling, leads to lower levels of CXCL10 expression and fibrosis-related transcripts (67).

Collectively, herein we demonstrate that the IFN-dependent lncRNA NRIR is a positive regulator of the LPS-induced IFN response in human monocytes and highlight, for the first time, that aberrant expression of NRIR can be involved in the dysregulation of immune system intertwined with SSc development.

ETHICS STATEMENT

All samples were obtained after patients provided written informed consent and after approval of the study by the institutional review board at each participating center.

AUTHOR CONTRIBUTIONS

BM performed experiments and analysis and contributed to write the paper, NS performed experiments and analysis on SSc monocytes, MR supervised research on SSc monocytes and wrote the paper, NT performed RNA-seq of LPS-treated monocytes, MAC supervised and critically discussed the results of the RNA-seq experiments in LPS-treated monocytes, MC, LB, and MvdK collected patients and clinical info, TR supervised the study and FB designed research and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00100/full#supplementary-material

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Transcriptome of Extracellular Vesicles: State-of-the-Art

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Exosomes and microvesicles are two major categories of extracellular vesicles (EVs) released by almost all cell types and are highly abundant in biological fluids. Both the molecular composition of EVs and their release are thought to be strictly regulated by external stimuli. Multiple studies have consistently demonstrated that EVs transfer proteins, lipids and RNA between various cell types, thus mediating intercellular communication, and signaling. Importantly, small non-coding RNAs within EVs are thought to be major contributors to the molecular events occurring in the recipient cell. Furthermore, RNA cargo in exosomes and microvesicles could hold tremendous potential as non-invasive biomarkers for multiple disorders, including pathologies of the immune system. This mini-review is aimed to provide the state-of-the-art in the EVs-associated RNA transcriptome field, as well as the comprehensive analysis of previous studies characterizing RNA content within EVs released by various cells using next-generation sequencing. Finally, we highlight the technical challenges associated with obtaining pure EVs and deep sequencing of the EV-associated RNAs.

Keywords: apoptotic bodies, microvesicles, circulating RNA, next generation sequencing, exosomes, extracellular vesicle (EV)

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INTRODUCTION

The "Extracellular Vesicles (EVs)" is a general term used to describe various types of spheroid structures, encircled by a lipid membrane bilayer, which are secreted by mammalian cells either passively or upon certain stimuli (1). Since their initial discovery more than 30 years ago (2, 3) EVs have been purified from nearly all mammalian cell types including cells of the immune system (1). Furthermore, EVs have been detected in almost all human biological fluids, and shown to mediate cell-cell communication, thus playing a key role in the regulation of various physiological processes in the body (4) including the immune response (5–8). Finally, it becomes increasingly evident that EVs may contribute to carcinogenesis, as well as the spread of viruses, toxic proteins, and prions (1, 9).

There are three distinct types of EVs (as classified by their origin and biogenesis)—apoptotic bodies (ABs), microvesicles (MVs, also known as shedding vesicles), and exosomes (**Figure 1A**). The ABs are on average 1–5 μm in diameter and are by-products of cell disassembling during the apoptosis (10, 11). The MVs are formed by outward budding of the plasma membrane and are between 100 and 1,000 nm in diameter (8). The exosomes are the smallest type of EVs, having a diameter of 30–150 nm, and are primarily formed as intraluminal vesicles (ILVs) within multi-vesicular bodies (MVBs). Upon fusing of MVBs with the plasma membrane, the ILVs are released as exosomes into the extracellular space (8). Both

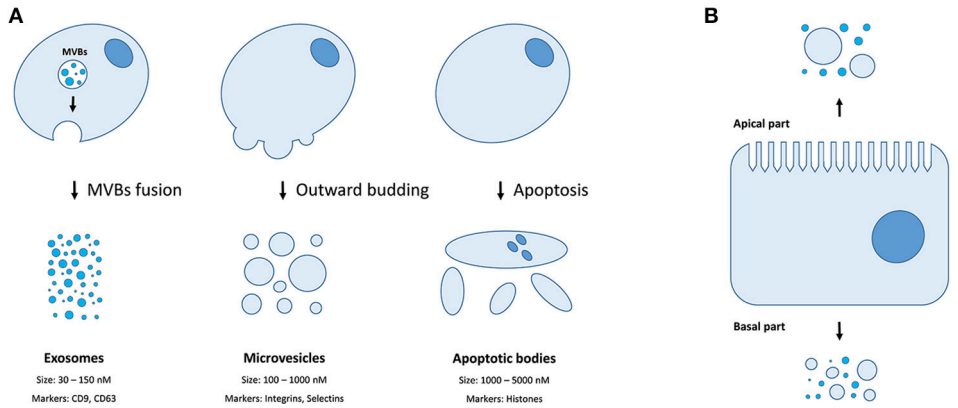

FIGURE 1 | Extracellular membrane vesicles. **(A)** The mechanisms of generation, size distribution, and common protein markers of different EVs types; **(B)** Different populations of EVs could be released depending on the side of the plasma membrane of a polar cell.

MVs and exosomes contain various cytoplasmic and membrane-associated proteins as well as lipids, sugars, and nucleic acids (9), while ABs may in addition include nuclear fractions and cell organelles (1, 10, 11). Well-characterized protein markers for exosomes include various tetraspanins such as CD9, CD63, and CD81; while the MVs contain transmembrane proteins common for the plasmalemma such as integrins and selectins (8). Concurrently, ABs could be differentiated by the presence of histones (10, 11). Recently, large oncosomes (LOs) have been identified as the fourth type of EVs which are generated by shedding of membrane blebs from tumor cells and have a size similar to ABs (12).

Importantly, multiple research reports have demonstrated that various RNA species (including mRNA, miRNAs, and lncRNAs) entrapped within EVs can be transferred from donor to acceptor cells and interfere in gene expression in the latter (13, 14). This mini-review is aimed to provide a state-of-the-art in the EVs field, focusing primarily on the reported RNA cargo in different subtypes of EVs as well as the methodological challenges associated with purification of membrane vesicles and deep sequencing of their RNA content. Furthermore, we elaborate on a putative contribution of vesicular RNA to the functioning of the immune cells.

THE CHALLENGES IN PURIFICATION OF EVS AND CHARACTERIZATION OF EXTRACELLULAR RNA

The techniques widely used so far for isolating EVs include ultracentrifugation, density gradient flotation, ultrafiltration, chromatography, polymer-based precipitation, and immunoprecipitation (15). Differential ultracentrifugation is the most commonly used approach for EVs purification and, in particular, for separating exosomes from ABs and MVs (16). A biological fluid is first depleted from living cells, cell debris, ABs, and MVs with a series of lower-speed centrifugation, and the exosomes fractions are ultimately pelleted by ultracentrifugation. However, the final exosomes pellets can be contaminated with low-sized MVs, large protein aggregates as well as viruses (17, 18). The method of density gradient flotation harnesses differences in size, shape, and density of different EVs types and allows much higher purity of isolated EVs especially when combined with ultracentrifugation. However, high-density lipoproteins (HDL) and low-sized MVs are ultimately co-isolated with exosomes when using density gradient ultracentrifugation (19). Size exclusion chromatography generally allows recovery of EVs populations free from ribonucleoproteins and other soluble contaminants, however, different EVs types with a similar size could co-elute (20). While, ultrafiltration can also efficiently remove soluble components from EVs preparations, the similarly sized particles (both membrane vesicles and protein aggregates) will co-purify (21). An alternative approach that is increasingly being applied is the use of co-precipitants such as polyethylene glycol combined with low-speed centrifugation to aggregate and pellet exosomes for subsequent processing (22). However, while precipitation techniques have generally very high exosomes recovery rates, they also co-precipitate various proteins (23). Finally, immunoprecipitation techniques utilize antibodies against certain proteins located on the surface of EVs and can specifically isolate CD63, CD9, and CD81 positive exosomes (24). However, large-scale separation of exosomes with immunoprecipitation is challenging due to their highly diluted state in the biological fluids.

While each of the above-mentioned approaches harnesses certain differences in biophysical or molecular properties of EVs (including the size, the density, and the content of the surface proteins), neither method can recover a pure material and allows only an enrichment for certain subpopulations of EVs in a sample (15, 25). As a result, the characterization of EV type-specific RNA cargo remains highly challenging and strongly depends on the purification method. In addition, the bovine serum that is used as a component of most cell culture media could be a source of contaminating extracellular RNAs in a sample that mask human-derived RNA species having a sequence similar to bovine RNAs

(e.g., miR-122) (26). The accuracy of the subsequent analysis of EV-associated transcriptome is also highly dependent on an RNA qualification method, including a DNA library preparation for deep sequencing (15). For instance, widely used commercial kits for RNA sequencing, by default, capture only 5′-phosphorylated 3′-OH short RNA molecules representing only a fraction of total RNA in the sample (27). Likewise, most whole-transcriptome sequencing techniques can incorporate only relatively long RNAs and, thus, overlook small RNAs.

Finally, certain cell types (e.g., cells of retinal pigmented epithelium and the intestine) exhibit a membrane polarity (**Figure 1B**). Therefore, EVs secreted by such cells might have distinct properties and molecular content depending on whether they derive from basal or apical parts of the membranes (28). While MVs and exosomes of polar cells have not yet been properly studied, the differences in structure, size, and lipid composition of apical and basal membranes could determine the features of the secreted EVs (28). The polarized trafficking machinery in certain cells suggests that additional care should be taken for isolating apical exosomes, including a careful control of the functional integrity of cell monolayers during preparation of conditioned media (29). On the contrary, an apical-only isolation approach risks missing important basolaterally released vesicles (28).

THE REPORTED RNA CONTENTS WITHIN DIFFERENT EVS CLASSES

The presence of mRNAs, miRNAs, and lncRNAs within exosomes and MVs have been consistently shown with microarrays and RT-qPCR techniques in multiple early (13, 30–36) as well as more recent reports (37–40). The application of more advanced high-throughput RNA sequencing methods revealed the presence of various other RNA species within subpopulations of EVs isolated from biological fluids and cell conditioned media (**Table 1**). Those RNA species include snRNA, snoRNA, piRNA, vault RNA, Y-RNA, scRNA, SRP-RNA, and 7SK-RNA; as well as short fragments originating from rRNA, tRNA, mRNA, lncRNAs, and various intergenic repeats (40–57).

In a pioneering work, Nolte-'t Hoen et al. characterized small RNA content in EVs released by the immune cells in culture using deep sequencing. Interestingly, the majority of total RNA isolated from EVs consisted of small RNAs (<200 nt), with minor amounts of 18S and 28S rRNA. Those short RNA fragments were primarily mapped to protein-coding regions and genomic repeats including SINE, LINE, and LTR sequences (**Table 1**). On the contrary, the majority of sequences present in the cellular small RNA population represented miRNAs, while the proportion of miRNAs in the daughter EVs was dramatically lower. Besides protein coding mRNA and repeats, the EV fractions contained all types of structural RNAs (such as vault RNA, Y-RNA, snRNA, snoRNA, SRP-RNA, and tRNA) as well as fragments deriving from lncRNAs and pseudogenes. Furthermore, many of the small non-coding transcripts were enriched in EVs relative to cellular RNA, indicated that cells might destine specific RNAs for extracellular release (41). A significant underrepresentation of miRNA over other RNA species in the exosomes released by various cultured cells have been also confirmed by multiple other studies (40, 42, 46, 50, 52, 55, 57). These data go in accordance with the previous observation that most individual exosomes does not carry any biologically significant number of miRNA copies (58). Nevertheless, other RNA sequencing experiments indicated that a significant proportion of small RNA-seq reads still correspond to miRNA in exosomes released by some cell lines (51, 53, 54, 57). Interestingly, several independent groups have observed a significant enrichment (15–50% of total reads) of RNA fragments mapped to genomic repeats comprising retroviral sequences, LTR, SINE, and LINE sequences (41, 42, 47, 50, 55). It has to be mentioned that the authors did not specify whether the small RNA library preparation protocols used in the above studies included the modifications to allow capturing 5′-OH and/or 3′-phosphorylated RNAs. Therefore, it remains unclear whether they actually characterized the full spectrum of small RNA in the corresponding EVs.

The sequencing of total (both long and small) RNAs in the EVs was reported by Jenjaroenpun et al. (46) and Miranda et al. (49) in the EVs present in conditioned media from MDA-MB cells and the urine, respectively, and showed a significant proportion of rRNA reads (87–97%) that was similar to the rRNA content in the cytoplasm. Out of the remaining 3–13% reads, approximately half was mapped to protein-coding transcripts while another half aligned to non-coding RNAs and genomic repeats. In another report by Beradrocco et al. the authors used both total RNA and small RNA sequencing protocols separately to characterize a spectrum of long RNAs encapsulated within the EVs released by four different liver cancer cell lines (55). The largest proportion (32–66%) of total RNA reads were mapped to rRNA, while 15–44% corresponded to the genomic repeats, and only 11–25% of reads were mapped to protein-coding and non-coding RNA genes. The small RNA sequencing performed on the same EVs preparations revealed only a slightly different distribution of RNA classes: rRNA (16–54%), genomic repeats (24–40%), and transcriptome (24–51%) (55). In another whole-transcriptome RNA-seq study paralleled with small RNA sequencing, Lasser et al. demonstrated that human mast and erythroleukemic cell lines release two exosomes populations (as separated by flotation on a density gradient into HD and LD fractions) (40). A clear lack of correlation between both long and short RNA cargo in HD and LD fractions suggests that extracellular RNA in these two fractions are associated with distinct pathways. Thus, reads mapped to mRNA transcripts were more abundant percentage-wise in the HD as compared to LD exosomes (75 vs. 20%), while the distribution of non-coding RNA reads was opposite (25 vs. 80%). In short RNA libraries, the HD fractions were enriched in mature miRNA (23%), while the LD fractions were dominated by tRNA (28%), and mature miRNA (10%) (40).

Another study investigated RNA content in three separate EVs types released by melanoma cells in culture and identified some non-coding RNAs to be enriched in every EV samples (53). Thus, RNA profiles indicated the presence of prominent 18S and 28S rRNA peaks in ABs and MVs with relatively moderate levels of small RNA. By contrast, exosomes contained predominantly small RNA and much less rRNA as compared to both ABs and MVs (53). Interestingly, a similar number of different miRNAs

TABLE 1 | The reports demonstrating a transcriptome content of the EVs using deep sequencing.

	EVs source cells or biofluid	EVs isolation method	Expected EVs types	NGS type and platform	RNA classes and alignment statistics
(41) PMID: 22821563	Co-cultures of dendritic and T-cells	DUC (S10 –> P100)	EXOs & small MVs	Small RNA-seq (Illumina)	Exons (6.7%) incl. small ncRNA (0.49%), introns (19.4%), genomic repeats (27.4%); Exons reads: protein coding (~84%), vault RNA (~3%), lincRNA (~2%), pseudogenes (~2%), SRP-RNA(~1%), rRNA (<1%), Y-RNA (<0.5%), miRNA (<0.5%), snRNA (<0.5%), snoRNA (<0.5%).
(42) PMID: 22965126	GT1-7 cells	DUC (S10 + 0.2 μm filtration –> P100)	EXOs & small MVs	Small RNA-seq (SOLID)	Genomic repeats (~50%), mRNA & ncRNA (~33%), small ncRNA (~15%), rRNA (~0.5%); Small ncRNA: tRNA (~90%), scRNA (~3%), siRNA (~2%), snRNA (~1%), miRNA (~1%), snoRNA (0.1%)
(43) PMID: 22849433	HEK293T cells	DUC (S2 –> P100)	EXOs, MVs & ABs	Small RNA-seq (Illumina) for miRNA only	Various miRNAs
(44) PMID: 23663360	Human plasma	Precipitation (ExoQuick)	EXOs & MVs	Small RNA-seq (Illumina)	miRNA (76.20%), rRNA (9.16%), DNA (5.63%), lncRNA (3.36%), mRNA (2.11%), piRNA (1.31%), tRNA (1.24%), snRNA (0.18%), snoRNA (0.01%)
(45) PMID: 23302638	Human saliva	UF (100 kDa) + GF	EXOs & small MVs	Small RNA-seq (Illumina)	miRNA (51-58%), rest: piRNA, snoRNA, genomic repeats
(46) PMID: 24255815	MDA-MB-231, MDA-MB-436 cells	DUC (S17 –> P100)	EXOs & small MVs	Total RNA-seq (Ion Torrent)	rRNA (~97%), protein coding (~1%), ncRNA (~1%)
(47) PMID: 23807490	U251 cells	DC (S1.8 –> P18)	Large MVs & ABs	Small RNA-seq (SOLID)	miRNA (38.7%), genomic repeats (>20%), rest: tRNA, vault RNA, miscRNA, intergenic and intronic
(48) PMID: 24352158	Human urine	DUC (S17 –> P200)	EXOs & small MVs	Small RNA-seq (Ion Torrent)	miRNA (35%), protein coding (3%), snRNA (0.02%), snoRNA (0.04%), lncRNA (0.19%), other non- coding RNAs (61%)
(49) PMID: 24816817	Human urine	DUC (S17 –> P118)	EXOs & small MVs	Total RNA-seq (Illumina)	rRNA (87%), protein coding (4.6%), ncRNA and genomic repeats (6.1%), mtRNA (0.1%)
(50) PMID: 26129847	Human mesenchymal stem cells	DUC (S10 –> P70)	EXOs & MVs	Small RNA-seq (Illumina)	tRNAs (23-50%), genomic repeats (17-40%), miRNA (2-5%), snoRNA (<0.6%), rest: miscRNA, rRNA, protein coding, snRNA, pseudogenes, mtRNA
(51) PMID: 26027894	HMEC-1 cells	DUC (S10 –> P100) + SDG	EXOs & small MVs	Small RNA-seq (Illumina)	miRNA (~80%), Y-RNA (~14%), mRNA (~1.5%), mtRNA (~1%), lncRNAs (~0.8%), vault RNA (~0.2%), other ncRNA (<0.1%)
(52) PMID: 25940616	MCF-7, MCF-10A cells	DC (S2 –> P16) DUC (S16 + 0.2 μm filtration –> P100)	Large MVs & ABs EXOs & small MVs	Small RNA-seq (Illumina)	Enriched in rRNA, Y-RNA, vault RNA, tRNA halves, much less miRNAs (<1%)
(53) PMID: 26176991	MML-1 cells	DC (S0.3 –> P2) DC (S2 –> P16.5) DUC (S16.5 + 0.2 μm filtration –> P120)	ABs Large MVs & ABs EXOs & small MVs	Small RNA-seq (Ion Torrent)	ABs: 3.3-6.5% miRNAs, rest: snRNA, snoRNA, mtRNA, Y-RNA, vault RNA MVs: 2.4-3.8% miRNAs, rest: snRNA, snoRNA, mtRNA, Y-RNA, vault RNA EXOs: 5.6-8.1% miRNAs, rest: snRNA, snoRNA, mtRNA, Y-RNA, vault RNA
(40) PMID: 27791479	HMC-1, TF-1 cells	DUC (S16.5 –> P120) + SDG	EXOs & small MVs (in LD and HD fractions)	Total RNA-seq and smRNA-seq (Illumina)	Total RNA-seq HD: protein coding (~75%), non-coding RNA (~25%); Total RNA-seq LD: protein coding (~20%), non-coding RNA (~80%); Small RNA-seq HD: miRNA (~23%), rest: vault RNA, snoRNA, snRNA; Small RNA-seq LD: tRNA (~28%), miRNA (~10%), rest: mtRNA, Y-RNA, piRNA

(Continued)

TABLE 1 | Continued

	EVs source cells or biofluid	EVs isolation method	Expected EVs types	NGS type and platform	RNA classes and alignment statistics
(54) PMID: 28381156	MKN45, SGC7901, NCI-N87, AGS, GES-1 cells	DUC (S2 –> P110)	EXOs, MVs & ABs	Small RNA-seq (Illumina)	miRNA (22-38%), rRNA (0.6-21%), snRNA (0.6-13%), rest: Y-RNA, piRNA, snoRNA, tRNA
(55) PMID: 29137313	HuH7, Hep3B, HepG2, HuH6 cells	DUC (S16 –> P120)	EXOs & small MVs	Total RNA-seq & small RNA-seq (SOLID)	Total RNA-seq: rRNA (32-66%), genomic repeats (15-44%), transcriptome (11-25%); Small RNA-seq: rRNA (16-54%), genomic repeats (24-40%), transcriptome (24-51%)
(56) PMID: 27858503	U87 cells	DUC (S10 –> P100)	EXOs & small MVs	Exome RNA-seq (Illumina)	Various mRNAs
(57) PMID: 30018314	HEK293T, RD4, C2C12, Neuro2a, C17.2 cells	DUC (S1.5 + 0.2 μm filtration –> P110)	EXOs & small MVs	Small RNA-seq (Illumina)	rRNA (~60%), small ncRNA (~22%), rest: tRNAs, protein coding, Y-RNA, miscRNA; Small ncRNA: piRNA (~33%), miRNA (~25%)

S, supernatant obtained after centrifugation at the corresponding g (in thousands); P, pellet obtained after centrifugation at the corresponding g (in thousands); DC, differential centrifugation; DUC, differential ultracentrifugation; SDG, sucrose density gradient ultracentrifugation; UF, ultrafiltration; GF, gel-filtration; EXOs, exosomes; MVs, microvesicles; ABs, apoptotic bodies.

have been identified in every EV type. Overall, a close relationship between miRNA profiles was found in ABs and MVs ($R = 0.91$), MVs and exosomes ($R = 0.86$), as well as MVs and parental cells ($R = 0.86$). While a less strong correlation was found between ABs and exosomes ($R = 0.79$) and exosomes and cells ($R = 0.75$). Despite the fact that EVs subsets were different only to a minor degree from the aspect of their miRNA cargo, a significant number of miRNAs were detected only in exosomes and were absent in both ABs and MVs, supporting the concept of specific RNA loading into exosomes. It has to be mentioned that other ncRNA species were not only significantly more abundant as compared to miRNA but also selectively enriched in different EVs subtypes released by melanoma cells, which adds another level of complexity to investigating extracellular vesicle RNA cargo and its function (53).

Only a few reports have so far investigated small RNA cargo in EVs isolated from human biological fluids with next generation sequencing (44, 45, 48). These studies indicated that exosomes isolated from human plasma, saliva, and urine contained a significant proportion of miRNA reads (35–76% of total). The rest RNA species in the EVs from the above mentioned biofluids included fragments of rRNA, lncRNAs, tRNA, mRNA, repeated regions as well as small noncoding RNA such as piRNA, snRNA, and snoRNA. It is important to mention that exosome isolations from biofluids may contain much higher amounts of large protein aggregates, including miRNA-loaded AGO complexes that are normally released upon cell death (59), as compared to "few-days" cell conditioned media. Therefore, it remains to be validated whether the miRNAs detected in human biofluids were indeed associated with the EVs. Interestingly, deep sequencing of total RNA purified from urea exosomes (49) revealed drastically different transcripts distribution than that observed by Cheng et al. (48). Specifically, a substantial proportion (∼87%) of total RNA reads was mapped to rRNA and only about 8% of reads aligned to non-coding RNA and DNA repeats, while the remaining ∼5% of reads corresponded to protein-coding RNA (49). Conversely, the mapping statistics and reads distribution reported by Miranda et al. were similar to those obtained upon total RNA sequencing of exosomes from cell conditioned media (46, 55).

To conclude, the collective evidence evolving from the above mentioned studies (**Table 1**) argue that EVs released by most cells indeed carry significant amounts of non-coding and protein-coding transcripts, as well as their parts, that should be considered when studying the effects of extracellular RNA on recipient cells. The differences in EVs RNA cargo content among the reported studies might be explained in part by: (1) cell type-specific RNA expression differences; (2) different EVs and RNA isolation methods; and (3) the use of different NGS library preparation protocols and sequencing platforms.

THE ROLE OF THE EVS RNA CONTENT FOR THE IMMUNE SYSTEM

While it was consistently shown that the exchange of exosomes and microvesicles among different immune cells contribute

to both adaptive and innate immune response, the impact of the EVs RNA cargo onto immune cells function remains obscure (60). Mittelbrunn et al. demonstrated that exosomes originating from T cells are loaded with certain miRNAs (e.g., miR-335) can be internalized by the antigen-presenting cells APCs at the immune synapses and that subsequently led to the reduction of target mRNA SOX4 expression (61). Likewise, miRNA transfer from one dendritic cell (DC) to another via EVs led to alterations in recipient-cell gene expression (62), and regulatory T cells reduce Th1 (CD4+ IFNg+) inflammatory responses by EV transfer of miRNA (especially let-7d) to Th1 cells (63). The discovery of the variety of different RNA species in MVs and exosomes secreted by the immune cells as well (41), added another level of complexity to the theory of cell-cell communication via cell-free RNA. In particular, very marginal levels of EVs-encapsulated miRNAs as compared to other RNA species not only question the contribution of miRNA to cell-cell communication but also suggests that other RNAs might play much more determining biological role.

Thus, previous experiments revealed that extracellular miRNA can activate Toll-like receptor (TLR) 8 signaling, which induces cytokine secretion, presumably by mimicking viral RNA (64). The TLRs are a family of innate immune system receptors which recognize various molecular patterns of microbial pathogens and induce antimicrobial immune responses (65, 66). Specifically, both free-floating AGO protein bound miRNAs and miRNA encapsulated in EVs have been hypothesized to mediate communication between immune cells via binding to extracellular or intracellular Toll-like receptors (TLRs) (64, 67). Among the major effects of the nucleic acids-mediated activation of intracellular TLRs is the induction of certain cytokines essential for the innate immune response. While multiple other reports link activation of TLR pathways and exosomes, it remains unclear whether the observed effects were indeed mediated by the encapsulated RNAs. However, the finding that intracellular TLRs located within endolysosomal compartments can bind both double-stranded and single-stranded nucleic acids derived from viruses and bacteria (68) strongly suggest that various RNA classes incorporated within the EVs could also activate the corresponding TLRs. Due to the largely sequence-independent impact of nucleic acids on the TLRs, it is feasible that more abundant non-miRNA classes could significantly contribute to such activation. Overall, it remains feasible that combined interactions of vesicular RNAs and TLRs within and between diverse immune and non-immune cells could contribute to the regulation of the complex nexus of immune responses.

CONCLUSION AND FUTURE PERSPECTIVES

Massive parallel sequencing has enabled characterization of the whole spectrum of nucleic acids in a given sample, and was consistently applied to demonstrate the presence of the complex RNA cargo within EVs populations released by various cells. Interestingly, the intravesicular miRNAs (which were well-documented previously using microarray and qPCR-based methods) represented only a very marginal proportion as compared to other RNA species including various small non-coding RNAs, lncRNAs, and mRNA fragments. A putative biological impact of the EVs-associated transcriptomes remains to be validated; however, multiple studies indicated that, at least, exosomal miRNA could mediate communication among various cell types including the immune cells. In addition, EVs-encapsulated miRNAs have been shown to serve as highly specific biomarkers for various pathological conditions and correlate with the presence of malignant tumors. Indeed, exosomes carrying a tumor-specific miRNA repertoire have been consistently detected in the venous blood of cancer patients and mouse models. The collective finding that non-miRNA species are in fact much more abundant in the isolated EVs populations, suggests that they could serve as even more promising non-invasive biomarkers for cancer and/or other disorders.

AUTHOR CONTRIBUTIONS

ATo and ATu conceived the study. ATu prepared the tables and figures and wrote the manuscript. ATo and OD participated in final manuscript design, and provided experts' opinion on the content and critical revision.

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MiR-135-5p-p62 Axis Regulates Autophagic Flux, Tumorigenic Potential, and Cellular Interactions Mediated by Extracellular Vesicles During Allergic Inflammation

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The objective of this study was to investigate the relationship between autophagy and allergic inflammation. *In vitro* allergic inflammation was accompanied by an increased autophagic flux in rat basophilic leukemia (RBL2H3) cells. 3-MA, an inhibitor of autophagic processes, negatively regulated allergic inflammation both *in vitro* and *in vivo*. The role of p62, a selective receptor of autophagy, in allergic inflammation was investigated. P62, increased by antigen stimulation, mediated *in vitro* allergic inflammation, passive cutaneous anaphylaxis (PCA), and passive systemic anaphylaxis (PSA). P62 mediated cellular interactions during allergic inflammation. It also mediated tumorigenic and metastatic potential of cancer cells enhanced by PSA. TargetScan analysis predicted that miR-135-5p was a negative regulator of p62. Luciferase activity assay showed that miR-135-5p directly regulated p62. MiR-135-5p mimic negatively regulated features of allergic inflammation and inhibited tumorigenic and metastatic potential of cancer cells enhanced by PSA. MiR-135-5p mimic also inhibited cellular interactions during allergic inflammation. Extracellular vesicles mediated allergic inflammation both *in vitro* and *in vivo*. Extracellular vesicles were also necessary for cellular interactions during allergic inflammation. Transmission electron microscopy showed p62 within extracellular vesicles of antigen-stimulated rat basophilic leukemia cells (RBL2H3). Extracellular vesicles isolated from antigen-stimulated RBL2H3 cells induced activation of macrophages and enhanced invasion and migration potential of B16F1 mouse melanoma cells in a p62-dependent manner. Extracellular vesicles isolated from PSA-activated BALB/C mouse enhanced invasion and migration potential of B16F1 cells, and induced features of allergic inflammation in RBL2H3 cells. Thus, miR-135-5p-p62 axis might serve as a target for developing anti-allergy drugs.

Keywords: P62, miR-135, extracellular vesicles, cellular interactions, allergic inflammation

INTRODUCTION

Impaired autophagy in myeloid cells has a causal role in eosinophilic inflammation and chronic rhino sinusitis (1). Dysregulation of autophagy and inflammasome activity contributes to the development of auto-inflammatory diseases (2). Autophagy plays a crucial role in degranulation of mast cells (3, 4). Histamine H3 receptor (H3R) blockade can inhibit mammalian target of rapamycin (mTOR) phosphorylation and reinforce autophagy (5). B cell autophagy aggravates experimental asthma through multiple mechanisms (6). Antibody-enhanced Dengue viruses (DENV) infection of KU812 cells (pre-basophil-like cell line) and immature human mast cell line (HMC-1) shows increases of autophagosome vesicles, light chain 3 (LC3) punctation, and LC3-II accumulation (7). MTOR, an inhibitor of autophagy, mediates metabolic adaptation of antigen presenting cells (APCs) in distinct tissues, thus influencing immunological characters of allergic inflammation (8). Inhibition of PI3K/Akt activity and subsequent blockade of mTOR-hypoxia inducible factor (HIF)-1α-vascular endothelial growth factor (VEGF) module can attenuate typical asthmatic attack in a murine model (9). Transglutaminase II (TGaseII) mediates passive cutaneous anaphylaxis and atopic dermatitis (10). TGase II through interaction with NF-kappaB can induce histone deacetylase-3 (HDAC3) by direct binding to promoter sequences (10). HDAC3 can interact with FcεRIβ and mediate allergic inflammation by increasing expression of monocyte chemo attractant protein 1 (MCP1) (11). Down-regulation of HDAC3 abrogates the ability of HDAC inhibitor valproic acid (VPA) to modulate AKT phosphorylation, suppress tumor cell growth, and induce autophagy (12). Thus, autophagy might play a role in allergic inflammation.

Scaffolding adaptor protein P62/SQSTM1 is an autophagy receptor that acts as a link between ubiquitination and autophagy machineries. Upon binding to its ligand, p62 acts as a modulator of macroautophagy and induces autophagosome biogenesis (13). Stimulation of TLR2/6 or TLR4 in primary human keratinocytes can activate autophagy pathways and increase p62 expression through induction of NADPH oxidases 2 and 4 and generation of reactive oxygen species (14). P62 acts downstream of TCR activation. It is important for Th2 polarization and asthma. P62 also plays a significant role in the control of sustained activation of NF-kappaB and late synthesis of GATA3 and IL-4 by participating in the activation of the IKK complex (15). Overexpression of p62 increases expression levels of pro-inflammatory cytokines, such as TNFα, CXCL10, and CCL2 (16). P62 can stabilize COX-2 protein through its ubiquitin-associated domain. P62 can also regulate prostaglandin E_2 production *in vitro* (17). It is known that miR-26a/-26b-COX-2 axis regulates allergic inflammation (18). These reports suggest a role of p62 in allergic inflammation.

Asthma shows enhanced secretion of extracellular vesicles by epithelial cells, not by macrophages, under the influence of IL-13 (19). Alveolar macrophages secrete SOCS1 and SOCS3 in extracellular vesicles and microparticles, respectively, for uptake by alveolar epithelial cells and subsequent inhibition of STAT activation (20). MiR-122-SOCS1 axis regulates allergic inflammation (21). Increased release of extracellular vesicles can induce autophagy (22). BALF extracellular vesicles from asthmatics might contribute to subclinical inflammation by increasing generation of cytokine and LTC (4) in airway epithelium (23). GW4869, an inhibitor of extracellular vesicles formation, can decrease Th2 cytokines and eosinophil counts in BALFs and reduce eosinophil accumulation in airway walls and mucosa (24). These reports suggest a role of extracellular vesicles in allergic inflammation.

In this study, we present a novel role of miR-135-5p-p62 axis in regulating allergic inflammation in conjunction with autophagic flux, cellular interactions, and allergic inflammation-promoted enhanced tumorigenic and metastatic potential of cancer cells. We showed the presence of p62 within extracellular vesicles and the role of p62 in cellular interactions mediated by extracellular vesicles during allergic inflammation. Thus, miR-135-5p-p62 axis can be employed to develop anti-allergy therapeutics.

MATERIALS AND METHODS

Materials

Oligonucleotides used in this study were commercially synthesized by the Bioneer Co. (Daejeon Korea). DNP-HSA (2,4-dinitrophenyl-human serum albumin), TNP-BSA (trinitrophenyl-bovine serum albumin), DNP-specific IgE antibody, and TNP-specific IgE antibody were purchased from Sigma. Chemicals used in this study were purchased from Sigma. All other antibodies were purchased from Cell Signaling Co. (Beverly, MA). Anti-mouse and anti-rabbit IgG-horseradish peroxidase-conjugated antibody was purchased from Pierce. Lipofectamine and PlusTM reagent for transfection were purchased from Invitrogen.

Cell Culture

Rat basophilic leukemia (RBL2H3) cells, B16F1 cells, and B16F10 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle's medium containing heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cultures were maintained in 5% CO_2 at 37°C. Lung mast cells and lung macrophages were isolated according to standard procedures (25).

Mice

Five-weeks-old female BALB/C mice were purchased from Nara Biotech (Seoul, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (KIACUC-160329-2) and conducted in accordance with the ethical committee guidelines for the care and use of laboratory animals. To measure tumorigenic potential, mouse melanoma B16F1 cells (1×10^6 cells in 100 μl of PBS), after induction of passive systemic anaphylaxis, were injected subcutaneously into the right flank of each mouse ($n = 5$).

β-Hexosaminidase Activity Assays

The β-hexosaminidase activity assay was performed according to standard procedures (26).

Immunoblot and Immunoprecipitation

Immunoblot and immunoprecipitation were performed according to the standard procedures (25).

The Levels of PGE2 and Histamine Release

The levels of PGE2 and the amount of histamine released were measured according to the manufacturer's instruction using commercially available ELISA kit (Abcam, UK). Reaction product was measured colorimetrically with a microplate reader.

Chemo Invasion and Migration Assays

The invasive potential was determined by using a transwell chamber system with 8-μm pore polycarbonate filter inserts (CoSTAR, Acton, MA). The lower and upper sides of the filter were coated with gelatin and matrigel, respectively. For determination of migration potential, the lower sides of the filters were coated with gelatin. Trypsinized cells (5×10^3) in the serum-free RPMI 1640 medium containing 0.1% bovine serum albumin were added to each upper chamber of the transwell. RPMI 1640 medium supplemented with 10% fetal bovine serum was placed in the lower chamber and cells were incubated at 37°C for 16 h. The cells were fixed with methanol and the invaded cells were stained and counted.

Immunofluorescence Staining

Cells were seeded onto glass coverslips in 24-well plates and were fixed with 4% paraformaldehyde (v/v) for 10 min and then permeabilized with 0.4% Triton X-100 for 10 min. Cells were incubated with primary antibody specific to LC3 (1:100; Santa Cruz Biotechnology), P62 (1:100; Santa Cruz Biotechnology), CD163 (1:100; Ab Cam) or iNOS (1:100; Santa Cruz Biotechnology) for 2 h. Anti-rabbit Alexa Fluor 488 (for detection of LC3 and iNOS) or anti-goat Alexa Fluor 546 (for detection of P62 and CD163) secondary antibody (Molecular Probes) was added to cells and incubated for 1 h. Fluorescence images were acquired using a confocal laser scanning microscope and software (Fluoview version 2.0) with a X 60 objective (Olympus FV300, Tokyo, Japan).

Matrigel Plug Assays

Seven weeks-old BALB/C mice (Nara Biotech) were injected subcutaneously with 0.1 ml of matrigel containing culture medium and 10 units of heparin (Sigma). After 8 days, the skin of the mouse was easily pulled back to expose the matrigel plug, which remained intact. Hemoglobin (Hb) content in the matrigel plugs was measured using the Drabkin reagent (Sigma, USA) for quantification of blood vessel formation.

Transfection

Transfections were performed according to the manufacturer's instructions. Lipofectamine and Plus reagents (Invitrogen) were used. For miR-135-5p knockdown, cells were transfected with 10 nM oligonucleotide (inhibitor) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The sequences used were 5′-UUCACAUAGGAAUAAAAA GCCAUA-3′ (miR-135-5p inhibitor) and 5′-TAACACGTCTATA CGCCCA-3′ (control inhibitor).

miRNA Target Analysis

Genes that contain the miRNA-binding site(s) in the UTR were obtained using the TargetScan program (http://www.targetscan.org/, http://pictar.mdc-berlin.de/, http://www.microrna.org/microrna/home.do).

RNA Extraction and Quantitative Real Time PCR (QRT-PCR)

Total miRNA was isolated using the *mir*VanamiRNA isolation kit (Ambion). MiRNA was extended by a poly (A) tailing reaction using the A-Plus poly (A) polymerase tailing kit (Cell Script). cDNA was synthesized from miRNA with poly(A) tail using a poly(T) adaptor primer and qScriptTM reverse transcriptase (Quanta Biogenesis). Expression level of miR-135-5p or p62 was quantified with SYBR Green quantitative real-time-PCR kit (Ambion) using miRNA-specific forward primer and a universal poly (T) adaptor reverse primer. Expression level of miR-135-5p was defined based on the threshold (*Ct*), and relative expression levels were calculated as $2^{-(Ct\text{ofmiR}-135-5\text{p})-(Ct\text{ofU6})}$ after normalization with reference to expression of U6 small nuclear RNA. For quantitative real-time PCR, SYBR PCR Master Mix (Applied Biosystems) was used in a CFX96 Real Time System thermocycler (Bio-Rad).

Constructs

To generate the pGL3–3′-UTR-P62 construct, a (136)-bp human p62 gene segment encompassing 3′-UTR was PCR-amplified and subcloned into the (XbaI) site of pGL3 luciferase plasmid. The mutant pGL3–3′-UTR-CAGE construct was made with the QuikChange site-directed mutagenesis kit (Stratagene). Luciferase activity assay was performed according to the instruction manual (Promega).

Passive Cutaneous Anaphylaxis

BALB/C mice were sensitized with an intradermal injection of IgE (0.5 μg/kg). Twenty four hours later, mice were challenged with an intravenous injection of DNP-HSA (250 μg/kg) and 2% (v/v) Evans blue solution. One hour after injection with evans blue solution after DNP-HSA challenge, the mice were euthanized, and the 2% (v/v) Evans blue dye was extracted from each dissected ear in 700 μl of acetone/water (7:3) overnight. The absorbance of Evans blue in the extracts was measured with a spectrophotometer at 620 nm. To determine the effect of p62 on the PCA, BALB/C mice were given an intradermal injection of DNP-IgE (0.5 μg/kg) and intravenous injection of p62 siRNA (100 nM). The next day, BALB/C mice were given an intravenous injection of PBS or DNP-HSA (250 μg/kg) along with 2% (v/v) Evans blue solution for determining the extent of vascular permeability accompanied by PCA.

Effect of Passive Systemic Anaphylaxis on Tumorigenic Potential

BALB/C mice were sensitized by intravenous injection of IgE (0.5 μg/kg). The next day, the sensitized mice were intravenously injected with DNP-HSA (250 μg/kg). Two days after injection of DNP-HSA, B16F1 mouse melanoma cells (1×10^6 cells) were injected into the flanks of each BALB/C mouse. To determine the effect of p62 on the enhanced tumorigenic potential by PSA, BALB/C mice were given an intravenous injection with p62 siRNA (100 nM) on the indicated days.

Effect of Passive Systemic Anaphylaxis on Metastatic Potential

Passive systemic anaphylaxis was induced as described. Three days after the injection of IgE, BALB/C mice were given an intravenous injection of B16F1 melanoma cells (2×10^5). To determine the effect of p62 on the enhanced metastatic potential of cancer cells by PSA, BALB/C mice were given an intravenous injection with p62 siRNA (100 nM) on days 5, 7, 10 and 12. On day 14, lung tumor tissues were harvested.

Monitoring of Rectal Temperature

Changes in core body temperature associated with systemic anaphylaxis were monitored by measuring changes in rectal temperatures using a rectal probe coupled to a digital thermometer.

Immunohistochemical Staining

Immunohistochemical staining was performed using avidin-biotin detection method (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA).

Electron Microscopic Observation of Autophagosomes

The IgE-sensitized RBL2H3 cells stimulated without or with DNP-HSA (100 ng/ml) for 2 h were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate solution (pH 7.0) for 1 h, and then followed with 2% osmium tetroxide for 2 h at 4°C.

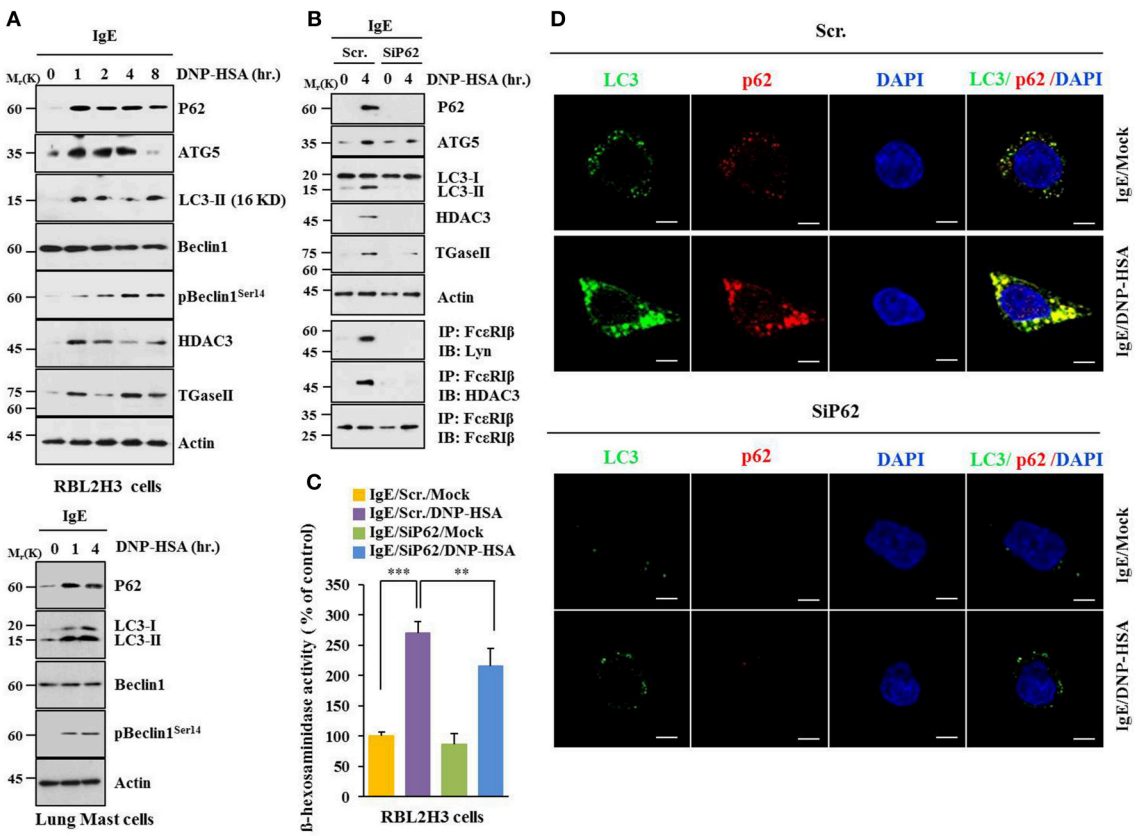

FIGURE 1 | P62 is necessary for allergic inflammation and regulation of autophagic flux**. (A)** IgE (DNP-specific)-sensitized RBL2H3 cells were treated without or with DNP-HSA (100 ng/ml) for various time intervals followed by immunoblot (upper panel). IgE-sensitized lung mast cells were treated without or with DNP-HSA for various time intervals followed by immunoblot (lower panel). Each blot is a representative of three independent experiments. **(B)** RBL2H3 cells were transfected with indicated siRNA (each at 10 nM). The next day, cells were sensitized with IgE for 24 h followed by stimulation without or with DNP-HSA. Scr. denotes scrambled siRNA. Each blot is a representative of three independent experiments. **(C)** Same as **(B)** except that ß-hexosaminidase activity was performed. $^{**}p < 0.005$; $^{***}p < 0.0005$. Each value represents average of three independent experiments. **(D)** Immunofluorescence staining shows co-localization of p62 with LC3 in RBL2H3 cells. Scale bars represent 10 μm.

Then, the cells were dehydrated with a graded acetone series, and embedded into Spurr medium (Electron Microscopy System). The samples were sectioned (60 nm) with an ultra-microtome (RMC MTXL, Arizona, USA), and double-stained with 2% uranyl acetate for 20 min and lead citrate for 10 min. The sections were then viewed under a Tecnai G2 (FEI, USA) TEM at 200 kV.

Isolation and Characterization of Extracellular Vesicles

Cells were cultured under serum-free medium (Invitrogen, Carlsbad, CA). The culture medium was harvested after 48 h of incubation, and the extracellular vesicles fraction was purified using Exoquick-TC reagent (System Biosciences, Mountain View, CA) according to the manufacturer's instructions. Extracellular vesicles were observed under a Tecnai T10 transmission electron microscope (FEI, USA).

Labeling and Internalization of Extracellular Vesicles

Extracellular vesicles from antigen-stimulated RBL2H3 cells were isolated and were labeled using PKH67 Fluorescent Cell Linker kits (Sigma-Aldrich, St. Louis, MO). To examine the uptake of extracellular vesicles, unstimulated RBL2H3 cells were plated out onto coverslip (2×10^4 cells). After 24 h, coverslips were washed three times in PBS, and each medium containing PKH67-labeled extracellular vesicles or PKH67-unlabeled extracellular vesicles were added into each well for 24 h. After incubation, the coverslips were washed three times in PBS, and 4% paraformaldehyde solution then added to the slides for 15 min. The coverslips were washed three times in PBS. Cells were visualized under a confocal laser scanning microscope LX70 FV300 05-LPG-193 (Olympus).

The Presence of P62 in the Extracellular Vesicles of Antigen-Stimulated RBL2H3 Cells

Extracellular vesicles extracted from antigen-stimulated RBL2H3 cells (REF, KIT model) were subjected to centrifugation at 60,000 g for 30 min to precipitate extracellular vesicles. Collected extracellular vesicles were fixed with 0.1% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.4) for 1 h at 4°C and then post-fixed in 2% osmium tetroxide for 30 min at 4°C. They were dehydrated with a graded series of ethanol

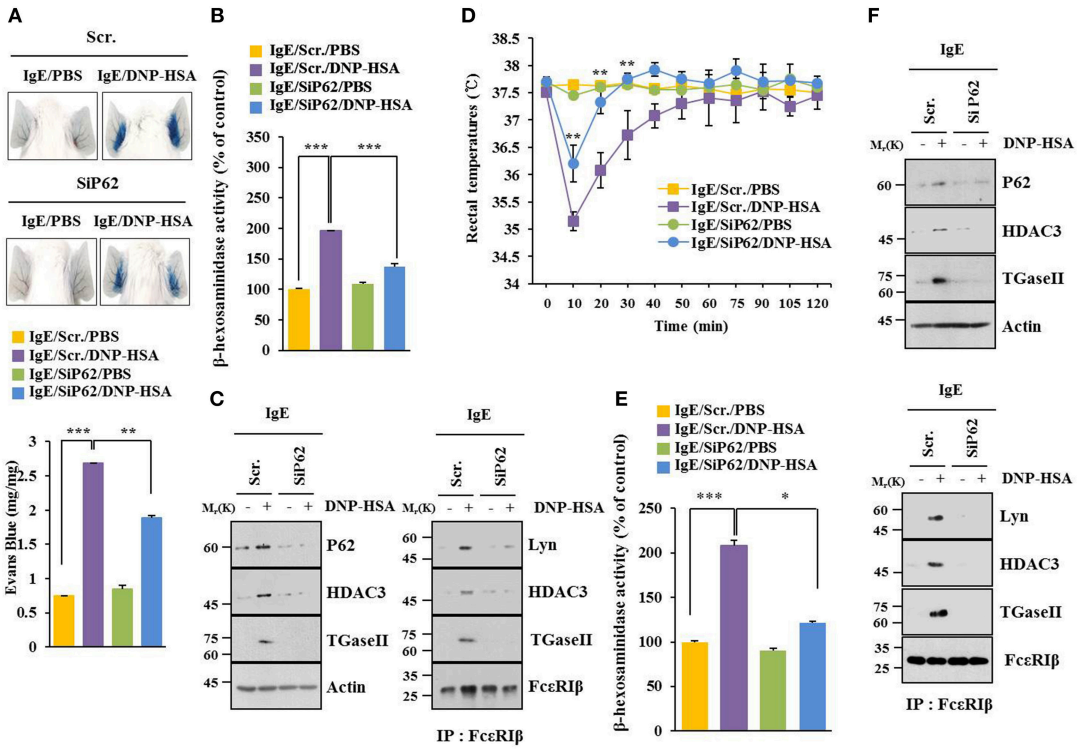

FIGURE 2 | P62 mediates anaphylaxis. **(A)** BALB/C mice were given an intradermal injection of IgE (0.5 μg/kg) and an intravenous injection of indicated siRNA (each 100 nM). The next day, BALB/C mice were given an intravenous injection of PBS or DNP-HSA (250 μg/kg) along with 2% (v/v) Evans blue solution. Representative images of each BALB/C mouse of each experimental group are shown. Each experimental group consisted of four BALB/C mice. **(B,C)** Ear tissue lysate from BALB/C mouse of each experimental group was subjected to β-hexosaminidase activity assay, immunoblot, and immunoprecipitation. **(D)** BALB/C mice were given an intravenous injection with indicated siRNA. The next day, BALB/C mice were given an intravenous injection with IgE. The following day, BALB/C mice were given an intravenous injection with DNP-HSA and rectal temperatures were measured. Each experimental group consisted of four BALB/C mice. Means ± S.E. of three independent experiments are depicted. Comparison was made between PSA-activated mice and mice injected with SiP62. **(E,F)** Tissue lysates were subjected to β-hexosaminidase activity assay, immunoblot, and immunoprecipitation. *$p < 0.05$; **$p < 0.005$; ***$p < 0.005$.

followed by treatment with graded propylene oxide series, and embedded into epoxy resin (PELCO, USA). Ultrathin sections (~80 nm) were obtained with Ultracut UCT (Leica, Germany), mounted on copper grids, and stained with 1% uranyl acetate and lead citrate (10 min) for the subsequent observations. For immune-gold labeling electron microscopy, ultrathin sections on the grids were treated with 0.02 M glycine for 10 min for quenching the reaction of free aldehyde group. Sections were then washed in deionized water, floated for 1 h in PBS containing 1% BSA, and incubated directly in the primary rabbit or/and mouse antibodies (Anti-P62 or/and Anti-CD63 antibodies) at 1:20 dilutions for overnight at 4°C. The grid were washed five time with 0.1% BSA in PBS, incubated in secondary antibodies, anti- Rabbit IgG conjugated to 10 nm and anti-mouse IgG conjugated to 25 nm (AURION, Holland) diluted 1:20 in 0.1% BSA-PBS. The sample grids were stained with uranyl acetate and lead citrate. The sectioned and immune-gold labeled grids were examined using a Tecnai T10 transmission electron microscope (FEI, USA) operated at 100 kV and JEOL-2100F transmission electron microscope (JEOL, USA) operated at 200 KV.

Statistical Analysis

Data were analyzed and graphed using GraphPad Prism statistics program (GraphPad Prism software). Results are presented as means ± S.E. Statistical analysis was performed using one way *t*-tests with differences between means considered significant when $p < 0.05$.

RESULTS

P62 Mediates Allergic Inflammation by Regulating Autophagic Flux

Based on a close relationship between allergic inflammation and autophagy (3, 4, 26) and the role of p62, a selective adaptor in autophagic processes (13), effect of p62 on allergic inflammation was examined. Antigen DNP-HSA increased autophagic flux, such as ATG5, LC3-II, pBeclin1^{Ser14}, and p62, along with HDAC3 and TGaseII in RBL2H3 cells (**Figure 1A**). Roles of HDAC3 and TGaseII in allergic inflammation have been previously reported (11, 25, 26). Expression levels of LC3-II, p62, and pBeclin1^{Ser14} were increased in antigen-stimulated lung mast cells (**Figure 1A**, lower panel). Down-regulation

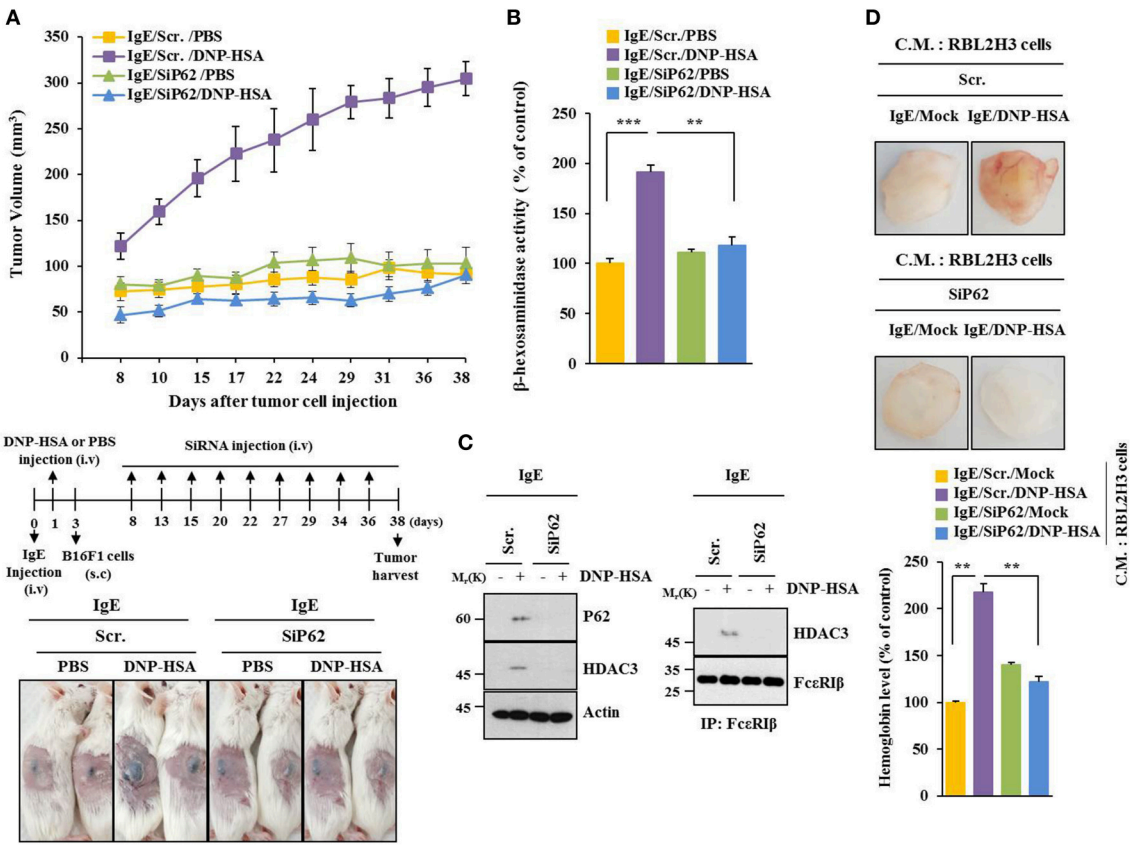

FIGURE 3 | P62 mediates tumorigenic potential of B16F1 cells enhanced by passive systemic anaphylaxis. **(A)** Passive systemic anaphylaxis (PSA) was induced as described. Each mouse received injection of B16F1 melanoma cells (2×10^5) on day 3. After tumor reached a certain size, BALB/C mice were given an intravenous injection of indicated siRNA. Each experimental group consisted of four BALB/C mice. **(B,C)** Tumor tissue lysate from each experimental group was subjected to β-hexosaminidase activity assays, immunoblot, and immunoprecipitation. **(D)** Culture medium of antigen-stimulated RBL2H3 cells transfected with each siRNA for 48 h was subjected to matrigel plug assays. C.M. denotes culture medium. $^{**}p < 0.005$; $^{***}p < 0.005$.

of p62 prevented antigen from increasing autophagic flux, HDAC3, and TGaseII. It prevented antigen from inducing interactions of FcεRIß with Lyn and HDAC3 (**Figure 1B**). It also prevented antigen from increasing ß-hexosaminidase activity (**Figure 1C**). Down-regulation of p62 only blocked the increase of p62 expression upon allergen stimulation, but not decreased it below the level detected in non-stimulated RBL2H3 cells (**Figure 1B**). Increased LC3 puncta expression (**Figure 1D**) and co-localization of p62 with LC3 were seen in antigen-stimulated RBL2H3 cells (**Figure 1D**). Antigen-stimulated RBL2H3 cells showed increased number of autolysosomes compared to un-stimulated RBL2H3 cells (**Figure S1**). 3-MA, an inhibitor of autophagic processes, prevented antigen from increasing levels of autophagic flux and hallmarks of allergic inflammation, prevented antigen from inducing interactions of FcεRIß with Lyn and HDAC3 (**Figure S2A**), prevented antigen from increasing ß-hexosaminidase activity (**Figure S2B**), inhibited passive cutaneous anaphylaxis (PCA) (**Figure S2C**), prevented antigen from increasing ß-hexosaminidase activity (**Figure S2D**), and prevented antigen from increasing autophagic flux and hallmarks of allergic inflammation in a mouse model of PCA (**Figure S2E**). Thus, allergic inflammation is mediated by p62 which regulates autophagic flux. Effect of p62 on allergic inflammation in conjunction with autophagic flux has not been reported previously.

P62 Mediates Anaphylaxis

BALB/C mouse model of passive cutaneous anaphylaxis (PCA) was employed to investigate the role of p62 in allergic inflammation. PCA increased vascular permeability (**Figure 2A**) and β-hexosaminidase activity (**Figure 2B**) in a p62-dependent manner. P62 was necessary for increased expression levels of HDAC3 and TGase II. It was also necessary for interactions of FcεRIβ with HDAC3, Lyn, and TGaseII in a mouse model of PCA (**Figure 2C**). Passive systemic anaphylaxis (PSA) decreased rectal temperatures of BALB/C mice (**Figure 2D**), but increased β-hexosaminidase activity (**Figure 2E**) in a p62-dependent manner. Down-regulation of p62 prevented antigen from increasing expression levels of HDAC3 and TGaseII. It also prevented antigen from inducing interactions of FcεRIβ with HDAC3, TGase II, and Lyn (**Figure 2F**). Thus, p62 can mediate anaphylaxis *in vivo*.

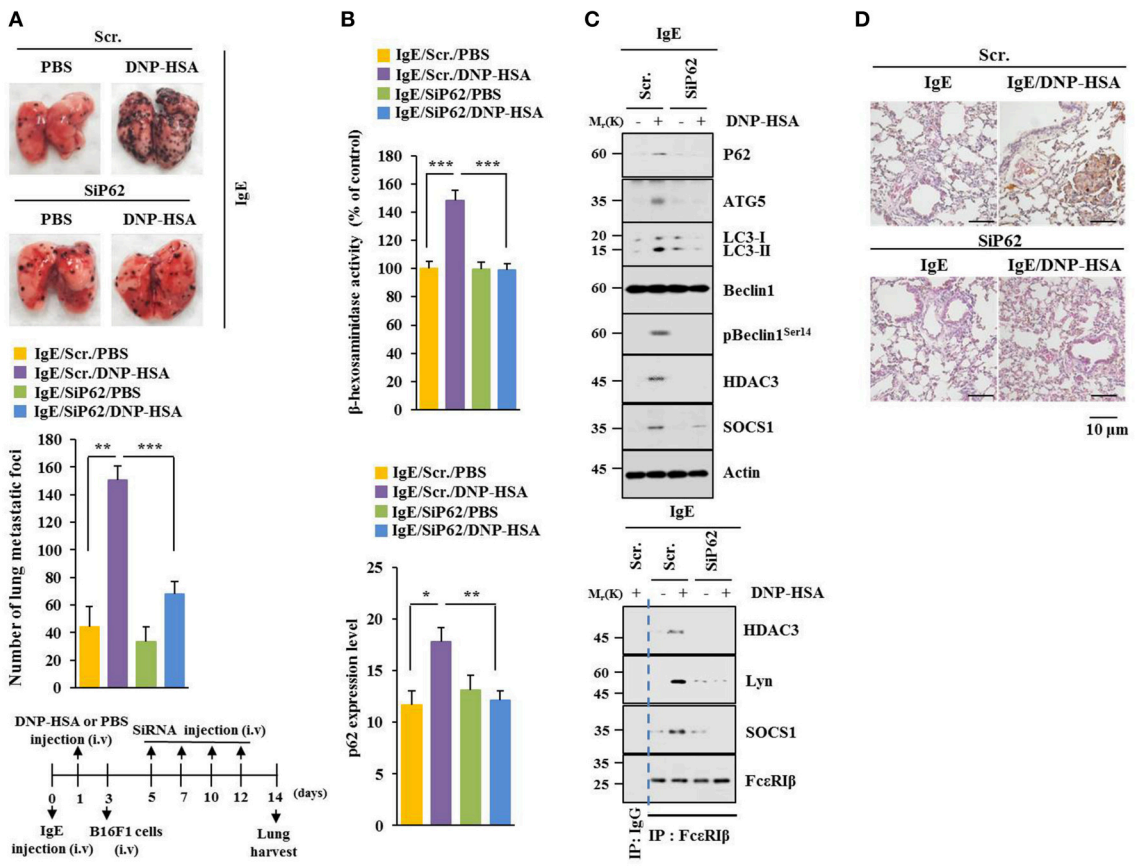

FIGURE 4 | P62 mediates metastatic potential of B16F1 cells enhanced by passive systemic anaphylaxis. **(A)** Each mouse received an injection of B16F1 melanoma cells (2×10^5) on day 3. The extent of lung metastasis was determined. H&E staining was also performed. Each experimental group consisted of four BALB/C mice. **(B,C)** Lung tumor tissue lysates were subjected to β-hexosaminidase activity assays, qRT-PCR analysis, immunoblot, and immunoprecipitation. **(D)** Immunohistochemical staining of lung tumor tissue employing p62 antibody was performed. $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.005$.

P62 Mediates Tumorigenic Potential of B16F1 Cells Enhanced by Passive Systemic Anaphylaxis

PSA enhanced tumorigenic potential of B16F1 cells (**Figure 3A**) and increased β-hexosaminidase activity in a p62-depedent manner (**Figure 3B**). PSA induced interaction between FcεRIβ and HDAC3 in a p62-dependent manner (**Figure 3C**). Enhanced tumorigenic potential by allergic inflammation is known to be due to enhanced angiogenic potential during allergic inflammation (21). Culture medium of antigen-stimulated RBL2H3 cells showed angiogenic potential in a p62-dependent manner based on matrigel plug assays (**Figure 3D**). Thus, p62 can mediate allergic inflammation-promoted enhancement in tumorigenic potential of cancer cells.

P62 Mediates Metastatic Potential of B16F1 Cells Enhanced by Passive Systemic Anaphylaxis

P62 can promote tumor cell growth and metastasis in a Twist1-dependent manner (27). PSA enhanced metastatic potential of B16F1 cells (**Figure 4A**) and increased β-hexosaminidase activity in a p62-dependent manner (**Figure 4B**). Down-regulation of p62 prevented PSA from increasing levels of autophagic flux and hallmarks of allergic inflammation. It also prevented PSA from inducing interactions of FcεRIβ with HDAC3, Lyn, and SOCS1 (**Figure 4C**). Immunohistochemical staining showed increased expression level of p62 by PSA (**Figure 4D**). Thus, p62 can mediate enhanced metastatic potential of cancer cells by allergic inflammation.

P62 Mediates Cellular Interactions During Allergic Inflammation

Allergic inflammation-enhanced tumorigenic and metastatic potentials of cancer cells are known to be due to interactions between cancer cells and immune cells, such as mast cells and macrophages (11, 25, 26). Antigen increased expression levels of HDAC3 and TGaseII in RBL2H3 cells in a p62-dependent manner (**Figure 5A**). When culture medium of antigen-stimulated RBL2H3 cells was added to B16F1 cells, it increased invasion (**Figure 5B**), migration potential (**Figure 5C**),

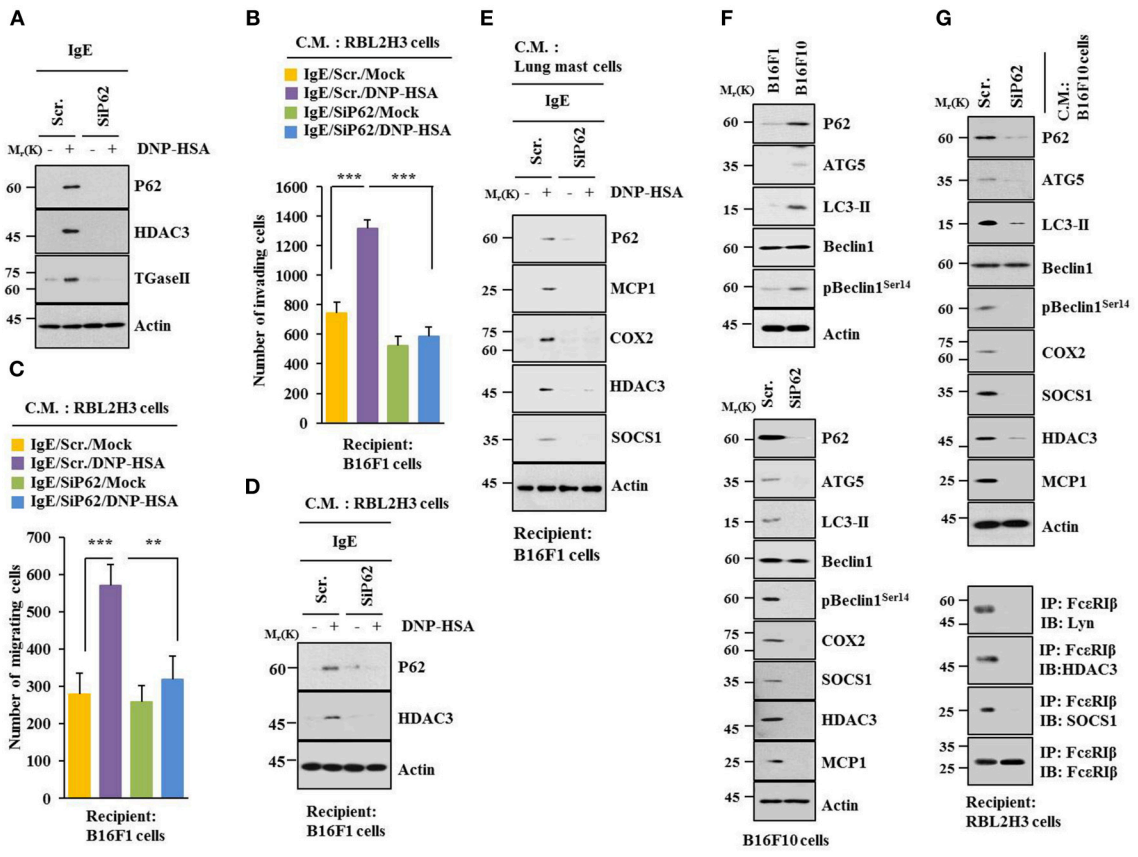

FIGURE 5 | P62 mediates cellular interactions during allergic inflammation. **(A)** Immunoblot was performed. **(B–D)** One hour after stimulation with DNP-HSA, culture medium was added to B16F1 cells and incubated for 8 h followed by migration, invasion assays, and immunoblot. C.M. denotes culture medium. **(E)** Same as **(D)** except that culture medium of lung mast cells was employed. **(F)** Cell lysates from indicated cells were subjected to immunoblot (upper panel). At 48 h after transfection with indicated siRNA, immunoblot was performed (lower panel). **(G)** At 48 h after transfection with indicated siRNA, culture medium of B16F10 cells was added to RBL2H3 cells and incubated for 8 h followed by immunoblot and immunoprecipitation. $^{**}p < 0.005$; $^{***}p < 0.005$.

and expression level of HDAC3 (**Figure 5D**) in a p62-dependent manner. When culture medium of antigen-stimulated lung mast cells was added to B16F1 cells, it increased expression levels of hallmarks of allergic inflammations, such as MCP1, COX2, HDAC3, and SOCS1 in a p62-dependent manner (**Figure 5E**). B16F10 cells showed higher level of autophagic flux than B16F1 cells (**Figure 5F**). Down-regulation of p62 decreased autophagic flux and hallmarks of allergic inflammation in B16F10 cells (**Figure 5F**). When culture medium of B16F10 cells was added to RBL2H3 cells, it increased hallmarks of allergic inflammation and autophagic flux. It also induced interactions of FcεRIß with HDAC3, Lyn, and SOCS1 in a p62-dependent manner (**Figure 5G**). When culture medium of B16F10 cells was added to lung macrophages, it increased hallmarks of allergic inflammation and autophagic flux in a p62-dependent manner (**Figure S3A**). When culture medium of B16F10 cells (**Figure S3B**) or RBL2H3 cells (**Figure S3D**) was added to lung macrophages, it increased expression level of CD163, but decreased expression level of iNOS in a p62-dependent manner. When culture medium of RBL2H3 cells was added to lung macrophages, it increased hallmarks of allergic inflammation, autophagic flux, and CD163, but decreased expression level of iNOS in a p62-dependent manner (**Figure S3C**). Thus, p62 can mediate cellular interactions during allergic inflammation.

MiR-135-5p Directly Targets p62

TargetScan analysis predicted binding of miR-135-5p to 3′-UTR of p62 (**Figure 6A**). Wild type and mutant 3′-UTR of p62 showed luciferase activities when they were transfected into RBL2H3 cells (**Figure 6B**). Antigen increased luciferase activities associated with wild type and mutant 3′-UTR of p62 (**Figure 6B**). MiR-135-5p mimic decreased luciferase activity associated with Luc-3′-wild type UTR of p62, but not luciferase activity associated with Luc-3′-mutant UTR of p62 in antigen-stimulated RBL2H3 cells (**Figure 6B**). Thus, miR-135-5p can directly regulate expression level of p62.

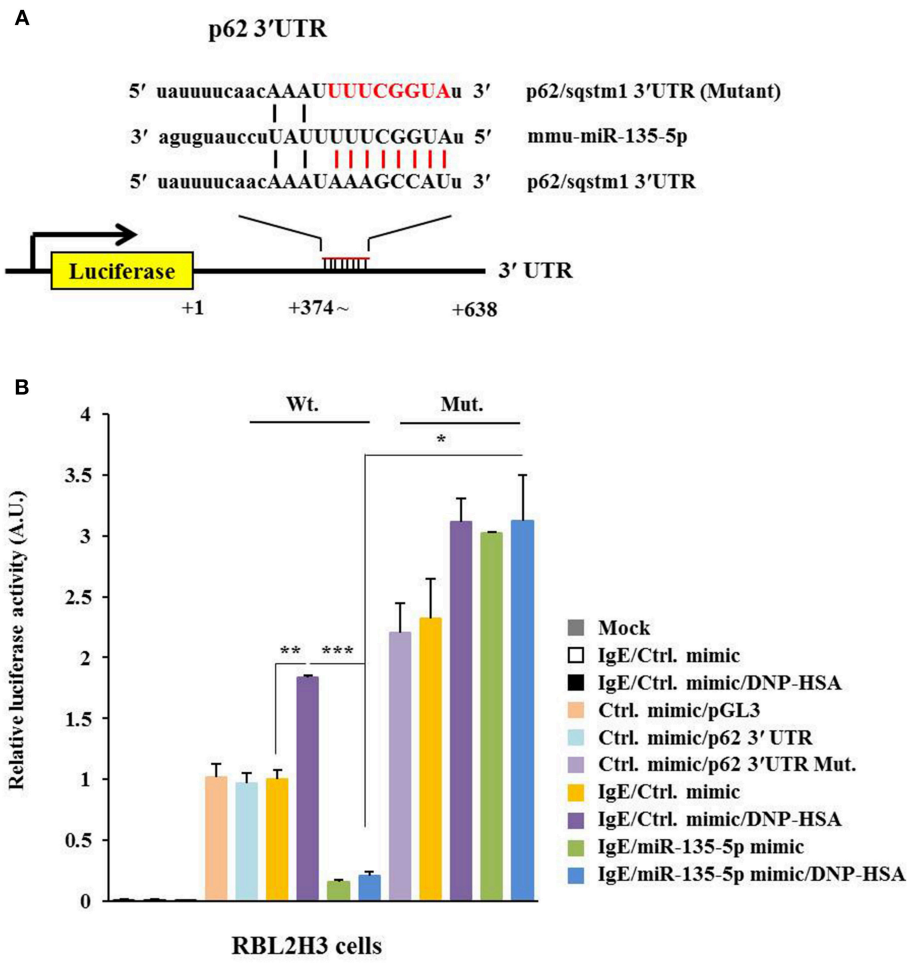

FIGURE 6 | miR-135-5p directly targets p62. **(A)** Potential binding of miR-135-5p to 3′-UTR of p62. **(B)** Wild type Luc-p62-3′-UTR or mutant Luc-p62-3′-UTR was transfected along with control mimic or miR-135-5p mimic (each at 10 nM) into the indicated cell line. At 48 h after transfection, luciferase activity assays were performed. *$p < 0.05$; **$p < 0.005$; ***$p < 0.005$.

MiR-135-5p Mimic Inhibits Allergic Inflammation Both *in vitro* and *in vivo*

MiR-135-5p mimic prevented antigen from increasing hallmarks of allergic inflammation and autophagic flux. It also prevented antigen from inducing interactions of FcεRIß with HDAC3, Lyn and SOCS1 in RBL2H3 cells (**Figure 7A**). Antigen decreased expression level of miR-135-5p in RBL2H3 cells (**Figure 7B**). MiR-135-5p mimic prevented antigen from increasing ß-hexosaminidase activity (**Figure 7C**). It also prevented culture medium of antigen-stimulated RBL2H3 cells from enhancing migration (**Figure 7D**) and invasion potential (**Figure 7E**) of B16F1 cells. MiR-135-5p mimic prevented culture medium of antigen-stimulated RBL2H3 cells from increasing hallmarks of allergic inflammation and autophagic flux in B16F1 cells (**Figure 7F**). It prevented antigen from enhancing vascular permeability (**Figure 8A**). It also prevented antigen from increasing ß-hexosaminidase activity (**Figure 8B**) and p62 expression (**Figure 8B**) in a mouse model of PCA. MiR-135-5p mimic also prevented antigen from increasing autophagic flux, hallmarks of allergic inflammation, and antigen from inducing interactions of FcεRIß with HDAC3, Lyn, and SOCS1 in a mouse model of PCA (**Figure 8C**). Thus, miR-135-5p mimic can regulate allergic inflammation both *in vitro* and *in vivo*.

MiR-135-5p Mimic Inhibits Allergic Inflammation-Enhanced Metastatic Potential and Tumorigenic Potential of B16F1 Melanoma Cells

MiR-135-5p mimic prevented PSA from enhancing metastatic potential of B16F1 melanoma cells (**Figure 9**). It also prevented antigen from increasing β-hexosaminidase activity, amount of histamine released, and PGE2 level in BALB/C mice (**Figure 9B**). PGE2 is known to contribute to the development of asthma by promoting IgE production (28). MiR-135-5p mimic prevented antigen from increasing hallmarks of allergic inflammation and autophagic flux. It also prevented antigen from inducing interactions of FcεRIβ with HDAC3 and Lyn (**Figure 9C**). Immunohistochemical staining showed that miR-135-5p mimic prevented antigen from increasing expression level of p62 (**Figure 9D**). MiR-135-5p mimic prevented PSA from enhancing

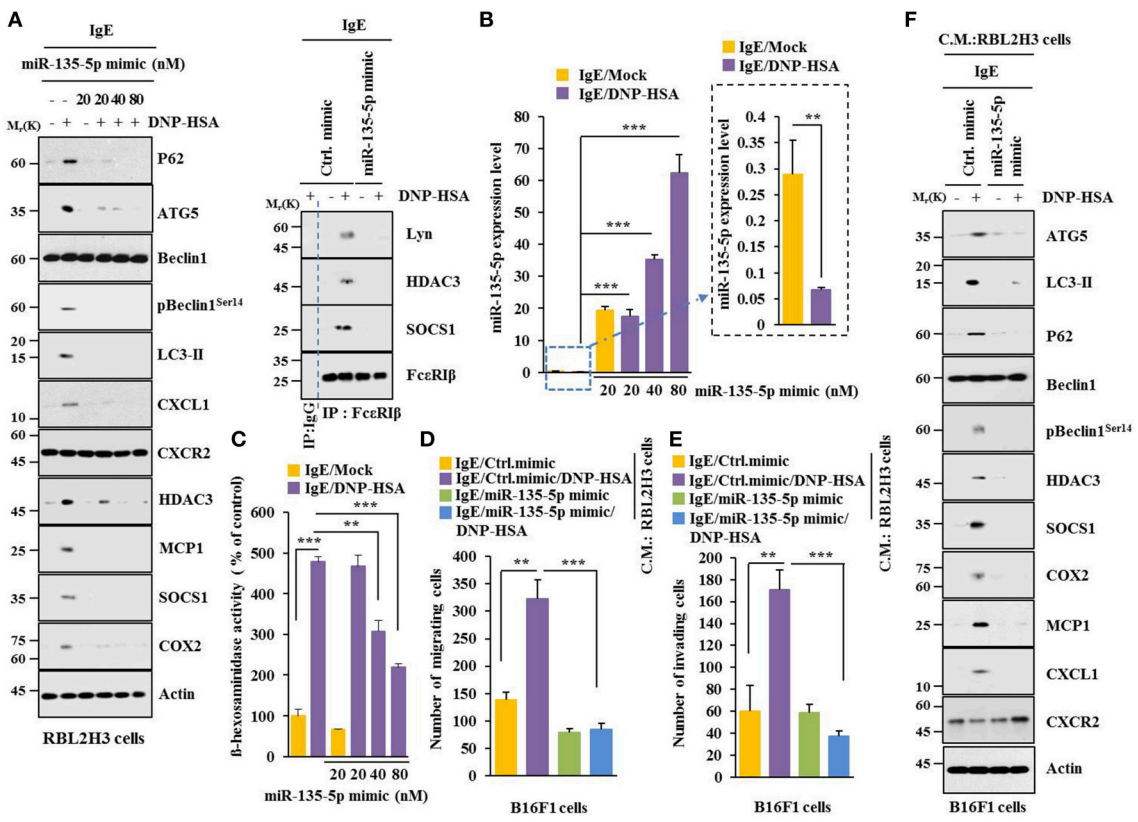

FIGURE 7 | miR-135-5p mimic inhibits allergic inflammation. **(A)** RBL2H3 cells were transfected with control mimic (80 nM) or miR-135-5p mimic at indicated concentration. The next day, cells were sensitized with IgE for 24 h, stimulated with DNP-HSA for 1 h, and subjected to Immunoblot. For immunoprecipitation, RBL2H3 cells were transfected with control mimic (80 nM) or miR-135-5p mimic (20 nM). **(B)** Same as **(A)** except that qRT-PCR analysis was performed. **(C)** Same as **(A)** except that ß-hexosaminidase activity assays were performed. **(D,E)** RBL2H3 cells were transfected with indicated mimic (each at 20 nM). The next day, cells were sensitized with IgE for 24 h followed by stimulation with DNP-HSA for 1 h. The culture medium was obtained and added to B16F1 cells and incubated for 8 h followed by migration or invasion potential assays. **(F)** Same as **(E)** except that immunoblot was performed. $^{**}p < 0.005$; $^{***}p < 0.005$.

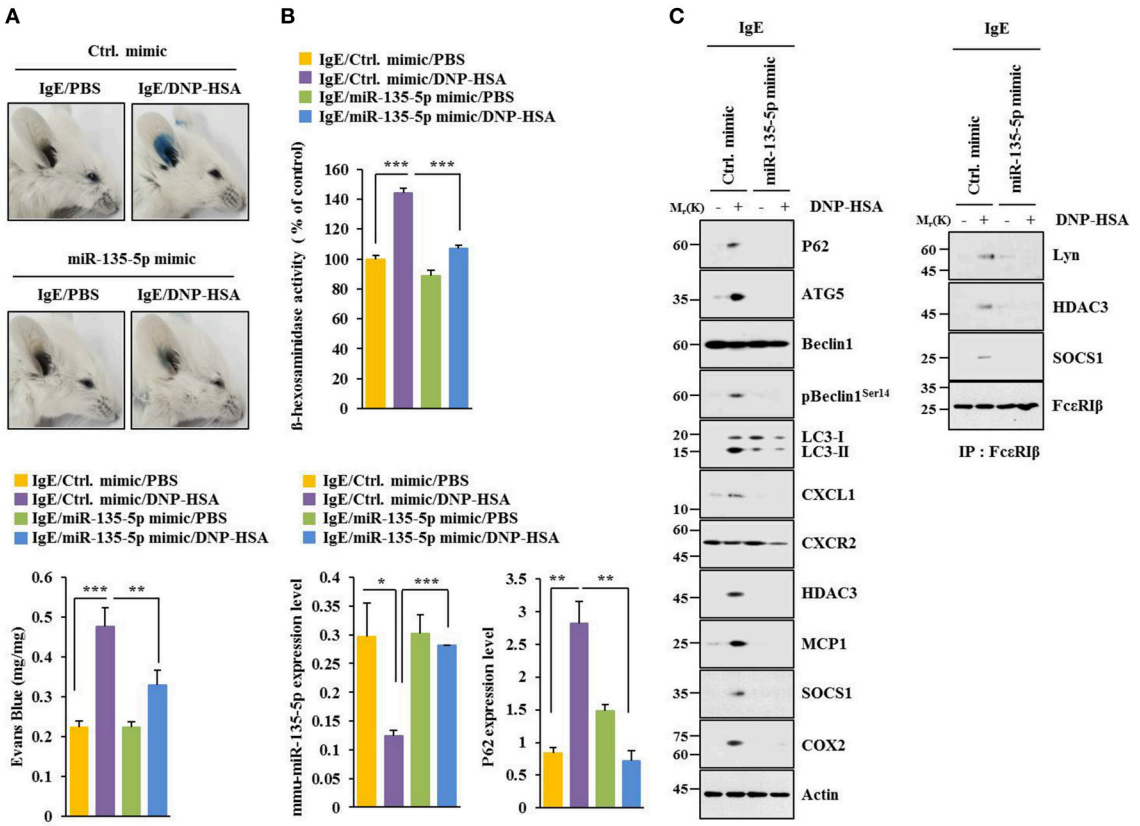

FIGURE 8 | miR-135-5p mimic inhibits passive cutaneous anaphylaxis. **(A)** BALB/C mice were given an intradermal injection of IgE antibody (0.5 μg/kg) or IgG (0.5 μg/kg) along with indicated mimic (each at 100 nM). The next day, BALB/C mice were given an intravenous injection of PBS or DNP-HSA (250 μg/kg) along with 2% (v/v) Evans blue solution. One hour after the injection, the extent of vascular permeability was determined. Each experimental group consisted of four BALB/C mice. Means ± S.E. of three independent experiments are depicted. **(B,C)** Ear tissue lysate from BALB/C mouse of each experimental group was subjected to β-hexosaminidase activity assays, qRT-PCR analysis, immunoblot, and immunoprecipitation. *$p < 0.05$; **$p < 0.005$; ***$p < 0.005$.

tumorigenic potential of B16F1 melanoma cells (**Figure 10A**). MiR-135-5p mimic prevented antigen from increasing hallmarks of allergic inflammation and autophagic flux. It prevented antigen from inducing interactions of FcεRIβ with HDAC3 and Lyn in tumor tissues (**Figure 10B**). It also prevented antigen from increasing β-hexosaminidase activity, the amount of histamine released, and PGE2 level (**Figure 10C**). Thus, miR-135-5p mimic can inhibit allergic inflammation- enhanced metastatic potential and tumorigenic potential of B16F1 melanoma cells.

Extracellular Vesicles Are Necessary for Cellular Interactions During Allergic Inflammation

Extracellular vesicles of multiple myeloma (MM) cells can stimulate secretion of cytokines, such as CXCL1, MCP1, IL6, IL-, IP-10, and CCL5 in mesenchymal stromal cells (MSCs) to promote MM cell growth and migration (29). GW4869, an inhibitor of extracellular vesicles formation, decreased hallmarks of allergic inflammation and autophagic flux. It also inhibited interactions of FcεRIβ with HDAC3 and Lyn in antigen-stimulated RBL2H3 cells (**Figure 11A**). It prevented antigen from increasing β-hexosaminidase activity (**Figure 11B**). GW4869 prevented culture medium of antigen-stimulated RBL2H3 cells from increasing hallmarks of allergic inflammation and autophagic flux (**Figure 11C**) or enhancing migration and invasion potential of B16F1 cells (**Figure 11D**). GW4869 prevented culture medium of antigen-stimulated RBL2H3 cells from regulating expression levels of CD163 and iNOS, hallmarks of allergic inflammation, and autophagic flux in lung macrophages (**Figure 11E**). GW4869 prevented antigen from inducing expression level of p62 in extracellular vesicles of RBL2H3 cells (**Figure 11F**). MiR-135-5p mimic decreased expression level of extracellular vesicular p62 in antigen-stimulated RBL2H3 cells (**Figure 11G**). When culture medium of antigen-stimulated RBL2H3 cells was added to lung macrophages, it increased expression level of CD163, but decreased expression level of iNOS, in the absence of GW4869 (**Figure S4A**). Pellet fraction of growth medium of RBL2H3 cells showed extracellular vesicles (**Figure S4B**). Thus, extracellular vesicles can mediate cellular interactions during allergic inflammation.

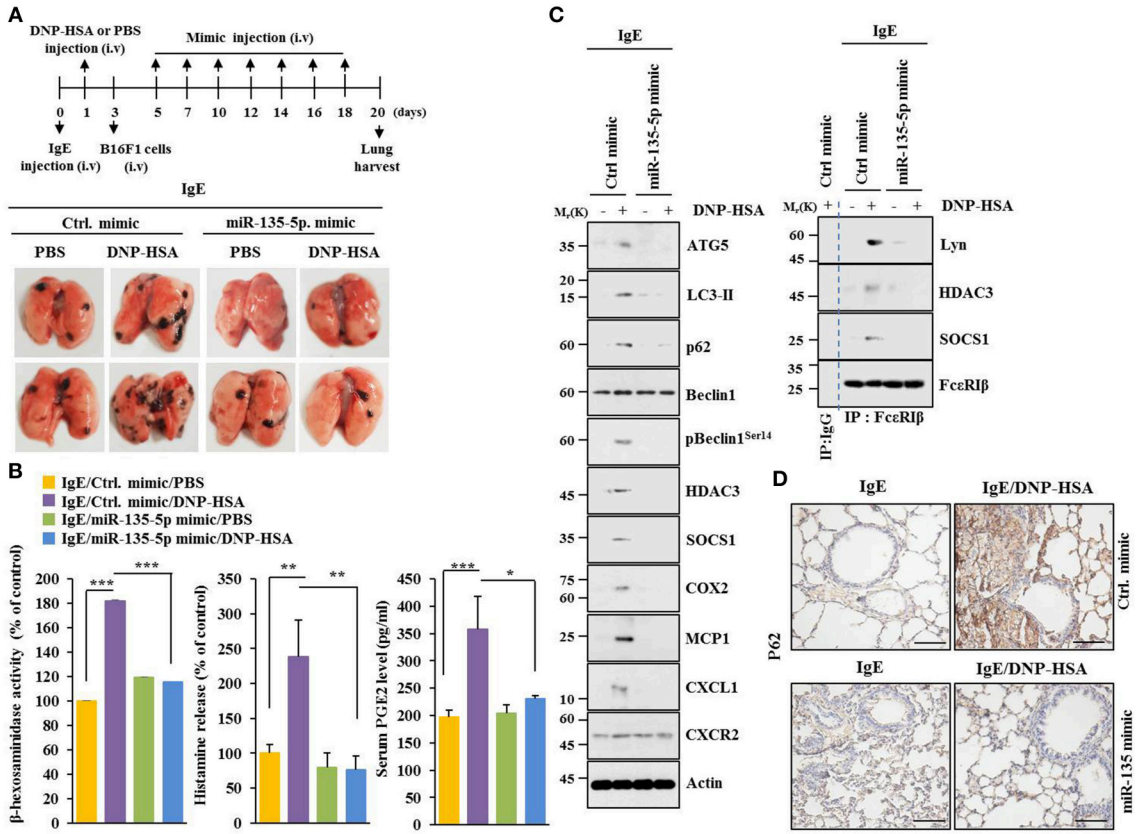

FIGURE 9 | MiR-135-5p mimic inhibits passive systemic anaphylaxis (PSA)-promoted metastatic potential of B16F1 melanoma cells. **(A)** Induction of passive systemic anaphylaxis was performed as described. Each mouse received an intravenous injection of B16F1 melanoma cells (2×10^5) on day 3 and intravenous injection of miR-135-5p mimic (100 nM) at indicated day. Each experimental group consisted of four BALB/C mice. **(B,C)** Tumor tissue lysates were subjected to β-hexosaminidase activity assays, immunoblot, and immunoprecipitation. Sera of BALB/C mice were employed to determine levels of histamine released and PGE2. **(D)** Immunohistochemical staining was performed. $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.005$.

Extracellular Vesicles Are Necessary for Anaphylaxis

GW4869 prevented antigen from decreasing rectal temperatures (**Figure S5A**). It also prevented antigen from increasing ß-hexosaminidase activity, the amount of histamine released, and PGE2 level in a mouse model of PSA (**Figure S5B**). GW4869 prevented antigen from increasing hallmarks of allergic inflammation and autophagic flux. It also prevented antigen from inducing interactions of FcεRIß with HDAC3, Lyn, and SOCS1 (**Figure S5C**). GW4869 prevented antigen from increasing expression levels of G-CSF and MCP1 in the sera of BALB/C mouse model of PSA (**Figure S5D**). GW4869 prevented antigen from increasing vascular permeability (**Figure S6A**), autophagic flux, and hallmarks of allergic inflammation (**Figure S6B**). It also prevented antigen from inducing interactions of FcεRIß with HDAC3, SOCS1, and Lyn (**Figure S6B**) in a mouse model of PCA. GW4869 also prevented antigen from increasing ß-hexosaminidase activity (**Figure S6C**). Thus, extracellular vesicles can mediate anaphylaxis *in vivo*.

Extracellular Vesicles Contain p62, Shuttle Between Cells and Induce Features of Allergic Inflammation

We next examined whether p62 exists in extracellular vesicles, by using immunogold-staining electron microscopy. Immunogold-conjugated p62 antibody was used to determine the location of P62 in the isolated vesicles, and P62 was detected in the lumen of the vesicle, whereas CD63, a known membrane marker of extracellular vesicles, was detected in the outer membrane of the vesicles (**Figure 12A**). This observation was confirmed by co-immunogold staining of p62 (as shown by 10 nm golds located in the inner of the vesicles) and CD63 (as shown by 25 nm golds located in the outer membrane of the vesicles). Visualized extracellular vesicles under negative staining electron microscopy demonstrated the existence of the vesicles in RBL2H3 cells regardless of antigen stimulation (**Figure 12B**).

PKH67-labeled extracellular vesicles were added to RBL2H3 cells to examine whether extracellular vesicles could shuttle between cells. Green fluorescence was observed in RBL2H3 cells that took up PKH67-labeled extracellular vesicles

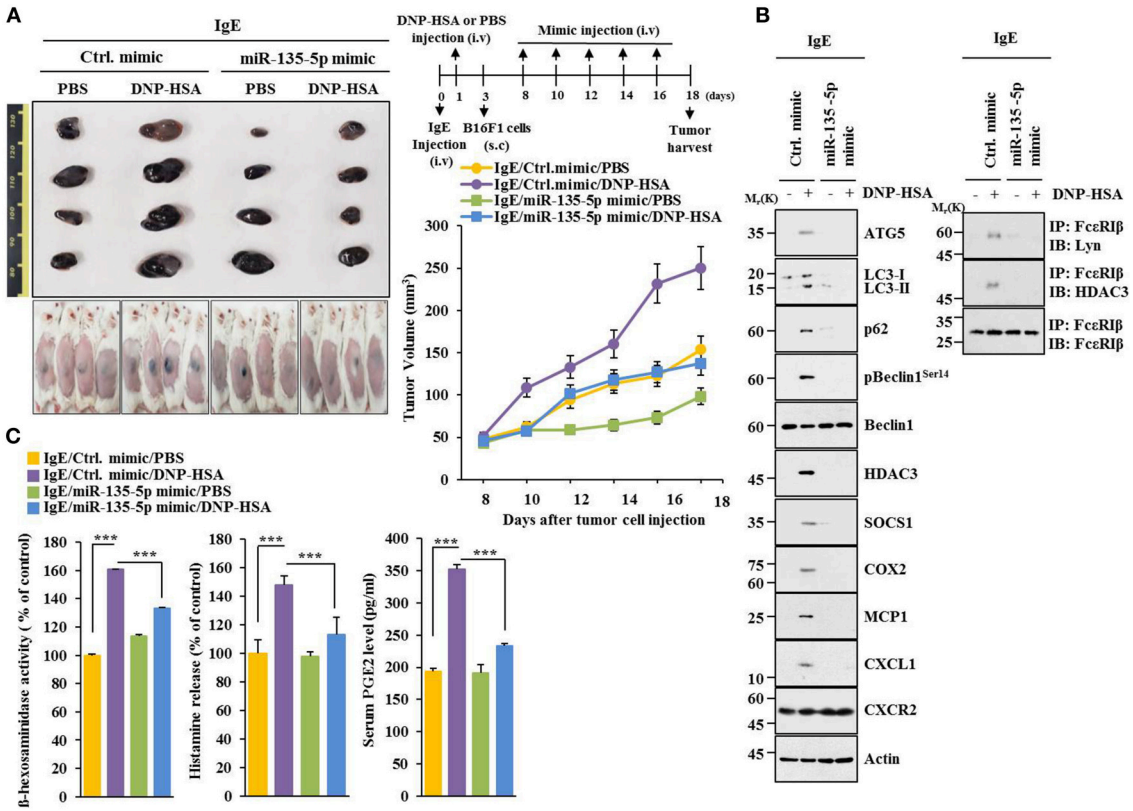

FIGURE 10 | miR-135-5p mimic inhibits enhanced tumorigenic potential of B16F1 melanoma cells induced by passive systemic anaphylaxis. **(A)** Each mouse received an intravenous injection of B16F1 melanoma cells (2×10^5) on day 3 and an intravenous injection of control mimic or miR-135-5p mimic (each at 100 nM) at indicated day. Each experimental group consisted of four BALB/C mice. **(B,C)** Tumor tissue lysates were subjected to immunoblot, immunoprecipitation, and β-hexosaminidase activity assays. Sera were employed to determine levels of histamine released and PGE2. ***$p < 0.0005$.

of un-stimulated RBL2H3 cells and antigen-stimulated RBL2H3 cells (**Figure S7A**). However, fluorescence was not observed in RBL2H3 cells that took up un-labeled extracellular vesicles of antigen-stimulated RBL2H3 cells (**Figure S7A**). Extracellular vesicles of antigen-stimulated RBL2H3 cells increased levels of histamine released and PGE2 in RBL2H3 cells (**Figure S7B**). Thus, extracellular vesicles can shuttle between cells and induce features of allergic inflammation.

Extracellular Vesicles Promote Features of Allergic Inflammation in a p62-Dependent Manner

Using GW4869, an inhibitor of extracellular vesicles formation, results showed that extracellular vesicles played a role in anaphylaxis (**Figure S5A**). Therefore, direct effect of extracellular vesicles on allergic inflammation was examined. Markers of extracellular vesicles, such as CD63, TSG101 and CD81 were found in the pellet fraction of growth medium, but not in the supernatant fraction of growth medium, of RBL2H3 cells (**Figure 13A**). P62 was also present in the pellet fraction of growth medium, but not in the supernatant fraction of growth medium, of antigen-stimulated RBL2H3 cells (**Figure 13A**). Extracellular vesicles isolated from antigen-stimulated RBL2H3 cells increased hallmarks of allergic inflammation and autophagic flux. They also induced interactions of FcεRIß with HDAC3 and Lyn in unstimulated RBL2H3 cells (**Figure 13B**). Extracellular vesicles isolated from antigen-stimulated RBL2H3 cells increased CD163 and hallmarks of allergic inflammation and autophagic flux, but decreased expression of iNOS in macrophages (**Figure 13C**). Extracellular vesicles isolated from antigen-stimulated RBL2H3 cells increased expression of p62, hallmarks of allergic inflammation, and autophagic flux in B16F1 cells (**Figure 13D**). They also enhanced migration and invasion potentials of B16F1 cells (**Figure 13E**). Extracellular vesicles of antigen-stimulated RBL2H3 cells contained p62 (**Figure S8A**). They increased autophagic flux and CD163, but decreased expression of iNOS in a p62-dependent manner in lung macrophages (**Figure S8B**). Extracellular vesicles of antigen-stimulated RBL2H3 cells increased autophagic flux in RBL2H3 cells (**Figure S8C**). They also induced interactions of FcεRIß with HDAC3 and Lyn in a p62-dependent manner (**Figure S8C**). Extracellular vesicles of antigen-stimulated RBL2H3 cells also increased levels of histamine released and PGE2 in RBL2H3 cells in a p62-dependent manner

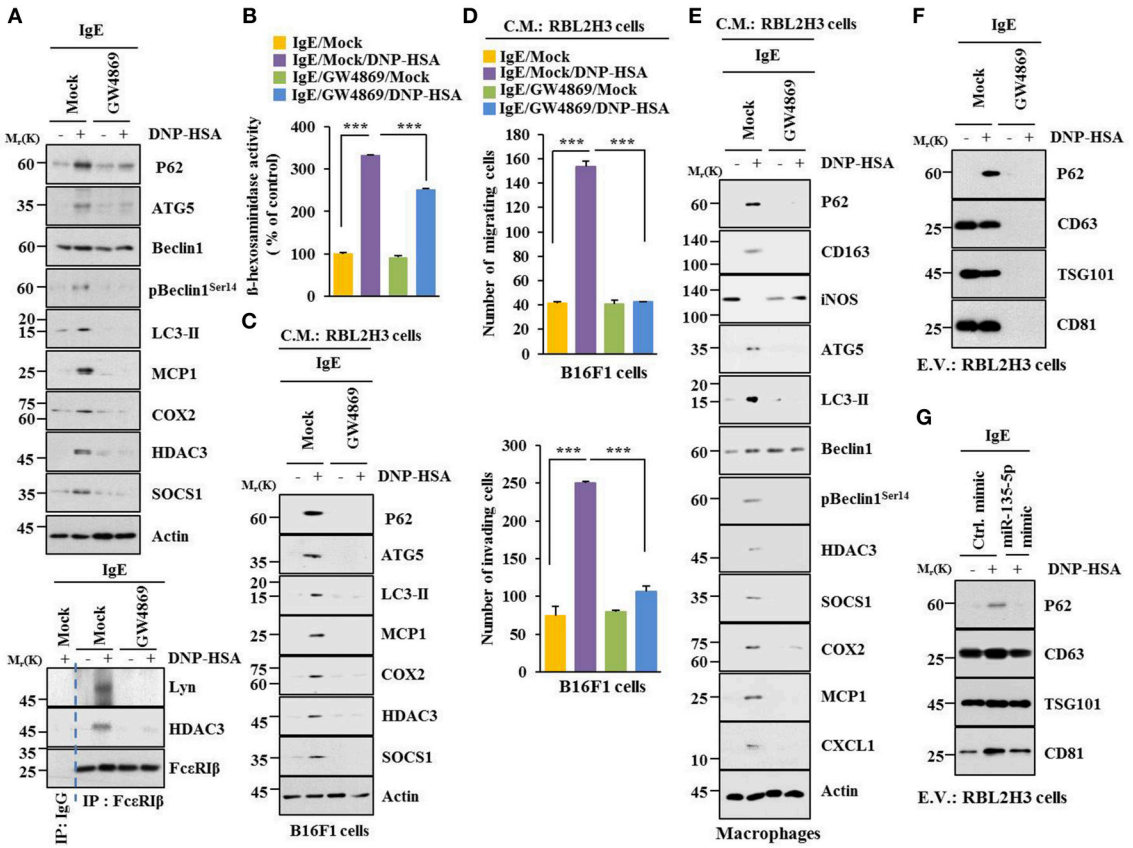

FIGURE 11 | Extracellular vesicles are necessary for cellular interactions in allergic inflammation. **(A,B)** IgE-sensitized RBL2H3 cells were treated without or with GW4869 (10 μM) for 24 h followed by stimulation with DNP-HSA for 1 h. Immunoblot, immunoprecipitation and ß-hexosaminidase activity assays were performed. **(C)** Culture medium of RBL2H3 cells was added to B16F1 cells and incubated for 8 h followed by immunoblot. **(D)** Same as **(C)** except that migration and invasion potentials of B16F1 cells were determined. **(E)** Same as **(C)** except that culture medium was added to lung macrophages. **(F)** Extracellular vesicles isolated from antigen-stimulated RBL2H3 cells without or with G4869 treatment were subjected to immunoblot. **(G)** RBL2H3 cells were transfected with indicated mimic (each at 20 nM). The next day, cells were sensitized with IgE for 24 h followed by stimulation with DNP-HSA. Extracellular vesicles were isolated and subjected to immunoblot. ***$p < 0.0005$.

(**Figure S8D**). Extracellular vesicles of antigen-stimulated RBL2H3 cells increased autophagic flux (**Figure S8E**) and enhanced migration and invasion potentials of B16F1 cells in a p62-dependent manner (**Figure S8F**). Effect of p62 on extracellular vesicles -mediated cellular interactions was further investigated. For this, we employed extracellular vesicles isolated from sera of PSA-activated BALB/C mouse (**Figure S9A**). Extracellular vesicles isolated from serum of PSA-activated BALB/C mouse showed expression of p62 (**Figure S9B**). Serum of each mouse of each experimental group in the mouse model of PSA showed extracellular vesicles (**Figure S9C**). Extracellular vesicles increased autophagic flux and hallmarks of allergic inflammation (**Figure S9D**) and induced interactions of FcεRIß with HDAC3, Lyn, and SOCS1 in unstimulated RBL2H3 cells (**Figure S9E**). They also enhanced migration and invasion potentials of B16F1 cells in a p62-dependent manner (**Figure S9F**). Thus, p62 is necessary for extracellular vesicles -mediated cellular interactions during allergic inflammation.

DISCUSSION

Neutrophil autophagy enhances asthma severity by damaging airway epithelium and triggering inflammatory responses (30). TLR2 confers a pivotal role in allergic airway inflammation via regulating PI3K/Akt signaling pathway-related autophagy in mice (31). Positive correlation between gene expression patterns of ATG5 and COL5A1 suggests that dysregulated autophagy may contribute to subepithelial fibrosis in airways of refractory asthmatic individuals (32). Expression of beclin-1 was upregulated in airways of patients with asthma and OVA-challenged mice, accompanied by airway EMT and remodeling (33). More autophagosomes are found in patients with asthma and OVA-challenged mice compared with healthy controls (33). Autophagy is closely correlated with the severity of asthma through eosinophilic inflammation (34). These reports suggest a close relationship between autophagy and allergic inflammation.

p62 was increased during allergic inflammation (**Figure 1A**). It regulated hallmarks of allergic inflammation and autophagic

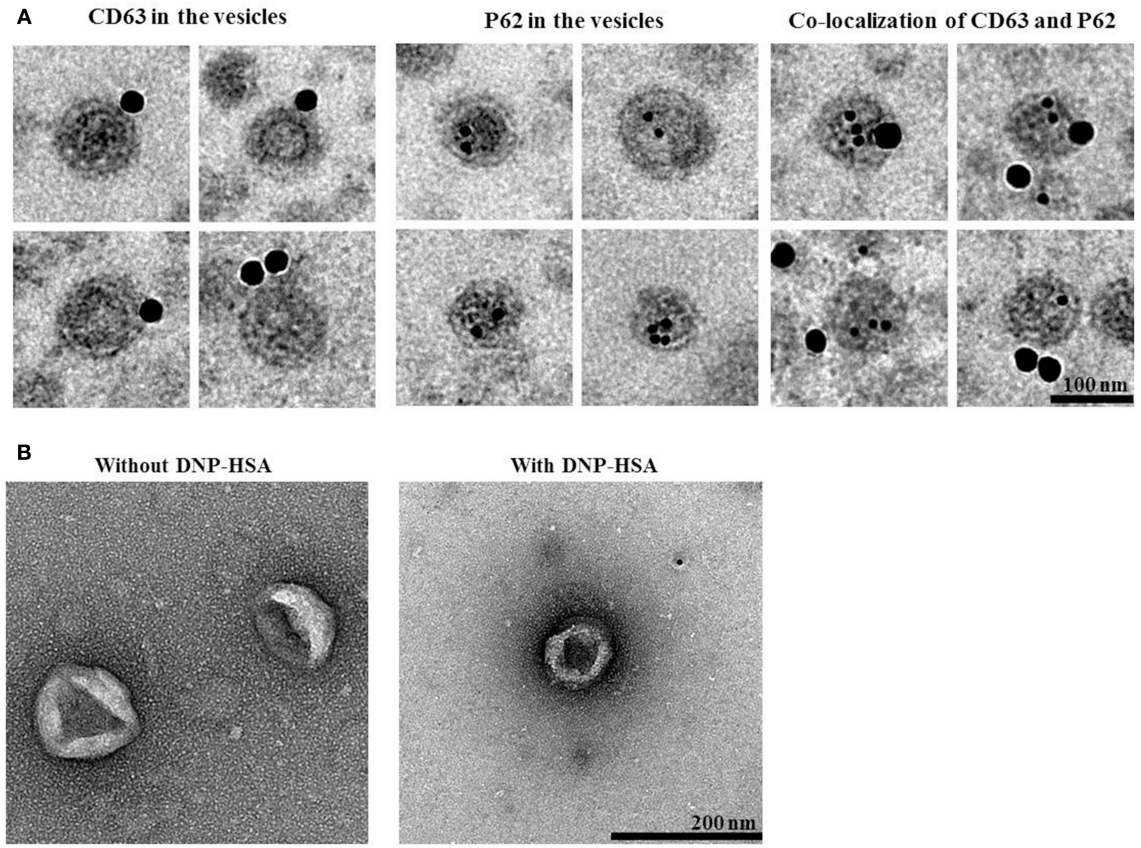

FIGURE 12 | P62 is present in extracellular vesicles of antigen-stimulated RBL2H3 cells. **(A)** General appearances of isolated extracellular vesicles and immuno-gold staining images using anti-CD63, a known membrane marker for the extracellular vesicles, and anti-p62 antibodies. Twenty-five and 10 nm gold particles indicate the localization of CD63 (outer membrane of the vesicles) and p62, respectively. Note that p62 is shown to locate in the lumen of the vesicles. **(B)** Extracellular vesicles isolated from un-stimulated RBL2H3 cells or antigen-stimulated RBL2H3 cells were visualized by negative staining electron microscopy. One hundred and 200 nm scale bars applied to the montages **(A)** and the fields **(B)**, respectively.

flux (**Figure 1B**). We also showed that allergic inflammation was accompanied by enhanced autophagosome formation (**Figure S1**). It will be interesting to examine the effect of p62 on autophagosome formation during allergic inflammation in future studies. It will also be necessary to identify molecule regulated by p62. 3-MA, an inhibitor of autophagy, prevented antigen from increasing expression of p62 and hallmarks of allergic inflammation in RBL2H3 cells (**Figure S2A**). 3-MA also negatively regulated PCA (**Figure S2D**). This indicates a role of autophagy in allergic inflammation. The role of autophagy in anaphylaxis has not been reported yet.

Antigen stimulation increased expression of HDAC3 in RBL2H3 cells (**Figure 1A**). Role of HDAC3 in allergic inflammation has been reported (11, 26). MiR-384 and HDAC3 can form a negative feedback loop to regulate allergic inflammation and cellular interactions during allergic inflammation (26). MiR-384, a negative regulator of HDAC3, can reduce augmentation of Beclin1-dependent autophagy of airway smooth muscle cells (35). Hdac3-deficient iNKT cells showed less Cyto-ID staining and lower LC3A/B expression, indicating reduced autophagy (36). HDAC3 may regulate autophagic flux during allergic inflammation. Further studies are needed to identify miRNAs that regulate expression of HDAC3 during allergic inflammation.

COX2 is known to be an asthma-associated gene (37). Allergic inflammation increased expression of COX2 in RBL2H3 cells (**Figure S2E**). COX2 and miR-26 can form a negative feedback loop and regulate allergic inflammation and cellular interactions during allergic inflammation (18). COX2 overexpression induced by the ATF4 ER stress pathway contributes to Lupus Nephritis-induced kidney autophagy and injury (38). It is probable that miR-26/COX2 axis may regulate autophagic flux during allergic inflammation.

TargetScan analysis predicted binding of miR-181a/-218/-122a to the 3′UTR of p62 (personal observation). MiR-181a/-218 can form a negative feedback loop with TGaseII and regulate allergic inflammation (25). Allergic inflammation increased expression of TGaseII in RBL2H3 cells (**Figure 1A**). Under stress, TGaseII mediates enhanced autophagy to promote Mantle cell Lymphoma (MCL) (39). Autophagy product ATG5 involved in autophagosome elongation can positively regulate TGase II/NF-κB/IL6 signaling (39). MiR-181a mimic prevented antigen from

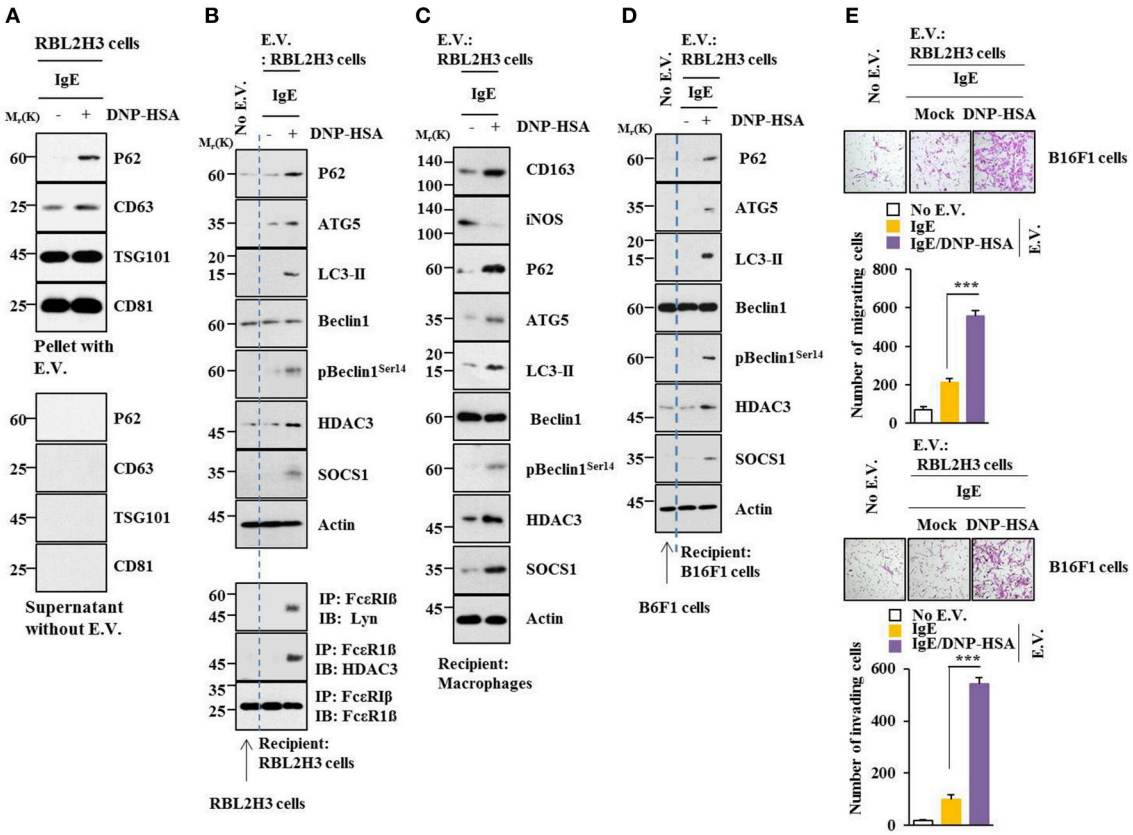

FIGURE 13 | Extracellular vesicles promote features of allergic inflammation**. (A)** Extracellular vesicles were isolated from growth medium of RBL2H3 cells without or with antigen stimulation for 1 h. Extracellular vesicular proteins were subjected to immunoblot. E.V. denotes extracellular vesicles. **(B,C)** Extracellular vesicles (20 μg) isolated from RBL2H3 cells without or with antigen stimulation for 1 h were added to RBL2H3 cells or lung macrophages and incubated for 24 h followed by immunoblot and immunoprecipitation. No extracellular vesicles denote immunoblot and immunoprecipitation of RBL2H3 cells. **(D)** Same as **(B)** except that extracellular vesicles were added to B16F1 cells. No extracellular vesicles denote immunoblot of B16F1 cells without extracellular vesicles treatment. **(E)** Same as **(D)** except that migration and invasion potential assays were performed. No extracellular vesicles denote migration or invasion potential of B16F1 cells without extracellular vesicles treatment. ***$p < 0.0005$.

increasing expression levels of TGaseII and p62 in RBL2H3 cells while miR-181a inhibitor increased expression levels of TGaseII and p62 in an antigen-independent manner in RBL2H3 cells (personal observations). It will be necessary to identify miRNAs that can regulate expression of TGaseII in the future.

Increased level of CXCL1 has been reported in a mouse model of allergic rhinitis (40). Allergic inflammation increased expression of CXCL1 in RBL2H3 cells (**Figure 7A**). MiR-135-5p mimic prevented antigen from increasing expression of CXCL1 in RBL2H3 cells (**Figure 7A**). Neutrophilic inflammation, a hallmark of allergic asthma, is mediated by CXCR2, a receptor of CXCL1 (41). CXCR2 can enhance neutrophilic inflammation and exacerbate IL-33-induced airway hyper responsiveness (41). Neutrophilic asthma in STAT6$^{-/-}$ mice that are steroid resistant is accompanied by elevated lung levels of TNF-α, CXCL1, CXCL2, and CXCL5 (42). Mast cell-derived CXCL1 mediates the protumorigenic role of mast cells (43). It is probable that CXCL1 can mediate cellular interactions during allergic inflammation. It would be interesting to examine signaling pathways of CXCL1-CXCR2 axis for better understanding of p62-promoted allergic inflammation. It is also important to identify cytokines/miRNAs that can serve as targets of CXCL1.

Intravitreal application of miR-135 facilitates retinal ganglion cell (RGC) axon regeneration after optic nerve injury in adult mice in part by repressing KLF4 (44). Lack of Kruppel-like factor 4 (KLF4) expression in monocytes and lung epithelial cells decreases Th2 cytokines in mice and airway hyper responsiveness (AHR) (45). Endogenous KLF4 can bind to promoter regions of p62 gene while upregulation of KLF4 induces expression of p62 (46). Thus, KLF4 might mediate allergic inflammation both *in vitro* and *in vivo* in association with autophagic processes by regulating expression level of p62. TargetScan analysis predicted that miR-135-5p was a negative regulator of p62 (i.e., miR-135-5p directly regulated expression of p62) (**Figure 6**). MiR-135-5p mimic had a negative regulatory role in *in vitro* allergic inflammation (**Figures 7A,C**). MiR-135-5p mimic negatively regulated cellular interactions during allergic inflammation (**Figures 7D,F**). It will be necessary to identify cytokines that are regulated by miR-135-5p mimic. These cytokines may

mediate cellular interactions during allergic inflammation. MiR-135-5p mimic can inhibit PCA (**Figures 8A,C**). MiR-135mimic negatively regulated metastatic potential of cancer cells enhanced by PSA (**Figures 9A,B**).

MiR-135-5p targets Smad5, a key transducer of the BMP2 osteogenic signal, and inhibits differentiation of osteoprogenitors (47). BMP2 is involved in allergic airway inflammation induced by house dust mite (48). Mast cells-derived histamine induces BMP-2 expression in human coronary artery endothelial cells (49). Thus, BMP2 might act as a target of miR-135-5p and mediates anaphylaxis in conjunction with autophagy.

Extracellular vesicles regulate anti-cancer drug-sensitivity by promoting autophagy (50). Human umbilical cord mesenchymal stem cells (MSC)-derived extracellular vesicles (hucMSC-Ex) can promote autophagy to prevent cisplatin-induced renal injury (51). Extracellular vesicles of mesenchymal stem cells activate regulatory T cells to suppress asthma (52). These reports suggest that extracellular vesicles have roles in allergic inflammation. GW4869, an inhibitor of extracellular vesicles formation, negatively regulated PSA (**Figure S5A**) and PCA (**Figure S6A**). Thus, extracellular vesicles can mediate anaphylaxis.

Extracellular vesicles isolated from infected macrophages can stimulate secretion of cytokines, such as RANTES, IL-1ra, MIP-2, CXCL1, MCP1, sICAM-1, and G-CSF (53). Thus, extracellular vesicles might mediate cellular interactions during allergic inflammation. GW4869, an inhibitor of extracellular vesicles formation, prevented antigen from increasing expression of hallmarks of allergic inflammation and autophagic flux in RBL2H3 cells (**Figure 11A**). GW4869 prevented culture medium of antigen-stimulated RBL2H3 cells from enhancing invasion and migration potentials of B16F1 cells (**Figure 11D**). These results indicate a role of extracellular vesicles in allergic inflammation.

GW4869 prevented antigen from increasing expression levels of MCP1 and CXCL1 in a mouse model of PSA (**Figure S5C**). GW4869 prevented antigen from stimulating secretion of MCP1 in serum of PSA-activated BALB/C mouse (**Figure S5D**). MCP1 in B16F1 cells was increased by culture medium of antigen-stimulated mast cells in a p62-dependent manner (**Figure 5E**). Thus, MCP1 and CXCL1 might mediate cellular interactions during allergic inflammation. It will be necessary to examine the presence of MCP1 and/or CXCL1 in extracellular vesicles of activated immune cells, such as mast cells and macrophages, during allergic inflammation. We showed the presence of p62 in extracellular vesicles of antigen-stimulated RBL2H3 cells (**Figures 12A**, **13A**). Extracellular vesicles of antigen-stimulated RBL2H3 cells activated macrophages (**Figure 13C**) and enhanced invasion and migration potentials of B16F1 cells (**Figure 13D**). These extracellular vesicles might induce features of allergic inflammation in antigen-independent manner. It will be necessary to further identify miRNAs and cytokines present within extracellular vesicles of antigen-stimulated RBL2H3 cells. It will also be necessary to identify molecules regulated by these extracellular vesicles.

MiRNA array analysis was performed to identify miRNAs regulated by p62. Our results showed that miR-154-5p and miR-31-5p were increased in RBL2H3 cells by antigen stimulation in a p62-dependent manner (personal observations). Increased expression level of miR-154-5p was also observed in extracellular vesicles of antigen-stimulated RBL2H3 cells (data not shown). Our results showed that miR-154-5p was necessary for allergic inflammation both *in vitro* and *in vivo* (data not shown). Promoter sequences of miR-154-5p and miR-31-5p contain binding sites for HDAC2, SP1, and YY (personal observations). Therefore, SP1 and YY1 might directly increase expression levels of miR-154-5p and miR-31-5p. TargetScan analysis predicted binding of miR-154-5p to the 3′-UTR of SOCS5 and binding of miR-31-5p to the 3′-UTR of oxidative stress responsive-1 (Oxsr-1). SOCS5 can reduce JAK2 phosphorylation (54). JAK2 is necessary for allergic inflammation (21). Therefore, SOCS5 might be a negative regulator of allergic inflammation. MiR-31-5p is a candidate master regulator of genes associated with neutrophil recruitment. It targets Oxsr-1 (55). It would be interesting to examine whether Oxsr-1 is a negative regulator of allergic inflammation in the future.

In summary, we showed novel roles of miR-135-5p-p62 axis in allergic inflammation in conjunction with autophagic flux. It would be necessary to further identify extracellular vesicular cytokines and miRNAs to better understand p62-mediated allergic inflammation and cellular interactions during allergic inflammation.

AUTHOR CONTRIBUTIONS

DJ conceived the study, contributed to experimental design, and wrote paper. MK performed *in vitro* and *in vivo* experiments. YP, YKw, and YKi contributed with *in vivo* experimental data. JM contributed with electron microscopic observations of autophagosomes. HJ, H-UK, and MJ contributed with extracellular vesicles isolation and electron microscopic observations of extracellular vesicles. JB contributed with luciferase constructs.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00738/full#supplementary-material

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REVIEW
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MicroRNAs as Molecular Switches in Macrophage Activation

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The efficacy of macrophage- mediated inflammatory response relies on the coordinated expression of key factors, which expression is finely regulated at both transcriptional and post-transcriptional level. Several studies have provided compelling evidence that microRNAs play pivotal roles in modulating macrophage activation, polarization, tissue infiltration, and resolution of inflammation. In this review, we highlight the essential molecular mechanisms underlying the different phases of inflammation that are targeted by microRNAs to inhibit or accelerate restoration to tissue integrity and homeostasis. We further review the impact of microRNA-dependent regulation of tumor-associated macrophages and the relative implication for tumor biology.

Keywords: macrophages, microRNA, endotoxin, Toll-like receptor, TAM

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INTRODUCTION

Inflammation plays a critical role in host defense to invading microbial pathogens and is also essential for the successful repair of tissue damage (1). In this process, the involvement of multiple immune cells is critical. Macrophages are important component of the innate immune response, with a prominent role in host defense and clearance of foreign microorganisms, and in tissue healing (2). The inflammatory response is elicited by the recognition of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns by specific receptors expressed on macrophages (DAMPs), such as Toll like receptors (TLRs). These receptors elicit a signaling cascade that enhances phagocytic activity and activates the production of pro-inflammatory cytokines, chemokines, reactive oxygen and nitrogen species, and antimicrobial peptides (3). The efficacy of macrophage-mediated inflammatory responses relies on the coordinated expression of key proteins involved in macrophage activation and polarization processes, whose expression is finely regulated at both transcriptional and post-transcriptional levels (4–9). Evidence accumulated over the last decade suggests a prominent role for microRNAs (miRNAs) as key regulators of macrophage differentiation, infiltration, and activation. In addition to initial studies indicating the capability of miRNAs to modulate the magnitude of the innate immune response, participating as integral components of feedback loop regulatory mechanisms, which significantly shape the inflammatory response, recent studies have also established their role in tuning macrophage differentiation and polarization. In particular, a complex and highly regulated network of miRNAs exerting a pervasive regulation of inflammatory pathways by targeting multiple component of the TLR signaling pathway and thus affecting the profile of inflammatory cytokines induced downstream has been defined (10). Collectively taken, miRNAs can be viewed as a new regulatory layer of inflammatory reactions operating as intracellular effectors of well-known pro- and anti-inflammatory mediators, including PAMPs and DAMPs, inflammatory, and anti-inflammatory cytokines and macrophage polarizing factors (e.g., IFNγ, TGFβ, glucocorticoids, IL-4) (10–13). In this review, we summarize the most recent

findings addressing the role of miRNAs in macrophage-mediated inflammatory response, with particular emphasis on the molecular pathways affected by miRNA-mediated regulation during macrophage polarization, bacterial infection, endotoxin tolerance, and tissue regeneration.

miRNA BIOGENESIS AND MECHANISM OF ACTION

Genome-wide sequencing approaches have lead to the discovery of non-coding RNAs (ncRNAs), which account for approximately 98% of the entire genome output, compared to the remaining 2% corresponding to protein-coding transcripts (14). Evolutionary studies have demonstrated that the increase in organisms complexity corresponds to a decrease in the abundance of protein-coding genes and a concomitant rise in the number of ncRNAs, indicating that regulatory RNA diversification has been critical to increase vertebrate complexity (15, 16). In this scenario, the most extensively studied class of ncRNAs is represented by miRNAs, short (20–24 nt in length) single-stranded RNA molecules which comprise 1–2% of all genes in worms, flies, and mammals (17, 18). According to information available in public repositories, at today 48885 mature different miRNAs have been reported in 271 species (miRBase 22, www.mirbase.org) (19). miRNAs essentially undergo the same regulatory mechanisms of any other protein-coding gene, being in normal conditions transactivated or silenced by specific transcription factors (4–7), affected by chromosomal deletions or amplifications (5, 8), and/or point mutations (9, 10). Moreover, their expression level is also affected by extensive epigenetic regulatory mechanisms, such as promoter methylation and histone modifications (20).

miRNAs are mainly transcribed by RNA polymerase II as primary miRNA (pri-miRNA) transcripts, with a local stem-loop structure and typically over 1 kb in length, in which the mature miRNA sequence is embedded (21). Following transcription, the pri-miRNA undergoes several steps of maturation. First, in the nucleus the RNAse III Drosha and its essential cofactor *DiGeorge syndrome critical region 8* (DGCR8) form a complex, called Microprocessor, that initiates the maturation process by cropping the pri-miRNA to release a small hairpin-structured miRNA precursor (pre-miRNA), of about 65 nucleotides in length (22). The pre-miRNA is then exported to the cytoplasm through an Exportin-5 (XPO5)-mediated system (22). There, a cytoplasmic RNAse III protein, called Dicer, generates the mature miRNA duplex, that is subsequently loaded onto an AGO protein (most commonly AGO2), to form the effector complex called RNA-induced silencing complex (RISC) (22, 23). miRNA-loaded RISC specifically recognizes short sequences of 6-7 nt, located in the 3' untranslated region (UTR) of target mRNAs, that are complementary to the so called "seed region" located in the 5' end of the miRNA molecule (18).

miRNAs are implicated in most biological processes, being able to regulate post-transcriptionally the expression of hundreds of transcripts in the same cells. According to computational analysis based on evolutionarily conservation of miRNA-mRNA target pairing, more than 60% of human coding transcripts are predicted to be regulated by one or more miRNAs (24). The biological outcome of the miRNA-mRNA interaction is strongly affected by several factors, including the binding strength of such interaction (e.g., perfect or imperfect complementarity between the miRNA seed region and the relative target site), sequence features (e.g., site accessibility, RNA secondary structure) (25–28) and the relative abundance in the cell of distinct RISC cofactors (e.g., deadenylase complexes, dsRNA binding proteins), which is itself influenced by the cell identity and its activation conditions (29–31). It was primarily acknowledged that miRNAs act as repressors of gene expression by multiple mechanisms (**Figure 1**). These include their ability to impair mRNA stability by recruiting factors and enzymes involved in mRNA cleavage and degradation (i.e., endo/exo-nucleases, decapping enzymes, deadenylase), to interfere with protein translation by blocking the initiation or elongation steps, to sequester and segregate the target mRNA into processing bodies (P-bodies), and finally to function as RNA decoy by competing with RNA-binding proteins for their binding to a specific mRNA target (18, 32–34) (**Figure 1**). Independently by the specific mechanisms adopted, increasing evidence suggests that miRNAs may act as a buffer system against internal and external cell perturbations and confer robustness to biological processes by reinforcing transcriptional programs and attenuating the effects of aberrant transcription (35). To further add to the complexity of miRNA-mediated regulation, it has been showed that, in specific cell contexts (e.g., proliferation or cell cycle arrest) miRNAs are also capable of activating gene expression, directly or indirectly (36). The post-transcriptional regulatory functions of miRNAs is key to allow cells to rapidly respond to different cellular cues, thus representing an important component of cellular networks defining the cell state (35). Emerging evidence also indicates biological activities of miRNAs unrelated to their role of intracellular regulators of mRNA transduction. Exosome-derived miRNAs released by immune cells have been demonstrated to operate as extracellular soluble mediators with regulatory effects on adjacent and remote cells or tissues through endocrine or paracrine signaling (37, 38). Few studies also reported the ability of miRNAs to directly bind proteins in a sequence-specific manner. As examples, some miRNAs released by cancer cells have been shown to bind to TLR7 and TLR8, inducing a pro-metastatic inflammatory response (39), while miR-328 directly binds to the poly(rC)-binding protein hnRNP E2, which normally interacts with the 5′-UTR of CCAAT/enhancer binding protein alpha (C/EBPα) mRNA, causing the release of C/EBPα from hnRNP E2-mediated translational inhibition and the consequent increased expression of C/EBPα expression (40). These findings strongly point out the multi-faceted role of miRNAs in regulating important cellular processes. In the following sections, we will focus on the effect of such regulation on macrophage-mediated immune responses.

MACROPHAGES POLARIZATION AND miRNAs

Macrophages possess a broad array of cell surface receptors, intracellular mediators, and essential secretory molecules for

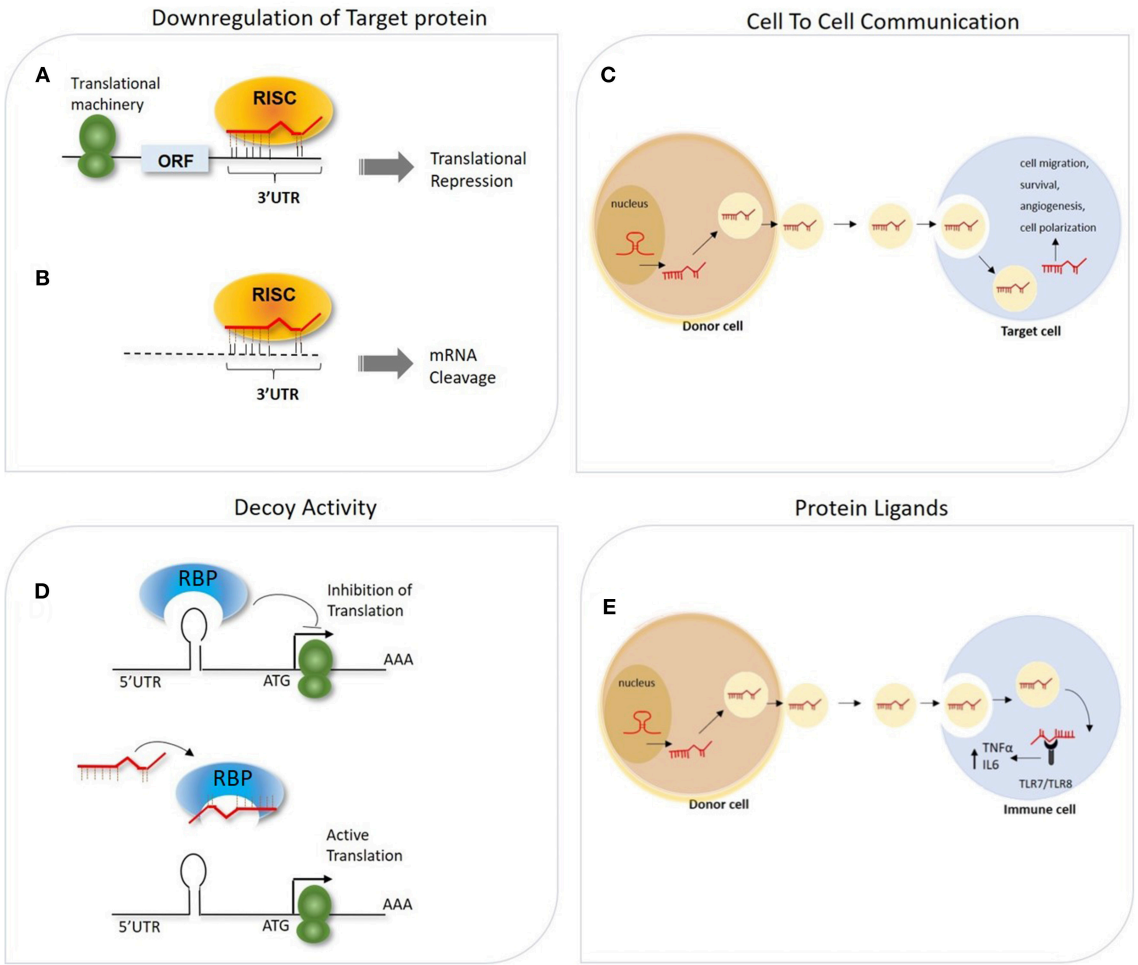

FIGURE 1 | General overview of different mechanisms of miRNA-mediated gene regulation. **(A,B)** The association of miRNA-RISC complex to the 3′ UTR of mRNA target can lead to miRNA-dependent downregulation of the targeted gene through two potential mechanisms, depending on the degree of complementarity between the seed region and the 3′UTR: **(A)** Translation initiation inhibition **(B)** mRNA target degradation. **(C)** Secreted miRNAs within exosomes can act as intercellular messenger released from donor cells to regulate gene expression in the recipient cells. **(D)** miRNAs can interfere with the activity of RNA-binding proteins (RBPs) by pairing with the RBP itself and impeding the mRNA-RBP interaction. **(E)** miRNA can also be transported via exosomes from donor cells to recipient cells, in which these miRNAs function as TLR ligands.

recognition, engulfment, and destruction of invading pathogens and also regulation of other type of immune cells and serve as sentinels of the immune system, sensing the presence of pathogens by means a variety of membrane anchored and cytosolic detectors. Macrophages participate in the inflammatory process by adapting their functional phenotype according to microenvironmental cues (41–43), and plasticity confers them the ability to coordinate host defense mechanisms to eliminate the pathogen and re-establish homeostasis in the host tissues (41, 44, 45). To efficiently induce protection from invading pathogens, macrophages mount an effective and balanced inflammatory response that mainly comprises four orderly stages: (a) recognition of foreign pathogens by pattern-recognition receptors (PRRs); (b) eradication of invading agents; c) resolution of inflammation through the involvement of suppressing cells and the release of anti-inflammatory mediators; e) tissue repair and restoration of tissue homeostasis. Macrophage remarkable heterogeneity results in the acquisition of an array of phenotypes and functional properties in response to different microenvironmental factors, and manifests as a spectrum of different functional states, oversimplified by the canonical dual distinction between classically- (M1) and alternatively-activated macrophages (M2) (5, 46–48). Classically activated macrophages respond to intracellular bacterial products (e.g., lipopolysaccharide, LPS) and several pro-inflammatory cytokines (including interferon gamma [IFNγ] and tumor necrosis alpha [TNF]) (8, 49). By contrast, the induction of alternative activated macrophages is favored by anti-inflammatory cytokines (IL-10, IL-4/IL-13), TGFβ, and glucocorticoids (GCs) (50) (**Figure 2**). M2-polarized macrophages are typically associated with immune response to parasites, wound healing and promotion of angiogenesis. They express tissue-remodeling

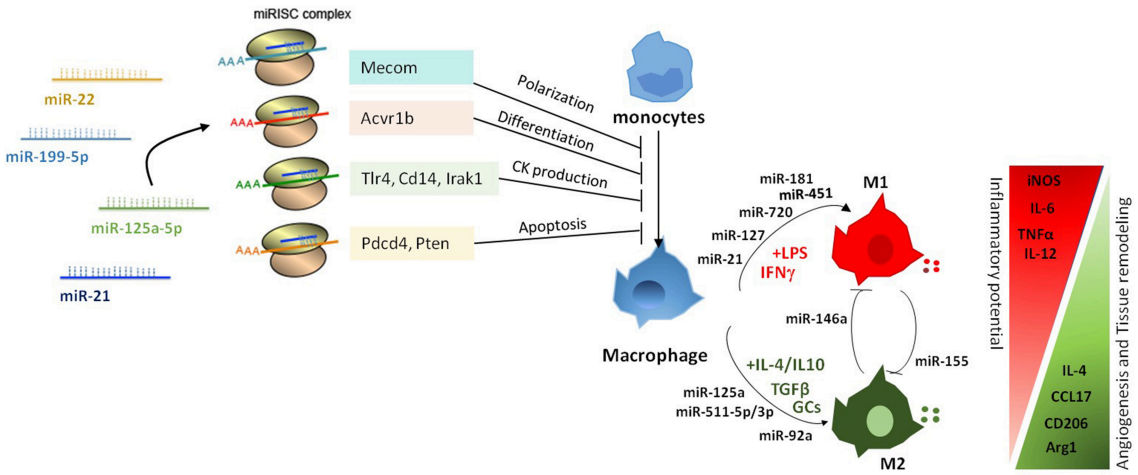

FIGURE 2 | Pleiotropic role of miRNAs in the regulation of macrophage activation and polarization. miRNAs affect macrophage activation and differentiation by exerting a multiple regulation of sets of genes involved in different biological processes. Differential expression of miRNAs in macrophages also modulates macrophage polarization from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype.

and pro-angiogenic factors and inhibit the secretion of pro-inflammatory cytokines (51–53).

Genome-wide studies profiling transcriptional and epigenetic modifications reveal profound dynamic changes at gene loci associated with macrophage polarization, resulting in the coordinated action of distinct signaling pathways and transcription factors (8, 43, 54, 55). Several studies identified specific subsets of miRNAs differentially expressed under distinct polarizing conditions and investigated the impact of miRNA deregulation in macrophage polarization. A study by Zhang et al. (56) identified 109 miRNAs differentially expressed in human and murine M1- and M2-polarized macrophages, focusing in particular on miR-155, miR-181, and miR-451 in M1 macrophages, and miR-146a, miR-125a, and miR-145-5p in M2 macrophages (**Figure 2**). Subsequent studies confirmed the higher expression of miR-155 in M1-polarized macrophages and of miR-146a, miR-125b, and miR-127 in M2-polarizing conditions (9), and investigated their relationship with target genes involved in macrophage activation. In particular, miR-155 has been extensively studied in the context of macrophage polarization and inflammation. Its expression levels largely increased when macrophages were polarized to the M1 phenotype; whereas in M2-polarized macrophages miR-155 levels were strongly decreased (57). Interestingly, miR-155 was not just an M1 phenotypic marker, but actually had a role in driving macrophage polarization as its inhibition resulted in impaired M1 polarization and its overexpression induced a re-polarization toward an M1 phenotype of M2-macrophages (57). Later on, studies in miR-155 knockout (KO) mice demonstrated that miR-155 drives the inflammatory phenotype of M1-macrophages by regulating the expression of approximately 650 genes (58). Although the mechanism by which miR-155 directs macrophage M1 polarization has not been completely elucidated, evidence indicates it directly targets the IL-13 receptor α1 (IL13Rα1) thus interfering with STAT6 activation and indirectly regulates the expression of other M2-related genes, including CD23, DC-SIGN, CCL18, and SERPINE (59, 60). M1 polarization is also supported by miR-127 and miR-125b, which have been shown to target Bcl6 and IRF4, respectively, with consequent increased expression of pro-inflammatory cytokines (61, 62). In particular, inhibition of Bcl6 by miR-127 led to reduced expression of the phosphatase Dusp1 and increased phosphorylation of JNK, and its knockdown resulted in reduced expression of M1 signature genes and promoted the transcription of M2-related genes (61). Finally, overexpression of miR-720, a miRNA downregulated by M2 stimulation, decreased the expression levels of GATA3 a transcription factor important in M2 macrophage polarization, thus resulting in the inhibition of M2 polarization. Consistently, ectopic expression of GATA3 restored the M2 phenotype in miR-720 overexpressing macrophages and enforced expression of miR-720 inhibited pro-migration behavior and phagocytic ability of M2-polarized macrophages (63).

The first miRNA associated with M2 polarization was miR-146a. Enforced expression of miR-146a in peritoneal macrophages resulted in reduced levels of M1-marker genes (e.g., iNOs, CD86, TNF, IL-12 and IL-6), and increased production of M2-phenotype markers (e.g., Arg1, CCL17, CCL22 and CD206). In contrast, miR-146a knockdown promoted M1 macrophage polarization and diminished M2 macrophage polarization (64). Mechanistically, it was demonstrated that miR-146a modulated macrophage polarization at least in part by targeting Notch1, PPARγ, and inhibin βA subunit of activin A (INHBA) (65). Other miRNAs highly expressed in M2 macrophages are miR-511-3p, miR-223, and let-7c (9, 66), all of which have been shown to promote M2 polarization. miR-511-3p, which was found highly expressed also in tumor-associated macrophages (67), targets Rho-associated coiled-coil containing protein kinase 2 (Rock2),

a serine-threonine kinase that phosphorylates IRF4 (67), thus supporting expression of M2-related genes. miR-223 targets the transcription factor Pknox1 and its overexpression led to the inhibition of LPS-dependent release of IL-1β and IL-6 (68), thus enhancing the alternative anti-inflammatory responses and limiting the pro-inflammatory activity of M1 macrophages (69). Similarly, enforced expression of let-7c reduced the expression of M1-related genes (i.e., iNOs and IL-12) and increased levels of M2 markers (*i.e.,* FR-β), *via* targeting of P21 activated kinase 1 (PAK1) (70) and C/EBP-δ (71). Also relevant for macrophage polarization is the miR-23a/27a/24-2 cluster, which is downregulated by M1-type stimuli and upregulated by M2-type stimuli. Interestingly, enforced expression of either miR-23a or miR-27a promoted the expression of pro-inflammatory cytokines and the concomitant inhibition of M2-type cytokines by acting on different signaling pathways. MiR-23a activated the nuclear factor-kappa B (NF-κB) pathway by targeting TNF-inducing protein 3 (TNFAIP3), and by targeting JAK1 and STAT6 directly suppressed the activity of this signaling pathway and reduced the production of M2 type cytokines, while miR-27a showed the same phenotype by targeting interferon regulatory factor 4 (IRF4) and peroxisome proliferator-activated receptor gamma (PPARγ) (72). Altogether, the examples illustrated above show complex regulatory networks between miRNAs and transcription factors driving macrophage polarization, strongly candidating miRNAs as prominent regulatory elements in macrophage biology.

miRNA AS MOLECULAR DETERMINANTS IN MACROPHAGE-MEDIATED INFLAMMATORY RESPONSE

As discussed above, plasticity is a peculiar trait of macrophages that render them critical innate immune cells with versatile functions (73–75). Excessive and inadequate macrophage activation may lead to inefficient elimination of invading pathogens and contributes to self-tissue damage in inflammatory and autoimmune disorders (74, 76–78). Therefore, fine regulation of macrophage activation is needed during inflammatory and infectious conditions. Macrophage response to infectious agents is elicited by a pro-inflammatory environment, which further promotes macrophage microbicidal activity by inducing the transcriptional activity of genes belonging to the M1 program (79, 80). Therefore, M1 macrophage polarization is usually associated with protection during acute infectious diseases. It is now apparent that the regulation of miRNA expression in response to bacterial pathogens is a crucial part of the host mechanism against infections. miRNA-induced expression can increase or inhibit macrophages activity against infection (81). miRNAs have been recently recognized as important modulators of multiple signaling pathways activated or repressed along the different phases of the inflammatory response (82). Of note, it has also been reported that some bacterial pathogens reprogram macrophage polarization and induce specific M2 programs in macrophages, to evade the innate immune response (83, 84).

A number of inflammation-related miRNAs have been reported to be regulated in response to bacterial infections. Upregulation of miR-146a/b was observed in human monocytes infected by *Salmonella* (85), whereas the concomitant downregulation of let-7 family members (e.g., miR-98) was correlated with the upregulation of IL-10 (68, 86). Similarly, *Listeria monocytogenes* infection induced upregulation of miR-155, miR-146a, miR-125a-3p/5p, and miR-149 (87), and a miRNA expression profile performed in human macrophages infected with *M. avium* showed upregulation of miR-155, miR-146a/b, miR-886-5p, let-7, and miR-29a and concomitant decreased expression of miR-20a, miR-191, miR-378, miR-185 (88). More in details, let-7e and miR-29a downmodulated, respectively, caspases 3 and 7, thus regulating the apoptosis process after mycobacteria infection (88). miR-15a/16 have been reported as upregulated in bone marrow-derived macrophages in sepsis (89). Interestingly, it was demonstrated that miR-15a/16 support macrophage antibacterial activities as their deletion resulted in increased phagocytosis and mitochondrial reactive oxygen species production (89). A prominent role in controlling macrophage activation in inflammatory conditions is exerted by miR-155, which is widely expressed in immune cells and has a pleiotropic role in regulating both innate and adaptive immunity. In macrophages, miR-155 expression is induced by TLR agonists and pro-inflammatory cytokines through an AP-1 and NF-κB-mediated mechanism (81, 90, 91). Consistent with its pattern of expression, miR-155 acts as an early regulator of the inflammatory response, being able to inhibit the expression of negative regulators of the TLR signaling, including suppressor of cytokine signaling-1 (SOCS1) and Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) (92–94). miR-155 also indirectly enhances TNFα production by increasing TNFα mRNA half-life and translation (81). In primary macrophages infected by *Francisella* spp., miR-155 directly repressed the expression of SHIP1 and consequently enhanced the release of the pro-inflammatory response (95). As for other pro-inflammatory genes, miR-155 expression is then repressed by IL-10, a prominent anti-inflammatory cytokine induced at late time points by LPS stimulation, which operates as a negative regulator of the acute inflammatory phase. IL-10 inhibits miR-155 transcription in a STAT3-depedent manner and this inhibitory effect of IL-10 leads to an increase in the expression of SHIP1 (96).

Insights on the importance of miRNA-mediated regulation of macrophage response to bacterial infection were provided in particular by extensive studies performed on *Mycobacterium tubercolosis* (TB) infection. TB infection has dramatic effects on gene expression in host cells and this is associated with significant changes in a distinct panel of miRNAs (4 upregulated miRNAs: miR-24, miR-142, miR-155, miR212; 3 downregulated miRNAs: miR-19a, miR-202, miR-376a) (97, 98). Another study revealed that TB also induced the expression of miR-125b, which directly downregulated TNFα expression, thus resulting in the increase of TB pathogenicity (81). Similarly, upregulation of miR-32-5p expression observed in THP-1 monocytic cells after TB infection resulted in the reduction

of inflammatory cytokine levels and a concomitant increase in the survival rate of intracellular mycobacteria. Conversely, the inhibition of miR-32-5p suppressed the intracellular growth of TB and promoted the expression of IL-1β, IL-6, and TNF (99). Furthermore, miR-23a-5p induction after TB infection also suppressed autophagy in infected macrophages through a mechanism that implicated the downregulation of TLR2 (100). Finally, TB downregulates let-7f with consequent upregulation of is target A20, a feedback inhibitor of NF-kB pathway (101). Indeed, let-7f overexpression increases the production of cytokines such us TNFα and IL-1β and diminishes TB survival (101).

REGULATORY FUNCTIONS OF miRNAs IN THE TLR SIGNALING PATHWAY

Macrophage activation requires recognition of various microbial components by means of specific families of pattern recognition receptors (PRRs), including Toll-like receptors (TLR) (102, 103). Several miRNAs have been shown to be upregulated in response to TLR ligands, and many of them directly target components of the TLR signaling system, revealing the involvement of miRNAs in feedback regulatory mechanisms. Over 10 years ago, a pioneering study documented the upregulated expression of miR-146a, miR-155, and miR-132 in the human monocytic cell line THP-1 upon treatment with pro-inflammatory stimuli, including the TLR agonists LPS and palmitoyl-3-cysteine-serine-lysine-4 (104), and later on with the identification as major miR-146a targets of TRAF-6 and IRAK1, key adaptor proteins of the TLR signaling cascade required for NF-κB activation, the first regulatory loop was revealed (105, 106). Since then, several TLR-responsive miRNAs have been identified, including miRNAs induced at early time points (e.g., miR-146a, miR-155, miR-9, miR-21, miR-147b, miR-181b) (90, 104, 107) as well as miRNAs regulated at later time-points by late-induced anti-inflammatory mediators (e.g., miR-146b, miR-187, the miR-125a∼99b∼let-7e cluster) (10–12). A subset of miRNAs downregulated by TLR stimuli have also been described (e.g., miR-92a, miR-29b, let-7i, miR-107, miR-27a*, miR-532-5p, and miR-322) (108–113).

An essential mediator of the inflammatory signaling pathway is the NF-κB family of transcription factors, which coordinates the expression of an array of genes involved in the inflammatory response (e.g., TNFα, IL-1β, IL-6, IL-12p40, cyclooxygenase-2). Notably, several of the miRNAs regulated by TLR agonists were directly controlled by the NF-κB-dependent pathway (e.g., miR-146a, miR-155, miR-9), and in most cases those miRNAs operated a feedback control of the NF-κB-dependent response by fine tuning the expression of key members of this pathway. For instance, miR-9 is early induced by TLR agonists *via* the MyD88-NF-κB-dependent pathway in human monocytes and neutrophils and directly targets the p50 precursor NF-κB1 (107). More recently, miR-322 was also reported as a negative regulator of NF-κB1 expression (110, 113). Finally, miR-147 expression is induced by TLR4 *via* NF-κB, which physically binds to miR-147 promoter region, and the induction of miR-147 in turn represses the release of pro-inflammatory cytokines (114).

PERVASIVE REGULATION OF TLR SIGNALING PATHWAY BY IL-10-DEPENDENT miRNAs

TLR triggering sets in motion a regulatory network of genes that tightly control the immune response by balancing pro- and anti-inflammatory signals. Negative regulation of TLR signaling by miRNAs represents an important step in setting this balance. This can be achieved by inhibiting the expression of genes required for LPS response, as discussed above, but also by inducing the expression of anti-inflammatory molecules, as in the case of miR-21, which indirectly increases IL-10 expression as a consequence of its targeted repression of PDCD4 (115).

A small subset of miRNAs late expressed during the course of the inflammatory response was shown to be induced by IL-10 and, more strikingly, to mediate the IL-10-driven anti-inflammatory response. The first IL-10-dependent miRNA identified was miR-187, whose ectopic expression selectively reduced the production of TNFα, IL-6, and IL-12p40 by LPS-activated monocytes (11). This effect was at least in part mediated by direct targeting of NFKBZ, a master regulator of IL-6 transcription (11). Later on, miR-146b and the miR-125a∼99b∼let-7e cluster were also demonstrated to be late-induced after LPS challenge by an IL-10-dependent regulatory loop mediated by STAT3, the main transcription factor downstream IL-10. Whereas, miR-146a is early induced by LPS in both human and murine macrophages, miR-146b is selectively induced upon stimulation with the anti-inflammatory cytokine IL-10 *via* an NF-κB-independent pathway. Dependency of miR-146b expression on endogenous IL-10 was formally demonstrated in IL-10 KO mice, which were compromised in miR-146b but not miR-146b upon LPS triggering (12). miR-146b then exerts an anti-inflammatory activity by direct targeting the LPS receptor TLR4 and key adaptors/signaling molecules, including MyD88, TRAF6, and IRAK1 (12) (**Figure 3**). These findings also represent an example of two miRNA isoforms induced by different factors at different moments in the same cell type, suggesting that miR-146a/b might operate as a relay system to buffer the expression of pro-inflammatory genes induced by TLR4 triggering. The miR-125a∼99b∼let-7e cluster is also late induced by TLR agonists *via* the IL-10 dependent regulatory loop, and is counter-regulated by IFNγ (10), which promotes macrophage classic pro-inflammatory activation and chronic inflammation (116). miR-125a-5p and let-7e, but not miR-99b, enter the RISC complex in human primary monocytes and operate a pervasive negative regulation on the TLR signaling pathway by downregulating multiple components of the TLR signaling pathway, including receptors (e.g., TLR4, CD14) and signal transducers (e.g., IRAK1), with the resulting effect of a global suppression of downstream inflammatory cytokine production (10).

ROLE OF miRNA IN INNATE IMMUNE MEMORY

Innate immune cells retain a memory of past stimulations and actions, allowing them to enhance (trained immunity) or repress

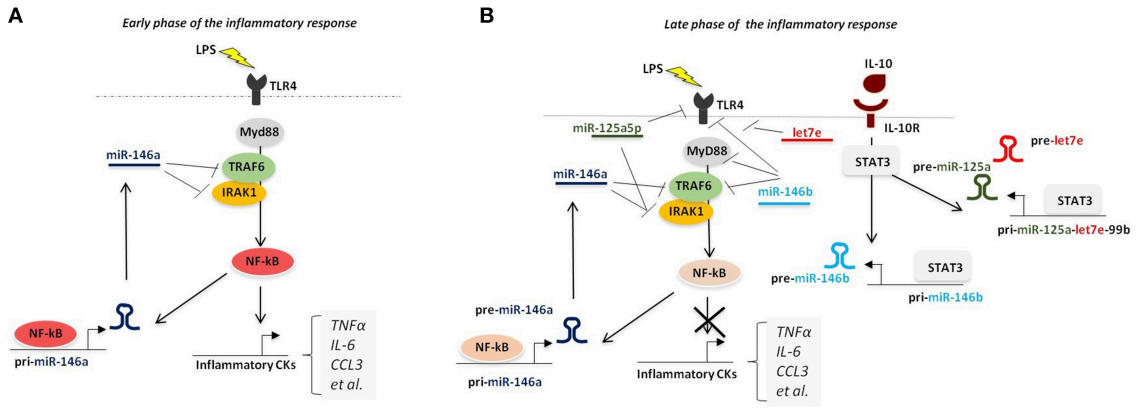

FIGURE 3 | Pervasive regulation of TLR signaling pathway by miR-146a/b family and miR-125a∼99b∼let-7e cluster. **(A)** During overt inflammation binding of LPS to TLR4 induces the expression of NF-κB pathway, that ultimately leads to the release of pro-inflammatory cytokines (including TNFα, IL-6, CCL3, and IL-12). NF-κB also positively regulates the expression of miR-146a, which operates a negative feedback control of the NF-κB-dependent inflammatory response by repressing TRAF-6 and IRAK1. **(B)** During the late phase of the inflammatory response, production of IL-10 induces the expression of miR-146b and miR-125a∼99b∼let-7e cluster, *via* STAT3 binding to their promoters, thus resulting into downregulation of multiple components of TLR signaling pathway.

(endotoxin tolerance; ET) the immune response when facing the same or different infectious agent (117, 118). At the base of both forms of innate memory is the establishment of a functional cell reprogramming resulting in the acquisition of new cellular properties, maintained long after the termination of the initial stimulus (119). The limited understanding of the molecular mechanisms behind this phenomenon points to an involvement of chromatin modifications and inducible regulatory molecules, such as miRNAs, which shape the different transcriptional programs and outcomes that characterize ET (120) and trained immunity (121).

During ET, monocytes and macrophages display reduced response to subsequent challenges after they have been exposed to low concentrations of endotoxin (111, 112). ET is a dynamic process that relies on the action of several negative regulatory loops resulting in repression of pro-inflammatory cytokines and chemokines (e.g., TNFα, IL-6, IL-12, CCL3, CXCL8, CCL2), that are transcriptionally silenced (that is, tolerized) upon LPS-re-exposure and concomitant upregulation of anti-inflammatory cytokines (e.g., IL-10, TGFβ, scavenging receptors (e.g., MARCO; CD64 and CLEC4a) and a variety of anti-microbial genes (e.g., RNASET2, FPR1) (112). We highlighted the impact of miRNAs in regulating TLR signaling pathways. Not far from the demonstration of miRNA-mediated regulation of the inflammatory response, further evidence suggested that specific miRNAs indeed play a role in the development of LPS tolerance (122). miR-146a was the first miRNA described as upregulated in tolerant THP-1 monocytic cells able to partially induce LPS desensitization (123). This effect has been related to its ability to downregulate the NF-κB pathway by acting on TRAF6 and IRAK1, and is consistent with evidence demonstrating high levels of miR-146a and impaired NF-κB activity in endotoxin-tolerant murine macrophages and human monocytes (124–126). Similar evidence has also been reported for miR-181b, which contributed to the downregulation of IL-6 after LPS exposure (127), and miR-155 (81, 90, 127). miR-155 expression is regulated by the phosphoinositide-3 kinase (PI-3K)-AKT kinase signaling pathway, which has a well-documented role in controlling macrophage sensitivity and ET (94, 128–130). Androulidaki et al. (94) demonstrated that the state of LPS tolerance was at least in part dependent on the regulation of let-7e and miR-155 expression by AKT1, which induced the former and suppressed the latter (94). As discussed, these miRNAs have opposite roles in controlling the inflammatory reaction, with let-7e inhibiting the inflammatory response by direct targeting of TLR4 (10, 94) and miR-155 promoting macrophage sensitivity to LPS response by SOCS1 downregulation (94). Of note, miR-155 is one of the few miRNAs induced not only by LPS, but also by the TLR3 agonist double stranded RNA and by IFNβ through TNF autocrine signaling (81, 90), and miR-155 knock-in mice are highly susceptible to LPS shock due to high levels of TNF (81, 90, 131).

Some miRNAs are differentially expressed during ET, but are not expressed or are late induced in LPS-primed macrophages. Among them miR-146b, (132), miR-125a∼99b∼let-7e cluster (10), miR-222 (133) and miR-511-5p (13). A prominent role in ET elicited by different anti-inflammatory stimuli (e.g., IL-10, GCs, TGFβ) was demonstrated for miR-146b (132), miR-125a∼99b∼let-7e cluster (10), and miR-511-5p (13). MiR-146b is late induced by LPS and higher expressed in monocytes tolerized by IL-10 and TGFβ. Regulation of miR-146b expression modulated ET in monocytes (132). Similarly, miR-125a∼99b∼let-7e cluster was found expressed at high levels in LPS tolerant monocytes. Of note, ET rescue by IFNγ, a cytokine known to suppress expression of miR-125a∼99b∼let-7e cluster, was impaired in cells overexpressing miR-125a∼99b∼let-7e cluster (10), indicating that IFNγ ability to prevent induction of LPS tolerance is at least in part mediated by the inhibition of this miRNA cluster (10). Similarly, miR-222 expression increased late during LPS response and is counteracted by IFNγ. In a

recent paper published in 2018 (133). Inhibition of miR-222 was also shown to reduce the duration and magnitude of ET, by restoring the expression of tolerized genes, such as IL-6 and IL-12p40 at levels comparable to those observed in non tolerized cells. Differently from other miRNAs involved in ET regulation, miR-222 did not modulate expression of ET genes by regulating TLR4 signaling. Instead, it targeted BRG1, a catalytic subunit of the chromatin remodeling complex SWI/SNF, recruited to the promoters of late LPS-response genes to induce their transcription. Thus, miR-222 represents a paradigmatic example of the existence of a crosstalk between miRNAs and chromatin modifications, both important components of the mechanistic framework that is at the base of short-term memory in ET.

Whereas, the role of miRNA in ET is well established, less defined is their relative contribution in the other form of innate immune memory, that is trained immunity. A distinguishing feature of trained innate immune cell is its ability to mount a qualitatively different- and to some extent quantitatively stronger- transcriptional response compared to untrained cells when rechallenged with infectious or danger signal (134). miRNAs known as activators of the inflammatory response, such as miR-155, might also contribute to trained immunity, possibly through the repression of phosphatases or other negative regulators. Further studies are needed to define which miRNAs induced during trained immunity play an active role in initiating and sustaining the hyper- sensitive state of trained cells.

ROLE OF miRNA IN MACROPHAGE ANTI-INFLAMMATORY AND TISSUE HEALING ACTIVITIES

The functional and phenotypic plasticity of macrophages become particularly apparent during the resolution of inflammation, which is initiated with the clearance of apoptotic neutrophils (efferocytosis) by tissue resident macrophages and their switch from a pro- to an anti-inflammatory phenotype (135–137). In this biological setting, miRNAs have been described as part of negative regulatory loops that keep inflammation in check by promoting production of anti-inflammatory mediators, tissue healing, and return to homeostasis (138, 139). In an *in vivo* murine model of peritonitis, treatment with resolving D1 (RvD1), an endogenous lipid mediator generated during the resolution phase of acute inflammation, regulated resolution indices and controlled specific miRNA expression in exudates. (140). In particular, RvD1 upregulated miR-21, miR-146b, and miR-219, and downregulated miR-208a (140), and miR-21 was candidate as a novel component of a RvD1-miRNA circuit (140). Two other studies further confirmed the key role for miR-21 in controlling inflammation and promoting the switch from a pro-inflammatory to a pro-resolving phenotype of macrophages in relation to different anti-inflammatory stimuli. It was demonstrated that miR-21 downregulates the translation of the pro-inflammatory tumor suppressor programmed cell death 4 (PDCD4), an inhibitor of IL-10 (141), and this miR-21/PDCD4/IL-10 circuit was shown to play an important role in efferocytosis (142). Interestingly, in addition to PDCD4, miR-21 also promoted downregulation of PTEN and GSK3, with consequent inhibition of NF-kB and AP-1 activity and TNF production, thus bolstering the anti-inflammatory response (139, 141–143). In a model of sepsis, increase circulating levels of miR-466l were detected (144). This miRNA is early expressed in polymorphonuclear neutrophils, where it promotes inflammation, and then is induced at later time points in macrophages engaged by apoptotic neutrophils, and in macrophages it contributes to resolution by promoting efferocytosis (144). Interestingly, the presence of miR-466l was also confirmed in septic patients. Further studies are required to fully disclose the role of miRNAs in the resolution of inflammation and to evaluate their potential for the development of novel therapeutic approaches for inflammatory diseases.

ROLE OF miRNA IN TUMOR- ASSOCIATED MACROPHAGE BIOLOGY

Tumor infiltrating myeloid cells are educated by the tumor milieu and exert a number of pro-tumoral functions, ranging from promotion of tumor growth, to angiogenesis and immunosuppression (145–147). The two major myeloid cells associated with cancer-related inflammation are tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (145). A detailed summary of miRNA-mediated regulation of myeloid-derived suppressor cells in the context of cancer-related inflammation has been recently reviewed elsewhere (20, 147). Here we focus on the participation of miRNAs in regulatory networks controlling the function of TAM, which are a double edge sword as they usually exert pro-tumoral functions but are potentially endowed with anti-tumoral activities. This crucial balance tightly depends on the macrophage activation status. As miRNAs modulate macrophage polarization in inflammatory conditions, it is not surprising that they also have been also implicated as intracellular determinants of the biology of tumor-educated macrophages (148, 149).

It has been reported that miRNAs are differentially expressed in myeloid cells during metastatic tumor progression in mouse models of melanoma and breast cancer, two biological contexts where the link between TAM and tumor progression and the relative molecular mechanisms are well-established. Colony-Stimulating Factor-1 (CSF-1) is a growth factor which modulates macrophage survival and functions during inflammation through the regulation of the transcription factor ETS2 (150–152). Genetic deletion of CSF-1 resulted in reduction of mammary TAMs and in lower incidence of lung metastasis in *in vivo* mammary tumor mouse models (153), deletion of ETS2 in macrophages resulted in reduced metastatic tumor burden (153) and its elevated expression has been correlated with human breast cancer (154). In TAM the CFS-1/ETS-2 pathway was associated with an oncogenic miRNA expression signature, including miR-223, miR-21, miR-29a, and miR-142-3p. These oncogenic miRNAs likely promote macrophage pro-tumoral functions, including tumor cell proliferation and

angiogenesis, as miR-21 and miR-29a target genes involved in M1-polarization and anti-angiogenic regulators (155). Further evidence suggesting that endogenous miRNAs may exert important roles in controlling the polarization and function of TAM was obtained in a transplanted breast cancer mouse model, where miR-146a and miR-222 were significantly downregulated, and this was associated with the upregulation of the NF-κB p50 subunit (156). Interestingly, inhibition of miR-146a resulted in decreased expression of M2 macrophage genes in TAM and reduced tumor growth *in vivo*, while overexpression of miR-222 reduced macrophage recruitment by targeting CXCL12 and CXCR4 (156). Similarly, in agreement with the aforementioned role of mir-155 in negative regulation of M2 polarization, miR-155 knockdown in myeloid cells accelerated spontaneous breast cancer development by impairing macrophage classical activation, with consequent imbalance toward a pro-tumoral microenvironment which favored the skew of tumor-associated immune cell toward an M2/Th2 response (157). miR-511-3p is another M2-associated miRNA, that was found to be also highly active in TAM, where it triggers a negative feedback response that inhibits tumor growth and attenuates TAM pro-tumoral genetic programs (67). Finally, exosome-secreted miRNAs have been described as an alternative mechanism adopted by TAM to modulate breast cancer invasion and metastasis, as miR-223 was detected within macrophage exosomes and was shown to promote invasiveness of breast cancer cells *via* the targeting of MEF2C-β-CATENIN pathway (158).

CONCLUSIONS

In the last decade, several efforts have been made to shed light into the molecular mechanisms driving the inflammatory response, in all its facets. miRNAs have been demonstrated to be pivotal players actively participating in the modulation of the early phase as well as the resolution of inflammation. Recent findings on their involvement in chronic inflammatory conditions, sepsis, and tumors strongly encourage the development of new miRNA-based therapeutic strategies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Antimicrobial Peptide LL-37 Facilitates Intracellular Uptake of RNA Aptamer Apt 21-2 Without Inducing an Inflammatory or Interferon Response

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RNA aptamers are synthetic single stranded RNA oligonucleotides that function analogously to antibodies. Recently, they have shown promise for use in treating inflammatory skin disease as, unlike antibody-based biologics, they are able to enter the skin following topical administration. However, it is important to understand the inflammatory milieu into which aptamers are delivered, as numerous immune-modulating mediators will be present at abnormal levels. LL-37 is an important immune-modifying protein upregulated in several inflammatory skin conditions, including psoriasis, rosacea and eczema. This inflammatory antimicrobial peptide is known to complex nucleic acids and induce both inflammatory and interferon responses from keratinocytes. Given the attractive notion of using RNA aptamers in topical medication and the prevalence of LL-37 in these inflammatory skin conditions, we examined the effect of LL-37 on the efficacy and safety of the anti-IL-17A RNA aptamer, Apt 21-2. LL-37 was demonstrated to complex with the RNA aptamer by electrophoretic mobility shift and filter binding assays. In contrast to free Apt 21-2, LL-37-complexed Apt 21-2 was observed to efficiently enter both keratinocytes and fibroblasts by confocal microscopy. Despite internalization of LL-37-complexed aptamers, measurement of inflammatory mediators and interferon stimulated genes showed LL-37-complexed Apt 21-2 remained immunologically inert in keratinocytes, fibroblasts, and peripheral blood mononuclear cells including infiltrating dendritic cells and monocytes. The findings of this study suggest RNA aptamers delivered into an inflammatory milieu rich in LL-37 may become complexed and subsequently internalized by surrounding cells in the skin. Whilst the results of this study indicate delivery of RNA aptamers into tissue rich in LL-37 should not cause an unwarranted inflammatory of interferon response, these results have significant implications for the efficacy of aptamers with regards to extracellular vs. intracellular targets that should be taken into consideration when developing treatment strategies utilizing RNA aptamers in inflamed tissue.

Keywords: LL-37, RNA aptamer, skin, inflammation, interferon response, safety, complexes

INTRODUCTION

RNA aptamers are synthetic single stranded RNA oligonucleotides that bind targets with high specificity and affinity. Whilst they function like antibodies, there are several advantages presented by these molecules over their protein-based counterparts, boasting improved thermostability, reduced immunogenicity, and cheaper, more tractable production by chemical synthesis (1, 2). RNA aptamers are becoming an increasingly attractive immune-modulating tool for the treatment of disease. In particular, they have great potential for use in topical treatment of inflammatory skin conditions as they are small in size and therefore may effectively penetrate the skin, allowing direct treatment of diseased tissue without delivering a systemic dose of antibody-based biologics. This has been illustrated recently by delivering an anti-IL-23 RNA aptamer into epidermal compartments of porcine and *ex vivo* skin (3). However, when treating diseased tissue, it becomes important to consider the altered inflammatory milieu into which the drug is delivered.

In pathologically inflamed tissue, the upregulation of immune-modifying cytokines and proteins may impact on the efficacy of delivered RNA aptamers. One such protein is the anti-microbial peptide cathelicidin (LL-37) (4–8). LL-37 is derived from the precursor hCAP18, which is proteolysed to generate a biologically active C-terminal peptide of 37 amino acids, of which the first two are leucines (9). LL-37 is produced in the skin primarily by keratinocytes in response to invading micro-organisms and, once proteolytically activated, functions as a microbicidal peptide. This cationic peptide (with an α-helical structure) can bind the membranes of bacteria and enveloped viruses, polymerise on membrane surfaces and cause membrane disruption, killing invading organisms (10). In recent years, it has become evident LL-37 possesses numerous functions aside from its anti-microbial activity; many of which are immunomodulatory. Interestingly with regards to RNA aptamers, LL-37 has a high affinity for single and double stranded nucleic acids and is capable of enhancing inflammation through promoting toll-like receptor (TLR) activation (11–13). Furthermore, LL-37 has been shown to shuttle complexed nucleic acids across cell membranes (12, 14), primarily via receptor-mediated endocytosis. However, in keratinocytes, uptake seems to occur by mechanisms that do not require activation of specific receptors (15, 16), promoting inflammatory and interferon responses via both TLR and cytosolic nucleic acid sensors such as the cGAS-STING and RIG-I Like Receptor (RLR) pathways (17, 18).

LL-37 is found over-expressed in many of the most common inflammatory skin conditions, including psoriasis, rosacea, and eczema (5, 6, 19). These conditions together account for a significant percentage of patients treated for skin-related illnesses, with ∼3%, 10–20%, and 10% of the population suffering from psoriasis, rosacea and eczema, respectively. Recently, the advent of biologic medicine has facilitated highly effective treatment strategies for these conditions, yet the expense and difficulties found in effective delivery limits biologic treatment to the most severe of cases. Topically applied aptamer-based treatments provide a cheaper and arguably more effective alternative to protein-based biologics that would open the field of biologic medicine to a much larger percentage of patients. IL-17A is a pro-inflammatory protein that plays a central role in initiating and perpetuating inflammation in psoriasis, and has been targeted with great success using antibody-based biologic treatments (20–22). Expressed by infiltrating immune cells in the skin, IL-17 cytokines act on surrounding keratinocytes and fibroblasts to induce expression of angiogenic and inflammatory mediators crucial in the development of psoriatic lesions (23). The anti-IL-17A RNA aptamer Apt 21-2 has also been shown to effectively bind IL-17A, and we and others have previously illustrated that Apt 21-2 may be suitable for use in treating psoriatic plaques (24, 25). Given the attractive notion of using RNA aptamers in topical medication and the prevalence of LL-37 in these inflammatory skin conditions, it is of great importance to examine the effect of LL-37 on RNA aptamer efficacy and safety. This work investigates the interaction between Apt 21-2 and LL-37, and the consequent effects on aptamer uptake and immune activation.

METHODS

Reagents

RNA Aptamer Apt 21-2 (25) was synthesized to order by Dharmacon GE Healthcare as 33 nucleotides (5′ GGU CUAGCCGGAGGAGUCAGUAAUCGGUAGACC 3′) with 2′ fluoro-modified cytosine and uracil. A fluorescently tagged Apt 21-2 was also synthesized by addition of a single Cy3 molecule on the 5′ end of the aptamer (Apt 21-2 Cy3) (24). Fluorochrome-conjugated antibodies were obtained from Miltenyi Biotech (HLA-DR-FITC, CD11c-VioBlue, CD14-VioBlue, CD19-VioBlue, IFNα-APC) or BioLegend (CD303-PE-Cy7, CD123-BV711). For analysis of intracellular cytokines by flow cytometry, cytokine secretion was inhibited by GolgiPlug (BD Biosciences).

Primary Cell Isolation, Cell Culture, and Ethics

Primary keratinocytes and fibroblasts from healthy donors were purchased from PromoCell or isolated from healthy volunteers respectively and were cultured as described previously (26). The participants' samples used for this study were collected under ethical approval, REC 10/H1306/88, National Research Ethics Committee Yorkshire and Humber-Leeds East. All experiments were performed in accordance with relevant guidelines and regulations.

PBMC Isolation

Whole blood anti-coagulated in heparin was collected from healthy volunteers and PBMCs were isolated by density gradient, followed by centrifugation using Greiner Leuco-Sep tubes (Sigma Aldrich, Gillingham, UK). Isolated PBMCs were washed in MACS buffer (D-PBS, 2 mM EDTA, 0.5% BSA) followed by 1x wash in PBS. Cells were resuspended in RPMI 1640 (10% FCS, 1% penicillin/streptomycin), plated in 24 well plates, and immediately stimulated.

Flow Cytometry

PBMCs were stimulated as detailed in the results in the presence of GolgiPlug. Following stimulation, cells were washed in PBS and resuspended in blocking buffer (10% mouse serum and 1% IgG) for 15 min at 4°C. Cells were then stained for surface antigens (HLA-DR, CD11c, CD14, CD19, CD303, CD123) for 30 min at 4°C. Cells were washed and fixed and permeabilized using IntraPrep kit (Beckman Coulter) according to the manufacturer's instructions. Cells were then washed and stained for intracellular IFNα for 30 min at 4°C. Finally, cells were washed, resuspended in PBS and analyzed by a BD LRSII flow cytometer (BD Biosciences). Plasmacytoid dendritic cells (pDCs) were identified as a HLA-DR^{high}, CD11c^{low}, CD14^{low}, CD19^{low}, CD303^{high}, CD123^{high} population (gating strategy outlined in **Figure S1**).

Confocal Microscopy

Cells were grown on poly-D-lycine coated coverslips to the desired confluency prior to stimulation as detailed in the results. Following stimulation, cells were subject to an acid wash (200 mM acetic acid, 150 mM NaCl), were fixed in 4% paraformaldehyde, and permeabilized with 0.3% saponin BSA PBST before mounting on glass slides in mountant containing DAPI. Cells were then imaged with a LSM880 confocal microscope. Images were processed in Zen software.

Quantitative PCR

Quantitative real-time PCR was carried out by a QuantStudio 5 Real Time PCR System (ThermoFischer) and a ΔΔCT-analysis formed from the generation of standard curves for the housekeeping genes and genes of interest. RNA isolation was carried out using the Direct-zol RNA MiniPrep kit (Zymo Research). cDNA was generated by SuperScript II reverse transcriptase (Thermo Fischer Scientific) according to the manufacturer's protocol. Qiagen QuantiFast SYBR green PCR was used to carry out the qRT-PCR according to the manufacturer's protocol.

ELISA

Nunc-ImmunoTM MicroWellTM 96 well plates (SIGMA) were coated with capture antibody and ELISA proceeded as detailed in manufacturer instructions using IL-8 ELISA MAX Standard ELISA kit (BioLegend, Hatfield, UK), IFNα ELISA using MT1/3/5 capture antibody and MT2/4/6 detection antibody (Mabtech), and CXCL10 ELISA DuoSet (R&D Systems).

5′ ^{32}P Labeling of Apt 21-2

Unlabeled Apt 21-2 (1 μg) synthesized by Dharmacon was incubated at 37°C for 30 min in a reaction containing 2 μl of T4 Poly nucleotide kinase (PNK) (NEB), 2 μl PNK buffer and ∼30 μCi of ^{32}p UTP in a total reaction size of 20 μl. Following incubation, the reaction was terminated by heating to 65°C for 10 min and purified by ethanol precipitation and resuspension in nuclease free water.

Electrophoretic Mobility Shift Assay

^{32}P labeled aptamer and LL-37 were mixed at the concentrations stated and incubated for 1 h on ice. Native polyacrylamide gel (7%) was prepared in TBE buffer and electrophoresis was carried out in TBE buffer. Samples were loaded with 60 mM KCl, 10 mM TRIS (pH 7.6), 40% glycerol and 0.01% bromophenol blue. Following completion of the separation, the gels were fixed in 12% methanol and 10% acetic acid in dH_2O, before drying in a vacuum pumped gel dryer (Biorad) and imaged by exposure to a phosphoscreen.

Filter Binding Assay

^{32}P labeled aptamer and LL-37 were mixed at the concentrations stated and incubated for 1 h on ice. Samples were drawn through stacked nitrocellulose and nylon membranes using a slot-blot device (Biorad). Filters were dried and imaged by exposure to a phosphoscreen.

RNA Urea Gels

Urea gels (7% polyacrylamide, 7 M urea) were prepared in TBE buffer for analysis of RNA stability. Samples were prepared by addition of equal volume 2x Novex® TBE-Urea Sample Buffer (ThermoFischer), incubated at 85°C, then cooled on ice prior to loading on the urea gels. Gels were stained with 0.2% methylene blue (0.4 M sodium acetate, 0.4 M acetic acid) for visualization of RNA.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software. Data were analyzed by one-way ANOVA to determine overall differences, and a Tukey *post hoc* test to determine statistical significance between groups.

RESULTS

LL-37 Interacts With RNA Aptamer Apt 21-2

LL-37 has been shown to interact with nucleic acids including single and double stranded RNA, and this interaction is thought to be mediated via positively charged residues on LL-37 (11, 13, 27). It therefore seems plausible for LL-37 to interact and complex with RNA aptamers. To explore whether this is the case, we incubated Apt 21-2 5' end labeled with ^{32}P UTP (100 nM) with increasing concentrations of LL-37 and separated samples by electrophoretic mobility shift assay (EMSA). A large observable shift in ^{32}P-labeled aptamer occurs as the concentration of LL-37 is increased, indicative of a higher order protein and aptamer complex (**Figure 1A**). This interaction was also observed when conducting a filter binding assay with the same samples (**Figure 1C**). There is evidence of protein:RNA complex formation at approximately the same concentration as the observed shift in EMSA. Densitometry analysis of band density in both EMSA and the filter binding assay show a 50% reduction in free aptamer at ∼2 μM, indicating an interaction between Apt 21-2 and LL-37 with an apparent K_D of 2 μM (**Figures 1B,D**).

LL-37-complexed RNA has been reported in the literature to be less susceptible to degradation. We questioned whether this would also be true of LL-37-complexed Apt 21-2. We therefore incubated Apt 21-2 in the presence or absence of

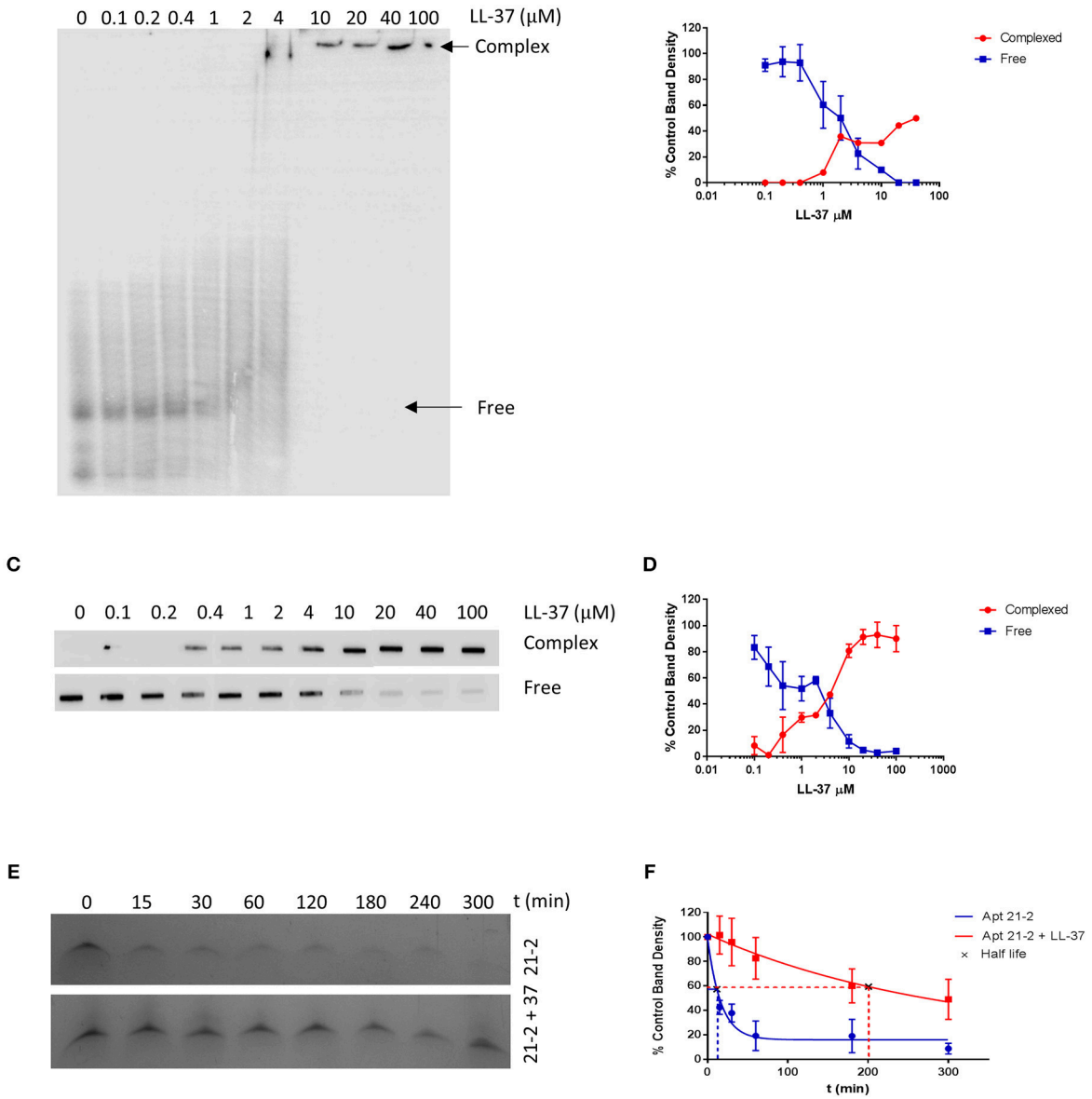

FIGURE 1 | Samples of Apt 21-2 (100 nM) combined with LL-37 (0 to 100 μM as indicated above lanes) were incubated for 1 h on ice. Samples were then separated on a 7% native polyacrylamide gel **(A)** or by filter binding assay **(C)**. Images are representative of 2 independent experiments. Densitometry was measured by ImageJ software and plotted in GraphPad Prism as percentage density of control RNA band with no added LL-37 to estimate K_D. Data shown are mean ± SD ($n = 2$) **(B,D)**. Apt 21-2 (1 μg) incubated with fetal calf serum at 37°C in the presence or absence of LL-37 (10 μg) for 5 h. Samples taken at indicated time points, separated on a 7% polyacrylamide urea gel and visualized using methylene blue stain. Image representative of 3 independent experiments **(E)**. Densitometry calculated by ImageJ software and normalized to t = 0 band density as control. Band density plotted and half-life calculated in GraphPad Prism. Data shown are mean ± SD ($n = 3$) **(F)**.

LL-37 in fetal calf serum for 5 h at 37°C and analyzed aptamer degradation by separation of the samples on denaturing (urea) gels. Within the 1st h Apt 21-2 alone had significantly degraded. However, addition of LL-37 reduced the extent of degradation (**Figure 1E**). Indeed, half-life of aptamer alone was calculated as 11.5 ± 5.65 min, whilst LL-37-complexed aptamer was calculated to have a half-life of 202.4 ± 82.95 min (**Figure 1F**).

LL-37 Facilitates Internalization of Apt 21-2 in Keratinocytes and Fibroblasts

LL-37 is known to cross plasma membranes through a variety of mechanisms, and in doing so can facilitate internalization of its binding partners. This has been shown to occur with poly(I:C) in keratinocytes and both viral dsRNA and self-RNA released from dying cells, as well as with other non-nucleic acid binding partners such as LPS (28). Once LL-37 was identified to associate

with Apt 21-2, we next examined whether this interaction (in the presence of primary keratinocytes and fibroblasts) might promote internalization of the RNA aptamer.

Primary keratinocytes and fibroblasts were treated with Cy3-labeled Apt 21-2 (Apt 21-2 Cy3) and FITC-labeled poly(I:C) in the presence or absence of LL-37 before analysis by confocal microscopy. As previously reported, evidence of uptake (to a low level) by keratinocytes was observable when Apt 21-2 Cy3 was added to cells alone (**Figure 2A**). Conversely, no uptake was observed by the fibroblasts (**Figure 2B**). However, with the addition of 2.5 μM LL-37 internalization of Apt 21-2 Cy3 was greatly enhanced in both keratinocytes and fibroblasts, with evidence of punctate aggregation and diffuse cytosolic staining closely resembling that observed upon addition of both LL-37 and FITC-poly(I:C). Indeed, whilst uptake of aptamer alone was not observed in fibroblasts, significant internalization was observed when added with LL-37 (**Figure 2**).

Given the hydrophobic nature of cyanine dyes and the propensity of LL-37 to interact with various molecular partners, it is important to confirm the observed interaction between LL-37 and Apt 21-2 Cy3 is due to interaction with the aptamer RNA rather than the cyanine label. To this end, reactions of FITC-LL-37 and Apt 21-2 Cy3 were spiked with increasing concentrations of unlabeled Apt 21-2 to compete for binding with LL-37 before addition to cells (**Figures 3B,C**). As shown in **Figure 3A**, Apt 21-2 Cy3 appears to co-localize with the aggregated FITC-LL-37, both in extracellular and intracellular aggregates. However, a decrease in Cy3 fluorescence was observed corresponding with increase in concentration of unlabeled Apt 21-2, suggesting the uptake observed in **Figures 2A,B** is due to interaction between LL-37 and RNA rather than LL-37 and Cy3. Furthermore, addition of LL-37-FITC suggests co-localization of LL-37 and Apt 21-2 (**Figure 3A**), corroborating the results observed by EMSA and the filter binding assay (**Figure 1**).

Apt 21-2 Does Not Induce an Inflammatory or Interferon Response When Combined With LL-37

Once associated with LL-37, dsRNA/LL-37 complexes can facilitate an inflammatory and interferon response by enhancing activation of TLRs after internalization of complexed RNA. This has been illustrated for co-stimulation of LL-37 with poly(I:C), self-RNA and DNA (29). Although small RNA aptamers are known to be immunologically inert, the possibility of their interaction with LL-37 allowing an immunological response must be considered. We therefore stimulated primary keratinocytes and fibroblasts with either Apt 21-2 or poly(I:C) in the presence or absence of LL-37 and measured both IL-8 secretion and mRNA expression of skin-relevant interferon stimulated genes (ISGs) MxA, CXCL10, GBP-1, and the tissue-derived IFNλ (in keratinocytes), which has a significant role in tissue-based antiviral activity (30, 31).

As has been previously reported, treatment with poly(I:C) alone induced a strong response from primary keratinocytes, eliciting both IL-8 secretion and ISG expression (**Figures 4A,C**). Whilst addition of LL-37 alone had little effect on either IL-8 secretion or ISG expression, an additive effect was observed in ISG expression when added in combination with poly(I:C) to keratinocytes, and a synergistic increase in both ISG expression and IL-8 secretion by fibroblasts. Primary keratinocytes, however, appeared to secrete less IL-8 when challenged with both LL-37 and poly(I:C) (**Figure 4**). Whilst this does not seem to fit the trend of our other results, this inhibition of poly(I:C)-induced IL-8 secretion by LL-37 in keratinocytes has been previously reported in the literature (13). Contrary to poly(I:C), stimulation with Apt 21-2 did not induce IL-8 secretion or up-regulation of measured ISGs. Furthermore, the addition of LL-37 and Apt 21-2 in combination had no significant effect on IL-8 secretion or ISG expression in either keratinocytes or fibroblasts (**Figure 4**).

In addition to keratinocytes and fibroblasts, immune cells also infiltrate into the dermis and epidermis, and are often found in increased numbers during inflammation. Of these, plasmacytoid dendritic cells (pDCs) and monocytes/macrophages are known as significant producers of type 1 interferon and are known to respond to LL-37-complexed nucleic acids (7, 11, 18, 27). We therefore examined the response of pDCs to LL-37-complexed Apt 21-2. Human PBMCs were stimulated with either Apt 21-2 or the TLR9 agonist CpG oligodeoxynucleotide (ODN) in the presence or absence of LL-37 for 12 h, and pDC intracellular IFNα production was assessed by flow cytometry. Additionally, PBMCs were treated with either Apt 21-2 or the TLR3 agonist poly(I:C) in the presence or absence of LL-37 for 24 h, and secreted IFNα and the interferon stimulated chemokine CXCL10 were measured by ELISA (**Figures 5C,D**). As shown in **Figure 5A**, whilst stimulation with CpG ODN induced a modest increase in the percentage of $IFN\alpha^+$ pDCs, no response was measured following Apt 21-2 stimulation. When stimulated with both LL-37 and CpG ODN, a significant increase in the percentage of $IFN\alpha^+$ pDCs was observed over CpG ODN stimulation alone, as has been previously reported (7) (**Figure 5B**). In contrast, no $IFN\alpha^+$ pDCs were identifiable following stimulation with LL-37 and Apt 21-2 in combination (**Figure 5A**). In agreement with these findings, PBMCs treated with poly(I:C) secreted significant amounts of both IFNα and CXCL10, and these levels increased when treated in combination with LL-37. However, treatment with Apt 21-2 did not cause an elevation in secretion of IFNα or CXCL10 above that of non-stimulated cells, and the addition of LL-37 in combination with Apt 21-2 had no significant effect on the secretion of either IFNα or CXCL10 (**Figures 5C,D**). These results suggest that despite the interaction between Apt 21-2 and LL-37 and the increase in internalization of the complexes, Apt 21-2 remains immunologically inert when present with LL-37, unable to elicit an interferon or inflammatory response. This remains true for both skin resident and infiltrating immune cells.

DISCUSSION

In this study, we initially sought to determine whether LL-37, a pro-inflammatory protein well documented to interact with nucleic acid, can also complex with a single stranded

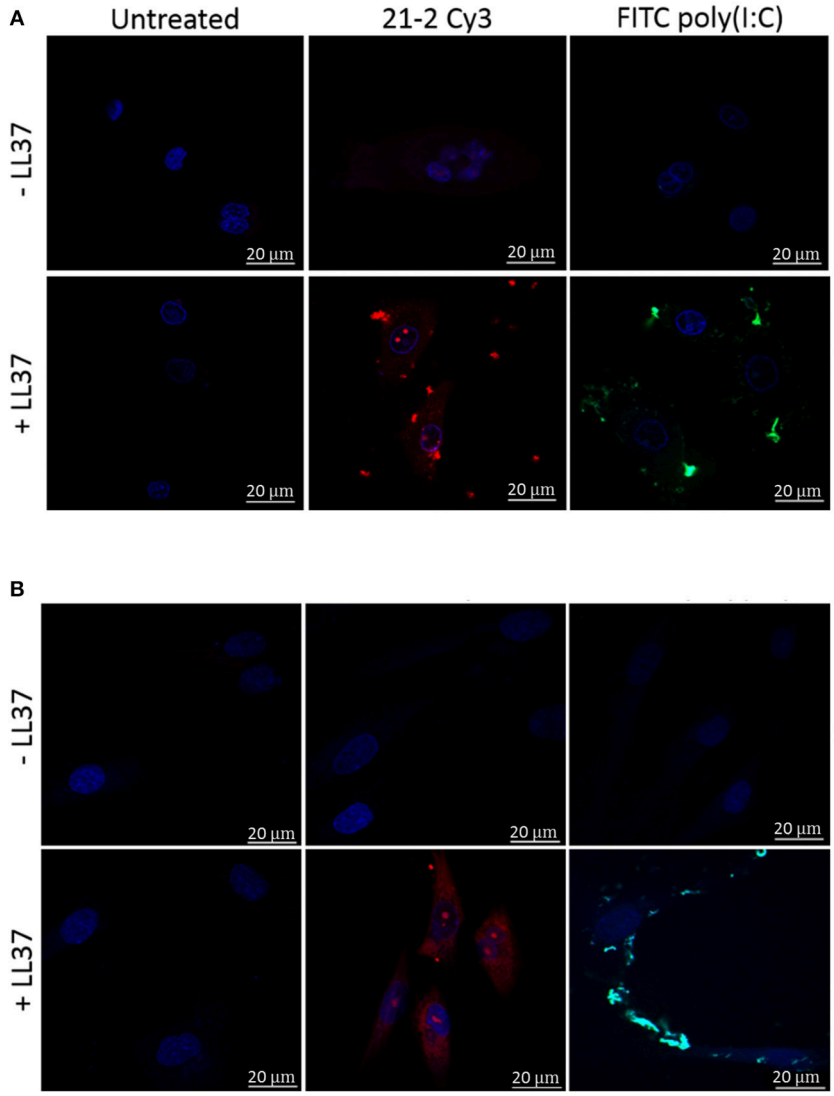

FIGURE 2 | Healthy primary keratinocytes **(A)** and fibroblasts **(B)** were treated with Apt-21-2 Cy3 (100 nM; red) or FITC-conjugated poly(I:C) (1 μg/ml; green) in the presence or absence of LL-37 (2.5 μM) for 24 h. Cells were washed with acid to remove extracellular RNA and imaged by confocal microscopy to assess uptake of Apt 21-2 and poly(I:C). Nuclei were visualized with 4′-6-diamidion-2-phenylidole (DAPI) (bars = 20 μm). Images are representative of three independent experiments.

RNA aptamer. We tested this by electrophoretic mobility shift assay, filter binding assay, and confocal microscopy, all of which provided evidence of interaction. Analysis by EMSA and filter binding established that, in a controlled reaction, LL-37 interacts with Apt 21-2 with an apparent K_D of ∼2 μM, and when added to cells in combination we observed strong co-localization of the aptamer and protein. This observation is perhaps not surprising as LL-37 is well documented to complex with both single and double stranded self-RNA and exogenous RNA, in addition to DNA (11, 13, 27). Indeed, Ganguly et al. postulated that LL-37 may preferentially bind structured RNA containing double stranded regions and stem loops. However, by illustrating the ability of LL-37 to complex small chemically modified RNA aptamers we bring to light the possibility that any RNA aptamers delivered into an environment rich in LL-37 may become complexed and potentially sequestered by the antimicrobial peptide. Keratinocytes have been shown to significantly increase production of LL-37 in response to cytokines associated with psoriasis (32). Whilst exact concentrations of LL-37 in the skin is unclear, it has been observed that psoriatic lesions contain a median of 304 μM LL-37 (5). Delivering an aptamer into such high concentrations, in this case ∼150 times higher than the apparent K_D, it seems likely that a proportion of the delivered aptamer will become complexed. Whilst this study was conducted in the context of skin-based inflammation, LL-37 expression is found over-expressed in several diseases and tissues, including inflammatory bowel disease and rheumatoid arthritis, and has been measured up to ∼6 μM in bronchoalveolar lavage

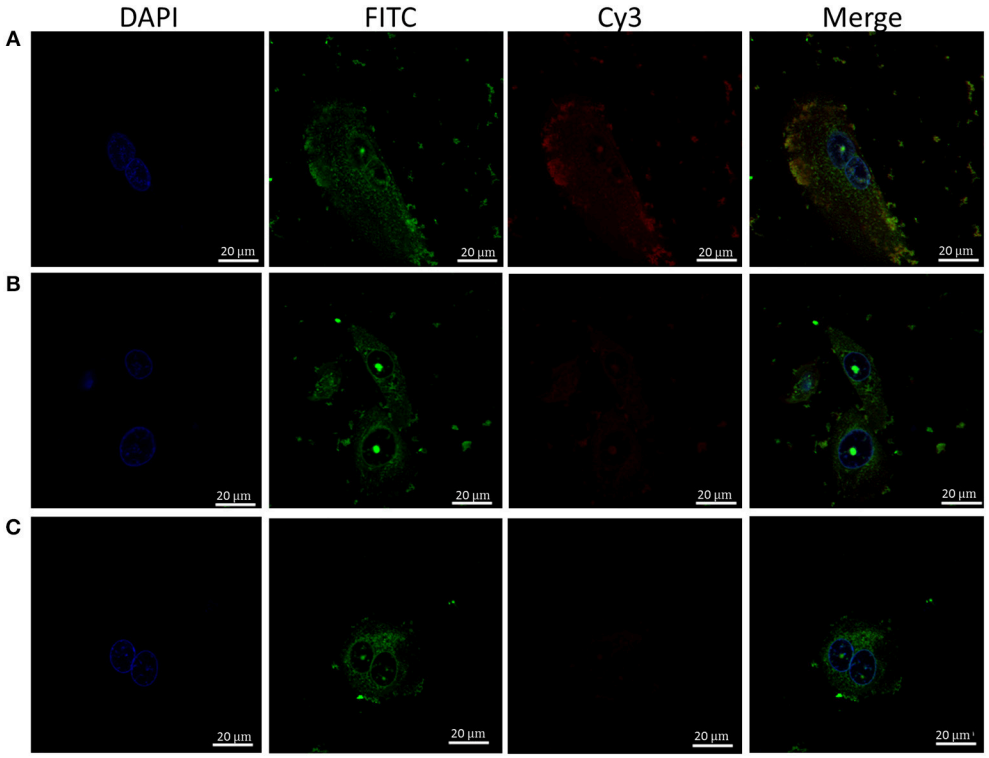

FIGURE 3 | Healthy primary keratinocytes were treated with Apt-21-2 Cy3 (100 nM; red) and FITC-labeled LL-37 (2.5 μM; green) with either 0 μM **(A)**, 1 μM **(B)**, or 5 μM **(C)** unlabeled Apt 21-2 to compete with Apt 21-2 Cy3 for binding with LL-37. Cells were washed with acid to remove extracellular RNA and imaged by confocal microscopy to assess uptake of aptamer. Nuclei were visualized with DAPI (bars = 20 μm). Images are representative of 2 independent experiments.

fluid extracted from infants suffering systemic inflammation, and so should be considered when treating any inflamed area (33–35). Even in healthy human sweat, LL-37 can be found at a concentration of ∼1 μM, which may have considerable implications when using a topically administered RNA aptamer (36).

A significant finding of the work conducted in this study is the observation that LL-37-complexed aptamer is efficiently internalized by both keratinocytes and fibroblasts. Whilst keratinocytes are known to actively take up extracellular components quite readily by macropinocytosis, fibroblasts are not known to do so. Indeed, our previous work has demonstrated that when added to keratinocytes and fibroblasts alone, keratinocytes take up RNA aptamers, but the fibroblasts do not (24). However, as demonstrated here, when complexed with LL-37, the aptamer is internalized by both fibroblasts and keratinocytes, with confocal microscopy exhibiting striking intracellular staining in both keratinocytes and fibroblasts with a slight punctate appearance in keratinocytes. Internalization of LL-37-complexed nucleic acid has been previously reported in the context of keratinocytes and dendritic cells, however, to our knowledge this is the first time it has been described in fibroblasts (11, 17, 27). Indeed, these novel observations have significant implications when considered in the context of using RNA aptamers to treat inflammatory skin conditions and may influence how and where RNA aptamers might be delivered for treatment of extracellular or intracellular targets. As the results presented in this work show both keratinocytes and fibroblasts will internalize RNA aptamers complexed by LL-37, an extracellular target in either the dermis or epidermis may prove difficult to treat in this manner in inflamed skin tissue. Conversely, as internalization appears to be so effective in the presence of LL-37, it may be possible to utilize this mechanism as a method of targeting intracellular pathways and molecules. Considering these observations, it seems natural to suggest when using RNA aptamers to treat inflammatory skin conditions where LL-37 is strongly expressed that intracellular targets may be more desirable than extracellular. We have previously reported that free RNA aptamers taken up by cells enter the endosomal/lysosomal pathway, however whether these aptamers escaped the endosomal network is unclear (37). It is also unclear as to the effect that LL-37 complexing might have on intracellular trafficking of internalized complexes, and whether complexed aptamers might be able to access cytosolic targets. These possibilities should be further explored by delineating the mechanism by which LL-37 facilitates entry of complexed aptamers to facilitate identification of the fate of internalized complexes.

LL-37 has been reported to increase stability of complexed RNA, inhibiting RNase-mediated degradation (27). By

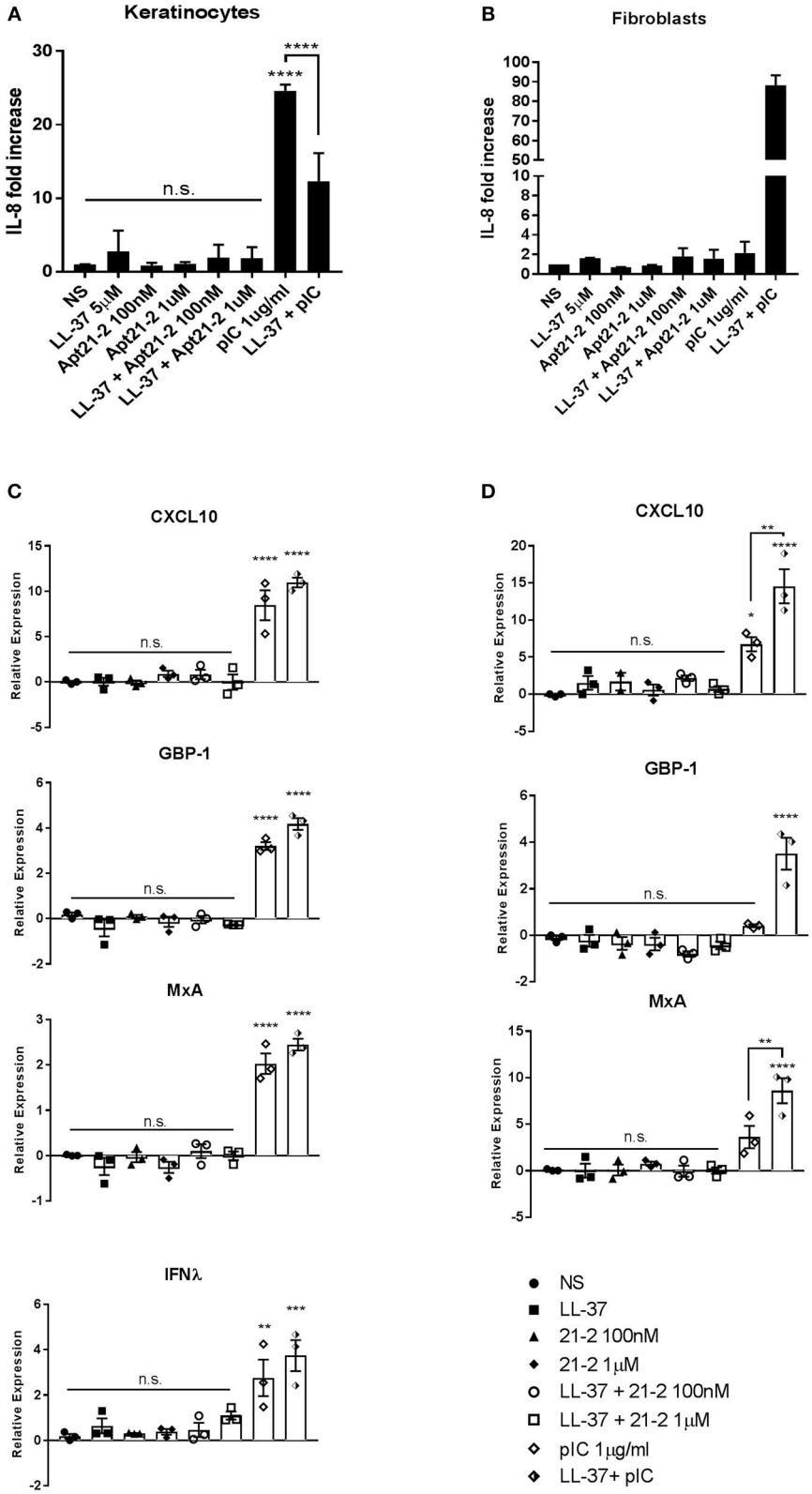

FIGURE 4 | Healthy primary keratinocytes **(A,C)** and fibroblasts **(B,D)** were grown to 80% confluence in 24 well plates and treated with LL-37 (5 μM), Apt 21-2 (100 nM, 1 μM), LL-37 + Apt 21-2 (5 μM + 100 nM and 1 μM, respectively), poly(I:C) (1 μg/ml), poly(I:C) + LL-37 (1 μg/ml + 5 μM), or left untreated (NS). To assess

(Continued)

FIGURE 4 | inflammatory response supernatants were harvested 24 h post-stimulation and IL-8 measured by ELISA **(A,B)**. Data shown are mean ± SD from independent experiments. $n = 3$, ANOVA, $^{****}p < 0.0001$ **(A)** $n = 2$ **(B)**. To assess interferon response RNA was harvested at 6 h post stimulation and mRNA expression of the interferon-stimulated genes CXCL10, GBP-1, MxA, and IFNλ was measured by qPCR normalized to U6 housekeeping gene presented as ΔΔCq. ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data shown are mean ± SEM with individual data points of independent experiments, $n = 3$ **(C,D)**.

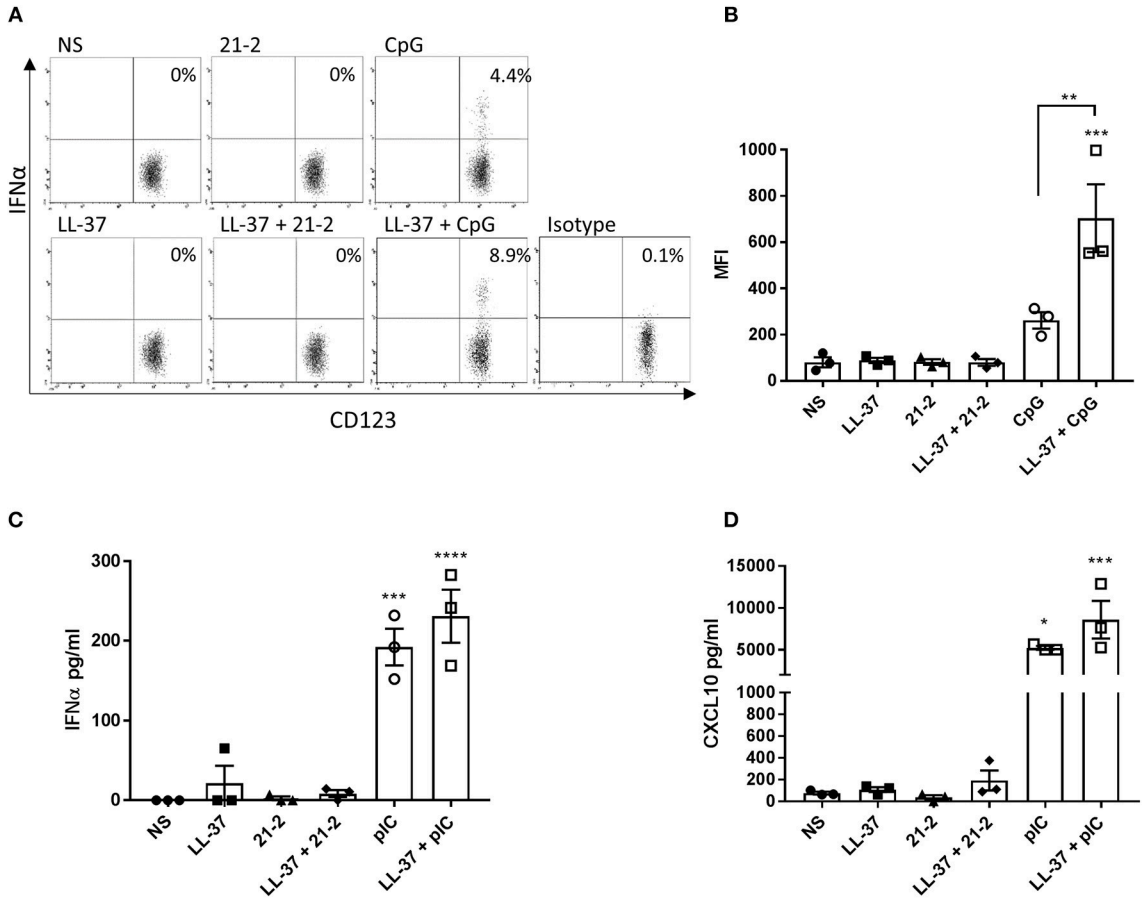

FIGURE 5 | PBMCs were isolated from whole blood and treated with LL-37 (2.5 μM), Apt 21-2 (100 nM; 21-2), CpG ODN (2.5 μM; CpG), LL-37 + Apt 21-2 (2.5 μM + 100 nM), LL-37 + CpG ODN (2.5 μM each), or left untreated (NS) for 12 h at 37°C 5% CO_2. The isotype control was treated with CpG (2.5 μM). After 1 h of stimulation, GolgiPlug was added to all cells (1 μl per ml of media). Following stimulation, the percentage of IFNα$^+$ pDCs was determined by flow cytometry. pDCs were identified using the gating strategy outlined in **Figure S1**. A representative set of dot plots for one donor is shown **(A)** with a graph plotting mean fluorescence intensity (MFI) of IFNα for each donor **(B)**. Data shown are mean ± SEM with individual data points of independent donors, $n = 3$, ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$. **C-** PBMCs were stimulated as in **A** but substituting CpG ODN with poly(I:C) (100 μg/ml) and without addition of GolgiPlug. Cells were incubated for 24 h at 37°C 5% CO_2. Supernatants were harvested and tested for IFNα **(C)** and CXCL10 **(D)** by ELISA. Data shown are mean ± SEM with individual points of independent donors. $n = 3$ $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

incubating LL-37-complexed and free Apt 21-2 with fetal calf serum we have illustrated this is also true for LL-37-complexed RNA aptamers. This may have implications for the efficacy of RNA aptamers in inflammatory milieu as they may persist for longer in a LL-37 rich environment if provided protection by complexing. Indeed, this may prove beneficial if complexed aptamers remain functional, however, this is currently unknown. As LL-37-nucleic acid complexes have been observed to dissociate once internalized into acidic endosomes, it seems plausible that complexed and internalized aptamers may also be released and so available to bind targets (38). It may, therefore, be interesting to examine the kinetics of binding between LL-37 and RNA aptamers under various physiological conditions as this may provide key information on the availability of RNA aptamers when present in LL-37-rich tissue.

An important consideration which comes to light from demonstrating that RNA aptamers are both complexed and internalized with LL-37 is the effect this has on immune activation. RNA aptamers are considered immunologically inert, however, with LL-37 known to be an immunomodulatory protein that can significantly enhance the inflammatory properties of nucleic acids through mechanisms that are not entirely characterized, it is important to explore whether LL-37-complexed RNA aptamers become immunologically stimulatory.

Previous work has shown that LL-37 complexed with ssRNA can initiate inflammatory signaling through TLR7 and TLR8 in pDCs once delivered into endosomal compartments, however, the results obtained in our study show that whilst LL-37-complexed aptamers were delivered intracellularly, no activation of cells was observed following treatment of either healthy pDCs, PBMCs, keratinocytes or fibroblasts with complexed aptamer (27). Whilst expression of TLR7 and TLR8 in healthy keratinocytes is not clearly defined, with contradictory evidence published in the literature, fibroblasts reportedly express both, and the pDC response to LL-37-complexed ssRNA has been previously described (27, 39). In addition to pDCs, monocytes are also known to infiltrate into the epidermis in inflamed tissue and can also contribute to IFN production in response to LL-37-complexed nucleic acids (18). Whilst pDCs effectively respond to ssRNA, they are poor expressers of TLR3 and therefore do not respond well to dsRNA (40). Monocytes/macrophages, however, express high levels of TLR3 and generate IFN in response to dsRNA (41). Despite this, neither pDCs nor isolated PBMCs (containing pDCs, conventional DCs and monocytes/macrophages) generated an IFN response to LL-37-complexed aptamer. These results therefore suggest aptamer 21-2:LL-37 complexes are unable to activate TLR7/8 or TLR3. LL-37 is thought to enhance activation of TLR3 through complexing dsRNA and producing crystalline structures that more effectively initiate TLR3 by engaging several receptors, inducing receptor clustering and immune amplification (42). The efficacy of these crystalline structures was found to depend on the length of dsRNA present in the crystals, so it is possible that Apt 21-2 does not contain long enough dsRNA tracts to form the correct crystal structure with LL-37 and so does not activate TLR3 in this manner. However, larger RNA aptamers may contain longer stretches of duplexed RNA, therefore further research to examine the effect of aptamer length on TLR activation may be necessary.

In conclusion, this work has illustrated the importance of understanding the environment into which an RNA aptamer is being delivered when treating inflammatory disease. In particular, it has shown that RNA aptamers delivered into inflamed tissue rich in the anti-microbial peptide LL-37 will become complexed and internalized by surrounding cells. Despite evidence of complexing and internalization, we did not observe any inflammatory or interferon response from keratinocytes, fibroblasts, or PBMCs, suggesting RNA aptamers should be safe for use when delivered into inflamed skin. However, the observation that LL-37-complexed aptamers are internalized by surrounding cells should be taken into consideration when developing an RNA aptamer-based treatment for an extracellular target in inflamed tissue with high levels of LL-37, as cells may sequester complexed aptamers away from their targets.

AUTHOR CONTRIBUTIONS

TM, JW, CB, and AA undertook experimental work. NS and MW designed and supervised the study. All authors contributed to writing the manuscript, with TM taking the leading role.

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SUPPLEMENTARY MATERIAL

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The Interaction Between Two Worlds: MicroRNAs and Toll-Like Receptors

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MicroRNAs (miRNAs) are critical mediators of posttranscriptional regulation via their targeting of the imperfect antisense complementary regions of coding and non-coding transcripts. Recently, researchers have shown that miRNAs play roles in many aspects of regulation of immune cell function by targeting of inflammation-associated genes, including Toll-like receptors (TLRs). Besides this indirect regulatory role of miRNAs, they can also act as physiological ligands of specific TLRs and initiate the signaling cascade of immune response. In this review, we summarize the potential roles of miRNAs in regulation of TLR gene expression and TLR signaling, with a focus on the ability of miRNAs bind to TLRs.

Keywords: microRNAs, Toll-like receptors, inflammation, TLR, TLR ligands

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INTRODUCTION

An efficient immune system is required for all multicellular organisms to detect and respond to pathogenic microorganisms or cells and/or tissue damage (1, 2). After discovery of the Toll-like receptor (TLR) family of pattern recognition receptors in the late 1990s, investigators showed that they recognize specific and distinct conserved endogenous and exogenous molecular patterns (3, 4). TLRs are crucial in recognition of microbial products, release of inflammatory mediators through the induction of transcription factors in immune response and inflammation and control of adaptive immune responses (5, 6). Emerging evidence has demonstrated that non-coding RNAs such as microRNAs (miRNAs) are involved in almost all known cellular processes, including innate, and adaptive immune responses, via modulation of gene expression (7–11). MiRNAs are secreted by several cell types, including tumor cells and macrophages within extracellular vesicles, such as exosomes and microvesicles; act as cell-to-cell communication vectors; and are taken up by recipient cells (12–17). Moreover, several miRNAs can bind to TLRs and initiate immune response by inducing immune and inflammatory gene expression. This review focuses on the inflammation-related miRNAs in the let-7 family, miR-21, miR-146b, and miR-155 and their involvement in TLR signaling pathways via regulation of TLRs and/or TLR signaling expression and binding to TLRs.

TLRS AND TLR SIGNALING

TLRs are evolutionarily conserved molecules that initiate the signaling cascade of immune response against a wide variety of pathogens (18). Moreover, TLRs are type I integral membrane proteins consisting of 10–30 leucine-rich repeats in the N-terminal portion of TLRs that participate in ligand recognition and a cytoplasmic domain of the Toll/interleukin (IL)-1 receptor (TIR) in the C-terminal portion of TLRs that is responsible for activation of downstream signaling. Both

pathogen-associated and damage-associated molecular patterns can be recognized by different TLRs and subsequently trigger signaling transduction pathways through adaptor molecules (19, 20). Damage-associated molecular patterns are endogenous molecules released from stressed or dying cells. Depending on the type of cells and tissues damaged, they can be classified as protein damage-associated molecular patterns, such as heat shock proteins, high-mobility group box 1 protein, or non-protein damage-associated molecular patterns, such RNA and DNA (3, 21, 22). Pathogen associated-molecular patterns are exogenous molecules derived from pathogens such as bacteria, fungi, parasites, and viruses and can be recognized by TLRs, leading to activation of the TLR signaling cascade, which regulates the expression of inflammation-related genes such as IL-1 receptor-associated kinase (*IRAK1*), tumor necrosis factor (*TNF*) receptor-associated factor 6 (*TRAF6*), and type I interferon (*IFN*) (3, 21, 22). Various TLRs are primarily or selectively expressed in specific cell types, including immune cells such as lymphocytes, dendritic cells (DCs), macrophages, and neutrophils and non-immune cells such as epithelial cells and fibroblasts (23–26). Recent studies identified that TLRs are also expressed in tumor cells and their microenvironments that composed cancer-associated fibroblasts, tumor-associated macrophages, marrow-derived suppressive cells, and regulatory T cells, adipocytes, and immune cells (23, 27). In mammals, the TLR protein family currently comprises 13 members (humans, TLR1-10; mice, TLR1-9, and TLR11-13), with the TLRs in humans and mice having some functional differences (28–31). Based on the subcellular localization of TLRs, they can be broadly divided into two subgroups. Those in the first group, including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, are expressed on the surface of cells and recognize microbially derived ligands. TLRs in the second group, including TLR3, TLR7, TLR8, and TLR9, are expressed intracellularly in vesicles such as endosomes and lysosomes and recognize microbial nucleic acids (32, 33). In contrast, TLR3 can be localized both on cell surfaces and in intracellular vesicles (34).

Upon activation of TLR signaling transduction pathways, TLRs interact with several TIR-containing intracellular adaptor molecules, including myeloid differentiation primary response gene 88 (*MYD88*), sterile alpha and TIR motif-containing protein 1, TIR domain-containing adaptor protein, TIR domain-containing adapter-inducing IFN-β (TRIF), TIR domain-containing adapter molecule 1 (TICAM1), and TICAM2, leading to transcription factor activation and ultimately causing the release of various proinflammatory cytokines, chemokines, and IFNs and activation of the adaptive immune system (35, 36). Depending on the adaptor protein recruited, TLR signaling can be activated via the MyD88-dependent pathway that leads to release of proinflammatory cytokines and a TRIF-dependent (MyD88-independent) pathway associated with production of IFN-β (37–40). TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, and TLR9 signaling are activated by the MyD88-dependent pathway, which typically leads to activation of nuclear factor (NF)-κB, whereas TLR3 signaling is activated by the TRIF-dependent pathway. In contrast, TLR4 is activated by both pathways simultaneously (37–40).

THE EFFECTS OF MIRNAS ON TLR EXPRESSION AND SIGNALING

A growing number of reports have stated that specific epigenetic processes such as histone modifications, DNA methylation, and non-coding RNAs may regulate the transcriptional responses of TLRs (41–43). MiRNAs make up one of the well-characterized non-coding RNA families that generally bind to the 3' untranslated regions of their target messenger RNAs (mRNAs) to suppress translation or degradation of the mRNAs (44, 45). MiRNAs have a fundamental role in many biological processes, including apoptotic cell death, cell-cycle, tumorigenesis, and inflammation. Also, dysregulation of miRNAs has been associated with prognosis for and progression of multiple human diseases, including cancer (46–51). An increasing number of studies have demonstrated that several miRNAs, including miR-21, miR-146, miR-155, and let-7 family, target TLRs or proteins in TLR signaling pathways (**Figure 1**) that are involved in the regulation of various processes, such as inflammation, T-cell activation, cellular infiltration, and immunity development (52, 53). We have selectively listed recent miRNAs and their regulator roles on TLRs in **Table 1**.

In one of the first studies demonstrating that miRNAs regulate immune response, researchers found that let-7i binds directly to TLR4 and regulates its expression in human cholangiocytes (54). In that study, infection of cultured cholangiocytes with *Cryptosporidium parvum* and lipopolysaccharide (LPS) stimulation of the cholangiocytes led to decreased let-7 expression via a MyD88/NF-κB–dependent mechanism, and low expression of let-7 was associated with upregulation of TLR4 in cholangiocytes. In concordance with this, upon *C. parvum* infection in non-malignant human biliary epithelial cells, inhibits expression of let-7 family miRNAs, including let-7i, let-7d, let-7f, let-7e, and miR-98, whereas induces the protein content of total SNAP23 and enhances phosphorylation of SNAP23. Activation of TLR4 signaling may induce SNAP23 protein expression by modulation of let-7-mediated gene regulation (57). Subsequently, investigators showed that let-7 and miR-98 target the 3' untranslated region of the cytokine-inducible Src homology 2-containing protein, resulting in translational

Abbreviations: AGO2, argonaute RISC catalytic component 2; CIS, cytokine-inducible Src homology 2-containing protein; DCs, dendritic cells; HMGB1, high-mobility group box 1; HSV-1, herpes simplex virus type 1; IFNs, interferons; IL, interleukin; IRAK1, interleukin 1 receptor associated kinase 1; IRF, interferon-regulatory factor; KSRP, KH-type splicing regulatory protein; LPS, lipopolysaccharide; MCP1, monocyte chemoattractant protein-1; miRNA, microRNA; mRNA, messenger RNA; MYD88, myeloid differentiation primary response protein; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cells; PDCD4, programmed cell death 4; PDCs, plasmacytoid dendritic cells; ROS, reactive oxygen species; SHIP1, inositol polyphosphate-5-phosphatase D; SNAP23, synaptosome associated protein 23; SOCS1, suppressor of cytokine signaling 1; TAB2, TGF-beta activated kinase 1 binding protein 2; TGF, transforming growth factor; TICAM, TIR domain-containing adapter molecule; TIR, Toll/interleukin-1 receptor; TLRs, Toll-like receptors; TNF, tumor necrosis factor.

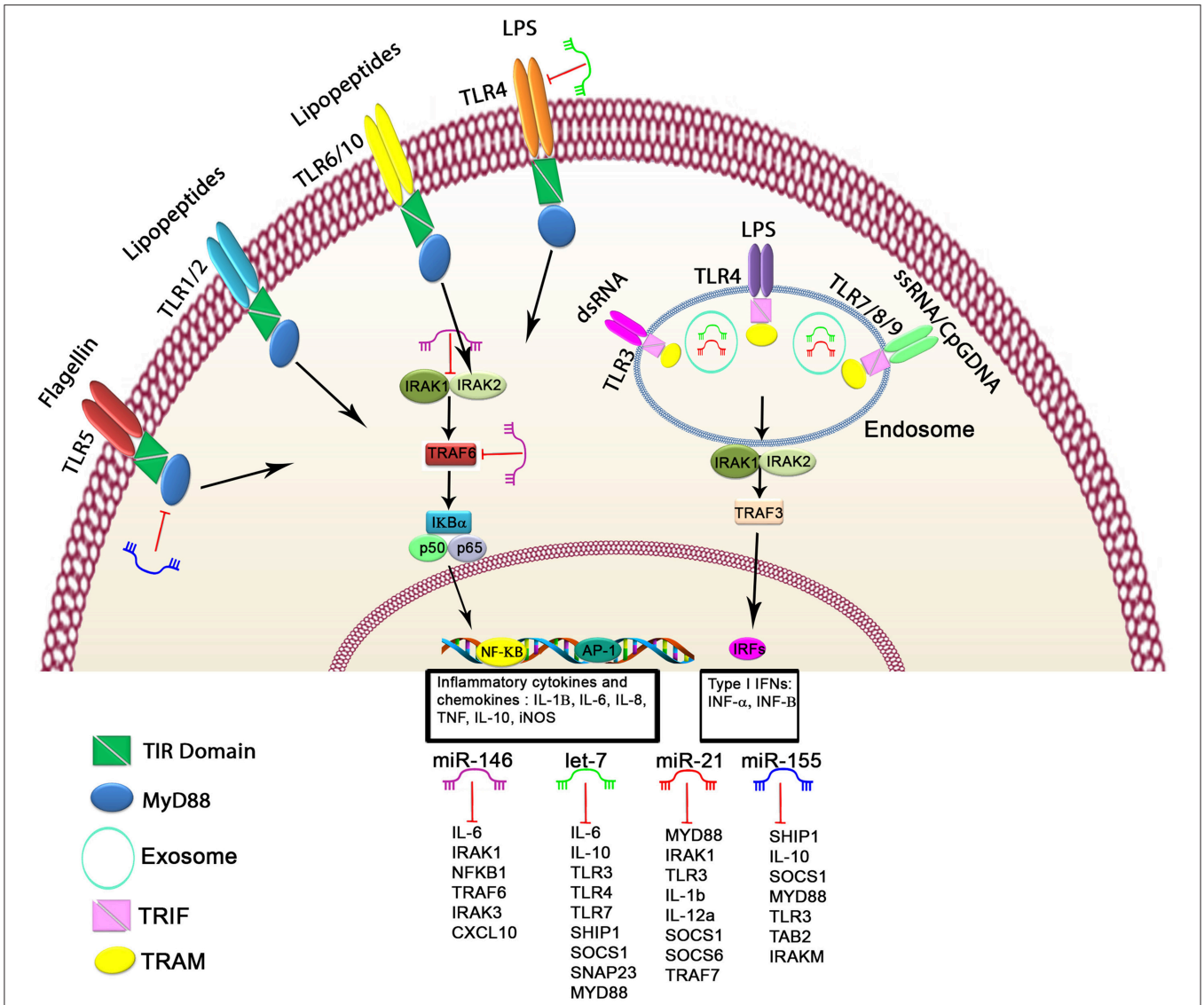

FIGURE 1 | Schematic of the regulatory mechanism of miRNAs in TLR signaling. Cell surface and cytoplasmic TLRs can be regulated by several miRNAs, including let-7 family members, miR-21, miR-146, and miR-155. First, miRNAs can bind directly to 3′untranslated region of TLRs or TLR-related genes, leading to modulated expression of TLRs through posttranscriptional regulation of TLR signaling. Second, miRNAs serve as physiological ligands of TLRs, such as miR-21, let-7 family members, and miR-29a, which can activate TLR signaling and stimulate the release of inflammatory cytokines and IFN genes in some cell types. Functional studies have demonstrated that these miRNAs may participate in activation of TLR signaling through regulating the *NF-κB* pathway and the production of inflammatory cytokines, which are shown here.

repression of this protein in cholangiocytes, and that this may be associated with modulation of inflammatory responses in epithelial cells during microbial infection (76). In addition to this regulatory role of let-7 regarding TLR4 activation the inflammation-associated transcription factors NF-κB p50 and C/EBPβ can interact with the let-7 promoter region and repress transcription following microbial stimulus in human cholangiocytes (91).

MiR-155 has a well-characterized oncogenic role in tumorigenesis (92, 93), and aberrant expression and function of miR-155 have been associated with inflammation and affect immune cell functions at various levels by targeting inflammation-related genes, including TLRs (87, 94). This miRNA suppresses the expression of the adaptor protein TAB2 in the TLR/IL-1 signaling cascade, thereby regulating the feedback mechanism of IL-1β and other inflammatory cytokines produced during LPS-mediated DC activation (72). MiR-155 can also target suppressor of cytokine signaling 1 (SOCS1) and consequently modulate transcriptional expression of SOCS1 in LPS-activated Akt1$^{-/-}$ murine macrophages (95). In addition, miR-155 and miR-M4 (virally encoded functional orthologs of miR-155) may target coding sequences of the TLR3 gene and regulate TLR3 expression in macrophages (61). In line with this, inhibition of miR-155 by antagomirs markedly increased

TABLE 1 | The regulatory effects of miRNAs on TLR signaling and the TLRs that regulate the miRNAs.

	miRNA	Target/pathway	Cell or tissue type	Function	References
The effects of miRNAs on TLR expression and signaling	let-7i	TLR4	Human cholangiocytes	Regulates TLR4 expression and contributes to immune responses against *C. parvum* infection	(54)
	let-7i	TLR4	Human monocytes	The let-7i mimic inhibits TLR4 expression	(55)
	let-7	TLR4	Human brain microvascular endothelial cells	Overexpression of let-7i reduces TLR4 expression and inflammation	(56)
	let-7/miR-98	SNAP23/TLR4	Human non-malignant biliary epithelial cells	The let-7 family reduces SNAP23 expression	(57)
	miR-155	TLR4 signaling	Murine Kupffer cells	Directly regulates expression of IRAK-M, SHIP1, SOCS1, and C/EBPβ	(58)
	miR-155	TLR3 signaling	Murine bone marrow macrophages	MiR-155 expression is dependent on TLR3/TRIF signaling	(59)
	miR-155	Caspase 3	Murine macrophages	MiR-155–mediated caspase 3 inhibition in LPS-activated macrophages suppresses apoptosis	(60)
	miR-155	TLR3	Avian macrophages	Inhibits IFN-β production in the TLR3 signaling pathway	(61)
	miR-155	CD1d	Human B cells	Directly targets CD1d upon TLR9 activation	(62)
	miR-155	TLR4 signaling	Murine ischemic cerebral tissue/microglial cells	Promotes TNF-α and IL-1β expression by upregulating TLR4 and downregulating SOCS1 and MyD88	(63)
	miR-155	MyD88 and SHIP1	Human primary monocyte-derived macrophages	Regulates downmodulation of MyD88 and SHIP1 expression and inhibits LPS-stimulated TNF-α secretion	(64)
	miR-155	SOCS1 and SHIP1	PBMCs	Suppresses expression of SOCS1 and SHIP1, which are negative regulators of TLR signaling	(65)
	miR-155	SHIP1	Murine macrophages	Represses SHIP1 expression and modulates ROS production	(66)
	miR-155	TGF-β and TLR3 signaling	Murine Kupffer cells and macrophages	Blocks the suppressive effect of IL-10 and TGF-β on TLR3 signaling	(67)
	miR-155	TNF-α and MCP1	Murine microglial cells	MiR-155 expression is induced by alcohol in the cerebellum in a TLR4-dependent manner	(68)
	miR-155	TLR3/4	Monocyte-derived macrophages	Restores infectivity in poly(I:C)-treated monocyte-derived macrophages	(69)
	miR-155	IRAK-M	Macrophages and PBMCs	Induces TLR7 stimulation and positively regulates IFN-α/β production in PDCs	(70)
	miR-155	SHIP1	Bone marrow-derived macrophages and PBMCs	IL-10 suppresses miR-155 expression in response to TLR4 stimulation	(71)
	miR-155	TAB2/TLR/IL-1	PBMCs	Controls the IL-1β pathway	(72)
	miR-21	PDCD4	Murine macrophages and human monocytes	Inhibits NF-κB activity and promotes IL-10 production	(73)
	miR-21	TLR4/ROS	Human primary lung cancer cells	Downregulation of miR-21 inhibits LPS-induced tumor growth	(74)
	miR-21	TLR4, IRAK3, and CXCL10	Human monocytes	Overexpression of miR-21 represses downstream transactivation of IL-1β and TNF-α	(75)
TLR signaling may modulate miRNA expression	let-7	CIS/TLR4	Human cholangiocytes	Activation of TLR4/MyD88 signaling downregulates miR-98 and let-7	(76)
	let-7	IL-6 and IL-10	Murine macrophages and human epithelial cells	Repression of let-7 activity relieves the cytokines IL-6 and IL-10	(77)

(Continued)

TABLE 1 | Continued

	miRNA	Target/pathway	Cell or tissue type	Function	References
	let-7	TLR4	Murine neuroblastoma cells and macrophages	TLR4 regulates let-7 repression through KSRP	(78)
	miR-155	TLR4 signaling	Murine Kupffer cells	TLR4 signaling regulates miR-155 expression	(79)
	miR-155	SOCS1	Murine macrophages	Mediates TREM-1–induced effects on TNF-α, IL-1β, and IL-6	(80)
	miR-155	IL-10	Murine bone marrow-derived macrophages	Downmodulation of Ets2 expression leads to suppression of miR-155 expression by IL-10	(81)
	miR-155	TLR2/MyD88	PBMCs	MiR-155 expression is regulated by HMGB1 in a MyD88-dependent manner	(82)
	miR-155	TLR4	Bone marrow-derived macrophages	Tenascin-C drives LPS-induced miR-155 expression	(83)
	miR-155	SHIP1	PBMCs and bone marrow-derived macrophages	*F. tularensis* infection induces miR-155 expression in a TLR-dependent manner through downregulation of SHIP1	(84)
	miR-155	SOCS1	Murine macrophages	Progesterone-based treatment inhibits LPS-induced IL-6 production by decreasing the activity of miR-155	(85)
	miR-21	PTEN	PDCs	MiR-21–deficient PDCs produce low levels of IFN-α and IFN-γ	(86)
	miR-146	IRAK1 and TRAF6	Human acute monocytic leukemia cells	LPS induces NF-κB expression through a MyD88-dependent pathway, resulting in upregulation of miR-146	(87)
	miR-146	TLR4 signaling	Human umbilical vein endothelial cells	Ang-1 triggers upregulation of miR-146b	(88)
The ability of miRNAs to bind to TLRs	miR-21	TLR7 signaling	Macrophages/microglial cells	In extracellular vesicles, miR-21 can activate TLR7 signaling	(89)
	let-7b	TLR7	Murine neurons	Activates TLR7 and causes neurodegeneration	(15)
	miR-21	TLR7/8 signaling	HEK-293 cells and murine macrophages	Functions as a human TLR8 or murine TLR7 ligand	(13)
	miR-21		Hematopoietic cell lines and PBMCs	Functions as an endogenous agonist for TLR8	(90)
	miR-29a	TLR7/8 signaling	HEK-293 cells and murine macrophages	Functions as a TLR7/8 ligand	(13)
	let-7	TLR7	Murine macrophages and microglia	Functions as a ligand for murine TLR7	(15)

CIS, cytokine-inducible Src homology 2-containing protein; KSRP, KH-type splicing regulatory protein; TREM-1, triggering receptor expressed on myeloid cells 1; ROS, reactive oxygen species; HMGB1, high-mobility group box 1; TGF, transforming growth factor; PDCs, plasmacytoid DCs.

TLR3 expression, whereas ectopic overexpression of miR-155 decreased IFN-β production in primary chicken embryo fibroblast cells (61). In another study, researchers found not only that miR-155 regulates TLR expression but also that miR-155 and caspase 3 mRNA can interact with AGO2 in LPS-activated murine macrophages (60).

MiR-21 is one of the multifunctional miRNAs and is mainly characterized by overexpression in many inflamed states, including lung inflammation in LPS-treated mice, allergic airway inflammation, and osteoarthritis (73, 96–98). Moreover, researchers detected high miR-21 expression in extracellular vesicles during simian immunodeficiency virus pathogenesis and increased miR-21 expression in mouse hippocampal neurons associated with neurotoxicity due to neuronal TLR7 expression (89). Furthermore, investigators showed that miR-21 expression was induced in murine macrophages by treatment with LPS, whereas proinflammatory protein PDCD4 expression was downregulated in these cells due to induction of miR-21 expression via the adaptor proteins MyD88 and NF-κB (73). In another study, miR-21 expression decreased in patients with primary graft dysfunction after lung transplantation, and incubation of human monocytes with bronchoalveolar lavage fluid obtained from patients with primary graft dysfunction induced miR-21 expression, suggesting that dysregulation of

miR-21expression is a novel regulator of TLR signaling during development of lung injury (75). Another study demonstrated that activation of TLR4 by treatment with LPS induced miR-21expression in primary human lung cancer cells and reactive oxygen species production by these cells (74). A more recent study demonstrated that miR-21 was upregulated in plasmacytoid DCs and that miR-21 deficiency significantly impaired production of IFN-γ and IFN-α in response to *HSV-1* infection through targeting of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling pathway in miR-21–knockout mice (86).

Importantly, an expression signature analysis of 200 miRNAs demonstrated that miR-146a/b, miR-132, and miR-155 were highly expressed in the acute monocytic leukemia cell line THP-1 after treatment with LPS as well as other microbial components and proinflammatory mediators (87). This finding suggests that miR-146 directly targets IRAK1 and TRAF6, which are key adapter molecules in the TLR4/NF-κB pathway (87). Furthermore, researchers found that miR-146 was significantly upregulated in hepatic stellate cells in mice infected with *Schistosoma japonicum* (99), is a negative regulator of NF-κB signaling in hepatic stellate cells, and acts by targeting TRAF6. Moreover, ectopic overexpression of the miR-146b-5p mimic significantly attenuated LPS-induced inflammatory responses and IRAK1 and TRAF6 expression in human umbilical vein endothelial cells (88).

Furthermore, miR-195 can regulate TLR2 expression through an indirect mechanism, as TLR2 expression was significantly reduced in miR-195–transfected THP-1 macrophages polarized toward the M1 phenotype (100). Treatment with LPS, synthetic lipid A, IL-2, IL-15, IL-1β, IFN-γ, and TNF-α similarly induced TLR2 gene expression in murine macrophages (101, 102), and acts is a key player in inflammation and atherosclerosis progression (103). Besides the role of cellular miRNAs in TLR signaling, authors recently reported that multiple viral miRNAs can activate production of proinflammatory mediators. For instance, Kaposi sarcoma herpes virus miRNAs such as miR-K-10b and miR-K12-12* are involved in sepsis as agonists of TLR8 through secretion of IL-6 and IL-10 (104, 105). Furthermore, Epstein-Barr virus miRNAs such as BHRF1-1 are expressed at higher levels in patients with chronic lymphocytic leukemia than in healthy individuals, and these viral miRNAs can serve as prognostic biomarkers for cancer (106, 107). Overall, these studies highlight miRNAs as central drivers of the TLR expression through transcriptional regulation of them, as indicated in **Table 1**.

HOW TLR SIGNALING MAY MODULATE MIRNA EXPRESSION

Initiation of the signaling cascade of immune response induced by TLR signaling can drive transcription of miRNAs during infection and inflammation. This is demonstrated by the fact that aberrant activation of TLR signaling after infection with microbial pathogens leads to dysregulation of miRNAs. Researchers have shown that infection of human peripheral blood monocytes (PBMCs) with *Francisella tularensis*, which is a highly pathogenic gram-negative bacterium that infects macrophages, induces expression of miR-155 in a TLR-dependent manner through downregulation of Src homology 2 domain-containing inositol 5-phosphatase 1 (SHIP1) (84). In concordance with this, authors reported significant differential expression of several miRNAs, including miR-155, after *F. tularensis* infection in primary human monocyte-derived macrophages and that *F. tularensis* infection leads to downmodulation of MyD88 and SHIP1 through an miR-155–dependent mechanism (64). Moreover, Leishmania RNA virus 1 was recognized by TLR3, and *Leishmania* infection induced miR-155 expression in murine bone-marrow macrophages (59). Concurrently, in that study, the pathogenesis of *LRV1+ Leishmania* infection decreased drastically in miR-155–deficient mice. In another study, let-7 and miR-98 were downregulated in murine macrophages upon Salmonella infection, whereas miR-155, miR-146a, and miR-21 were upregulated (77).

The immune-regulatory cytokine IL-10 may regulate transcription of miR-155 from the *BIC* gene in a signal transducer and activator of transcription 3-dependent manner in immortalized bone marrow-derived macrophages, and downmodulation of miR-155 expression leads to increased expression of SHIP1, which is one of the targets of miR-155 (71). Furthermore, investigators found that Ets2 is a critical transcription factor for the induction of miR-155 expression by LPS, and downmodulation of Ets2 leads to suppression of miR-155 by IL-10 (81). Another example miRNA signature is involved in human plasmacytoid DC activation (70), and miR-155 and its star form, miR-155*, were the most upregulated miRNAs in in primary human plasmacytoid DCs after TLR7 stimulation. MiR-155* induced IFN-α/β expression by suppressing IRAK-M expression, whereas miR-155 suppressed TAB2 expression (70). TLR3-dependent antiviral as well as inflammatory activity can be regulated by IL-10, transforming growth factor-β, and miR-155 in non-parenchymal liver cells *in vitro* (67). Other studies of macrophages demonstrated that chronic alcohol exposure induces TNF-α secretion through increased miR-155 expression both *in vitro* and *in vivo* (108), and miR-155 deficiency can protect against alcohol-induced liver injury, oxidative stress, steatosis, and inflammation in miR-155–knockout mice (79). In a similar study, after chronic ethanol feeding, miR-155 induced TNF-α and MCP1 expression in the cerebellum in a TLR4-dependent manner (68). Researchers have also observed aberrant expression of miR-155 in macrophages after stimulation by poly(I:C) and IFN-β and that miR-155 expression is induced by other TLR ligands through MyD88- or TRIF-dependent signaling pathways (109). However, investigators found TLR-independent upregulation of mature miR-155 in the murine macrophage cell line J774A and murine primary bone marrow-derived macrophages during *Helicobacter pylori* infection (110). Authors reported that treatment with progesterone augmented LPS- and poly(I:C)-induced miR-155 expression in macrophages through inhibition of NF-κB activation and led to downmodulation of IL-6 and IFN-β production in TLR-activated macrophages by increasing SOCS1 expression (85). A recent study identified that miR-155 expression increased in monocyte-derived macrophages

upon TLR3/4 but not TLR7 stimulation and that inhibition of miR-155 expression partially restored infectivity in poly(I:C)-treated monocyte-derived macrophages (69). Furthermore, miR-155 in PBMCs of systemic lupus erythematosus patients was specifically upregulated by high-mobility group box 1 protein in a MyD88-dependent manner during induction of anti-double-stranded DNA antibody, which is the central pathogenic autoantibody involved in pathogenesis of systemic lupus erythematosus (82). Authors reported that cold exposure (32°C) induced miR-155 expression in human monocytes and that increased miR-155 expression was associated with suppressed SOCS1 and SHIP1expression (65). Additionally, miR-155 was upregulated in ischemic cerebral tissue and promoted TNF-α and IL-1β expression by upregulating TLR4 and downregulating SOCS1 and MyD88 (63). Negative regulator proteins for the TLR4 pathway (IRAK-M, SHIP1, and SOCS1) were upregulated in Kupffer cells isolated from miR-155–deficient mice (58). Researchers have shown that miR-155-3p and miR-155-5p (the two mature miRNAs processed from the precursor miR-155 transcript) were highly expressed in mice after treatment with LPS, whereas expression of both was decreased in the lungs of triggering receptor expressed on myeloid cells 1- (TREM-1) knockout mice. Deficiency of TREM-1 significantly inhibited neutrophils and proinflammatory chemokines and cytokines, particularly IL-1β, TNF-α, and IL-6 (80). Researchers also identified that protein kinase Akt1 activated by LPS positively regulates the expression of let-7e and miR-181c but negatively regulates that of miR-155 and miR-125b revealing that let-7e inhibits the expression of TLR4, whereas miR-155 inhibits the expression of SOCS1; both proteins TLR4 and SOCS1 are critical for TLR signaling after LPS stimulation (95). In another study, investigators showed that exposure to angiopoietin-1 significantly decreased IRAK1 and TRAF6 protein expression but did not affect TLR4, MYD88, IRAK4, or TAK1 expression in human umbilical vein endothelial cells (88).

THE ABILITY OF MIRNAS TO BIND TO TLRS

MiRNAs may bind to TLRs and activate TLRs involved in intercellular communication in the tumor microenvironment (12, 13, 111). Authors reported that guanosine- and uridine-rich single-stranded RNA oligonucleotides derived from HIV-1 and influenza virus are recognized by murine TLR7 and human TLR8. Subsequently, activation of DCs and macrophages lead to the production of proinflammatory mediators such as IFN-α and cytokines (112, 113). Recently reported evidence demonstrated that extracellular vesicles such as exosomes and shed microvesicles isolated from different cell types may be novel mediators of cell-cell communication these vesicles can contain mRNAs, miRNAs, long non-coding RNAs, lipids, and DNA fragments. These active cargo molecules are packaged and released in exosome-derived cells and taken up by neighbor cells, where they are functionally active (114–116). MiRNAs are ubiquitously expressed in exosomes and are involved in modulation of the host immune response, expression of some activated molecules, enhanced tumor cell invasion, and mediation of intercellular communication (12, 117).

In 2012, researchers discovered that tumor-secreted exosomes in supernatants of lung cancer cells and exosomes loaded with miRNAs are physiological ligands for TLR7 and TLR8 (9, 12, 13). Expression of miR-21, miR-27b, and miR-29a was higher in exosomes derived from lung cancer cells than in those derived from HEK-293 cells (13). Upon co-culture of HEK-293 and RAW macrophages *in vitro*, labeled exosomes released from HEK-293 cells were incorporated with RAW macrophages, and miR-29a co-localized with TLR7 and TLR8 in the RAW macrophages (9, 12, 13). In addition, these investigators reported that cancer cell-derived exosomal miRNAs can bind to and activate TLR8 in macrophages and stimulate TLR8-mediated activation of NF-κB and NF-κB–mediated release of the proinflammatory and prometastatic cytokines IL-6 and TNF-α (13). Therefore, malignant cells release signals via exosomes loaded with miRNAs to the surrounding cells in their microenvironments that promote tumorigenesis and dissemination by different TLRs in humans (TLR8) and mice (TLR7) (13, 118). In our recent study, we demonstrated that exosomal miR-1246 released in abundance from ovarian cancer cells and miR-1246 transmit molecular signals to M2-type macrophages but not M0-type macrophages by shuttling exosomes (119).

Moreover, treatment with liposome-encapsulated miR-21 significantly induced human TLR8 expression in hematopoietic cell lines and PBMCs obtained from patients with systemic lupus erythematosus (90). Single-stranded RNAs containing twenty-nucleotide guanosine- and uridine-rich regions derived from human immunodeficiency virus and the influenza virus are physiological ligands for TLR7 and TLR8 that bind to TLR and activate TLR signaling (113, 120). Furthermore, the let-7 family contains a specific GU-rich motif GUUGUGU, which is present in the core of single-stranded RNA40 and responsible for murine TLR7 activation (15, 113, 120). Researchers recently discovered that let-7 may interact with TLR7 and activate TLR signaling in murine macrophages and microglia (15). They found that six nucleotide exchanges in the seed sequence of let-7b dramatically diminished induction of TNF-α expression in microglia and macrophages. In addition to let-7b, let-7a, −7c, and −7g induced a dose- and time-dependent cytokine response in wild-type immortalized bone marrow-derived macrophages but not TLR7-deficient cells (15). Consistent with these findings in macrophages, these investigators showed that neuronal loss was induced by let-7a, let-7c, let-7g, and miR-599 through TLR7. To further understand the role of let-7 in neurodegeneration *in vivo*, Lehmann et al. (15) showed that treatment with let-7b significantly induced marked axonal injury and neuronal loss in wild-type mice, whereas mutant let-7b rescued this phenotype. In contrast, TLR7 deficiency in Tlr7 knockout mice can protect against let-7b's induced neurotoxic effects. These findings suggest that endogenous miRNAs such as let-7b can be released during neuroinflammation and may cause further spread of central nervous system damage in patients with neurodegenerative disorders such as Alzheimer disease (15, 121).

CONCLUSION AND FUTURE PERSPECTIVES

MiRNAs are strongly implicated to have roles in the development and progression of inflammation-related diseases. Increasing numbers of studies have identified that miRNAs can act as physiological ligands for TLRs. The next challenges are to understand the complex mechanisms behind these integrated networks of interactions and, more importantly, determine whether therapeutic modulation of TLR-regulated and TLR-regulating miRNAs is beneficial for patients with cancer or inflammatory diseases. At present, MRG-106 therapy, is an oligonucleotide inhibitor of miR-155, for being tested in phase 1 clinical studies in Cutaneous T-cell Lymphoma, Mycosis Fungoides, Chronic Lymphocytic Leukemia, Diffuse Large B-Cell Lymphoma and Adult T-Cell Leukemia/Lymphoma (clinicaltrials.gov identifier NCT02580552). Therefore, researchers expect more preclinical and clinical studies regarding this new therapeutic avenue (93, 122, 123).

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