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Non-coding RNA in immunotherapies and immune regulation

Edited by
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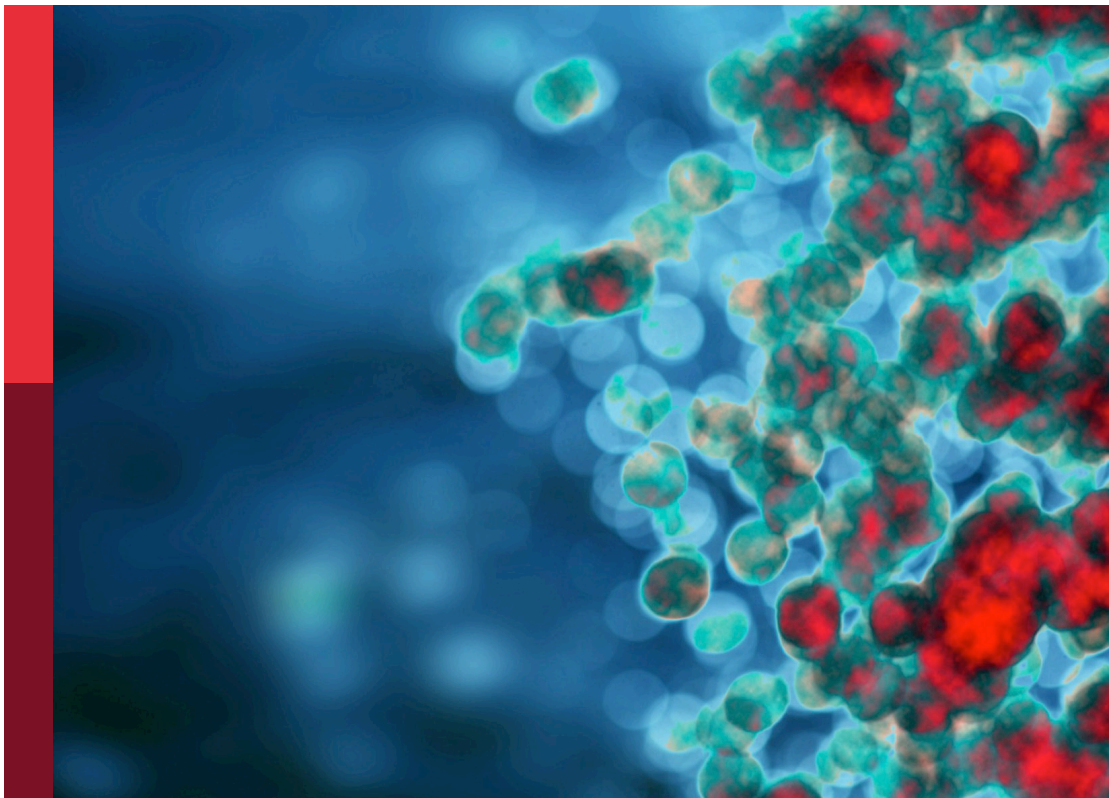

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Non-coding RNA in immunotherapies and immune regulation

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Editorial: Non-coding RNA in immunotherapies and immune regulation

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Editorial on the Research Topic
Non-coding RNA in immunotherapies and immune regulation

Introduction

The immune system is a key player in mammal homeostasis, where non-coding RNAs are involved in the differentiation and regulation of immune cells as well as in epigenetics mechanisms. Non-coding RNAs are divided into short-chain noncoding RNAs with a length of 18-200 nt and long-chain noncoding RNAs (lncRNAs) with a length >200 nt (1). Short-chain noncoding RNAs mainly include microRNAs (miRNAs), piwi-interacting RNAs, small nucleolar RNAs, small nuclear RNAs and repeat RNAs. The description of lncRNAs is in expansion, and some have been linked to epigenetic mechanisms. microRNAs bind to sequences with partial complementarity on target RNA transcripts, called microRNA recognition elements (MREs). Coding and non-coding RNA targets can cross-talk through their ability to compete for microRNA binding. Through MREs, transcripts can actively communicate to each other to regulate their respective expression levels with consequences on a large proportion of the transcriptome (2).

This e-book through the valuable contributions of the authors is paving the way for the development of RNA-based therapeutics in cancer, metabolic and infectious diseases (Figure 1).

Müller et al. propose that miR-125a-5p acts as a hub for endothelial barrier permeability and monocyte migration. Inflammatory stimulation of endothelial cells induces miR-125a-5p expression, which consecutively inhibits a regulatory network consisting of the two adhesion molecules VE-Cadherin and Claudin-5, two regulatory tyrosine phosphatases (PTPN1, PPP1CA) and the transcription factor ETS1. During macrophage polarization, miR-125a-5p also reduces the expression of M1 phenotype induced by LPS and promotes the expression of M2 induced by IL-4 by targeting

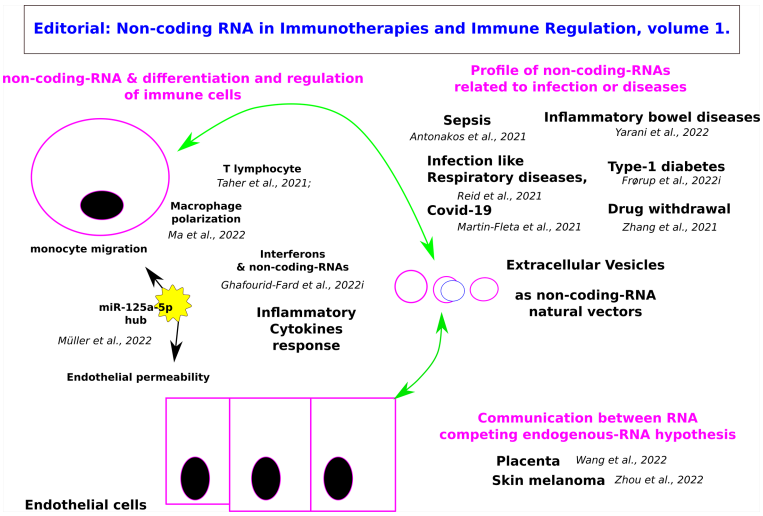

FIGURE 1
Graphical abstract of the Editorial: Non-coding RNA in Immunotherapies and Immune Regulation.

KLF13 to regulate the phagocytosis and bactericidal activity of macrophages (Ma et al.). Interferon-gamma (IFNG) is one of the most important mediators of immunity and inflammation and plays a key role in macrophage activation, inflammation, host defense against intracellular pathogens, Th1 cell response, and tumor surveillance. T-lymphocytes play a major role in adaptive immunity and current immune checkpoint inhibitor-based cancer treatments (Taheri et al.; Antonakos et al.; Ghafouri-Fard et al.). IFNG-antisense-1 (IFNG-AS1) is a lncRNA that participates in the regulation of IFN responses. Located downstream of the IFNG locus, its expression correlates strongly with the expression of IFNG. CD4+ and CD8+ T cells as well as NK cells express this lncRNA. The impact of lncRNAs on interferon signaling has also been assessed in the context of diabetes mellitus. Lnc10 contains a type I diabetes-associated single nucleotide polymorphism. This lncRNA can regulate the expression of the IRF7-driven inflammatory network regulating gene Ebi2 in immune cells IRF (Interferon Regulatory Factor). IFNG-AS1, lnc-ITSN1-2, lncRNA-CD160, NEAT1, MEG3, GAS5, NKILA, lnc-EGFR, and PVT1 are among lncRNAs that efficiently influence the function of T cells. Moreover, the effects of a number of circular RNAs, namely circ_0001806, hsa_circ_0045272, hsa_circ_0012919, hsa_circ_0005519 and circHIPK3 are ascertained in the modulation of T-cell apoptosis, differentiation, and secretion of cytokines (Taheri et al.). The circular RNAs, novel non-coding RNAs produced by reverse splicing of mRNA precursors, are currently explored for therapeutic applications (3).

The apoptosis, proliferation, or migration of endothelial cells, as well as the inflammatory status, are regulated by endogenous or microvesicle-derived miRNAs. For example, miR-155 increases in pulmonary endothelial cells of sepsis mice targeting the tight junction protein Claudin-1, and induces capillary leakage during infection (Antonakos et al.) but this miRNA is also involved in IFNG regulation (Ghafouri-Fard et al.). Likewise, platelet microparticles containing miR-223 reduce intercellular adhesion molecule 1 expression and binding to peripheral blood mononuclear cells by endothelial cells, providing a possible protective role against excessive sepsis-induced vascular inflammation. miR-223-3p regulates peroxisome proliferator-activated receptor-γ mediated M2 macrophage activation. Overall, many miRNAs are associated with the polarization/activity of M1 macrophages like miR-155-5p, and M2 macrophages like miR-125a-5p, and miR-223. Yarani et al. show that miR-223-3p, miR-16-5p, and miR-24-3p are upregulated in both mucosa and blood of Ulcerative Colitis patients. In Crohn Disease, miR-223-3p and miR-21-5p are upregulated in both mucosa and blood of patients. miR-223-3p is involved in the activation of granulocytes and is overexpressed in naive CD4+ T- lymphocytes. miR-223-3p abundance in macrophages can change macrophage activation and modulate the response to stimuli *via* effects on the TLR4/FBXW7 axis. miR-223-3p is now considered a biomarker in Intestinal Bowel Disease that seems to be conserved between different species.

Coronavirus Disease 2019 (COVID-19) pneumonia is a life-threatening infectious disease, especially for elderly patients with multiple comorbidities (4). Multivariate analysis displayed a combination of 4 miRNAs (miR-106b-5p, miR-221-3p, miR-25-3p and miR-30a-5p) that significantly discriminated between both pathologies (Martinez-Fleta et al.). miR-335-5p is

significantly downregulated in COVID-19. EGFR, a known target of miR-27b-3p, miR-146a-5p, miR-16-5p, miR-335-5p and miR-30a-5p, is found at higher levels in patients with COVID-19. Increased levels of the chemokine CXCL12 in Community-Acquired Pneumonia vs. COVID-19 patients are found. CXCL12, which is the CXCR4 ligand, is necessary for effective hematopoiesis, T cell, and memory B cell homing to the lymph nodes or monocyte recruitment. Inhibition of this axis is used by several viruses in order to increase their proliferation by reducing the number of circulating immune cells. Reid et al. have explored the potential role of Extracellular vesicles (EVs) as circulating inflammatory mediators which could propagate systemic inflammation and multimorbidity in response to shared risk factors including aging, inhaled noxious stimuli and respiratory infections. The intercellular transfer of EV miRNA has therefore been implicated in mediating a range of pathophysiological processes including in the development of COPD. Profiling miRNAs through Evs in the plasma of type-1 diabetic mothers has been done with hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p, and hsa-miR-30d-5p (Frørup et al.). The sorting mechanisms for MicroRNAs into Extracellular Vesicles and their Associated Diseases have been recently reviewed (5). Likewise, the dynamics of lncRNAs and mRNAs transported by EVs in Heroin addicts during acute and protracted withdrawal have been explored as a first step toward improved therapy (Zhang et al.).

In cancer, the potential diagnostic and prognostic markers related to Skin melanoma of the OIP5-AS1-hsa-miR-186-5p/hsa-miR-616-3p/hsa-miR-135a-5p/hsa-miR-23b-3p/hsa-miR-374b-5p-PTPRC/IL7R/CD69andMALAT1-hsa-miR-135a-5p/hsa-miR-23b-3p/hsa-miR-374b-5p-IL7R/CD69 ceRNA networks have been proposed (Zhou et al.). On placenta of mothers suffering from Pregnancy-related intrahepatic cholestasis, immune-related genes KLRD1, BRAF, and NFATC4 might have a potential ceRNA mechanism by individual lncRNA sponging miR372-3p, miR-371a-3p, miR-7851-3p, and miR-449a to control downstream the level of TNF-a, IFN-g, and IL-10 (Wang et al.).

In conclusion, RNAs are able to interact with DNAs, RNAs, and proteins bringing up biochemical networks within a single cell or connecting multiple cell types. Extensive validation studies will be needed to consider the side effects of RNA-based drugs (6), as well as detailed descriptions of the Competing Endogenous RNA network (ce-RNA) underpinning immune cell regulation. Designing new therapeutics from complex biological regulation driven by RNA machinery will benefit from the reappraisal of selective pressure on the machinery at the level of genome regulation or to ward off the cell from transposable elements or viruses (7). Likewise, designing new mathematical models of immunity (Bocharov et al.) integrating non-coding RNAs will be of utmost importance.

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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Conflict of interest

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Integration of Molecular Inflammatory Interactome Analyses Reveals Dynamics of Circulating Cytokines and Extracellular Vesicle Long Non-Coding RNAs and mRNAs in Heroin Addicts During Acute and Protracted Withdrawal

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Heroin addiction and withdrawal influence multiple physiological functions, including immune responses, but the mechanism remains largely elusive. The objective of this study was to investigate the molecular inflammatory interactome, particularly the cytokines and transcriptome regulatory network in heroin addicts undergoing withdrawal, compared to healthy controls (HCs). Twenty-seven cytokines were simultaneously assessed in 41 heroin addicts, including 20 at the acute withdrawal (AW) stage and 21 at the protracted withdrawal (PW) stage, and 38 age- and gender-matched HCs. Disturbed T-helper(T_h)1/T_h2, T_h1/T_h17, and T_h2/T_h17 balances, characterized by reduced interleukin (IL)-2, elevated IL-4, IL-10, and IL-17A, but normal TNF-α, were present in the AW subjects. These imbalances were mostly restored to the baseline at the PW stage. However, the cytokines TNF-α, IL-2, IL-7, IL-10, and IL-17A remained dysregulated. This study also profiled exosomal long non-coding RNA (lncRNA) and mRNA in the plasma of heroin addicts, constructed co-expression gene regulation networks, and identified lncRNA-mRNA-pathway pairs specifically associated with alterations in cytokine profiles and T_h1/T_h2/T_h17 imbalances. Altogether, a large amount of cytokine and exosomal lncRNA/mRNA expression profiling data relating to heroin withdrawal was obtained, providing a useful experimental and theoretical basis for further understanding of the pathogenic mechanisms of withdrawal symptoms in heroin addicts.

Keywords: heroin, withdrawal, exosome, non-coding RNA, cytokine

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INTRODUCTION

Globally, an estimated 40 million people with substance-use disorders (SUDs) require addiction treatment services, accounting for approximately 0.6 million drug-related deaths each year (1). To date, heroin remains one of the most abused illegal drugs in China. It is associated with a wide range of deleterious health problems and harmful economic consequences to individuals and communities (2). As an opiate drug, the administration of heroin suppresses some of the central nervous system (CNS) functioning, including heart rate, breathing, and blood pressure. The withdrawal effects are usually the opposite of the suppressive effects. In other words, instead of euphoria, reduced heart rate, and sedation, heroin addicts may experience irritable mood, anxiety, and rapid heart rate (3).

Dysregulated inflammation and increased incidence of infectious diseases like Acquired Immunodeficiency Syndrome, hepatitis, and pneumonia have been documented in heroin addicts (4). Previous studies also revealed that chronic heroin administration causes apparent suppression of some of the major components in the cellular immune system due to the presence of opioid receptors on immune cells (5, 6). Although, in the past decade, the effects of heroin and other opioids on cytokine production have been mainly investigated using cellular and animal models (7, 8), little information is available from studies on heroin addicts during withdrawal. It is noteworthy that some data from existing datasets exhibit contradictory results. For instance, a shift in T-helper(T_h)1/T_h2 cytokine balance toward the T_h2 response and a suppression of T_h1 cytokine tumor necrosis factor alpha (TNF-α) by heroin were often reported (9, 10), but some studies showed no effect or even an increase in TNF-α (11, 12). Similarly, the production of the T_h1 signature cytokine interferon gamma (IFN-γ) could be either enhanced or suppressed by various opioids in *in vitro* studies, whereas, in animal experiments, the IFN-γ production was often diminished after exposure to opioids (10, 13). Compared with the healthy controls (HCs), the T_h2 cytokine interleukin (IL)-4 was reported to be reduced significantly in teenage heroin addicts (14), but was also reported as unchanged in adult addicts (15). Also, the levels of the IL-10 were elevated in heroin addicts compared to controls (16). Nevertheless, it was significantly decreased in rodents within two hours after heroin administration (17). The comparison of *ex vivo* cytokine production after stimulation of whole blood between heroin users and healthy controls (HCs) revealed that heroin reduces IL-8 production, but IL-8 was reported to be significantly increased in methadone-maintained heroin addicts (12). Overall, the cytokine profiles for heroin use or abrupt cessation in individuals remain uncertain. Confounders such as age, BMI, duration of heroin exposure, or duration of withdrawal are rarely addressed.

With the advances in genome-wide sequencing and related technologies, recent studies demonstrated the influence of a systematic level regulatory network of non-coding RNAs in orchestrating a disease-specific signature (18). Long non-coding RNAs (lncRNAs) have attracted particular attention in research on the pathogenesis of various diseases, such as tumors, cardiovascular diseases, and neurodegenerative/psychiatric disorders (19). Abnormal lncRNA expression was detected not only in brain tissues but also in the peripheral blood and cerebrospinal fluid of patients with Parkinson's disease (20). In parallel, several studies have posited that lncRNAs BACE1-AS, 17A, 51A, and NDM29 may directly or indirectly increase amyloid β protein formation and play a major role in the pathology of Alzheimer's disease (21). Recent peripheral blood profiling studies also identified alterations in lncRNA expression patterns in patients with major depressive disorder (22, 23) and schizophrenia (24). In short, mounting evidence suggests that lncRNAs may comprise valuable diagnostic utilities and therapeutic targets in psychiatric disorders such as SUDs (25).

Exosomes are membrane-bound extracellular vesicles, usually 40–100 nm in diameter, presenting in blood, urine, saliva, cerebral spinal fluid, and breast milk (26). We and others have previously shown that the exosomal miRNAs are involved in the development of psychiatric withdrawal symptoms in patients with SUDs (27, 28), although the role of exosomal (Ex)-lncRNAs/mRNAs in SUDs remains undetermined. In addition, when evaluating the psychoneuroimmunologic factors involved in heroin addicts, there is a need to differentiate between the acute and protracted withdrawal (PW) stages. Acute withdrawal (AW) from heroin dependence induces a state of significant physiological activation and disruption of immunocompetence in heroin addicts, which seems to gradually reverse the immunological parameters to normal levels when withdrawal is sustained for an extended period (29). However, the possible mechanisms underlying molecular inflammatory interactome during heroin withdrawal are complicated and have not yet been well understood. Therefore, a careful examination of the transcriptome regulatory network, especially its impact on the immune system, is warranted.

In the present study, to investigate the molecular inflammatory interactome, particularly the cytokine associated transcriptome regulatory network in heroin addicts during withdrawal, we simultaneously profiled circulating cytokines and Ex-lncRNAs/mRNAs in plasma. We constructed gene co-expression networks based on differentially expressed Ex-lncRNAs/mRNAs in heroin addicts compared to HCs. To provide centrality between connected networks, we also identified several probable biological key drivers in the form of "hub" genes specifically associated with cytokine alterations. Consequently, our present approach uncovers the mechanism associated with the peripheral inflammatory cytokine and transcriptome regulatory network in developing withdrawal symptoms.

MATERIALS AND METHODS

Participants and Ethics Statement

A total of 79 male participants, including 41 heroin addicts currently undergoing withdrawal (n = 20 at the AW stage and n = 21 at the PW stage) and 38 healthy control subjects aged between 22 to 53, were recruited from a joint program of drug detoxification and rehabilitation in the First Affiliated Hospital of Kunming Medical University and the Kunming Drug

Rehabilitation Center between January 2018 and October 2019. All protocols and recruitment procedures described in the present study were approved by the Research Ethics Committee of the First Affiliated Hospital of Kunming Medical University (2018-L-42). All participants provided written informed consent before enrollment. A clear history of heroin use and current medication was obtained *via* self-report and verified with caregivers and was noted in the medical record when possible. Samples were collected as previously described (27). For the heroin addicts during withdrawal, inclusion criteria were as follows: (1) diagnosed with heroin dependence and (2) age between 20 and 55 years. Participants were excluded if (1) medical or neurological illness or trauma that would affect the central nervous system; (2) reported history of a seropositive test for human immunodeficiency virus or other infectious diseases; (3) severe endocrine, cardiovascular system, or history of loss of consciousness of more than 30 min; (4) take antibiotics for about a month.

Detection of Cytokine Levels

In brief, fresh whole blood samples from study participants were collected into 10 mL EDTA-2Na vacuum tubes and the blood was centrifuged at 1500 g for 15 min. The plasma was transferred into a new tube and centrifuged at 20,000 g at 4°C for 15 min. The supernatant was harvested, aliquoted and stored at −80°C until cytokine and other assays. Plasma was assessed for protein levels of 27 cytokines including bFGF (Basic Fibroblast Growth Factor), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IP-10, IFN-γ, MCP-1 (Monocyte Chemoattractant Protein-1), MIP (Macrophage Inflammatory Protein)-1α, MIP-1β, PDGF-BB (Platelet-Derived Growth Factor-BB), RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted), TNF-α and VEGF (Vascular Endothelial Growth Factor) using a Luminex Human Magnetic Assay Kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Standard curves were generated by Bio-plex Manager software to determine unknown sample concentration.

Exosome Purification and Characterization

The exosomes were isolated by size exclusion chromatography method as described previously with minor modifications (30). In brief, 2 ml of 0.8 μm-filtered plasma sample was purified using Exosupur® columns (Echobiotech, China). The exosome samples were eluted with Phosphate-buffered saline and 2 mL eluate fractions were collected. Fractions were concentrated to 200 μl by 100 kDa molecular weight cut-off Amicon® Ultra spin filters (Merck, Germany). Total RNAs containing lncRNAs from exosomes were isolated using the miRNeasy® Mini kit according to the manufacturer's protocol.

LncRNA and mRNA Expression Profiling by Deep Sequencing

A total amount of 1.5 μg RNA per sample was used as input material for rRNA removal using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). Sequencing libraries were generated using NEBNextR UltraTM Directional RNA Library Prep Kit for IlluminaR (NEB, USA) following manufacturer's recommendations. The RNA samples were barcoded by ligation with unique adaptor sequences to allow pooling of samples. The elution containing pooled DNA library was further processed for cluster generation and sequencing using a NovaSeq 6000 platform. Library quality was analyzed using a Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Raw reads were filtered using fastQC and aligned to the GRCh38 human genome assembly using HISAT2 (31). Annotations of mRNA and lncRNA in the human genome were retrieved from the GENCODE (v.25). The mRNAs and lncRNAs were quantified and analyzed using DESeq2 R package (32) and StringTie 1.3.1 (31), respectively. The number of mRNAs and lncRNAs were calculated if the fpkm >= 0.05. The mRNAs and lncRNAs with a *p*-value < 0.05 and log2 fold change > 2 were considered differentially expressed between groups.

RT-qPCR Validation

Reverse transcription and quantitative real-time PCR (RT-qPCR) was performed to validate the results of RNA-seq results. Total RNA extraction from the exosomes was performed with the miRNeasy® Mini kit (Qiagen, MD, USA) according to the manufacturer's instructions. The concentration and quality of the RNA was determined using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA). Single strand cDNA was synthesized with the PrimeScript™ RT reagent Kit (Takara, Dalian, China). The RT-PCRs were conducted on an ABI Prism 7500 HT using the TaqMan Fast Advanced Master Mix. Real-time PCR amplification was performed in triplicate and a negative control was included for each primer. The gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

Gene Ontology and KEGG Enrichment Analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the clusterProfiler R packages (33). Enrichment analysis uses hypergeometric testing to find GO entries that are significantly enriched compared to the entire genome background. Meanwhile, we used clusterProfiler R packages to find KEGG pathway that are significantly enriched compared to the entire genome background.

Statistical Analysis and Bioinformatics Analysis

All statistical analyses were performed in R 3.6.3 Statistical Software (R Core Team, 2020). All tests were two-tailed with alpha set at $p < 0.05$. The immunological data was significantly skewed and was analyzed using parametric tests. Specifically, analysis of variance (ANOVA) were implemented comparing all three groups, followed by pairwise *Student's tests*. Pearson's correlation coefficients and Spearman's rank correlation were implemented to analyze the correlations between heroin addicts' clinical characteristics and cytokines. Statistical analysis and significance were calculated using various tests and adjusted for multiple testing. Sequencing data have been deposited in the Gene Expression Omnibus database under GSE172306.

RESULTS

Clinical Characteristics

This study recruited 41 male heroin addicts, including 20 AW subjects at 7~14 days after the initiation of abstinence and 21 protracted PW subjects who had abstained from heron for approximately one year, and 38 age-matched healthy male volunteers. The main clinical characteristics of the study participants are summarized in **Table 1**. Although heroin was reported as the primary abused substance and all individuals in the two addicted groups had a diagnosis of Opioid Use Disorder, 25% of AW subjects (n = 5) and 28.6% of PW subjects (n = 6) were also methamphetamine users. Also, 45% of AW subjects (n = 9) and 61.9% of PW subjects (n = 13) used drugs *via* injection, whereas 50% of AW subjects (n = 10) and 33.3% of PW subjects (n = 7) used drugs *via* snorting. The others (n = 1 per group) used both routes. There were no significant differences for any variables, including age, BMI, substance-use history, and education level, between the two groups of heroin addicts and HCs.

Alterations in Cytokine Profiles in Heroin Addicts During Withdrawal

In the present study, to investigate the alterations of circulating cytokine associated with heroin addiction and withdrawal, we analyzed the concentrations of traditional cytokines in the peripheral blood from heroin addicts and age-matched HCs. Blood samples were collected, processed, and investigated using Luminex Human Cytokine 27-plex assay (**Materials and Methods**). In the cytokines measured, the IFN-γ and GM-CSF fell below the lower level of quantitation in > 20% of samples and were excluded from analysis. The analysis revealed that the levels of IL-1ra, IL-1β, IL-5, IL-12p70, IL-13, IL-15, MIP-1β, bFGF, and RANTES in the plasma of heroin addicts in the AW and PW stages were not significantly different from those in the plasma of the control group subjects (data not shown). Significantly lower levels of IL-2 (*adj.p* = 2.78E-03) and PDGF-BB (*adj.p* = 2.01E-05) were observed in AW subjects compared with those in HCs (**Table 2**). Simultaneously, significantly higher levels of IL-4 (*adj.p* = 1.59E-04), IL-10 (*adj.p* = 8.74 E-06), G-CSF (*adj.p* = 8.75E-09), Eotaxin (*adj.p* = 4.31E-09), MCP-1 (*adj.p* = 1.61E-05), IL-17A (*adj.p* = 3.85E-03), and VEGF (*adj.p* = 7.64E-07) were found in AW subjects than in the HCs (**Table 2**). A similar analysis was conducted for cytokine profiles in PW heroin addicts. Compared to HCs, there were significant fluctuations in the levels of IL-2 (*adj.p* = 0.047), IL-7 (*adj.p* = 1.58E-04), IL-10 (*adj.p* = 0.047), IL-17A (*adj.p* = 5.41E-03), MIP-1α (*adj.p* = 8.93E-03), and TNF-α (*adj.p* = 3.45E-03) in PW subjects (**Table 2**). We next compared the cytokine profiles of PW subjects with those of AW subjects. Significant differences in IL-2 (*adj.p* = 4.48E-03), IL-4 (*adj.p* = 1.59E-09), IL-10 (*adj.p* = 8.74E-06), IL-17A (*adj.p* = 3.85E-03), G-CSF (*adj.p* = 8.75E-09), Eotaxin (*adj.p* = 4.31E-09), MCP-1 (*adj.p* = 1.61E-05), MIP-1α (*adj.p* = 8.93E-03), PDGF-BB (*adj.p* = 7.25E-04), TNF-α (*adj.p* = 6.62E-4), and VEGF (*adj.p* = 7.64E-07) were detected (**Table 2**), suggesting that the majority of heroin acute discontinuation induced cytokine abnormalities (e.g., IL-4, Eotaxin, G-CSF, MCP-1, PDGF-BB, and VEGF) could be restored to normal range after a certain period of withdrawal (**Figures 1A–E**).

TABLE 1 | Demographic and Clinical Characteristics of all participants.

	Healthy Control	Acute Withdrawal	Prostrated Withdrawal	*p*
	(n=38)	(n=20)	(n=21)	
Age (year)	37.32 ± 8.29	37.7 ± 7.30	37.62 ± 8.7	0.7502
Marriage				1
Married	15 (39.5%)	8 (40.0%)	9 (42.9%)	
Unmarried	11 (28.9%)	6 (30.0%)	6 (28.6%)	
Divorced	12 (31.6%)	6 (30.0%)	6 (28.6%)	
Education				0.9597
Illiteracy	6 (15.8%)	4 (20.0%)	3 (14.3%)	
Primary school	14 (36.8%)	7 (35.0%)	7 (33.3%)	
Junior high school	10 (26.3)	6 (30.0%)	9 (42.9%)	
High school	6 (15.8)	2 (10/0%)	2 (9.5%)	
College	2 (5.3%)	1 (5.0%)	0	
Height (cm)	165.84 ± 7.38	166.65 ± 6.95	166.7 ± 6.76	0.8702
Weight (kg)	60.76 ± 9.23	60.34 ± 8.97	60.24 ± 7.77	0.9634
BMI	22.03	21.64	21.63	0.7513
Drug type				1
Heroin	NA	15 (75.0%)	15 (71.4%)	
Heroin & Meth	NA	5 (25.0%)	6 (28.6%)	
Route				0.6623
injection	NA	9 (45.0%)	13 (61.9%)	
snorting	NA	10 (50.0%)	7 (33.3%)	
both		1 (5.0%)	1(4.8%)	
Drug history (year)	NA	7.25 ± 3.8	7.2 ± 3.39	0.8859

Age-matched healthy male volunteers (HC, n=38) and heroin addicts including acute withdrawal (AW, n=20) subjects with 7~14 days after initiation of abstinence and protracted withdrawal (PW, n=21) subjects who had abstained from heron for approximately 1 year were recruited in this study. Values are showed as mean ± SD, medians (interquartile ranges) or numbers of participants (%). BMI, Body Mass Index; NA, Not Applicable.

TABLE 2 | Alterations of cytokine levels in heroin addicts (AW and PW) *versus* healthy controls (HC).

	mean	mean	mean	Healthy control *vs* Heroin AW			Healthy control *vs* Heroin PW			Heroin PW *vs* AW		
	Healthy Control	Heroin AW	Heroin PW	Fold change	*p.value*	*adj.p*	Fold change	*p.value*	*adj.p*	Fold change	*p.value*	*adj.p*
IL-2	7.02 ± 2.86	2.57 ± 4.93	14.17 ± 15.30	2.74	**9.26E-04**	**2.78E-03**	0.50	**0.0467**	**0.0467**	0.18	**2.98E-03**	**4.48E-03**
IL-4	3.58 ± 1.43	15.56 ± 4.80	4.86 ± 2.82	0.23	**4.58E-10**	**1.38E-09**	0.74	0.06	0.06	3.20	**1.05E-09**	**1.59E-09**
IL-5	13.85 ± 11.10	17.90 ± 28.34	21.71 ± 25.27	0.77	0.55	0.65	0.64	0.19	0.56	0.82	0.65	0.65
IL-6	2.16 ± 1.66	6.71 ± 6.12	6.85 ± 16.04	0.32	**3.77E-03**	**0.01**	0.31	0.20	0.29	0.98	0.97	0.97
IL-7	82.10 ± 27.61	236.96 ± 103.74	188.34 ± 100.88	0.35	**1.96E-06**	**5.89E-06**	0.44	**1.05E-04**	**1.58E-04**	1.26	0.14	0.14
IL-8	21.67 ± 12.07	21.63 ± 11.37	29.89 ± 13.75	1.00	0.99	0.99	0.73	**0.03**	0.06	0.72	**0.04**	0.06
IL-10	5.15 ± 4.11	39.11 ± 15.41	12.56 ± 15.31	0.13	**2.97E-08**	**8.91E-08**	0.41	**0.0461**	**0.0461**	3.12	**5.83E-06**	**8.74E-06**
IL-13	7.26 ± 8.53	5.81 ± 5.41	6.83 ± 6.04	1.25	0.43	0.82	1.06	0.82	0.82	0.85	0.57	0.82
IL-17A	14.03 ± 6.97	37.17 ± 17.67	22.02 ± 111.03	0.38	**1.12E-05**	**3.37E-05**	0.64	**5.41E-03**	**5.41E-03**	1.69	**2.56E-03**	**3.85E-03**
Basic FGF	261.77 ± 140.75	257.16 ± 75.53	302.48 ± 144.97	1.02	0.87	0.87	0.87	0.30	0.45	0.85	0.22	0.45
G-CSF	254.41 ± 92.42	839.87 ± 264.43	290.23 ± 107.94	0.30	**3.19E-09**	**8.75E-09**	0.88	0.21	0.21	2.89	**5.83E-09**	**8.75E-09**
Eotaxin	169.89 ± 85.67	914.96 ± 329.08	149.04 ± 57.95	0.19	**2.87E-09**	**4.31E-09**	1.14	0.27	0.27	6.14	**1.93E-09**	**4.31E-09**
MCP-1	63.13 ± 30.94	94.41 ± 30.14	49.02 ± 23.99	0.66	**4.30E-04**	**6.45E-04**	1.27	0.08	0.08	1.93	**5.52E-06**	**1.66E-05**
MIP-1α	4.02 ± 3.05	4.37 ± 1.21	6.39 ± 2.84	0.92	0.54	0.54	0.63	**4.59E-03**	**8.93E-03**	0.68	**5.95E-03**	**8.93E-03**
PDGF-BB	10095.47 ± 8850.47	2440.12 ± 1814.73	10243.75 ± 8533.97	4.14	**6.71E-06**	**2.01E-05**	0.99	0.95	0.95	0.24	**4.83E-04**	**7.25E-04**
TNFα	77.91 ± 18.43	69.50 ± 18.56	104.23 ± 33.11	1.12	0.11	0.11	0.75	**2.30E-03**	**3.45E-03**	0.67	**2.21E-04**	**6.62E-04**
VEGF	578.83 ± 547.43	1244.71 ± 498.93	405.09 ± 241.34	0.47	**3.81E-05**	**5.71E-05**	1.43	0.11	0.11	3.07	**2.55E-07**	**7.64E-07**

Values are showed as mean ± SD. P-values marked with bold indicate statistically significant differences between the groups.

However, several cytokines, IL-2, IL-7, IL-10, and IL-17A were dramatically altered at the AW stage, and could be only partially restored even a year after the initiation of abstinence (**Figures 1E–I**). In addition, it was noteworthy that the TNF-α and MIP-1α were only dysregulated in PW heroin addicts, not at the AW stage, but within 7~14 days after initiating abstinence (**Figure 1J**).

We then sought to examine the $T_h1/T_h2/T_h17$ balance in heroin addicts across two withdrawal stages. We computed the T_h1/T_h2, T_h1/T_h17, and T_h2/T_h17 using the ratios among the following three categories of cytokines, T_h1 (TNF-α or IL-2), T_h2 (IL-4 or IL-10), and T_h17 (IL-17A). Notably, the ratio of T_h1/T_h2 and T_h1/T_h17 was significantly decreased in AW subjects, relative to PW heroin addicts and HCs (**Figures 1K, L**). Conversely, the ratio of T_h2/T_h17 was significantly increased in heroin addicts at the AW stage, compared to the subjects in the other two groups (**Figure 1M**). Taken together, the analyses of cytokine profiles indicate a dramatic disturbance in the immune system function and a shift in $T_h1/T_h2/T_h17$ balance at the stage of acute discontinuation and a partial restoration after long-term withdrawal (**Table 3**).

We further analyzed the correlation between clinical characteristics (age, BMI, etc.) and the above dysregulated cytokines identified in heroin addicts. As shown in **Supplementary Table 1**, bFGF (ρ = -0.34, ρ = 0.0235) was significantly negatively correlated with the type of drug. IL-8 ρ = 0.34, ρ = 0.0387), bFGF (ρ = 0.42, ρ = 0.0085), MIP-1α (ρ = 0.34, ρ = 0.0001), and PDGF-BB (ρ = 0.36, ρ = 0.026) were significantly positively correlated with the routes of heroin administration. IL-7 (ρ = 0.36, ρ = 0.028), IL-13 (ρ = -0.32, ρ = 0.048), bFGF (ρ = 0.39, ρ = 0.0165), and PDGF-BB (ρ = 0.40, ρ = 0.0147) were significantly positively correlated with education. IL-7 is important in the homeostatic maintenance and peripheral expansion of naïve T cells. It was significantly linearly negatively correlated with age (ρ = -0.34, ρ = 0.0407), in line with the previous report showing that IL-7 might play a role in age-related changes (34). Furthermore, the ratios of T_h1/T_h17 and T_h2/T_h17 in the heroin addicts did not correlate with any variable, while the ratios of T_h1/T_h2 (TNF-α/IL-10, (γ= -0.34, ρ = 0.0401), and IL-2/IL-10 (γ = -0.33, ρ = 0.0434), were linearly correlated with the years of drug-intake (**Supplementary Table 1**).

Differential Expression Analysis of Ex-mRNAs and Ex-lncRNAs Between Heroin Addicts and Healthy Controls

To gain insight into the transcriptional dynamics of extracellular vesicles, we utilized the size exclusion chromatography approach to isolate exosomes derived from the peripheral blood of selected heroin addicts (n = 20) and HCs (n = 10) for RNA sequencing (27). Exosomes were characterized and verified using transmission electron microscopy, nanoparticle tracking analysis, and western blotting assays. CD63 and CD81 served as exosome surface markers, while Calnexin was used as a negative marker, and their expression patterns were verified by western blot results (**Supplementary Figure 1**).

Next, the total RNA of plasma exosomes extracted from heroin addicts and HCs were subjected to RNA sequencing using the Illumina NovaSeq platform. A total of 94, 361

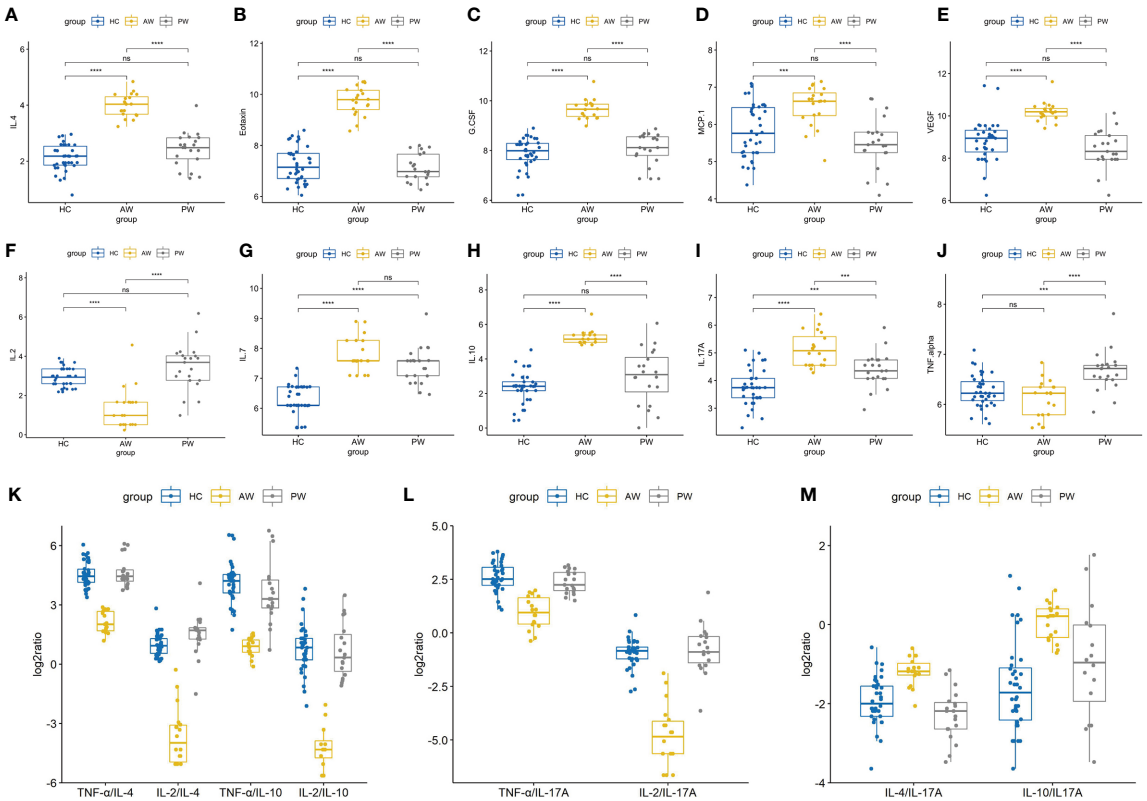

FIGURE 1 | Alterations in cytokine profiles in heroin addicts during withdrawal. **(A–J)** The levels of various cytokines in the plasma samples of healthy controls, heroin addicts from AW and PW groups was investigated, including IL-4 **(A)**, Eotaxin **(B)**, G-CSF **(C)**, MCP-1 **(D)**, VEGF **(E)**, IL-2 **(F)**, IL-7 **(G)**, IL-10 **(H)**, IL-17A **(I)**, TNF-α **(J)**. **(K–M)** Th1/Th2/Th17/Treg balance in heroin addicts across two withdrawal stages (AW and PW). The ratio of T_h1/T_h2, T_h1/T_{reg} **(K)** and T_h1/T_h17 **(L)** was significantly decreased in AW stage, and recovered in PW stage. The ratio of T_h2/T_h17 and T_{reg}/T_h17 **(M)** was significantly increased in AW stage, and recovered in PW stage. Each dot represents one patient in each group. Values of all significant correlations ($p < 0.05$) are given with degree of significance indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). All intergroup comparisons by Mann-Whitney U-test.

TABLE 3 | Th1/Th2/Th17 balance in heroin addicts across two withdrawal stages (AW and PW).

		mean	mean	mean	Healthy control *vs* Heroin AW		Healthy control *vs* Heroin PW		Heroin PW *vs* AW	
		Healthy Control	Heroin AW	Heroin PW	*p.value*	*adj.p*	*p.value*	*adj.p*	*p.value*	*adj.p*
Th1/Th2	TNF-α/IL-4	25.52 ± 12.28	4.63 ± 1.68	28.93 ± 17.83	**8.92E-13**	**2.68E-12**	0.4599	0.6899	**1.25E-05**	**3.78E-05**
	IL-2/IL-4	2.16 ± 1.04	0.14 ± 0.2	3.67 ± 3.44	**1.24E-14**	**1.18E-13**	0.0753	0.2260	**2.91E-04**	**6.56E-04**
Th1/Th17	TNF-α/IL-17A	6.7 ± 3.05	2.15 ± 1.04	5.48 ± 1.96	**6.01E-11**	**1.35E-10**	0.0718	0.2260	**5.29E-07**	**2.38E-06**
	IL-2/IL-17A	0.57 ± 0.28	0.06 ± 0.07	0.78 ± 0.78	**3.45E-14**	**1.55E-13**	0.2706	0.4871	**7.43E-04**	**1.34E-03**
Th1/Treg	TNF-α/IL-10	34.69 ± 56.64	1.92 ± 0.57	31.89 ± 48.08	**1.02E-03**	**1.02E-03**	0.8460	0.8763	**1.42E-02**	**1.58E-02**
	IL-2/IL-10	2.43 ± 2.64	0.05 ± 0.06	2.55 ± 2.84	**2.54E-06**	**4.57E-06**	0.8763	0.8763	**1.22E-03**	**1.84E-03**
Th2/Th17	IL-4/IL-17A	0.28 ± 0.12	0.45 ± 0.11	0.22 ± 0.10	**5.58E-06**	**8.37E-06**	0.0524	0.2260	**9.65E-08**	**8.68E-07**
Th2/Treg	IL-4/IL-10	1.19 ± 1.20	0.43 ± 0.09	0.82 ± 0.76	**4.31E-04**	**4.85E-04**	0.1607	0.3615	**4.16E-02**	**4.16E-02**
Th17/Treg	IL-17A/IL-10	4.92 ± 5.65	1.01 ± 0.33	5.42 ± 6.44	**1.36E-04**	**1.75E-04**	0.7742	0.8763	**7.91E-03**	**1.02E-02**

P-values marked with bold indicate statistically significant differences between the groups.

transcripts were assembled, including 25,932 mRNA (19,986 known and 5,946 new genes) and 6,8429 lncRNA transcripts (32,485 known and 35,944 putative new lncRNAs), which were then eventually filtered down to 12,158 mRNA and 10,297 lncRNA transcripts for final analysis. A total of 185 differentially expressed Ex-mRNAs exhibited a more than two-fold change, $p < 0.05$, of which 108 were upregulated, and 77 were downregulated comparing the AW and HC groups; they are indicated in a volcano plot and an MA plot (**Figures 2A, D**). In the comparison of the PW group and the HCs, a total of 186 differentially expressed Ex-mRNAs were identified, of which 57 were upregulated, and 129 were downregulated (**Figures 2B, E**). When comparing PW and AW, we identified a total of 181 differentially expressed Ex-mRNAs, of which 75 were

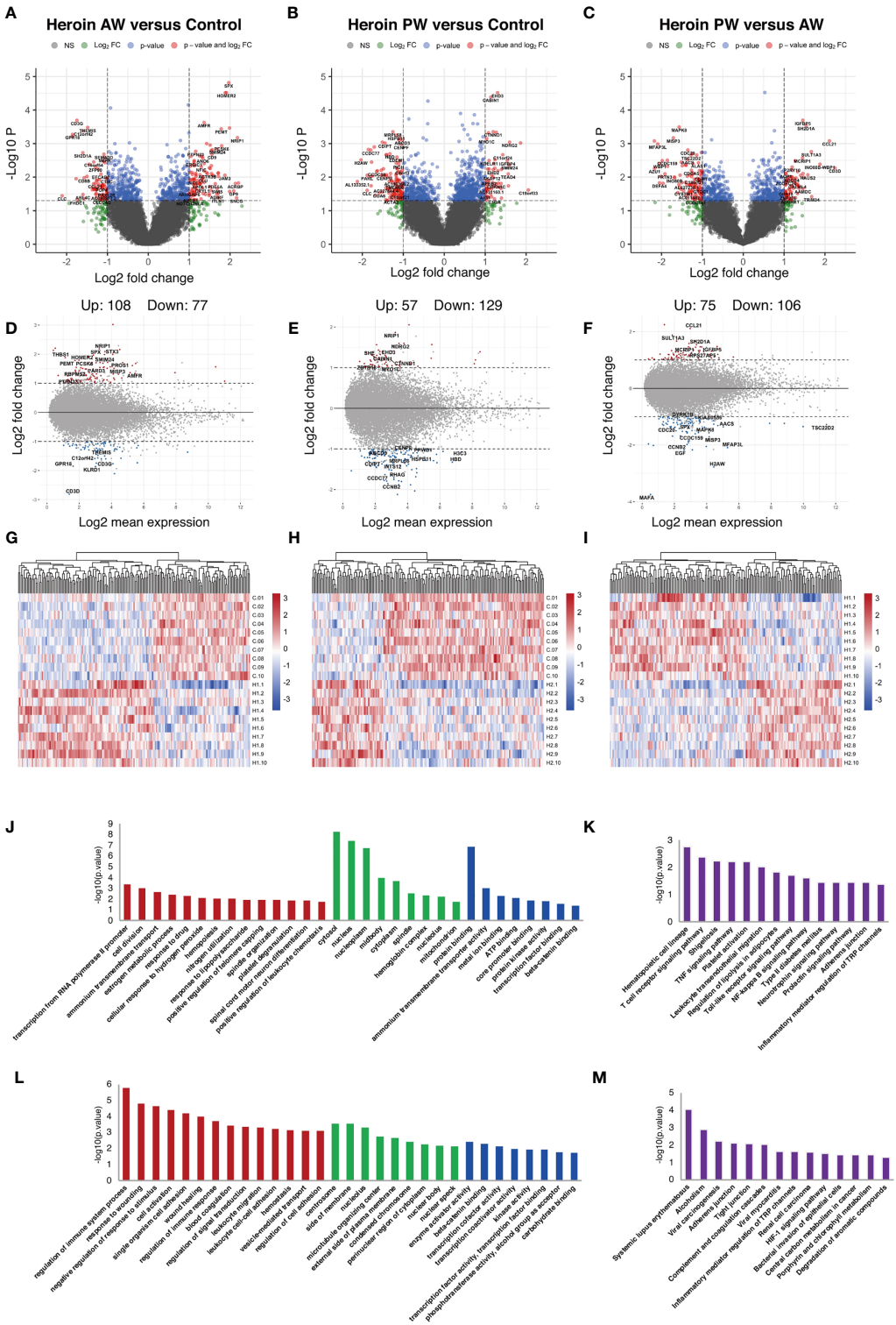

FIGURE 2 | Identification and functional enrichment analysis of differentially expressed Ex-mRNA. **(A, D, G)** The volcano plot and MA plot **(A, D)** and hierarchical clustering heatmap **(G)** of differentially expressed exosome mRNAs in Heroin AW versus HC ($P < 0.05$, fold change > 1.5). **(B, E, H)** The volcano plot and MA plot **(B, E)** and hierarchical clustering heatmap **(H)** of differentially expressed exosome mRNAs in Heroin PW versus HC ($P < 0.05$, fold change > 1.5). **(C, F, I)** The volcano plot and MA plot **(C, F)** and hierarchical clustering heatmap **(I)** of differentially expressed exosome mRNAs in Heroin AW versus Heroin PW ($P < 0.05$, fold change > 1.5). **(J–M)** Top enrichment of KEGG pathways related to differentially expressed exosome mRNAs in Heroin AW versus HC **(J, K)** and in Heroin PW versus HC **(L, M)**.

upregulated, and 106 were downregulated (**Figures 2C, F**). The differentially expressed Ex-mRNAs are shown in heatmaps (**Figures 2G–I**).

Similar analyses were performed for the Ex-lncRNAs, and we identified 231 lncRNA transcripts exhibiting a more than two-fold change, $p < 0.05$, 123 of which were upregulated, and 108 were downregulated when comparing the AW group and the HCs (**Figures 3A, D**). A comparison of PW group and the HCs shows that we identified 375 Ex-lncRNA differentially regulated transcripts, of which 143 were upregulated, and 232 were downregulated (**Figures 3B, E**). Moreover, when comparing PW and AW, we identified 365 differentially expressed Ex-lncRNA transcripts, of which 143 were upregulated, and 222 were downregulated (**Figures 3C, F**). These differentially expressed Ex-lncRNAs are shown in heatmaps (**Figures 3G–I**). Correspondingly, the fold changes of the 20 most significant differentially expressed EV-mRNAs and Ex-lncRNA are listed in **Supplementary Tables 2** and **3**, respectively.

Functional Annotation and Identification of Differentially Expressed Ex-mRNAs and Ex-lncRNAs

GO and KEGG pathway enrichment analyses were conducted to gain insight into the biological characteristics of the differentially expressed Ex-mRNAs. When comparing AW subjects to HCs, the GO terms associated with differentially expressed Ex-mRNAs are shown in **Figures 2J, K**: For the biological process, the differentially expressed Ex-mRNAs appeared to be most significantly enriched in *regulation of immune system process* (p = 1.64E-06), and *response to wounding* (p = 1.56E-05); for the cellular component, the most significantly enriched term was *centrosome* (p = 2.72E-04); and for the molecular function, the most significantly enriched term was *enzyme activator activity* (p = 0.0038) and *β-catenin binding* (p = 0.0047). The KEGG results indicated that differentially expressed Ex-mRNAs between the AW group and HCs were involved in *Hematopoietic cell lineage* (p = 0.0017), *T cell receptor signaling pathway* (p = 0.004), *Shigellosis* (p = 0.0058), and *TNF signaling pathway* (p = 0.0061).

As is shown in **Figures 2L, M**, the same analyses were applied to the differentially expressed Ex-mRNAs for the PW group and the HCs: For the biological process, the most significantly enriched term was transcription from RNA polymerase II promoter (p = 4.06E-04) and cell division (p = 9.14E-04); for the cellular component, the most significantly enriched term was cytosol (p = 4.97E-09); and for the molecular function, the most significantly enriched term was protein binding (p = 1.23E-07). Moreover, the KEGG analysis indicated that differentially expressed Ex-mRNAs in the PW group compared to the HCs were involved in *Systemic lupus erythematosus* (p = 8.60E-05) and *Alcoholism* (p = 0.0012).

GO and KEGG pathway enrichment analyses were conducted to investigate the potential function of genes related to differentially expressed Ex-lncRNAs. When comparing AW to HCs, the enriched GO terms associated with genes related to differentially expressed Ex-lncRNAs are shown in **Figures 3J, K**: For the biological process, the most significantly enriched term was *O-glycan processing* (p = 3.40E-04) and *cytoplasmic translation* (p = 3.71E-04); for the cellular component, the most significantly enriched term was *cytosolic large ribosomal subunit* (p = 6.74E-04); and for the molecular function, the most significantly enriched term was *extracellular matrix constituent, lubricant activity* (p = 2.63E-04, **Figure 3J**). The KEGG results indicated that genes related to differentially expressed Ex-lncRNAs between AW and HCs were mainly involved in *Phototransduction* (p = 0.0067) and *Ribosome* (p = 0.0099, **Figure 3K**).

The same analyses were applied to the differentially expressed Ex-lncRNAs between the PW group and the HCs (**Figure 3L, M**): For the biological process, the majority of differentially expressed Ex-lncRNAs appeared to be associated with *negative regulation of dendritic cell apoptotic process* (p = 3.15E-07), *negative regulation of interferon-β secretion* (p = 1.45E-06), and *positive regulation of γ-δ T cell activation involved in immune response* (p = 1.45E-06); for the cellular component, the most significantly enriched term was *proteinaceous extracellular matrix* (p = 1.76E-04); and for the molecular function, the most significantly enriched term was *HLA-A specific inhibitory MHC class I receptor activity* (p = 1.39E-06) and *serine-type endopeptidase activity* (p = 3.13E-06). Furthermore, the KEGG results indicated that differentially expressed Ex-lncRNAs in PW and HCs were significantly involved in *Osteoclast differentiation* (p = 1.65E-04), *Complement and coagulation cascades* (p = 0.004), and *Fc γ R-mediated phagocytosis* (p = 0.011). Altogether, these analyses indicate that the unique pattern of altered immune-related signaling pathways in AW and PW represents the pathogenesis of disrupted immunocompetence associated with heroin withdrawal stages.

Correlation Analysis of Differential Expressed Ex-lncRNAs/mRNAs and Cytokines

A transcriptome regulatory network was constructed using a combination of Ex-lncRNAs and Ex-mRNAs differentially expressed across various comparisons to explore the association and possible mechanism of inflammatory interactome between exosomal transcriptome and cytokine profiles during heroin withdrawal. We implemented a dynamic tree-cutting algorithm with a robust measure of pairwise interconnectedness in the WGCNA R package to construct a correlation network (35, 36). We then created a cluster tree and defined modules as branches (37–40). WGCNA analysis with a soft-thresholding power value equal to 7 revealed the co-expression of genes/transcripts in a total of five modules. Each module was assigned a unique color label: blue, turquoise, green, red, and brown, as shown under the cluster dendrogram (**Figure 4A**). The matrix with the module-trait relationships and corresponding p values between these five identified modules on group traits are shown in a heatmap (**Figure 4B**). The Module eigengenes (ME) were calculated and could represent each module. Among the five modules, the MEs of two modules MEblue (r = 0.79, p = 2.0E-11) and MEred (r = 0.87, p = 9.0E-10) were positively correlated with the heroin addicts in

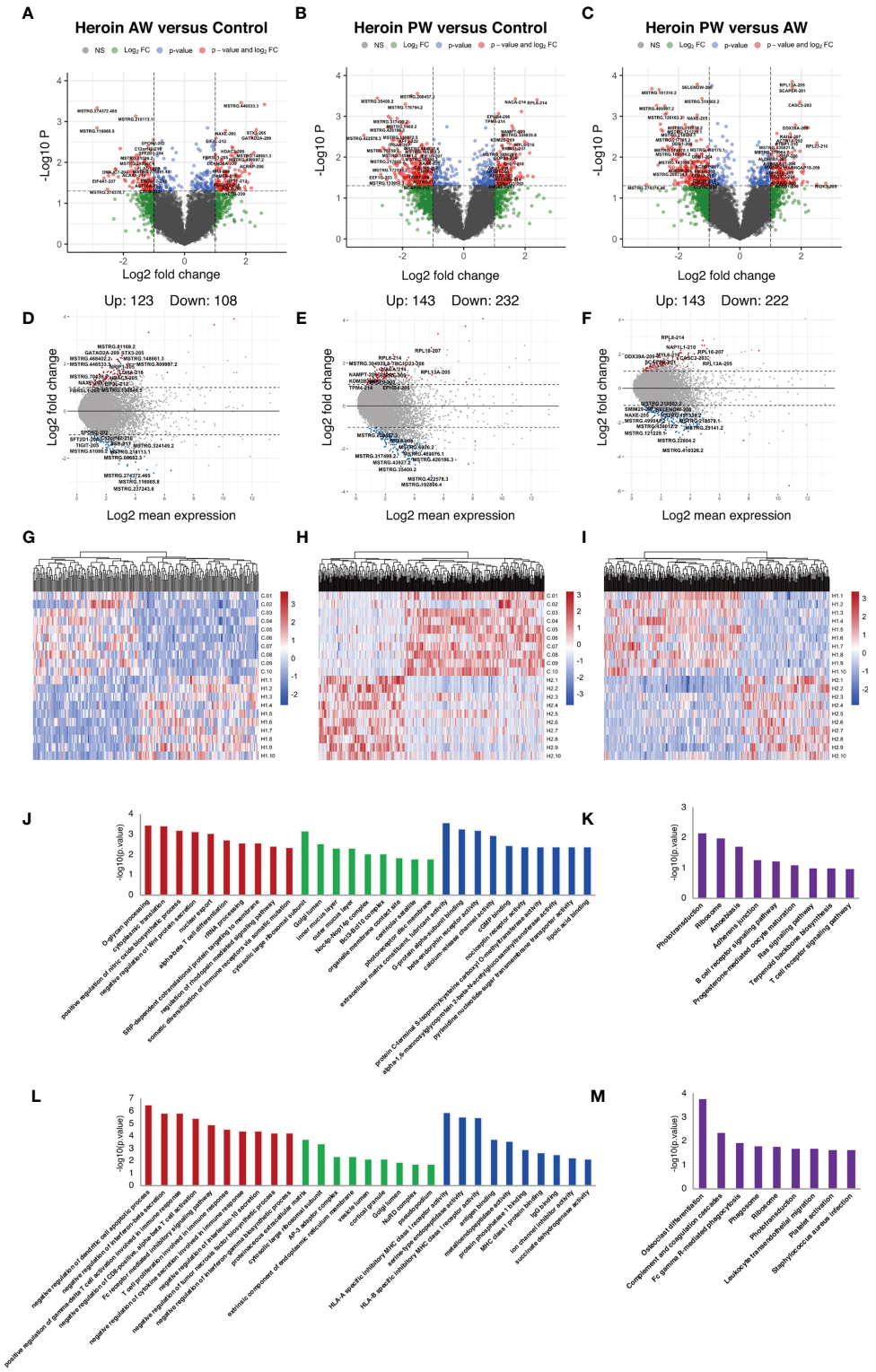

FIGURE 3 | Identification and functional enrichment analysis of differentially expressed exosome lncRNAs. **(A, D, G)** The volcano plot and MA plot **(A, D)** and hierarchical clustering heatmap **(G)** of differentially expressed exosome lncRNAs in Heroin AW versus HC ($P < 0.05$, fold change > 1.5). **(B, E, H)** The volcano plot and MA plot **(B, E)** and hierarchical clustering heatmap **(H)** of differentially expressed exosome lncRNAs in Heroin PW versus HC ($P < 0.05$, fold change > 1.5). **(C, F, I)** The volcano plot and MA plot **(C, F)** and hierarchical clustering heatmap **(I)** of differentially expressed exosome lncRNAs in Heroin AW versus Heroin PW ($P < 0.05$, fold change > 1.5). **(J, M)** Top enrichment of KEGG pathways related to differentially expressed exosome lncRNAs in Heroin AW versus HC **(J, K)** and in Heroin PW versus HC **(L, M)**.

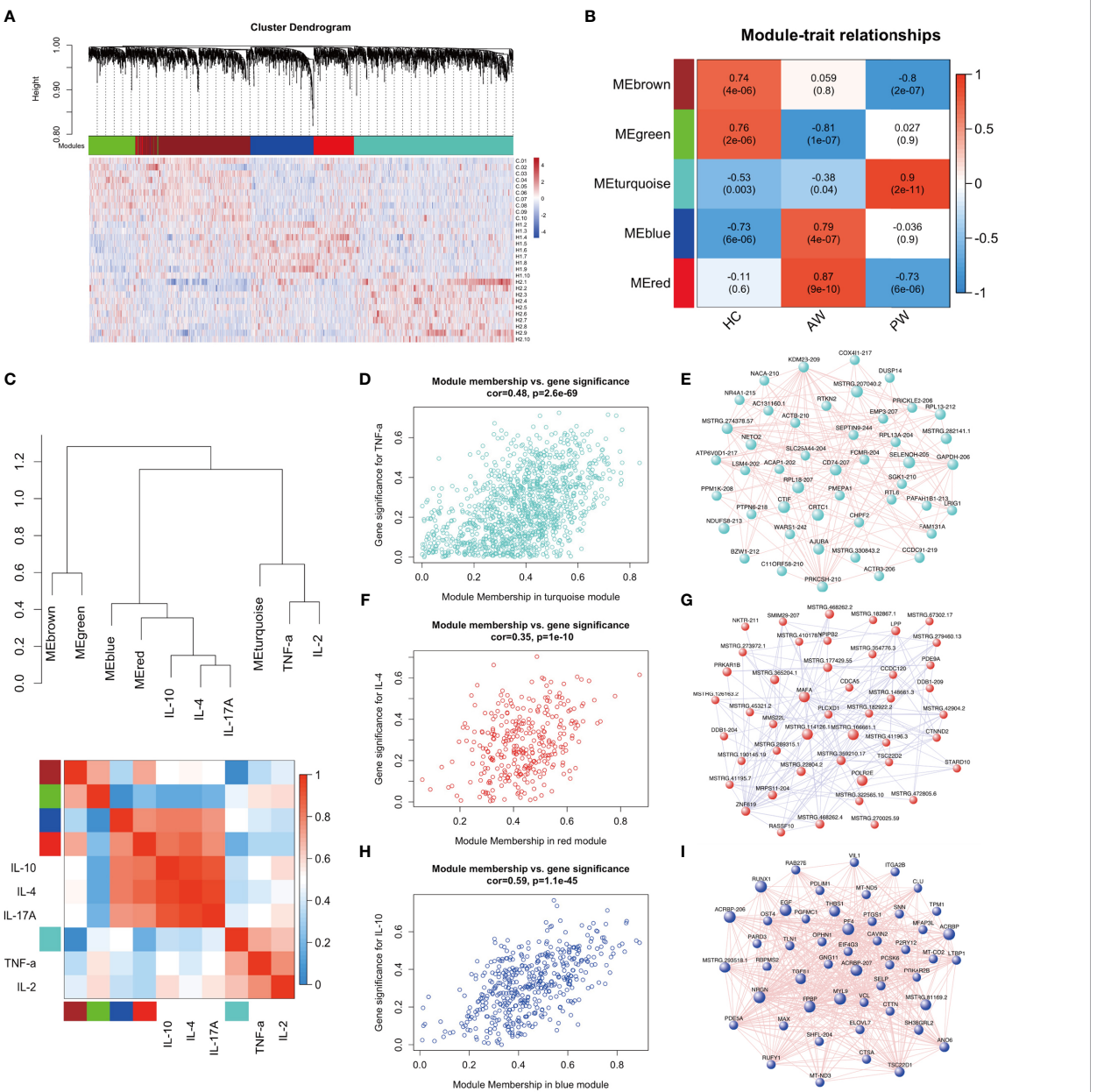

FIGURE 4 | WGCNA of plasma exosome samples uncovered dynamic changes of exosome mRNAs and lncRNAs signatures during substance withdrawal. **(A)** Hierarchical cluster dendrogram using WGCNA analyzing RNA sequencing data. A total of 5 modules were identified after 0.25 threshold merging. **(B)** Module trait correlation analysis revealed that 5 key modules were significantly correlated with Heroin AW and Heroin PW. **(C)** The eigengene dendrogram and heatmap showed the relevancies of cytokines and identified RNA-seq modules. TNF-a, IL-2, IL-4, IL-10 and IL-17A were selected. **(D)** The correlation and connectivity of the turquoise module and TNF-a (r = 0.48, p = 2.6E-69). **(E)** The hub-gene network of turquoise module. The network of Top 50 lncRNAs/mRNAs (Rankings related to turquoise module) were showed. **(F)** The correlation and connectivity of the red module and IL-4 (r = 0.35, p = 1.1E-10). **(G)** The hub-gene network of red module. The network of Top 50 lncRNAs/mRNAs (Rankings related to red module) were showed. **(H)** The correlation and connectivity of the blue module and IL-10 (r = 0.59, p = 1.1E-45). **(I)** The hub-gene network of blue module. The network of Top 50 lncRNAs/mRNAs (Rankings related to blue module) were showed.

the AW group, comprising of Ex-lncRNAs/Ex-mRNAs with relatively higher expression levels in the AW group compared to the control and PW groups. In another module, turquoise, the MEturquoise was positively correlated with the heroin addicts in the PW group (r = 0.9, p = 2.0E-11) and comprised transcripts with higher expression levels in the heroin addicts in the PW group relative to the other two groups. In contrast, in the other two modules, the MEgreen (r = -0.81, p = 1.0E-7) and MEbrown (r = -0.8, p = 2.0E-7) were correlated inversely with the AW and PW groups, respectively.

Next, we selected the TNF-α, IL-2, IL-4, IL-10, and IL-17A to determine their relevancies to those identified modules. As shown in **Figure 4C**, the eigengene dendrogram and heatmap indicated that the turquoise module was clustered with TNF-α and IL-2, whereas the red and blue modules were clustered with IL4, IL-10, and IL-17A, which were also supported by gene significance and module membership assays. The turquoise, red, and blue modules were selected for subsequent analyses. The turquoise module, which was comprised of 1,185 differentially expressed genes/transcripts including 497 Ex-lncRNAs and 688 Ex-mRNAs, displayed a strong correlation between gene significance for TNF-α and connectivity (r = 0.48, p = 2.6E-69, **Figure 4D**). The genes/transcripts in the turquoise module were ranked by their degree of gene co-expression connectivity, and the 50 genes with the highest connectivity were classified as candidate hub genes for further analysis, including CD74, major histocompatibility complex, class I, E (HLA-E), SELENOH-205, and RPL18-207 (**Figure 4E**). These genes were highly expressed in the heroin addicts of the PW group relative to the other two groups. Similar analyses were conducted on the red and blue modules. The blue (473 genes) and the red (322 genes) modules showed strong correlations between gene significance for IL-4 (r = 0.35, p = 1.1E-10, **Figure 4F**) and IL-10 (r = 0.59, p = 1.1E-45, **Figure 4H**) and their connectivity, respectively. Hub lncRNAs/mRNAs were then identified in the red module, including RNA polymerase II, I and III subunit E (POLR2E), MSTRG.462778.1, MSTRG.114126.1, and MSTRG.166661.1 (**Figure 4G**), and in the blue module, including pro-platelet basic protein (PPBP), platelet factor 4 (PF4), myosin light chain 9 (MYL9), prostaglandin-endoperoxide synthase 1 (PTGS1) and MSTRG.293518.1 (**Figure 4I**). These hub lncRNAs/mRNAs of the red and blue modules were significantly upregulated in the AW group of heroin addicts compared to the control and PW groups.

These hub lncRNAs/mRNAs were analyzed for their expression by qPCR. It was found that the lncRNAs/mRNAs from modules blue and red were significantly upregulated in the AW group of heroin addicts compared to the two other groups. The three lncRNAs/mRNAs from the turquoise module were significantly upregulated in the PW subjects compared to those from the other two groups (**Supplementary Tables 2** and **3**), consistent with the observations from RNA-seq analysis. To gain an insight into the function of immune-related lncRNAs and mRNA, we further examined the lncRNA/mRNA-pathway pairs identified in each module. We identified a lncRNA/mRNA-pathway regulatory network. This regulatory network involves 17 lncRNA, 43 mRNA, and 11 immune-related pathways. The majority of Ex-lncRNA/mRNA from red and blue modules and their targeted mRNAs were correlated with *Platelet activation*, *Chemokine signaling pathway*, and *Leukocyte trans-endothelial migration*. In contrast, the majority of Ex-lncRNA/mRNA from the turquoise module and their targeted mRNAs were significantly correlated with *Antigen processing and presentation*, *Fc γ R-mediated phagocytosis*, and *RIG-I-like receptor signaling pathway* (**Figure 5**). These results suggest a complex mechanism of a transcriptome regulatory network with distinct roles in modulating immune function between the AW and PW stages of heroin addiction.

DISCUSSION

Heroin addiction is attracting increasing attention from all elements of the basic, clinical, and public health science-related research communities. Accumulating evidence suggests that the non-coding RNAs involved in the transcriptome regulatory network play significant roles as immune regulators. However, research studies on the mechanism have been rare and there is still much to be learned concerning the molecular basis in the immunosuppression defects frequently observed in heroin addicts. In the present study, using luminex human magnetic assay and high-throughput sequencing technologies, we report the short-term and long-term alterations of circulating cytokines and Ex-lncRNAs/mRNAs in heroin addicts during substance withdrawal. After controlling for various factors (age, BMI, substance dependence history and education), we successfully identified a set of critical lncRNAs and mRNAs in circulating exosomes associated with $T_h1/T_h2/T_h17$ cytokine balance and switch in heroin addicts.

Over 20 years ago, Govitrapong et al. suggested that heroin addicts in short withdrawal periods experienced decreases in immune system functioning compared to healthy subjects (29). Since then, animal or *ex vivo* models revealed the immunomodulatory effects of heroin and other opioids on cytokine production (5). However, to date, clinical studies investigating immune responses to substance withdrawal and detoxification treatment in human subjects have been limited, and the results have been variable (12, 13). In the present study, it is evident that heroin AW could trigger the T_h2 cytokines IL-4 and IL-10, and diminish the T_h1 cytokine IL-2, indicating that heroin has a significant short-term damaging effect on a healthy immune balance, and that the effects of AW are mainly correlated with acute inflammation. Kelschenbach et al. reported that morphine withdrawal contributes to immunosuppression by polarizing T_h cells toward the T_h2 lineage, which is similar to our findings (41). The shift in the T_h1/T_h2 cytokine pattern could be reversed in mice by the opioid antagonist naloxone (42). Nevertheless, our results support the concept that the disturbed balance in the T_h1/T_h2 ratio contributes to the pathogenesis of heroin withdrawal symptoms. Furthermore, we found a high expression of IL-17A in heroin AW for the first time, indicating that the T_h17 cells were activated in the early withdrawal period. Zara et al. reported an increase in VEGF in heroin addicts, which is consistent with our data. The level of VEGF increased in heroin AW, indicating the protection of a vessel barrier during withdrawal (43). A prospective study reported that the platelet-lymphocyte ratio was increased in male heroin addicts. In contrast with our data, the level of PDGF-BB decreased in AW. Therefore, it seems that acute inflammatory processes play an important role in the pathophysiology of heroin addicts. However, they are suppressed in AW, perhaps as a self-protection mechanism in acute phase responses (44). Therefore, heroin AW is involved in immune dysregulation, presenting as synchronously activated pro- and anti-inflammation.

In previous studies, the recovery time needed to return to baseline levels varies. Withdrawal-induced immune suppression

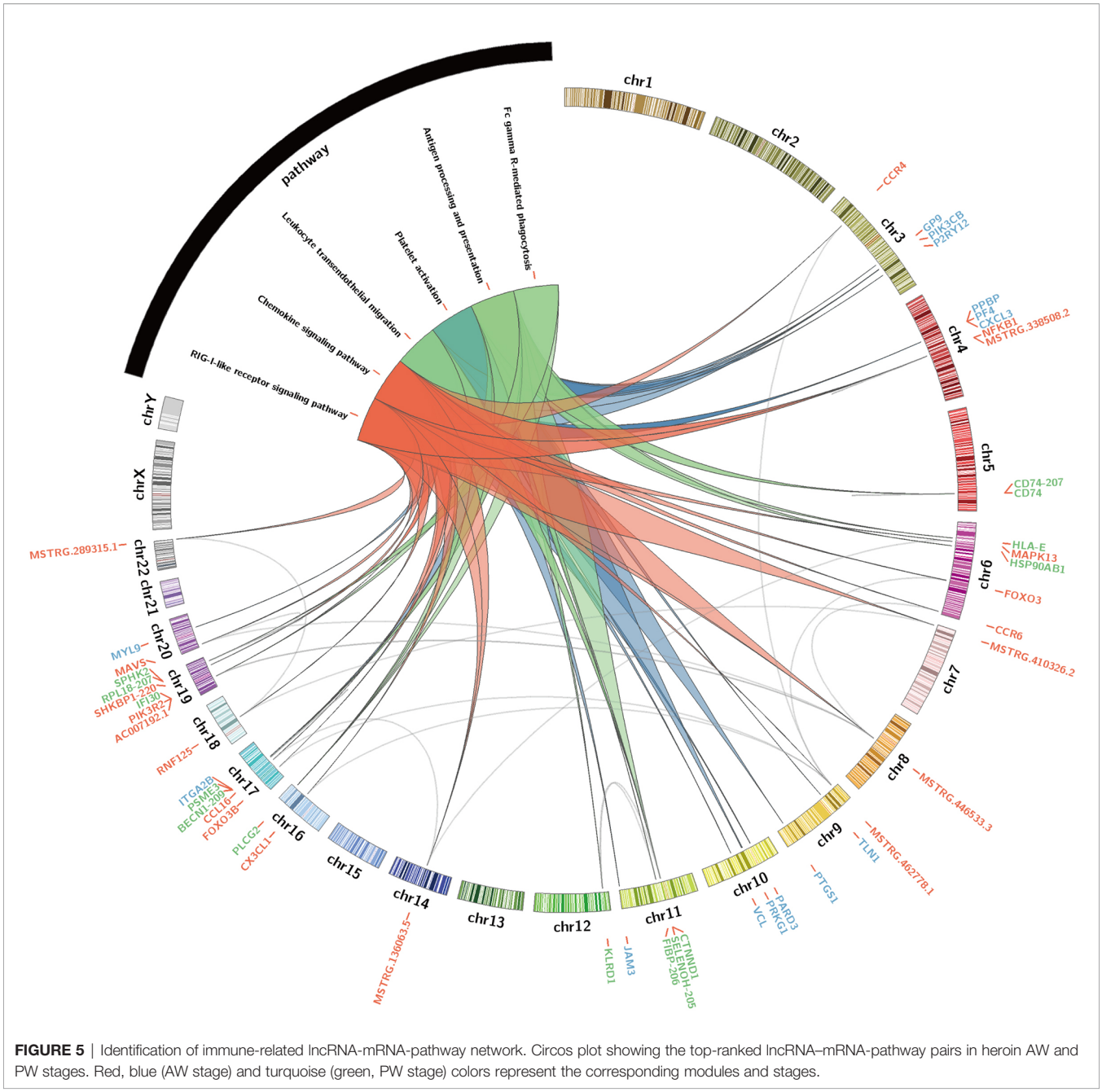

FIGURE 5 | Identification of immune-related lncRNA-mRNA-pathway network. Circos plot showing the top-ranked lncRNA–mRNA-pathway pairs in heroin AW and PW stages. Red, blue (AW stage) and turquoise (green, PW stage) colors represent the corresponding modules and stages.

could last for up to three years in some patients (45). Our study analyzed the alterations of circulating cytokines and the impact of heroin withdrawal on immune system function in a longer time dimension to elucidate the differential cytokine patterns in heroin addicts between the AW and PW groups. Surprisingly, although the dramatically disturbed $T_h1/T_h2/T_h17$ balance in heroin addicts at the AW stage has been mostly restored to the baseline around one year after the initiation of abstinence, the levels of cytokines of T_h1 (IL-2, *adj.p* = 0.0467; TNF-α, *adj.p* = 3.45E-03), T_h2 (IL-4, *adj.p* = 0.06; IL-10, *adj.p* = 0.0461), and T_h17 (IL-17A, *adj.p* = 5.41E-03) remained elevated in PW heroin subjects relative to HCs with a statistical significance or a considerable trend toward significance. TNF-α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for various signaling events. An increase in circulating TNF-α indicated a chronic inflammation state in heroin addicts even after a year of withdrawal. Meanwhile, the anti-inflammatory cytokine IL-10, which could be produced by virtually all T cells and could serve to antagonize the proinflammatory effects of other cytokines, thereby maintaining the immune balance, remained elevated in PW heroin subjects relative to HCs, suggesting the circulating levels of IL-10, a putative immune biomarker in surveying heroin withdrawal symptoms. Moreover, T_h17 cells mediate immune

responses against extracellular bacteria and fungi, and the dysregulation of IL-17 signaling has been implicated in the pathogenesis of autoimmune diseases and brain disorders. For example, IL-17 serum levels have been linked to morphine withdrawal syndrome in a rat model (46). Our study provides first-in-human evidence indicating that IL-17A may play an important role in the AW and PW stages. In short, we present data showing the dynamics of cytokines in regulating the immune system and inflammatory pathways during different periods of withdrawal. The potential role of these cytokines in immune response suggests that modulating cytokine may be an effective strategy in coping with withdrawal.

In recent years, small, nanosized vesicles (extracellular vesicles) have been identified as a new facet of micro-communication between different organs or cells in the human body, particularly in neurodegenerative and inflammatory diseases (47, 48). These vesicles gain more and more attention and are considered to serve as novel clinical biomarkers and as novel targets for therapeutic drug development (49). By analyzing the differential expression and the regulatory effects of lncRNAs, many studies have identified lncRNAs in association with Alzheimer's disease, schizophrenia, and depression (50), as well as rheumatoid arthritis and systemic lupus erythematosus (48). However, in the SUD field, the lncRNA biomarker and related mechanistic studies have been lacking. By exploring exosomal RNA contents from peripheral blood, the present study not only identified novel lncRNA biomarkers associated with withdrawal stages in heroin addicts but also revealed withdrawal-stage specific transcriptome regulatory networks.

We also investigated the inflammatory interactome and established associations between dysregulated cytokines and the lncRNA/mRNA co-expression network, which is of great significance to the in-depth study of the influence of heroin addiction and withdrawal on the immune system. To the best of our knowledge, this is the first study to simultaneously characterize the circulating lncRNA and cytokine profiling in plasma samples from heroin addicts and provide evidence that the cytokine-dependent transcriptome network is the functional link between a disturbed immune balance and differential acute and protracted heroin withdrawal symptoms. Moreover, identifying differentially expressed lncRNAs/mRNAs is a commonly used method to explore biomarkers or underlying mechanisms. We identified several novel candidates that may play critical roles in heroin withdrawal symptoms by regulating immune-related pathways. For example, major platelet-derived immune molecules, including the chemokine PF4 and PPBP (**Figure 5**), were significantly increased at the transcription level in AW heroin addicts. A similar situation was reported in a hypothermic mouse model (51). Considering that the functions of PF4 are complex, in generating pro- and anti-coagulant actions and differentially affecting immune cells (52), we speculate that in response to platelet activation during heroin AW, the platelet-derived PF4 and PPBP transcripts could be encapsulated in circulating exosomes and participate in monocyte recruitment and proinflammatory macrophage differentiation across multiple body sites. Acute inflammation is the protective response of body tissues to harmful stimuli caused by several kinds of tissue injury. The period of acute inflammation was tightly regulated by the immune system; the leukocyte trans-endothelial migration pathway was one of the regulating mechanisms, limiting vascular leakage during leukocytes across the endothelium and promoting leukocyte chemotaxis to the lesion site (53). Consistent with the level of VEGF, the leukocyte trans-endothelial migration pathway was enriched in AW. In addition, trans-endothelial migration of leukocytes involves the spatiotemporal regulation of chemokines (54), hence, *platelet activation, chemokine signaling pathway*, and *leukocyte trans-endothelial migration* point to acute inflammation triggering the process that modulates inflammatory reactions and assists immunocyte migration toward lesions.

Our results show that during heroin PW, the hub lncRNAs/mRNAs CD74, HLA-E, SELENOH-205, RPL18-207, and MSTRG.207040.2 in the turquoise module were significantly upregulated in PW heroin addicts and may also play critical roles in biological processes that are highly correlated with immune responses. CD74 is a cell-surface receptor for the cytokine macrophage migration inhibitory factor and is involved in antigen processing and presentation. MSTRG.207040.2 targets ABCA7 and ARHGAP45 (**Figure 5**). It has been reported that ABCA7 haplodeficiency disturbs microglial immune responses in the mouse brain, and that ARHGAP45 controls naïve T- and B- cell entry into lymph nodes and T cell progenitor thymus seeding (55). FcγR triggers inflammatory reactions in response to antigen-antibody complexes. It has been reported that the cytokine milieu, such as IL4/10/13, TNF-α, regulated FcγR-mediated uptake and inflammation (56). The combination with an *Antigen processing and presentation* enriched pathway suggests that an antigen-related immune response was reactivated by cytokines. INPP5D, also known as SHIP1, was one of the key factors of the FcγR activated receptor pathway. It is also a multifunction protein. In SHIP1 deficient mice, its loss affects platelet aggregation (57). INPP5D and PLCG2 both participate in the FcγR-mediated pathway. They are also involved in the progression of Alzheimer's disease, indicating that targeted INPP5D and PLCG2 may have potential in the treatment of heroin addicts (58, 59). Furthermore, MSTRG.136063.7, FIBP-206, MSTRG.7282.1, and IRF7-210 were increased, enriching the RIG-1-like receptor signaling pathway in PW. The activation of RIG-1-like receptor signaling was first identified in heroin addicts. RIG-1 is the important sensor of cytoplasmic viral RNA, and activation of the RIG-1-like receptor was an innate immunity pathway participating in response to viral RNA (60). In sum, it is a signaling pathway for anti-infection in the PW stage. Therefore, although the AW and PW stages are both correlated with immune responses, the molecular inflammatory interactome analyses suggest that unique lncRNA/mRNA-pathway regulatory network results can help to prioritize immune function-related lncRNAs/mRNA and provide a pathway-based view to improve our understanding of their regulatory function in heroin addiction and withdrawal.

A major advantage of this study is the well-balanced clinical characteristics of the two groups of heroin addicts and the HCs, resulting in a true reflection of the relative cytokine quantification in

certain withdrawal conditions. Similarly, lncRNA/mRNA in exosomes were enriched and quantitated using a validated protocol (30), offering an opportunity to dissect the correlations between dysregulated cytokine and transcriptome. However, this study has limitations. First, the study was limited by the relatively small sample size. Although we investigated a rather small number of patients, the characteristics of heroin addicts and HCs studied were well balanced, and the two groups of heroin addicts were representative of two typical withdrawal stages (61). This research should be further discussed and validated in large-scale or longitudinal studies. Second, cytokines are also important for brain development and have been implicated in the pathology of a series of brain diseases (47). The impact of heroin or other substances on the brain-immune-axis warrants further investigation.

Moreover, the characterization of the lncRNA-mRNA crosstalk network was mainly based on theoretically supported lncRNA-target relationships. WGCNA algorithm, this method has been widely used in high-throughput sequencing technologies, still has some shortcomings. WGCNA has the advantage of computing complex network relationships but suffers from the disadvantage of ignoring modularity in module identification process and no flexibility to adjust the algorithm in the calculation. For the important role of modularity in network clustering and community detection, high modularity raising more reliable clustering results (62). Considering that WGCNA cannot identify modules with overlapping genes. Once one gene is involved in multiple functional modules, it is difficult to classify it (39), therefore, additional experimental and clinical validation were required. Future studies are necessary to investigate the role of specific lncRNAs and their interactions with relevant mRNAs and to explore the impact of targeted therapeutics on heroin-induced disrupted immunocompetence and associated transcriptome regulatory network changes. In the future, co-administration of anti-inflammatory and anti-infection drugs might be an alternative and more efficient option for heroin addicts during withdrawal. For example, IL-6/7/10/17A inhibitors could be recommended during the entire withdrawal period. However, anti-IL-2 was only used in PW subjects, and the drugs for chemokines/platelet/EGF were only used in AW subjects. Anti-infection agents, especially antiviral infection agents are recommended in PW, depending on the circumstances.

In summary, our study provides a new concept of inflammatory interactome mediated by circulating cytokines and Ex-lncRNA/mRNA, indicating the value of exocellular vesicle encapsulated transcriptome profiling as a biomarker for heroin addiction and withdrawal. These findings open a wide range of future diagnostic and therapeutic options for opioid withdrawal management.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO, accession no: GSE172306.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the First Affiliated Hospital of Kunming Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZZ, HW, QP and JHY performed the experiments, researched the data, and wrote the manuscript. ZX, FC, YM, YZZ, YZ, JQY, CC, SL, YJZ, WWT, YX, HL, MZ and Y-QK researched the data and/or helped design experiments. JHY and KW designed the study, supervised all work, and helped write the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.730300/full#supplementary-material

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Plasma Exosome-Enriched Extracellular Vesicles From Lactating Mothers With Type 1 Diabetes Contain Aberrant Levels of miRNAs During the Postpartum Period

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Type 1 diabetes is an immune-driven disease, where the insulin-producing beta cells from the pancreatic islets of Langerhans becomes target of immune-mediated destruction. Several studies have highlighted the implication of circulating and exosomal microRNAs (miRNAs) in type 1 diabetes, underlining its biomarker value and novel therapeutic potential. Recently, we discovered that exosome-enriched extracellular vesicles carry altered levels of both known and novel miRNAs in breast milk from lactating mothers with type 1 diabetes. In this study, we aimed to characterize exosomal miRNAs in the circulation of lactating mothers with and without type 1 diabetes, hypothesizing that differences in type 1 diabetes risk in offspring from these groups are reflected in the circulating miRNA profile. We performed small RNA sequencing on exosome-enriched extracellular vesicles extracted from plasma of 52 lactating mothers around 5 weeks postpartum (26 with type 1 diabetes and 26 age-matched controls), and found a total of 2,289 miRNAs in vesicles from type 1 diabetes and control libraries. Of these, 176 were differentially expressed in plasma from mothers with type 1 diabetes (167 upregulated; 9 downregulated, using a cut-off of abs(log2FC) >1 and FDR adjusted p-value <0.05). Extracellular vesicles were verified by nanoparticle tracking analysis, transmission electron microscopy and immunoblotting. Five candidate miRNAs were selected based on their involvement in diabetes and immune modulation/beta-cell functions: hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p. Real-time qPCR validation confirmed that hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p, and hsa-

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miR-30d-5p were significantly upregulated in lactating mothers with type 1 diabetes as compared to lactating healthy mothers. To determine possible target genes and affected pathways of the 5 miRNA candidates, computational network-based analyses were carried out with TargetScan, mirTarBase, QIAGEN Ingenuity Pathway Analysis and PantherDB database. The candidates showed significant association with inflammatory response and cytokine and chemokine mediated signaling pathways. With this study, we detect aberrant levels of miRNAs within plasma extracellular vesicles from lactating mothers with type 1 diabetes during the postpartum period, including miRNAs with associations to disease pathogenesis and inflammatory responses.

Keywords: extracellular vesicles, exosomes, miRNAs, plasma, small RNA-Seq, type 1 diabetes

INTRODUCTION

Type 1 diabetes is an immune-mediated disease, characterized by immune-cell targeting of the insulin producing beta cells of the pancreatic islets of Langerhans, leading to their demise and resulting insulin deficiency (1, 2). Several studies have emphasized the occurrence of residual beta-cell mass, years after diagnosis, suggesting a potential for beta-cell preservation and regeneration in type 1 diabetes (3–5). This calls for further exploration into understanding the molecular drivers in type 1 diabetes. And while this to date remains largely unknown, it has been well established that some environmental and genetic risk factors can both contribute to and protect against disease development (2, 6).

Remarkably, the risk of developing type 1 diabetes in offspring of parents with preexisting type 1 diabetes seems to be different dependent on which parent is affected, i.e. the frequency is lower if the mother has type 1 diabetes, compared to the father (7), indicating an alteration in the genetic or molecular milieu in women with type 1 diabetes during pregnancy and postpartum. This could associate to the major molecular changes occurring during and after pregnancy, e.g. pro-inflammatory cytokines, c-peptide levels, pregnancy-associated growth hormones and lactating hormones, affecting both the woman and the developing fetus or breastfed infant (8, 9). Some changes are normalized at delivery or shortly thereafter, while others purposely persist (8). Interestingly, breastfeeding has shown to be a protective factor against the infant's risk of developing type 1 diabetes (10–12). However, the molecular mechanisms underlying this remain largely unexplored, and has never been studied in a setting comparing healthy women and women with preexisting type 1 diabetes. We speculate that circulating factors hold the ability to modulate type 1 diabetes risk in offspring of mothers with type 1 diabetes, still reflected after delivery.

Recently, we investigated the breast milk from lactating mothers with and without with type 1 diabetes, focusing on the exosome-enriched extracellular vesicle microRNA (miRNA) signature (13). We found that the breast milk from mothers with type 1 diabetes carry altered levels of both known and novel miRNAs, compared to healthy mothers, and that these miRNAs were associated with potentially immunomodulatory effects in the breastfed infant (13). Several other studies have previously highlighted the implication of distorted miRNA levels in the pathogenesis of type 1 diabetes (14–18). Particularly, the exosomal miRNAs are currently under investigation for their use as potential disease biomarkers (19, 20).

miRNAs target around 60% of all transcribed genes, exerting their function within the intracellular compartments of various cells (21, 22). However, they are also extensively being transported out of the cells in membrane-bound particles, broadly known as extracellular vesicles: the ~30-150 nm exosomes; the ~100-1,000 nm microvesicles; and the up to 5,000 nm large apoptotic bodies (19, 23, 24). While apoptotic bodies are thought to merely be a product of decayed cell packaging, microvesicles, arising from pinching off the cell membrane, and exosomes, formed by intracellular invagination, are both actively released by the cells. These are known to carry a broad variety of molecular cargo; protein, lipid and RNA-species, including unique miRNA profiles (19, 23). Therefore, these vesicles have been linked to cell-to-cell signaling, transporting specific and actively selected miRNAs from their parent cell to a recipient cell or tissue, while ensuring high abundance, availability and protection against degradation of the miRNAs (25, 26).

In this study, we aimed to characterize putative differences in circulating miRNA species in mothers with or without type 1 diabetes during the postpartum period. We hypothesized that differences in type 1 diabetes risk in offspring from these groups are reflected in the circulating miRNA profile. To investigate this, we extracted extracellular vesicles and its miRNA content from plasma samples of the study participants and performed small RNA sequencing (RNA-Seq) with unique molecular identifiers (UMIs). With this novel technology, we identify more than 2,000 miRNAs, 176 being differentially expressed in lactating mothers with type 1 diabetes.

Abbreviations: ARF6, ADP-ribosylation factor 6; CFH, complement factor H; HSP70, heat shock protein 70; INS, insulin; IPA, Ingenuity Pathway Analysis; MAFA, MAF bZIP transcription factor A; MDS, multidimensional scaling; miRNA, microRNA; MR1, major histocompatibility complex class I-related gene protein; NTA, nanoparticle tracking analysis; Pmaip1, phorbol-12-myristate-13-acetate-induced protein 1; RNA-Seq, RNA-sequencing; TEM, transmission electron microscopy; TNF, tumor necrosis factor; UMIs, unique molecular identifiers.

MATERIALS AND METHODS

Study Design and Sample Collection

The study was approved by the Ethical Committee for the Capital Region, Denmark (H-4-2013-008) and performed in accordance with the Declaration of Helsinki. All the participants signed the informed consent. The study included 52 lactating mothers; 26 mothers with pre-existing type 1 diabetes and 26 healthy mothers. Inclusion criteria were healthy, normal birth-weight infants born at gestational age ≥ 37 weeks and continuing breastfeeding. Exclusion criteria were type 2 diabetes, smoking, and complications during delivery. All mothers were recruited from Herlev and Gentofte Hospital and Rigshospitalet. None of the participants were treated for infections, inflammation or presented with symptoms thereof. None of the participants suffered from diabetes related complications. Clinical characteristics (age, BMI, blood glucose, HbA_{1c}, insulin dose, gestational age at delivery) were collected for all participants [**Table 1** (13)]. The blood samples were collected around five weeks after delivery from a cubital vein into EDTA Vacutainer tubes and all variables were measured. The samples were immediately centrifuged at 2,500 x g at 4°C for 10 min. and plasma were collected and stored in aliquots at −80°C prior to experimental processing and analysis. The study design is outlined in (**Figure 1**).

Extraction and Characterization of Plasma Exosome-Enriched Extracellular Vesicles

Briefly, 3 ml plasma per sample was diluted with 3 ml 1X PBS (pH 7.5) followed by centrifugation at 5,000 x g for 3 min. Next, 3.5 ml of the supernatant was filtered with a nitrocellulose filter (0.8 μm pore size) (VWR, Radnor, PA, USA). The extracellular vesicles were extracted by ExoEasy Serum Plasma Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Vesicle concentration per ml and size distribution were determined using Malvern NanoSight LM10 instrument (Malvern Panalytical technologies, Malvern, UK) and analyzed using nanoparticle tracking analysis (NTA) 3.1 Build 3.1.46. Extracellular vesicles were diluted in 1X PBS buffer for the analysis. Visualization of vesicles was carried out using transmission electron microscopy (TEM) by staining with 2% phosphotungstic acid and imaged with a CM 100 electron microscopy at 100 kV.

The presence of exosome particles was determined by immunoblotting of exosome markers CD63, CD9, CD81 and heat shock protein 70 (HSP70) and microvesicle markers Annexin A1 and ADP-ribosylation factor 6 (ARF6) in the plasma-derived extracellular vesicle samples from mothers with and without type 1 diabetes. Proteins were extracted after vesicle disintegration with cold RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein concentrations were determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA) and 25 μg/well were denatured with 2 μl dithiothreitol solution (AppliChem, Germany) and/or 5 μl NuPage LDS buffer (Thermo Fisher Scientific), dependent on protein of interest, at 75°C for 10 min. Immunoblotting was carried out by electrophoresis with Bolt 4–12% Bis-Tris Plus gels (Thermo Fisher Scientific) in MES running buffer (Thermo Fisher Scientific). Proteins were transferred onto a 0.45 μm nitrocellulose membrane (Thermo Fisher Scientific) and blocked in 5% skim milk in Tris-buffered saline with 0.1% Tween 20. Membranes were probed with primary antibodies for exosome surface markers: anti-CD63 diluted 1:500, anti-CD9 diluted 1:500, anti-CD81 diluted 1:1000, anti-HSP70 diluted 1:3000, anti-Annexin A1 diluted 1:1000, anti-ARF6 diluted 1:500 (all from Invitrogen, Carlsbad, CA, USA) and following secondary probing with HRP-conjugated anti-rabbit IgG antibody (Cell Signaling, Danvers, MA, USA) diluted 1:2000 and anti-mouse IgG antibody diluted 1:1000 (Cell Signaling). Bands were visualized by chemiluminescence with LumiGLO and peroxide reagents (Cell Signaling) and a FUJI LAS4000 Imager (GE Healthcare Chicago, IL, USA).

TABLE 1 | Clinical characteristics with mean ± SD and calculated p-values between the 52 participants; lactating mothers with type 1 diabetes (n=26); lactating healthy mothers (n=26).

Clinical characteristics			
Characteristic	**Control (n=26)**	**type 1 diabetes (n=26)**	**p-value**
Age (years)	31.8 ± 4.5	32.2 ± 4.8	0.74
BMI (kg/m^2)	26.9 ± 4.5	27.0 ± 4.4	0.95
BG (mmol/l)	5.6 ± 0.7	10.0 ± 4.9	6.78E-05
HbA_{1c} (mmol/mol)	27.7 ± 4.1	42.4 ± 8.1	7.30E-08
Insulin dose (IU/24 hours)	–	31.7 ± 12.3	–
Sampling (days after delivery)	36.9 ± 8.5	35.8 ± 7.7	0.76
Gestational age at delivery (weeks)	39.8 ± 1.2	37.9 ± 0.6	1.84E-06

BMI, body mass index; BG, blood glucose.

Small RNA Sequencing and Analysis

The exosomal RNA was extracted by exoRNeasy Serum Plasma Kit (Qiagen), and small RNA sequencing was performed by Qiagen's QIAseq miRNA sequencing platform (NextSeq 500), generating only miRNA specific UMIs. Prior to the small RNA library preparation, the quality of RNA extracted from exosome-enriched extracellular vesicles was assessed by miScript II RT Kit (Qiagen) and miScript SYBR Green PCR Kit (Qiagen) following manufacturer's instructions (**Supplementary Figure 1A**). In addition, RNA sample quality was assessed using miScript miRNA QC PCR Array (Qiagen) (**Supplementary Figure 1B**). Hemolysis was checked with the assessment of the relative expression of the erythrocyte specific hsa-miR-451a and plasma stable hsa-miR-23a-3p (27). Libraries were prepared using QIAseq miRNAseq library kit following the manufacturer's instructions. The raw fastq files were analyzed using GeneGlobe Data Analysis Software, and the reads were processed as follows. First, the miRNA entries were calibrated based on identical or near-identical sequences in miRBase mature database. Reads were then processed by trimming off the 3' adapter and low-quality bases using cutadapt (cutadapt.readthedocs.io/en/stable/

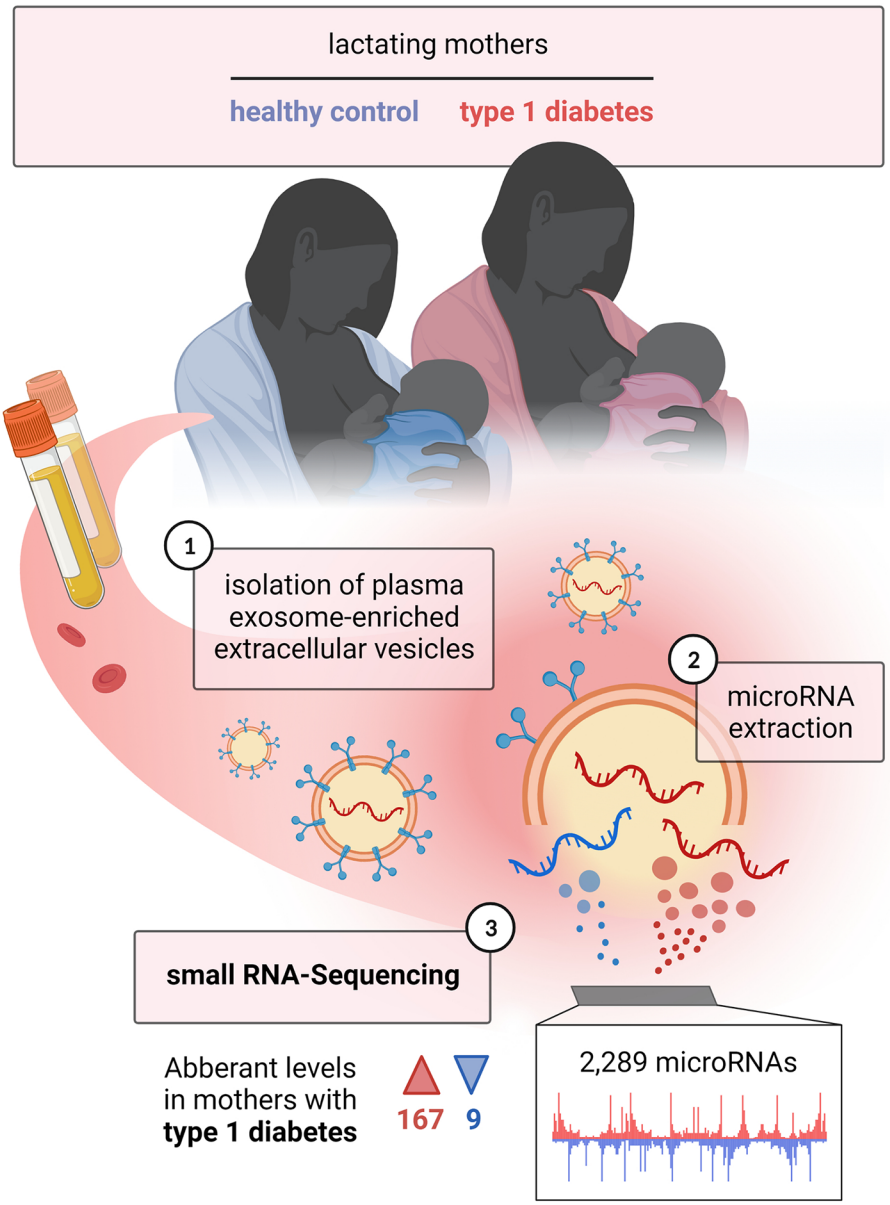

FIGURE 1 | Study overview. Lactating mothers with and without type 1 diabetes were included in the study, from whom blood samples were taken; 1) isolation of plasma exosome-enriched extracellular vesicles was performed; 2) microRNAs were extracted; 3) small RNA sequencing was carried out. The figure is intended to briefly summarize the study design, workflow and results in a simplified schematic. Created with BioRender.com

guide.html). Following trimming, UMI sequences were identified. Reads with less than 16 bp insert sequences or less than 10 bp UMI sequences were discarded. Reads were aligned using bowtie (bowtiebio.sourceforge.net/index.shtml), with up to two mismatches allowed. Read counts for each RNA category (miRBase mature, miRBase hairpin, piRNA, tRNA, rRNA, mRNA and other RNA) were calculated from the mapping results using miRBase V21 and piRNABank. All reads assigned to a particular miRNA were counted, and the associated UMIs were aggregated to count unique molecules. The UMI reads were normalized using Trimmed Mean of M (TMM) method in edgeR (28). To filter the lowly expressed miRNAs, a cut-off of UMI >10 in at least 40% of the samples was used. The differential expression analysis was performed on the two groups using GLM approach in edgeR. The differentially expressed miRNAs were identified using a cut-off of abs(log2FC) >1 and FDR adjusted p-value <0.05. The sample size (n=26 in each group) had a power >85% to demonstrate a 0.5-fold change between groups with a significance level of 5%. All statistical analyses were conducted using various bioconductor packages in R (29).

miRNA Expression Profiling by Real-Time qPCR in Plasma

To verify the RNA-Seq data, we wanted to select few miRNA candidates based on top hit differentially expression and known roles in immune-modulating pathways and beta-cell functions, for validation by real-time qPCR: hsa-miR-127-3p (477889_mir), hsa-miR-146a-5p (478399_mir), hsa-miR-26a-5p (477995_mir), hsa-miR-24-3p (477992_mir) and hsa-miR-30d-5p (478606_mir). Limited material available after exosome isolation allowed this to be carried out on 46 of the 52 samples (n=23 for each group). miRNAs were extracted and purified from the plasma of mothers with and without type 1 diabetes using the miRNeasy serum/plasma kit (Qiagen). RNA was quantified by NanoDrop Spectrophotometry (Thermo Fisher Scientific) and cDNA synthesis was performed using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific). TaqMan Advanced miRNA Assays and TaqMan Advanced Master Mix (Thermo Fisher Scientific) were used to validate the 5 selected miRNAs by real-time qPCR on the thermal cycler CFX384 system (Bio-Rad), with conditions: 20 sec. at 95°C and 40 cycles of 1 sec. at 95°C and 20 sec. at 60°C. Data were analyzed using the $2^{-\Delta Ct}$ method (30) and normalized to a geomean of stable and highly expressed reference genes: hsa-miR-16-5p (477860_mir) and hsa-miR-30e-5p (479235_mir).

Network and Pathway Analysis of miRNA Targets

The target genes for the 5 differentially expressed miRNAs that were selected for validation were retrieved using TargetScan (31) and mirTarBase (32). In total, 36,665 targets were retrieved for the 5 miRNAs: hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p. Targets that were common between TargetScan and miRTarBase (n=431) were selected for further analysis. To identify potential molecular interactions, networks and relationships for these 5 differentially expressed miRNAs, we next performed the Core Analysis using QIAGEN Ingenuity Pathway Analysis (IPA) software (33). The network of differentially expressed miRNAs and their targets was visualized and grouped using the IPA module Path Designer. The PantherDB classification system (34) was used to determine pathway based functional annotations of the gene targets belonging to the 5 miRNAs. This was done using a binomial test, with FDR for multiple corrections.

Statistical Analysis

All the statistical analyses and graphs were created using bioconductor packages in R and GraphPad Prism version 8. Correlations between the differentially expressed exosome-enriched extracellular vesicle-derived miRNAs from plasma (log2UMIs) and HbA_{1c} (mmol/mol) of mothers with type 1 diabetes were calculated using a two-tailed Pearson's correlation and adjusted p-value <0.05 by correcting for multiple testing with Benjamini-Hochberg procedure. Real-time qPCR-based validation of selected miRNA candidates was evaluated using Student's *t* test. P-values <0.05 were considered statistically significant. Validation data are expressed as means ± SEM.

RESULTS

Study Population Characteristics

The clinical characteristics of the study population of the 52 mothers are presented in (**Table 1**). There was no difference in age or body weight between the two groups of mothers. Expectedly, blood glucose and HbA_{1c} were different between the groups. Also, gestational age at delivery was lower for women with type 1 diabetes. Quality control showed stable hsa-miR-451a and hsa-miR-23a-3p ratios, verifying that samples were not affected by hemolysis.

Characterization of Exosome-Enriched Extracellular Vesicles Derived From Plasma

To investigate the particle population enriched in the plasma-derived extracellular vesicle samples, we performed NTA and TEM. Size and concentration of the extracellular vesicles were specified by NTA to cover vesicles, including adsorbates and electric double layer, with a mean hydrodynamic diameter of 240 nm and concentration of 3.7 x 10^9 particles/ml (**Supplementary Figure 2A**). TEM verified the enrichment of particles smaller than 200 nm in diameter (**Supplementary Figure 2B**). The expression of exosome surface markers CD63, CD9, CD81 and HSP70 were verified in the plasma-derived extracellular vesicles by immunoblotting (**Supplementary Figure 2C**). Microvesicle surface markers Annexin A1 and ARF6 were not detectable in the plasma-derived extracellular vesicle samples (data not shown). This supports that the isolation predominantly comprises exosome particles. Antibodies were validated in supplementary samples to account for specificity bias. Strong non-specific binding was detected by the ARF6 antibody.

miRNA Expression Profiles From Exosome-Enriched Extracellular Vesicles Derived From Plasma

From small RNA-Seq, a yield of miRNA-specific UMIs of 1.5 million were obtained. Library sizes, sample correlations and multidimensional scaling (MDS) plot are shown in (**Supplementary Figures 3A–C**). Quality control results are shown in (**Supplementary Figure 1**). A total of 2,289 miRNAs were detected in the libraries from the two groups using a cut-off of >10 UMI counts in at least 40% of the samples. In total, 176 differentially expressed miRNAs were identified when comparing lactating mothers with type 1 diabetes with lactating healthy mothers; 167 upregulated; 9 downregulated [**Figure 2A**, abs (log2FC) ≥1, FDR adjusted p-value <0.05].

hsa-miR-146a-5p, -26a-5p, -24-3p and -30d-5p Are Upregulated in Plasma of Mothers With Type 1 Diabetes

We selected 5 significantly upregulated miRNAs based on disease relevance and a known role in beta cell and immune modulation for validation. By real-time qPCR, hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p were validated in plasma. hsa-miR-146a-5p; 2.1-fold p<0.001, hsa-

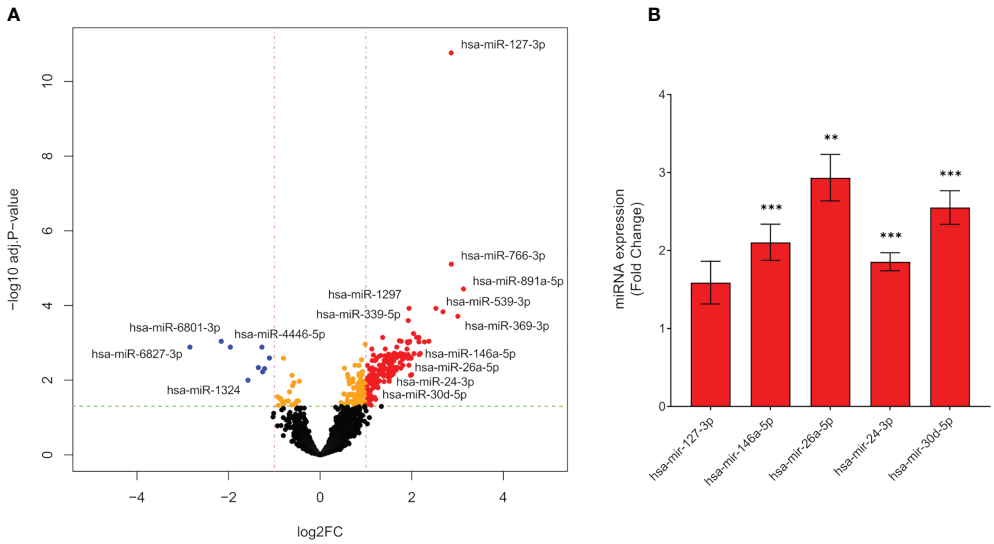

FIGURE 2 | Differentially expressed miRNAs from lactating mothers with type 1 diabetes compared to lactating healthy mothers. **(A)** Volcano plot of differentially expressed miRNAs in mothers with type 1 diabetes compared to control mothers by RNA-Seq (upregulated miRNAs: red dots, downregulated miRNAs: blue dots). Dotted red lines represent the log2FC cut-off., dotted green lines represent -log10 adjusted p-value cut-off (n=52). **(B)** mRNA expression by real-time qPCR of miRNA hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p in plasma from mothers with type 1 diabetes compared to control. The mRNA expression levels are presented as Fold Change +/- SEM based on $2^{-\Delta Ct}$ (n=46), **p < 0.01, ***p < 0.001.

miR-26a-5p; 2.9-fold p<0.01, hsa-miR-24-3p; 1.9-fold p<0.001 and hsa-miR-30d-5p; 2.6-fold p<0.001, were significantly upregulated in plasma from mothers with type 1 diabetes (**Figure 2B**). There was no difference in the level of hsa-miR-127-3p between the groups.

Differentially Expressed Exosome-Enriched Extracellular Vesicle-Derived miRNAs Correlate Positively With HbA_{1c} in Mothers With Type 1 Diabetes

We next investigated the effect of the differentially expressed miRNAs on the clinical characteristics, BMI, blood glucose and HbA_{1c}. Of the 176 differentially expressed miRNAs, 5 miRNAs showed a positive correlation to HbA_{1c} in mothers with type 1 diabetes (hsa-miR-6839-5p; hsa-miR-891a-5p; hsa-miR-1260a; hsa-miR-7977; hsa-miR-874-3p) after adjusting for multiple testing with Benjamini-Hochberg procedure, with a positive correlation above 0.6 (adj.p<0.05; **Supplementary Table 1**). However, as HbA_{1c} is known to be highly affected after delivery, introducing a bias to this observation, we did not pursue further analysis of these candidates. There was no significant association observed for miRNAs with BMI and blood glucose levels of the mothers with type 1 diabetes.

Network and Pathway Analysis of miRNA Targets

To understand the potential molecular interactions for the 5 selected miRNAs (hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p), we performed a network-based analysis using IPA (**Figure 3A**). IPA network analysis identified a network consisting of 26 nodes (genes) and 79 edges (interactions). For the 5 miRNAs we used IPA Path Designer tool to find the interacting partners which were grouped into three main pathways, i) Inflammatory response, ii) Cytokine and Chemokine mediated signaling and iii) Diabetes, based on their functional annotations. The diabetes-related notes for the 5 miRNAs were: the major histocompatibility complex class I-related gene protein (MR1), MAF bZIP transcription factor A (MAFA), insulin (INS), complement factor H (CFH), phorbol-12-myristate-13-acetate-induced protein 1 (pmaip1) and tumor necrosis factor (TNF). The entire network had a core around TNF, with 35 edges associated to TNF alone.

We next performed pathway-based enrichment analysis of all target genes for the 5 selected miRNAs using PantherDB annotations. In total, 431 targets commonly identified by both TargetScan and miRTarBase were used for the pathway analysis (**Figure 3B**). The pathway analysis identified 11 significantly enriched pathways for the selected miRNA target genes. The top two significant pathways included Angiogenesis and CCKR signaling, further supporting the results from IPA analysis, suggesting a potential role of these miRNAs in cytokine-mediated inflammatory responses.

DISCUSSION

In the present study, we analyzed the miRNA profiles derived from plasma exosome-enriched extracellular vesicles by a newly developed sequence-based technique (QIAseq) designed to detect each copy of the miRNA present, with high specificity,

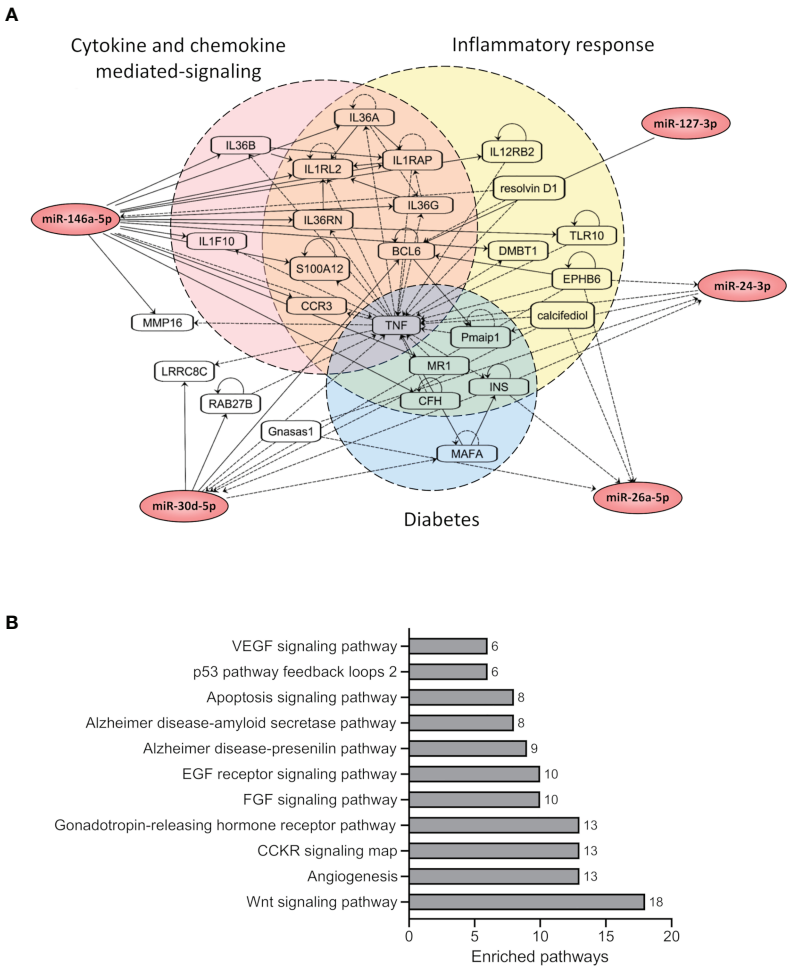

FIGURE 3 | Network and pathway-based analysis of miRNA targets revealed enrichment for cellular signaling pathways. **(A)** The figure shows the network of the target genes associated with selected 5 miRNAs (hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p, hsa-miR-30d-5p, and hsa-miR-127-3p). The target genes were grouped based on their pathway annotations in IPA. **(B)** The figure shows enriched pathways associated with the target genes of the selected 5 miRNAs based on PantherDB annotations. Only significant pathways with FDR <0.05 are shown. The total number of target genes from the input list associated with each enriched pathway are also shown.

sensitivity and dynamic range, through the integration of UMIs (35). QIAseq is one of the most efficient sequencing methods for miRNAs. It provides higher miRNA enrichment than other available small RNA sequencing technologies (36). With this method, we detected a total of 2,289 miRNAs and identified 176 differentially regulated miRNAs in plasma from lactating mothers with type 1 diabetes as compared to lactating healthy mothers around 5 weeks postpartum. Of these, 167 miRNAs were significantly upregulated and 9 were downregulated in the mothers with type 1 diabetes.

Five significantly upregulated candidates, known to be involved in diabetes and play roles in beta- and immune-cell functions: hsa-miR-127-3p, hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p, were further investigated. By pathway analysis, we found these miRNAs to be associated with inflammatory response, chemokine and cytokine mediated signaling and diabetes-related pathways, with a core around TNF, a known modulator of immune response and a key player in immune-mediated diseases, including type 1 diabetes (37, 38).

Disease-associated circulating miRNAs have been suggested to carry direct clinical relevance, as they can be extracted from conventionally collected blood samples in the clinic, with the potential of improving disease prediction, diagnosis and stratification (39, 40). Thus, analyzing and interpreting the circulating miRNA signatures may constitute a feasible clinical application in the future. By real-time qPCR, we confirmed the significant upregulation of hsa-miR-146a-5p, hsa-miR-26a-5p, hsa-miR-24-3p and hsa-miR-30d-5p in plasma samples from mothers with type 1 diabetes, compared to healthy control mothers. The expression level of hsa-miR-127-3p, however, was unchanged in plasma between the two groups, even though this miRNA was significantly upregulated in the extracellular vesicles from mothers with type 1 diabetes. This discrepancy could be explained by the

expression of distinct repertoires of some miRNA species in the respective biofluids of exosomes, which has previously been highlighted (41, 42). hsa-miR-127a-3p has also been found upregulated in the plasma extracellular vesicle fractions rather than free in plasma in individuals with Hodgkin lymphoma (42). Hence, the potential biomarker value of circulating hsa-miR-127-3p seems restricted to the extracellular vesicles. Previously, hsa-miR-127a-3p was found enriched in human pancreatic islets as compared to liver and skeletal muscle, where it was associated with insulin secretion and beta-cell function (43).

Consistent with the present pathway analysis, other studies highlight the role of miR-26a-5p in inflammatory response in several immune-mediated diseases (44–46), including type 1 diabetes pathogenesis (47). Circulating levels of hsa-miR-26a have previously been found upregulated in serum of children with newly diagnosed type 1 diabetes (18). Consistently, another study found it upregulated in plasma and showed a positive correlation of hsa-miR-26a-5p to HbA_{1c} at type 1 diabetes diagnosis (48).

Also miR-146a-5p has been well-studied for its role as an immune modulator in adaptive and innate immune responses, inflammation and apoptosis (16, 49–51), confirming the present study's pathway analysis, i.e. hsa-miR-146a-5p's association to inflammatory responses and chemokine and cytokine mediated signaling pathways. miR-146a-5p is expressed in various immune cells, participating in the regulation of inflammatory response, and has also been found to be upregulated by pro-inflammatory cytokines in human pancreatic islets (51, 52). Several studies found hsa-miR-146a-5p to be modulated in plasma and serum from individuals with type 1 diabetes (16, 53, 54). Polymorphisms in the gene encoding this miRNA have also been investigated for its protective role for type 1 diabetes, comparing the genotype of individuals with and without type 1 diabetes (55).

Altered levels of circulating hsa-miR-24-3p have been reported in newly diagnosed type 1 diabetes (54, 56). However, one study found hsa-miR-24-3p to be upregulated at newly diagnosed type 1 diabetes but found no difference at later stage type 1 diabetes, when compared to healthy controls (57). We have previously reported correlations between the plasma level of both hsa-miR-24-3p and hsa-miR-146a-5p to residual beta-cell function in children 6-12 months after diagnosis with type 1 diabetes (17). Furthermore, miR-24-3p has been associated with beta-cell failure, as overexpression of mmu-miR-24-3p in MIN6 cells inhibited beta-cell proliferation and insulin secretion (58).

Also, miR-30d has been shown to regulate insulin, as its overexpression in MIN6 cells induces insulin gene expression and silencing miR-30d inhibits glucose stimulated insulin expression (59). hsa-miR-30d has been studied in circulation, however, mostly in relation to type 2 diabetes, where it is upregulated in serum and plasma (54). Interestingly, one study found hsa-miR-30d-5p to be significantly upregulated in plasma from individuals with gestational diabetes (60).

In this study, we also observed that hsa-miR-6839-5p, hsa-miR-891a-5p, hsa-miR-1260a, hsa-miR-7977 and hsa-miR-874-3p positively correlated to HbA_{1c} in the group of mothers with type 1 diabetes. These have not previously been reported as markers for HbA_{1c}. However, due to known effects on HbA_{1c} postdelivery, the biomarker potential of these miRNAs remains uncertain. Further analyses are needed to determine associations of these candidates to HbA_{1c}.

Previously, we profiled miRNAs derived from exosome-enriched extracellular vesicles in breast milk from mothers with type 1 diabetes and healthy control mothers (13). Here, we found that miRNA levels in breast milk were not reflected in plasma. Only one candidate, hsa-miR-133a-3p exclusively overlapped between the two datasets, being significantly upregulated in both milk and plasma from the mothers with type 1 diabetes (13). However, while this differential expression was highly significant in milk, it was only nominally changed in the plasma samples. Overall, we conclude that the miRNA profiles depicted in these tissues hold distinct signatures, highlighting the biofluid or tissue-specific biomarker value of miRNAs (41).

From a general comparison, there are both discrepancies and similarities between our current findings and other studies investigating the miRNA profiles of individuals with type 1 diabetes. Garcia-Contreras et al. and Tesovnik et al. are to our knowledge the only studies that have investigated miRNAs verified to be derived from extracellular vesicles from plasma of individuals with type 1 diabetes (14, 15). The design and technologies used, however, differ greatly, giving rise to distinct identification profiles and quantities of detected miRNAs. These discrepancies most likely reflect the dynamic nature of miRNAs. Inconsistencies in design and methodologies are apparent in other studies investigating circulating miRNAs from individuals with and without type 1 diabetes (54, 61). Some candidates, however, are recurrent between studies and overlap with the present study's findings, e.g. upregulation of hsa-miR-21-3p, hsa-miR-21-5p, hsa-miR-24-3p, hsa-miR-25-3p and hsa-miR-93-5p in plasma of individuals with type 1 diabetes compared to healthy controls (54, 61).

The design of the present study poses the risk that the phenotype under study could have considerable impact on immune state and disease status, reducing the chance to observe diabetes-related differences between the two study groups. Despite this limitation, we succeeded in detecting significant alterations in the miRNA profile between lactating mothers with and without type 1 diabetes, including miRNA candidates with positive correlations to HbA_{1c} and associations to disease pathogenesis and inflammatory responses. These observations should be verified in a supplementary cohort of lactating mothers with and without type 1 diabetes, ideally including continuous follow-up sampling before pregnancy, before and after delivery, and/or sampling from the infant. Hence, further such analyses are left to explain the full impact of altered circulating and exosome-entrapped miRNAs on lactating mothers with type 1 diabetes, as well as its potential of modulating type 1 diabetes risk in offspring. The results of this study further warrant investigations to ascribe functional importance of these miRNAs, and to elucidate the overall consequence of their alteration in type 1 diabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/arrayexpress/, E-MTAB-10458.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committee for the Capital Region, Denmark (H-4-2013-008). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK and FP conceived and managed the study. LBN, HBM, and FP suggested the overall hypothesis and contributed to study design. LBN, ERM, PD, and JSv recruited participants and assembled phenotypic data. CF, AHM, RY, CE, JSt, JJ, FP, and SK carried out the sample preparation and interpretation of the data. HBM and FP secured resources and facilities for the research. CF, AHM, FP, and SK analyzed the data and drafted the manuscript. All authors provided critical scientific input and revised the manuscript. FP and SK had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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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.2021.744509/full#supplementary-material

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REVIEW
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Emerging Role of Non-Coding RNAs in Regulation of T-Lymphocyte Function

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T-lymphocytes (T cells) play a major role in adaptive immunity and current immune checkpoint inhibitor-based cancer treatments. The regulation of their function is complex, and in addition to cytokines, receptors and transcription factors, several non-coding RNAs (ncRNAs) have been shown to affect differentiation and function of T cells. Among these non-coding RNAs, certain small microRNAs (miRNAs) including miR-15a/16-1, miR-125b-5p, miR-99a-5p, miR-128-3p, let-7 family, miR-210, miR-182-5p, miR-181, miR-155 and miR-10a have been well recognized. Meanwhile, IFNG-AS1, lnc-ITSN1-2, lncRNA-CD160, NEAT1, MEG3, GAS5, NKILA, lnc-EGFR and PVT1 are among long non-coding RNAs (lncRNAs) that efficiently influence the function of T cells. Recent studies have underscored the effects of a number of circular RNAs, namely circ_0001806, hsa_circ_0045272, hsa_circ_0012919, hsa_circ_0005519 and circHIPK3 in the modulation of T-cell apoptosis, differentiation and secretion of cytokines. This review summarizes the latest news and regulatory roles of these ncRNAs on the function of T cells, with widespread implications on the pathophysiology of autoimmune disorders and cancer.

Keywords: miRNA, lncRNA, circRNA, T cell, cancer, autoimmune

INTRODUCTION

T-lymphocytes (T-cells) play a central role in adaptive immunity and are involved in the pathogenesis of immune-related disorders and cancer, thus several therapeutic strategies have been developed to stimulate their effector functions (1). During the process of maturation in the thymus, T cells express T cell receptors (TCR) on their surface. Moreover, they can express either CD8 or CD4 glycoproteins, thus being categorized as glycoprotein on their surface and are called CD8+ (cytotoxic) or CD4+ cells (helper) T cells (2). Based on the distinctive cytokine profiles, T helper (Th) cells can be categorized to Th1, Th2, Th9, Th17, Th22, regulatory T cells (Tregs), and follicular helper T cells (Tfhs) subtypes (3). Each cell type can be recognized by epigenetic and genetic signatures. For instance, Treg cells are described by over-expression of the FOXP3

transcription factor (4) Demethylation of the intronic conserved non-coding sequence 2 is required for maintenance of FOXP3 expression and regulation of stability of Tregs upon re-exposure to cytokines (5). In Tregs, this intronic sequence acts a sensor for IL-2 and STAT5 (5). The expression of a number of transcription factors has been shown to be altered in CD8+ T cells during clearing an Bacterial or Viral infection (6). Notably, it is possible to predict the potential of these cells to make memory cells based on gene signatures (6). For instance, expressions of Bcl-2 and Cdh-1 have been shown to be surged in the memory subset of CD8+ T cells (6). In addition, chromatin configurations have been found to influence the function of T cells (6). Non-coding RNAs (ncRNAs) carry a regulatory function in several biological processes including implications in immune checkpoint inhibitor treatment (7). Recent studies have highlighted the impact of different classes of non-coding RNAs in T cell functions. In this review, we highlight the function of microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) in regulation of T cells. These three classes of ncRNAs have regulatory effects on expression of mRNA coding genes. In fact, lncRNAs and circRNAs can sequester miRNAs and decrease availability of miRNAs. Since miRNAs can inhibit expression of target mRNAs, the sequestering effects of circRNAs and lncRNAs on miRNAs release miRNA targets from inhibitory effects of miRNAs (8).

miRNAs AND T CELL REGULATION

miRNAs are about 22 nucleotides in length and regulate expression of their target transcripts mostly through binding to their 3' UTR (9, 10). These small molecules are generated in the forms of precursors by RNA polymerases II and III. The mature miRNA is generated through a series of cleavage events in the nucleus and cytoplasm (9). Given their various regulatory functions, miRNAs are important players in the regulation of several physiologic and pathophysiologic processes (11). As for the regulation of T-cell differentiation, several examples of important miRNAs have been reported. For instance, miR-15/16 hampers T cell cycle, their survival and differentiation to memory T cells. Experiments in miR-15/16 deficient T cells have shown that these miRNAs directly inhibit the expression of a broad network of genes contributing in the regulation of cell cycle progression, survival, and development of memory cells (12). Another study has shown miR-15a/16-1 silencing in CD4+ T cells leads to the production of higher levels of IL-22, while up-regulation of miR-15a/16-1 results in down-regulation of the IL-22 expression through suppression of the aryl hydrocarbon receptor. miR-15a/16-1 silenced CD4+ T cells were superior to wild-type CD4+ T cells in terms of tissue repair capacity because of their higher capability in production of IL-22. Furthermore, IL-22 has been shown to decrease miR-15a/16-1 levels through activation of phosphorylated STAT3-c-myc signaling (13).

A high throughput miRNA profiling in human peripheral γδ T cells of healthy subjects has led to identification of 14 differentially expressed miRNAs between αβ and γδ T cells. While miR-150-5p, miR-450a-5p, miR-193b-3p, miR-365a-3p, miR-31-5p, miR-125b-5p and miR-99a-5p have been up-regulated in γδ T cells, miR-34a-5p, miR-16-5p, miR-15b-5p, miR-24-3p, miR-22-3p, miR-22-5p and miR-9-5p have had the opposite trend (14). Notably, miR-125b-5p and miR-99a-5p have been found to attenuate the activity of γδ T cells and decrease their cytotoxic effects against tumor cells. Up-regulation of miR-125b-5p or miR-99a-5p in γδ T cells was shown to suppress the activity of γδ T cells and induced their apoptosis. Moreover, miR-125b-5p silencing has increased cytotoxic effects of γδ T cells against tumor cells through enhancing the production of IFN-γ and TNF-α (14). Overexpression of miR-125b has also promoted Treg cells differentiation and suppressed Th17 cell differentiation (15). In addition, miR-125a, a miRNA which has only recently been reported to be involved in myelogenous leukemogenesis (16), could inhibit production of proinflammatory cytokines in CD4+ T cells and Th1/Th17 cell differentiation by targeting ETS-1 (17).

Let-7 family miRNAs are also involved in the regulation of T cells functions. *In vivo* experiments demonstrated that, let-7g-5p may attenuate the frequency of Th17 cells of rheumatoid arthritis (RA) and block Th17 differentiation (18). Let-7f-5p inhibits Th17 differentiation through targeting STAT3. This miRNA has been found to be downregulated in CD4+ T cells of patients with multiple sclerosis (MS) (19). Finally, let-7d-3p regulates the expression of IL-17 in CD4 + T cells by targeting AKT1 and modulation of AKT1/mTOR signaling pathway (20).

miR-210 is another miRNA whose deletion enhances T cell differentiation and Th17 polarization under hypoxic situation through modulation of HIF-1α expression (21). Expression of this miRNA has also been found to be over-expressed in both psoriasis patients and psoriasis animal models where it stimulates Th17 and Th1 cell differentiation but suppresses Th2 differentiation *via* inhibiting expressions of STAT6 and LYN. Ablation of miR-210 in animals and intradermal injection of miR-210 antagonist has reversed the immune imbalance and blocked the development of psoriasis-like inflammatory response in an animal model of psoriasis. TGF-β and IL-23 have been shown to increase the expression of miR-210 through the induction of HIF-1α, and subsequent recruitment of P300 and enhancement of histone H3 acetylation in miR-210 promoter (22).

miR-181c has been shown to enhance Th17 differentiation and promote autoimmunity through targeting Smad7 and modulating TGF-β pathway and IL-2 expression (13). Overexpression of miR-181c has suppressed activation of T cell, impaired cytoskeleton arrangement in T cells by targeting BRK1 (23). Meanwhile, miR-181a has been reported to restrict IFN-γ production by targeting Id2 so regulating IFN-γ-mediated CD8+ T cell responses mediated by (24). This miRNA also promotes expression of TGF-β and IL-10 and inhibits function of Tregs through modulating the PI3K/Akt pathway (25). **Figure 1** illustrates the role of various ncRNAs in regulating the differentiation of T cells *via* the PI3K/Akt/mTOR and MAPK/ERK signaling pathways. **Table 1** summarizes the impact of miRNAs on regulation of function of T cells.

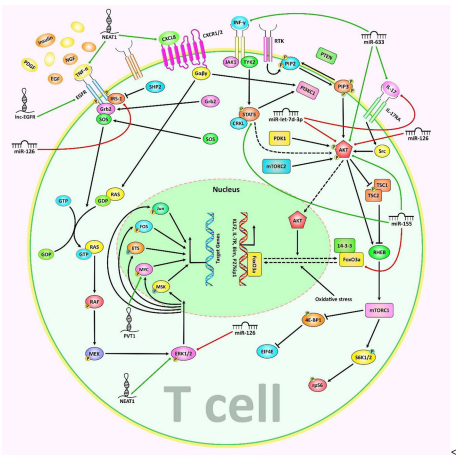

FIGURE 1 | A schematic representation of the role of various non-coding RNAs in modulating the differentiation of T cells *via* the PI3K/Akt/mTOR and MAPK/ERK signaling cascades. a) The MAPK/ERK pathway can be triggered *via* several growth factors including PDGF, EGF, NGF, and insulin. Upon receptor dimerization, activation of its tyrosine kinase module could be triggered, subsequently recruiting Grb2 and SOSto the phosphorylated domain, thus creating the Grb2-SOS complex. Furthermore, the GTP binding protein RAS interacts with the Grb-2-SOS complex that in turn leads to the activation of RAS. Activated GTP-bound RAS plays an effective role in upregulating the phosphorylation of MEK1/2 (MAPKK), which then phosphorylates ERK1/2 (MAPK). Eventually, ERK is transferred to the nucleus where it triggers the activation of various target genes involved in a variety of cellular processes (26–29). b) The PI3K/Akt/mTOR signaling is activated by a subset of growth factors such as PI3KCI, which phosphorylates PIP2 to PIP3. PIP3 has an important role in recruiting AKT which gets activated through double phosphorylation (via PDK1 and mTORC2). In addition, activated AKT suppresses TSC2 through phosphorylation. Inactive TSC1/2 complex is able to bind RHEB, which eventually triggers the activation of mTORC1. The mTORC1 has a significant impact on many downstream proteins, such as S6K1/2 and 4EBP1 (30, 31). Besides, exposure to IL-17 results in receptor-mediated activation of Src, MAPKs, and PI3K/Akt signaling cascades (32). Moreover, subsequent to JAK activation, CRKL is phosphorylated by TYK2 that could result in CRKL complexation with STAT5. STATs in turn interacts with individual mediators of the PI3K/AKT signaling cascade (33). Accumulating finding has demonstrated that miR-let-7d-3p *via* directly suppressing AKT1 could regulate expression level of IL-17 in CD4+ T cells through the AKT1/mTOR signaling pathway (20). In addition, another research has authenticated that overexpression of lncRNA NEAT1 could promote the expression levels of CXCL8 and TNF-α in Sjögren's syndrome *via* positively regulating MAPK signaling (34). Green arrows indicate upregulation of target genes modulated *via* ncRNAs (lncRNAs, and miRNAs), red arrows depict inhibition by these ncRNAs. All the information regarding the role of these ncRNAs in modulating T call differentiation can be seen in and.

LncRNAs AND T CELL REGULATION

LncRNAs are typically longer than 200 nucleotides and may also be several kilobases long (69). They exert diverse effects on chromatin structure, transcription of genes and post-transcriptional regulation of gene expression (70). These effects are exerted through both chromatin-based mechanisms and the interaction with other types of transcripts. Moreover, by serving as decoy, scaffold, and enhancers, lncRNAs influence genes expressions though various mechanisms (71). Several lncRNAs have been found to influence the function of T cells. For instance, IFNG-AS1 is up-regulated in the intestinal tissue of patients with active inflammatory bowel disease (IBD). Specific over-expression of IFNG-AS1 in T cells has led to significant enhancement of inflammatory cytokines, while attenuation of production of anti-inflammatory cytokines. Media from IFNG-AS1-overexpressing T cells has induced a potent inflammatory response in primary human peripheral blood mononuclear cells (PBMCs) (72). Lnc-ITSN1-2 is another lncRNA that affect T cells differentiation. This lncRNA has been shown to increased proliferation and activation of CD4+ T Cells and promote their differentiation to Th1/Th17 through targeting miR-125a and upregulating IL-23R (73).

The regulatory role of NEAT1 on T cells functions has been validated in different contexts, including sepsis, primary Sjögren's syndrome, RA and hepatocellular carcinoma (HCC) (74, 75). Downregulation of NEAT1 has restricted immune response in mouse model of sepsis and induced T cell apoptosis through modulating miR-125/MCEMP1 axis (76). This lncRNA has been shown to promote expression of CXCL8 and TNF-α and activate MAPK signaling pathway. NEAT1 expression has been up-regulated in CD4+ and CD8+ T cells of patients with primary Sjögren's syndrome (34). Similarly, this lncRNA has been found to be up-regulated in peripheral blood mononuclear cells of RA patients. Its silencing has led to inhibited differentiation of Th17 cells from CD4+ T cells by downregulating STAT3 through modulating its ubiquitination (77). Finally, NEAT1 has been found to be up-regulated in PBMCs of HCC patients parallel with up-regulation of Tim-3. NEAT1 silencing has blocked apoptosis of CD8+T cells and increased their cytolysis function. Further, NEAT1 has been shown to exert such effects through miR-155/Tim-3 pathway. Taken together, NEAT1 has been suggested as an important target for enhancing the efficiency of immunotherapy (78).

MALAT1 is another lncRNA with prominent role in the regulation of T cell function. This lncRNA regulates Th1/Th2 ratio by sponging miR-155 and modulating expression of CTLA4 (79). On the other hand, MEG3 has been found to enhance proportion of Th17 cells and regulate Treg/Th17 ratio by sponging miR-17 and upregulating RORγt (80). Moreover, this lncRNA decreases proliferation of CD4+T cell and inhibits Th1 and Th17 differentiation by absorbing miR-23a and modulating expression of TIGIT (81). **Figure 2** represents the role of various ncRNAs in regulating the JAK2/STAT3 and NF-κB signaling pathways in the regulation of function of T cells. **Table 2** summarizes the impact of lncRNAs on T cell function.

CircRNAs AND T CELL REGULATION

CircRNAs are another group of ncRNAs that can be occasionally translated into proteins. The enclosed structure of circRNAs has endowed them a certain resistance to RNases and thus increases the stability in different body compartments

TABLE 1 | miRNAs and T cell regulation.

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
miR-15/16	–	–	miR-15/16 deficient mouse model	CD4(+) T cells obtained from mice	–	–	Constrains formation of memory T cells and confines T cell survival and cell cycle through modulating complex network of their target genes implicated in cell cycle and survival	(12)
miR-15a/16-1	–	–	C57BL/6 mice	Naïve CD4+ T cells	AHR	–	Decreasing IL-22 secretion of CD4+ T cells through targeting AHR	(13)
miR-125b-5p miR-99a-5p	Downregulated (in γδ T cells compared with αβ T cells)	–	Peripheral blood obtained from 21 healthy donors	αβ T cells and γδ T cells purified from peripheral blood	–	–	upregulation inhibits activation of γδ T cells and cytotoxicity to tumor cells by decreasing secretion of IFN-γ and TNF-α.	(14)
miR-125b	Downregulated (in in PBMCs and CD4+ T cells of patients)	Juvenile idiopathic arthritis (JIA)	Peripheral blood obtained from 16 JIA patients and 22 healthy volunteers, 24 male DBA/1J mice	CD4+ T cells	–	–	overexpression promotes Treg cells differentiation and suppresses Th17 cell differentiation.	(15)
miR-125a	Downregulated (in PBMC of IBD patients)	Inflammatory bowel diseases (IBD)	Blood samples from 106 IBD patients and 16 healthy controls, Female C57BL/6 mice	CD4+ T cells	ETS-1↑	–	Inhibited production of proinflammatory cytokines in CD4+ T cells and Th1/Th17 cell differentiation by targeting ETS-1	(17)
miR-128-3p	Upregulated (in T cells RA patients)	Rheumatoid arthritis (RA)	Blood samples from 20 patients with RA and 20 healthy subjects, C57BL/6 mice	Patient derived T cells	TNFAIP3	NF-κB signaling pathway	silencing represses activation of T cells by upregulating TNFAIP3 and inhibiting NF-κB signaling pathway	(35)
let-7g-5p	Downregulated (in plasma of RA patients)	Rheumatoid arthritis (RA)	Plasma samples from RA patients and healthy controls, C57BL/6 mice, DBA 1/J mice	CD4+ T cells	–	–	Upregulation attenuates Th17 frequency in RA mouse model and blockes Th17 differentiation.	(18)
let-7f-5p	Downregulated (in CD4+ T cells of patients with MS)	Multiple sclerosis (MS)	Blood samples from 16 RRMS patients and 16 healthy controls, Female C57BL/6J mice	CD4+ T cell	STAT3↑	–	Overexpression inhibits Th17 differentiation through targeting STAT3.	(19)
miR-let-7d-3p	–	Primary Sjögren's syndrome (pSS)	Blood samples from pSS patients and healthy controls	CD4+ T cells	AKT1	AKT1/mTOR signaling pathway	Regulates expression of IL-17 in CD4 + T cells by targeting AKT1 and modulation of AKT1/mTOR signaling pathway	(20)
miR-183 miR-96	Upregulated Upregulated (in patients' T cells and	Graves' orbitopathy (GO)	Blood samples from patients with GO and normal subjects, TCR-HA/Thy.1.1 transgenic mice, INS-HA/Rag2KO	CD4(+) T cells from human blood samples and mice	EGR-1	–	overexpression was associated with lowered EGR-1 expression and augmented	(36)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
	activated T cells from controls)		transgenic mice and BALB/c mice				proliferation while their downregulation had reverse effects	
miR-210	Upregulated (in activated T cells)	Chronic colitis	Mir210 conditional knockout mice	Naive T cells, TH17 cells	HIF-1α	–	deletion potentiates T cell differentiation and TH17 polarization by modulation of HIF-1α expression	(21)
miR-210	Upregulated (in psoriasis patients)	Psoriasis	Blood samples and skin tissues specimens from 63 psoriasis patients and 80 normal volunteers, C57BL/6J and BALB/c mice	CD4+ T	STAT6, LYN	–	Enhances Th1 and Th17 differentiation and represses Th2 differentiation by targeting STAT6 and LYN	(22)
miR-182-5p	Downregulated (in Th17 cells of EAU mice)	Uveitis	Blood samples from 15 patients with Behçet's disease with uveitis, 15 patients with active sympathetic ophthalmia with uveitis and 15 healthy subjects, C57BL/6 mice	CD4+ T-cells, EL4 murine T cell line	TAF15	STAT3 signaling pathway	overexpression inhibits Th17 development and lowers diseased severity in experimental autoimmune uveitis by targeting TAF15 and modulating STAT3 pathway	(37)
miR-182	Upregulated (in CD4+ T cells of RRMS patients)	Relapse and remitting multiple sclerosis (RRMS)	Blood samples from RRMS patients and healthy controls, female C57BL/6 mice	CD4+ T cells	HIF-1α	–	Its overexpression led to promoted differentiation of naïve T cells to Th1 and Th17 through targeting HIF-1α and rising IFN-γ expression.	(38)
miR-181c	–	Multiple sclerosis (MS)	Female C57BL/6 mice	CD4+ CD62L+ T helper cells	Smad7	TGF-β signaling pathway	Enhanced Th17 differentiation and promoted autoimmunity through targeting Smad7 and modulating TGF-β pathway and IL-2 expression	(13)
miR-181c	Downregulated (in activated T cells)	–	–	MCF7, HeLa, CD3+ T cells, (Jurkat T cells	BRK1	–	Its overexpression suppressed activation of T cell, impaired cytoskeleton arrangement in T cells by targeting BRK1.	(23)
miR-181a	–	–	C57BL/6J mice	CD8+ T cell	Id2	–	Restricted IFN-γ production by targeting Id2 so regulated CD8+ T cell responses mediated by IFN-γ	(24)
miR-181a	–	Allergic rhinitis (AR)	C57BL/6 mice	CD4+ T cells, Treg cells	–	PI3K/Akt pathway	Promoted expression of TGF-β and IL-10 and inhibited function of Tregs through	(25)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
							modulating PI3K/Akt pathway	
miR-202-5P	Upregulated (in PBMCs, Tregs, and CD4+ T cells of AR patients)	Allergic rhinitis (AR)	Blood samples from 30 AR cases and 10 normal controls	Tregs cells, CD4+ T cells	MATN2	–	Repressed differentiation of Tregs by targeting MATN2	(39)
miR-155	–	Allergic rhinitis (AR)	C57BL/6 mice	CD4+ T cells, Treg cells	–	SOCS1 and SIRT1 signaling pathway	Elevated proliferation of Treg cells by modulating SOCS1 and SIRT1 signaling pathway but no influence on T cell function suppression	(25)
miR-155	Upregulated (in donor T cells in aGVHD patients)	Acute graft versus host disease (aGVHD)	C57BL/6 (B6, $H2^b$), C57BL/6-Tg(CAG-EGFP) 1Osb/J (B6 GFP, $H2^b$), Cg-miR-155tm1.1Rsky/j (miR-155–/–, $H2^b$), B6D2F1 (F1, $H2^{b/d}$), BALB/c ($H2^d$), and C3.SW-$H2^b$/SnJ ($H2^b$)	–	–	–	Its expression in CD8+ and CD4+ T cells is necessary for pathogenesis of aGVHD through regulation of migration, expansion and effector function of T cell	(40)
miR-155	–	Viral infection	C57BL/6, MiR-155–/–, wild-type (WT) and ovalbumin-specific Tcrα/Tcrβ transgenic (OTII) mice	CD4+ T	–	–	Is implicated in regulation of proliferation, activation and cytokine production of CD4+ T	(41)
miR-155	–	Vitiligo	Blood samples from one vitiligo patient and one healthy donor	naïve T and CD8+ T cells	–	–	Its overexpression decreased proliferation of CD8 + T cells and enhanced Treg percentage	(42)
miR-155	–	Glioma	C57BL/6 mice	GL261, T cell	FoxO3a	Akt and Stat5 signaling pathway	Its upregulation promoted proliferation and activation of T cells and increased their cytotoxicity by targeting FoxO3a and modulating Akt and Stat5 signaling pathway	(43)
miR-149-3p	Downregulated (in CD8+ T cells overexpressing PD-1)	Breast cancer	Female BALB/c mice	4T1, CD8+ T cell	–	–	Its overexpression reduced T cell apoptosis and expression of T cell inhibitor receptors, also promoted activation of T cells	(39)
miR-143	Upregulated (in naïve and memory T cells compared with effector T cells)	Esophageal squamous cell carcinoma (ESCC)	13 tumor tissues and adjacent normal tissues from 13 ESCC patients and blood samples from 10 healthy donors	CD8+ T cell, HER2-CAR T cells	Glut-1	–	Its upregulation promoted differentiation of CD8+ T cell to memory T cells, raised T cell cytotoxicity and decreased apoptosis by	(44)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
							targeting Glut-1 and regulation of metabolism	
miR-17-92	–	Chronic graft-versus-host disease (cGVHD)	miR-17-92 conditional knockout (KO) mice	CD4+ T	–	–	Increased differentiation of Th1 and Th17 cells, elevated production of follicular Th cells and associated with scleroderma and bronchiolitis	(45)
miR-10a	–	–	3 Adipose Tissue healthy subjects, female C57BL/6 mice	Naïve CD4+ T cell, adipose tissue derived mesenchymal stem cells (AD-MSCs)	–	–	Transfection with miR-10a-loaded exosomes derived from AD-MSCs elevated expression of RORγt and Foxp3 and reduced expression of T-bet and led to differentiation of naive T cells to Th17 and Treg	(46)
miR-10a-3p	Downregulated (in PBMC of LN patients)	Lupus nephritis (LN)	Blood samples from 94 LN patients and 38 healthy subjects	–	REG3A↑	JAK2/STAT3 pathway	Its upregulation enhanced Treg cells and lessened Th17/Treg ratio and alleviated renal function by targeting REG3A	(47)
miR-633	Downregulated (in CD4+T cells of SLE patients)	Systemic lupus erythematosus (SLE)	Blood samples from 20 SLE patients and 19 healthy controls	CD4+T cells, Jurkat cells	AKT1↑	AKT/mTOR pathway	Its downregulation increased IL-17, and IFN-γ production and activated AKT/mTOR pathway in CD4+T cells through modulating AKT1	(48)
miR-142-3p	Upregulated (in CD4+ T cells of T1D patients)	Type 1 diabetes (T1D)	Blood samples form T1D patients, CBy.PL(B6)-Thy1^{a}/ScrJ (CD90.1 BALB/c), Balb/cByJ (CD90.2 BALB/c), Balb/c.Cg-Foxp3tm2Tch/J (BALB/c Foxp3GFP), and NOD/ShiLtJ mice, NOD.Cg-Prkdc^{scid} H2-Ab1^{tm1Gru} Il2rg^{tm1Wjl} Tg (HLA-DQA1,HLA-DQB1) 1Dv//Sz mice	CD4+ T cells	Tet2	–	Inhibited differentiation of Treg cells and decreased stability of Tregs by targeting Tet2 and its depletion collapsed islet autoimmunity in mouse models of diabetes	(49)
miR-142-3p	–	Acute graft versus host disease (GVHD)	Blood samples form volunteer donors, NOD/SCID/mice	Thymic-derived regulatory T cell (tTreg) (CD4 + CD25 + CD127- tTreg)	ATG16L1	–	Its knockdown enhances survival and proliferation of tTregs by upregulating expression of ATG16L1 and modulating autophagy	(50)
miR-142-3p	–	–	Blood samples from healthy volunteers, NOD	Naïve CD4+CD45RA+ T cells	KDM6A	–	Its knockdown improved regulatory	(51)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
			CRISPR Prkdc Il2r gamma (NCG) mice				function and expression of cytokines and suppressed apoptosis in iTregs by upregulating KDM6A	
miR-26b-5p	Downregulated (in HCC tissues and CD4+ and CD8+ T cells)	Hepatocellular carcinoma (HCC)	42 HCC tissues and ANTs, SPF C57BL/6 and nude mice	CD4+ and CD8+ T cells	PIM-2	–	Its overexpression improved cytokine secretion of CD4+ and CD8+ T cells by targeting PIM-2	(48)
miR-34a	–	–	Blood samples from healthy donors	CD4+ and CD8+ T cells	PLCG1, CD3E, PIK3CB, TAB2, NFκBIA	NF-κB signaling pathway	Its overexpression suppressed expression of its target genes in CD4 + and CD8+ T cells and lowered cytotoxic ability of T cells through modulating NF-κB signaling	(52)
miR-34a	Downregulated (in tumor-infiltrating T cells)	Gastric cancer (GC)	Blood samples from 73 GC patients and 58 healthy controls	Jurkat cell	LDHA	–	Its overexpression decreased lactate level in T cells and increased IFN-γ expression through targeting LDHA	(53)
miR-140-5p	Downregulated (in encephalomyelitic CD4+T cells)	Experimental autoimmune encephalomyelitis (EAE)	Female C57BL/6 mice	CD4+T cells	–	–	Its upregulation constrained Th1 differentiation through regulating methylation of STAT1 and Tbx and modulation of mitochondrial respiration	(17)
miR-130a–3p	Downregulated (T cells AS patients)	Ankylosing spondylitis (AS)	Blood samples from 30 HLA-B27-positive AS patients and 30 HLA-B27-negative healthy controls	Jurkat T cells	HOXB1	–	Its overexpression resulted in increased proliferation and decreased apoptosis rate in T cells through targeting HOXB1	(54)
miR-126	–	Acute autoimmune colitis	Friend leukaemia virus B (FVB)/N miR-126 knock down mice	CD4+ T cells	IRS-1	AKT and NF-κB pathways	Its knockdown was associated with elevated proliferation and activation of CD4+ T cells and augmented expression of IFN-γ	(55)
miR-425	Upregulated (in PBMC of IBD patients)	Inflammatory bowel disease (IBD)	Blood samples from 124 IBD patients and healthy controls, Female BALB/c mice	CD4+ T cells	Foxo1↓	–	Promoted Th17 differentiation from CD4+ T cells through targeting Foxo1	(56)
miR-219a-5p	Downregulated (in CD4+ T cells of IBD patients)	Inflammatory bowel disease (IBD)	Blood samples from 33 IBD patients and 23 healthy individuals, female BALB/c mice	CD4+ T cells	ETV5↑	–	Its overexpression inhibited Th1/Th17 cell differentiation by targeting ETV5 and	(57)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
							regulating phosphorylation of STAT3 and STAT4	
miR-22	Upregulated (in intestinal tissues and CD4+ T cells of IBD patients)	Inflammatory bowel disease (IBD)	Intestinal tissues and blood samples from 99 IBD patients, 15 intestinal tissues from patients with colonic polyps and 20 blood samples from healthy controls	CD4+ T cells	HDAC4	–	Elevated Th17 differentiation and inflammatory cytokines production by targeting HDAC4	(58)
miR-21-5p	Downregulated (in PBMC of vitiligo patients)	Vitiligo	Blood samples from 15 vitiligo patients and 15 healthy controls	CD4+ T cells	STAT3↓	–	Its overexpression increased Treg cells proportion and decreased effector T cells (Teff), so balanced Treg/Teff ratio by targeting STAT3	(59)
miR-223-3p	Upregulated (in Th17 cells)	Experimental autoimmune uveitis	Female C57BL/6	CD4+ T cells	FOXO3	–	Induced autoreactive Th17 responses by targeting FOXO3 and modulation of IL-23 receptor expression	(60)
miR-669b-3p	–	–	C57BL/6 (H-2b) and BALB/c (H-2d) mice	CD4+ T cells	–	–	Increased proliferation of CD4 + T cells and restrained apoptosis of these cells by negative regulation of IDO	(61)
miR-146a	Upregulated (in CD27- γδ T cells)	–	C57BL/6J and CD45.1 mice, Rag2–/– mice, Il17a-GFP knock-in mice, miR-146a–/– mice, Nod1 –/– and Atf2–/– mice	CD27- γδ T cells and CD27+ γδ T cells, CD4 + T cells	NOD1		Decreased IFN-γ production and restricted functional plasticity of γδ T cells through targeting NOD1	(62)
miR-29b	Upregulated (in CD4+ T cells of OLP patients)	Oral lichen planus (OLP)	Blood samples form 18 OLP patients and 18 age- and gender-matched controls	CD4+ T cells	–	–	Inhibited IFN-γ expression and secretion in CD4+ T cells, also suppressed expression of DNMT1 induced global DNA hypomethylation in CD4+ T cells to Th1 responses	(63)
miR-31	Upregulated (in peripheral blood of CHD patients)	Coronary heart disease (CHD)	Blood samples from 56 CHD patients and 47 non-CHD individuals	CD4+ T cells	Bach2	–	Increased Th22 differentiation by targeting Bach2	(64)
miR-653	Downregulated (in thymic tissues of MG mice)	Myasthenia gravis (MG)	Thymic tissues from 42 MG patients, BALB/c male nude mice	Thymocytes obtained from thymic tissues	TRIM9	–	Its overexpression decreased viability of thymocytes and induced cell cycle arrest and apoptosis in these cells by targeting TRIM9	(65)
miR-192	Downregulated (in plasma and CD4+	Childhood asthma	Blood samples from 18 children with childhood	CD4+ T cells	CXCR5	–	Its overexpression impeded activation	(66)

(Continued)

TABLE 1 | Continued

microRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
	T cells of asthma patients)		asthma and 15 healthy children				of T follicular helper cells by targeting CXCR5	
miR-23a-3p	Downregulated (in CD4+ T cells of GD patients)	Graves' disease (GD)	Blood samples from 32 GD patients and 20 healthy individuals, female Balb/c mice	CD4+ T cell, 293T	SIRT1	–	Its overexpression enhanced Treg frequency and improved function of Tregs by targeting SIRT1 and modulating FOXP3 expression and acetylation	(67)
miR-133a/133b	Upregulated (in PBMC of IgAN patients)	IgA nephropathy (IgAN)	Blood samples form 20 IgAN patients and heakthy controls	CD4+ T cells	FOXP3	–	Inhibited Treg differentiation and decreased Treg frequency by downregulating FOXP3	(68)

(108). A genome wide transcriptome profiling of circRNAs has revealed that the median length of circRNAs is about 530 nt (109). Four categories of circRNA shave been identified: exonic circRNAs, circRNAs from introns, exon-intron circRNAs and intergenic circRNAs (110). The impact of this group of transcripts on T cell functions has been discovered during the recent decade. Several studies have shown that circRNAs can bind with miRNAs, thus decreasing bioavailability of miRNAs and releasing miRNA targets from their inhibitory effects. This kind of interactions between circRNAs and miRNAs has critical biological impacts. A high-throughput microarray study found down-regulation of circ_0001806 in patients with cryptococcal meningitis as compared to healthy controls. Circ_0001806 silencing has increased the intensity of fungal infection in the animal models and decreased their survival. Circ_0001806 has been suggested to regulate molecular cascades associated with the host antimicrobial response in T cells. Functionally, circ_0001806 has been shown to increase ADM level, decrease cell apoptosis and reverse G1/S arrest in T cells through sequestering miR-126. Thus, circRNA-1806/miR-126 cascade has an essential role in the regulation of cell cycle and apoptosis in T cells (111).

Another high throughput circRNA profiling in patients with systemic lupus erythematosus (SLE) has led to identification of 127 differentially expressed circRNAs in T cells of these patients. Among them, circRNA hsa_circ_0045272 has been reported to be the down-regulated. Functional studies have shown that hsa_circ_0045272 silencing increases early apoptosis of Jurkat cells and enhances production of IL-2 by activated Jurkat cells. Binding of this circRNA with hsa-miR-6127 has been validated through functional studies (112). Hsa_circ_0012919 has been reported to be up-regulated in CD4+ T cells of SLE patients in two independent studies. In a microarray study of circRNAs signature in these patients, hsa_circ_0012919 has been among differentially expressed circRNAs between SLE patients and healthy subjects. Expression of this circRNA has been associated with SLE features. Down-regulation of hsa_circ_0012919 has enhanced expression of DNMT1, decreased CD70 and CD11a levels and inverted the DNA hypomethylation of these genes in CD4+ T cells of SLE. Hsa_circ_0012919 has been found to regulate expressions of KLF13 and RANTES through sequestering miR-125a (113). This circRNA has also been found to increase the expression of MDA5 in CD4+ T cells through downregulating miR-125a-3p (114). Hsa_circ_0005519 is another circRNA influencing T cell function. This circRNA has been found to be up-regulated in CD4+ T cells of asthmatic patients. Expression of this circRNA has been inversely correlated with hsa-let-7a-5p levels. Expression of hsa_circ_0005519 in CD4+ T cells has been correlated with fraction of exhaled nitric oxide and eosinophil ratio in the circulation of these patients. Hsa_circ_0005519 has been predicted to sequester hsa-let-7a-5p and release IL-13/IL-6 from its inhibitory effect (115). Being up-regulated circRNA in nasal mucosa of patients with allergic rhinitis, circHIPK3 has been found to promote differentiation of CD4+ T cells to Th2 by targeting miR-495 and increasing expression of GATA-3 (116). **Figure 3** illustrates the role of different ncRNAs in Th2-cell differentiation through modulating the IL-4-STAT6-GATA3 axis. **Table 3** shows the impact of circRNAs on T cell function.

SUMMARY

Numerous miRNAs, lncRNAs and circRNAs have been found to influence activity, survival or differentiation of T cells under physiologic or pathologic conditions. These molecules can affect pathophysiology of autoimmune conditions such as MS, SLE, RA, IBD and asthma *via* this route. Moreover, several of these non-coding RNAs influence immune evasion of cancer cells and their response to immunotherapeutic modalities.

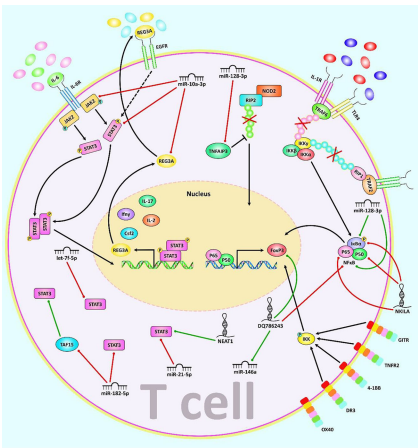

FIGURE 2 | A schematic illustration of the role of various noncoding-RNAs in modulating the JAK2/STAT3 and NF-κB signaling pathway as major regulators of T cell function. a) In JAK/STAT pathway, JAKs bind to the receptor, and upon multimerization, upregulation of JAK proteins is mediated *via* trans-phosphorylation. Consequently, JAKs have a significant part in STATs phosphorylation. After dimerization of STATs, they translocate to the nucleus, where they either activate or suppress several target genes. This cascade is remarkably involved in the control of immune responses. Dysregulation of JAK-STAT signaling is associated with different immune disorders (82, 83). Besides, REG3A, acts as a key molecule for overexpression of the JAK2/STAT3 pathway which effectively contributes to triggering inflammation (84). b) The NF-kB canonical or classical signaling pathway is initiated from the cell surface receptor of pro-inflammatory cytokines and PAMPs containing TNFR, TLR and T/B cell receptor. The activation of IKK complex is triggered *via* binding of ligand molecules to transfer the signal across the cell surface. This complex generally comprises heterodimer of IKKα and IKKβ catalytic subunits and an IKKγ regulatory subunit. The released NF-kB dimers (most generally the p50–P65 dimer) could be translocated to the nucleus, and bind to DNA to trigger activation of the down-stream gene transcription (85–87). In addition, NF-κB signaling cascade could be regulated *via* TNFAIP3 through deubiquitinating TNFR1-RIP1, IL-1R/TLR4-TRAF6, and NOD2-RIP2 pathways (88). Moreover, canonical NF-kB cascade could be activated by various members of the TNFRSF including GITR, TNFR2, 4-1BB, and DR3 but not OX40 in Treg cells and modulates induction of Foxp3, markers of Th2/Th17 response (89). Mounting studies have revealed that multiple ncRNAs (lncRNAs and miRNAs) have an effective role in as major regulators of T cell function through regulating the JAK/STAT and NF-κB cascades. As an illustration, recent research has detected that downregulation of miR-128-3p could notably reduce the inflammation response of rheumatoid arthritis *via* attenuating the activity of NF-κB pathway and promote expression of TNFAIP3 (35). Another study has figured out that reducing the expression of lncRNA NEAT1 could lead to suppression of Th17/CD4+ T cell differentiation *via* downregulating STAT3 expression in rheumatoid arthritis patients (77). Furthermore, upregulation of DQ786243 could play a remarkable role in elevating the expression level of miR-146a through modulating Foxp3, and thereby suppressing the NF-κB signaling cascade in oral lichen planus disease (90). Green arrows indicate upregulation of target genes modulated *via* ncRNAs (lncRNAs, and miRNAs), red arrows depict inhibition by these ncRNAs. All the information regarding the role of these ncRNAs in modulating T call differentiation can be seen in **Tables 1**, **2**.

Notably, both lncRNAs and circRNAs can serve as sponges for miRNAs. Through this molecular mechanism, lncRNAs and circRNAs bind with certain miRNAs to decrease their bioavailability. Thus, circRNAs and lncRNAs can indirectly affect expression of miRNAs targets. Circ_0001806/miR-126, hsa_circ_0045272/hsa-miR-6127, hsa_circ_0012919/miR-125, hsa_circ_0005519/hsa-let-7a-5p, circHIPK3/miR-495 are examples of circRNA/miRNA axes regulating T cell functions. In addition, lnc-ITSN1-2/miR-125a, NEAT1/miR-125, NEAT1/miR-155, MALAT1/miR-155, MEG3/miR-17, MEG3/miR-23a, Gm15575/miR-686 are examples of lncRNA/miRNA pairs in this regard. These examples not only indicate the intricate network between these classes of transcripts, but also provide clues to find the most important modules in the regulation of T cell functions. Contribution of miR-125, miR-155, MEG3 and NEAT1 in more than one of these molecular axes suggests their significance in the regulation of T cell function. Most notably, all of these four non-coding RNAs have essential roles in cancer development or suppression (118–120), further highlighting the intercalation of cancer-related and immune-related molecular pathways.

GATA3, RORγt, NF-κB, SIRT1, STAT3 and FOXO3 as major regulators of T cell function have been shown to be influenced by non-coding RNAs. For instance, GATA3 is influenced by Dreg1 and GATA3-AS1 lncRNAs; RORγt is regulated by MEG3; SIRT1 is modulated by miR-155 and miR-23a-3p; STAT3 is regulated by let-7f-5p, miR-182-5p and miR-10a-3p, miR-21-5p and NEAT1; and FOXO3 is controlled by miR-155. Therefore, non-coding RNAs affect T cells functions through different routes.

Consistent with the important roles of lncRNAs, circRNAs and miRNAs in the regulation of function of T cells and their impact on differentiation of different classes of T cells, therapeutic targeting of these ncRNAs represent an efficient tool for management of disorders related with abnormal function of T cells. Forced up-regulation or silencing of these transcript can affect signaling pathways that modulate T cell responses, thus alleviating tissue damage caused by abnormal T cell responses. Moreover, assessment of ncRNAs signature is a probable strategy for prediction of course of T cell-related disorders.

Taken together, the significant impact of non-coding RNAs on differentiation, survival, cytokine production and activity of T cells potentiates these molecules as important targets for treatment of various disorders, particularly cancer. Moreover, non-coding RNAs participate in the pathogenesis of autoimmune disorders *via* affecting epigenetic regulation of genes with crucial roles in the regulation of effector T cells as well as Tregs (121). Thus, identification of the role of these transcripts in the regulation of T cell functions can provide new modalities for treatment of this kind of disorders as well. High throughput sequencing method and assessment of the competing endogenous RNA network through bioinformatics methods is an efficient strategy in identification of appropriate targets for therapeutic interventions.

FUTURE PERSPECTIVES

High throughput sequencing strategies and identification of differential expressions of lncRNAs, circRNAs, miRNAs

TABLE 2 | LncRNAs and T cell regulation.

lncRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
IFNG-AS1	Upregulation (in colonic tissues of IBD patients)	Inflammatory bowel diseases (IBD)	Colonic tissues from 11 IBD patients,	PBMCs from anonymous donors, Jurkat cells	–	–	Its overexpression augmented inflammatory cytokines expression and decrease anti-inflammatory cytokines expression in T cells.	(72)
lnc-ITSN1-2	Upregulation (in intestinal mucosa and PBMC of IBD patients)	Inflammatory bowel diseases (IBD)	Blood samples and intestinal mucosa specimens from 120 IBD patients and 30 healthy controls	CD4+ T Cells	miR-125a, IL-23R	–	Increased proliferation and activation of CD4+ T Cells and promoted their differentiation to Th1/Th17 by targeting miR-125a and upregulation of IL-23R	(73)
lncRNA-CD160	Upregulated (in CD8+ T cells of HBV infected patients)	Chronic hepatitis B virus (HBV) infection	Blood samples from 164 patients with chronic HBV infection and 67 healthy volunteers, C3H/HeN mice	CD160– CD8 + T cells and CD160+ CD8 + T cells	–	–	Decreased secretion of IFN-γ and TNF-α and repressed function of CD8 + T cells by recruiting HDAC11 to promoters of IFN-γ and TNF-α and elevating methylation of H3K9Me1	(91)
NEAT1	–	Sepsis	130 specific pathogen-free C57BL/6 male mice	CD4+ CD25+ T cells	miR-125, MCEMP1	–	Downregulation of NEAT1 has restricted immune response in mouse model of sepsis and induced T cell apoptosis through modulating miR-125/ MCEMP1 axis	(76)
NEAT1	Upregulated (in CD4+ and CD8 + T cells of pSS patients)	Primary Sjögren's syndrome (pSS)	Blood samples from 20 pSS patients and 10 healthy subjects	CD4+, CD8+ and CD19+ T cells, Jurkat cells	–	MAPK signaling pathway	Promoted expression of CXCL8 and TNF-α and activated MAPK signaling pathway	(34)
NEAT1	Upregulated (in the PBMCs of patients with RA)	Rheumatoid arthritis (RA)	Blood samples from 25 RA patients and 20 healthy controls, Male DBA/1J mice	CD4+ T cell	STAT3	–	Its silencing prevented differentiation of Th17 cells from CD4+ T cells by downregulating STAT3 through modulating its ubiquitination.	(77)
NEAT1	Upregulated (in PBMCs of HCC patients)	Hepatocellular carcinoma (HCC)	Blood samples from 20 HCC patients and 20 healthy controls	CD8+ T cells	miR-155, Tim-3↑	–	Its knockdown decreased apoptosis and raised cytotoxicity of CD8+ T cells by miR-155-mediated downregulation of Tim-3.	(78)
lnc-EGFR	Upregulated (in Treg cells of HCC patients)	Hepatocellular carcinoma (HCC)	Blood and tissue samples from 70 HCC patients and 55 healthy controls	CD4+ T cells, tumor infiltrated lymphocytes (TIL), 97H, Huh7	EGFR	–	Induced differentiation of Treg cells and impeded CTLs function through stabilizing EGFR by interfering with its ubiquitination	(92)
PVT1	Upregulated (in the CD4+T cells of patients with SS)	Sjögren's syndrome (SS)	Blood samples and labial salivary gland tissues from SS patients and healthy controls, female C57BL/6 mice, NOD/ShiLtj mice and wild-type ICR mice	CD4+ T cell	Myc	–	Its downregulation decreased CD4+ T cells proliferation and impeded effector function in these cells through downregulation of Myc and controlling glycolysis	(93)
lncRNA Morrbid	–	Viral infection	C57BL/6 (WT), B6.SJL-Ptprc^{a} Pepc^{b}/Boy (B6.SJL), and B6.129S1-Bcl2l11$^{tm1.1Ast/J}$ (Bcl2l11 knock-out) mice, Ifnar1$^{tm1.1Ees}$ (Ifnar1^{flox}),	CD8+ T cells	–	PI3K-AKT signaling pathway	Regulates proliferation, survival and effector functions of CD8+ T cells by modulating Bcl2l11 expression and PI3K-AKT signaling pathway	(94)

(Continued)

TABLE 2 | Continued

lncRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
			TgCD4-Cre (CD4-cre), and Tg(TcrLCMV) (P14) mice					
RP11-340F14.6	Upregulated (in JIA patients)	Juvenile idiopathic arthritis (JIA)	Blood samples from JIA and healthy controls	T cell	P2X7R	–	Increased Th17 differentiation and inhibited Treg distribution by binding to P2X7R and inducing its expression	(95)
MALAT1	–	Asthma	Blood samples from 772 asthma patients and 441 healthy controls	CD4+ T cells	miR-155, CTLA4	–	Regulated Th1/Th2 ratio by sponging miR-155 and modulating expression of CTLA4	(79)
MEG3	Upregulated (in CD4 + T cells of patients with asthma)	Asthma	Blood samples from 52 asthma patients and 45 healthy controls	CD4 + T cells	miR-17↓, RORγt	–	Elevated proportion of Th17 cells and regulated Treg/Th17 ratio by sponging miR-17 and upregulating RORγt	(80)
MEG3	Downregulated (in CD4 + T cells of AA patients)	Aplastic anemia (AA)	Blood samples from 15 AA patients and 10 healthy controls	CD4+T cell	miR-23a, TIGIT	–	Its overexpression decreased proliferation of CD4+T cell and inhibited Th1 and Th17 differentiation by absorbing miR-23a and modulating expression of TIGIT	(81)
DQ786243	Upregulated (in CD4+ cells of OLP patients)	Oral lichen planus (OLP)	Blood samples from 10 OLP patients and 10 healthy volunteers	CD4+ T cell	miR-146a, Foxp3	NF-κB signaling pathway	Its overexpression increased Treg cells percentage and Foxp3 expression and promoted suppressive function of these cells by modulating Foxp3-miR-146a-NF-κB axis	(90)
AW112010	Upregulated (in activated CD4+ T cells)	–	Female C57BL/6J mice	CD4+ T cells	KDM5A	–	Induces differentiation of inflammatory T cells through inhibiting expression of IL-10 *via* interacting with KDM5A and histone demethylation	(96)
GAS5	Downregulated (in CD4+ T cells of HIV infected patients)	AIDS	Blood samples from 142 HIV infected patients and 58 healthy controls	CD4+ T cells	–	–	Regulated apoptosis rate and function of CD4+ T cells during HIV infection by modulating miR-21	(97)
LINC00176	Upregulated (in CD4+T cells of patients with SLE)	Systemic lupus erythematosus (SLE)	Blood samples from SLE patients and healthy individuals	CD4+ T cells	WIF1	WNT5a signaling pathway	Raised proliferation and adhesion of CD4+T cells by reducing WIF1 levels and WNT5a pathway activation	(98)
lncRNA028466	Downregulated (in CD4+ T cells of mice immunized with rEg.P29 antigen)	–	Female BALB/c mic	CD4+ T cell, CD8+ T cell	–	–	Implicated in regulation of cytokine expression from CD4+ T cells	(99)
NONHSAT196558.1 (TANCR)	–	–	Blood samples normal volunteers	Primary γδ T cells, Jurkat cells	TRAIL	–	Increased activation and cytotoxicity of γδ T cells by modulating expression of TRAIL in *cis* manner	(100)
Dreg1	–	–	Male C57BL/6 mice	splenic CD4+ T cells from mice and human	Gata3	–	Its expression was associated with expression of Gata3 during Th2 differentiation and	(101)

(Continued)

TABLE 2 | Continued

lncRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
							regulated Gata3 expression during development of immune system	
Gm15575	Upregulated (in Th17 cells and spleen tissues of EAE mice)	Multiple sclerosis (MS)	C57BL/6 mice	CD4+ T cells	miR-686, CCL7	–	Promoted Th17 differentiation by sponging miR-686 and upregulating expression of CCL7	(102)
lncDDIT4	Upregulated (in CD4+ T cells and PBMCs of patients with MS)	Multiple sclerosis (MS)	Blood samples from 36 MS patients and 26 healthy controls	naive CD4+ T cells	DDIT4	DDIT4/ mTOR Pathway	Suppressed Th17 differentiation by targeting DDIT4 and inhibiting DDIT4/mTOR signaling	(103)
linc-MAF-4	Upregulated (in PBMCs of patients with MS)	Multiple sclerosis (MS)	Blood samples from 34 MS patients and 26 healthy subjects	Naive CD4+ T cells	MAF	–	Suppressed Th2 differentiation and promoted Th17 differentiation by inhibiting MAF expression	(104)
NKILA	Upregulated (in CTLs and TH1 cells of patients with breast and lung cancer)	Non-small cell lung cancer (NSCLC) and breast cancer	Tissue samples and blood samples from 576 h invasive breast carcinoma patients and 256 NSCLC patients, blood samples from healthy donors, NOD.SCID mice	CD8+ and CD4+ T cells, cytotoxic T lymphocyte (CTL), Th1, Th2 and Treg	NF-κB	–	Sensitized CTLs and Th1 cells to activation-induced cell death in tumor microenvironment and facilitated tumor immune evasion through suppression of NF-κB activity	(105)
IFNA-AS1	Downregulated (in PBMCs of patients with MG)	Myasthenia gravis (MG)	Blood samples from 32 MG patients and 20 healthy volunteers, female C57/BL6 mice	CD4+ T cell, Jurkat T cell	HLA-DRB1	–	Is implicated in regulation of Th1/Treg cell proliferation and activation of CD4 + T cells by influencing HLA-DRB1	(106)
GATA3-AS1	–	–	Blood samples from healthy volunteers	Human PBMC	GATA3	–	Regulated polarization of Th2 cells by increasing expression of GATA3	(107)

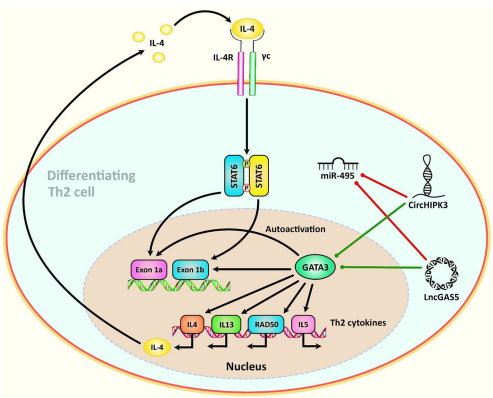

FIGURE 3 | A schematic diagram of the role of some ncRNAs in modulating the IL-4-STAT6-GATA3 axis in Th2-cell differentiation. Th2 cell differentiation requires considerable metabolic reprogramming. Upon encountering cognate antigen in the lymph node, naive CD4 T helper cells are differentiated into Th2 cells under the effect of the IL-4-STAT6-GATA3 axis. GATA3 could, in turn, alter the IL4– IL13–IL5 locus to generate a conformation that is reachable by different other transcription factors that are involved in triggering the differentiation of T cells into T H2 cells (117). Growing evidence has confirmed that the interactions between CircHIPK3, LncGAS5, and miR-495 could play a crucial role in the modulation of Th2 differentiation in allergic rhinitis (116). Green arrows indicate upregulation of target genes by ncRNAs (lncRNA, and circRNA), red arrows depict inhibitory effects of by these ncRNAs.

TABLE 3 | CircRNAs and T cell regulation.

circRNA	Expression pattern	Disease	Sample	Cell line	Interaction	Signaling pathway	Function	Reference
circ_0001806	Downregulated (in PBMCs of CM patients)	Cryptococcal meningitis (CM)	Blood samples from 20 CM patients and 18 healthy donors, female C57BL/6 mice	Jurkat T, CD3+ T cells	miR-126, ADM	–	Reduced apoptosis rate in T cells by sponging miRNA-126 and positive regulation of ADM	(111)
hsa_circ_0045272	Downregulated (in T cells of SLE patients)	Systemic lupus erythematosus (SLE)	Blood samples from 24 patients and 12 healthy controls	293T, Jurkat cells	hsa-miR-6127	–	Its knockdown resulted in increased early apoptosis in T cells and elevated production of IL-2	(112)
hsa_circ_0012919	Upregulated (in CD4+ T cells of SLE patients)	SLE	Blood samples from 28 SLE patients and 18 healthy controls	CD4+ T cells	RNATES, KLF13, miR-125	–	Its downregulation led to upregulation of DNMT1 and decreased expression and hypermethylation of CD11a and CD70 in CD4+ T cells.	(113)
hsa_circ_0012919	Upregulated (in CD4+ T cells of SLE patients)	SLE	Blood samples from 20 SLE patients and 12 healthy controls	CD4+ T cells	miR-125a-3p, MDA5↑	–	Increases expression of MDA5 in CD4+ T cells through downregulating miR-125a-3p	(114)
Hsa_circ_0005519	Upregulated (in CD4+ T cells form patients with asthma)	Asthma	Blood samples from 65 asthmatic patients and 30 healthy individuals	CD4+ T cells	hsa-let-7a-5p	–	Augments expression of IL-13 and IL-6 by targeting hsa-let-7a-5p in CD4+ T cells	(115)
CircHIPK3	Upregulated (in nasal mucosal tissues of AR patients)	Allergic rhinitis (AR)	Blood samples and nasal mucosa specimens from 10 AR patients and 10 healthy controls, male BALB/c mice	CD4+ T cells	miR-495, GATA-3	–	Promotes differentiation of CD4+ T cells to Th2 by targeting miR-495 and increasing expression of GATA-3	(116)

and mRNAs in different stages of T cells development would help in recognition of role of each transcript in development of this group of blood cells. Further knock-in and knock-out studies in different disease conditions can help in identification of specific treatment strategies for related disorders.

AUTHOR CONTRIBUTIONS

SG-F, DB, and JK wrote the draft and revised it. MT and MP designed and supervised the study. OR and MT designed the figures and tables. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Extracellular Vesicles as a Shared Disease Mechanism Contributing to Multimorbidity in Patients With COPD

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Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death worldwide. Individuals with COPD typically experience a progressive, debilitating decline in lung function as well as systemic manifestations of the disease. Multimorbidity, is common in COPD patients and increases the risk of hospitalisation and mortality. Central to the genesis of multimorbidity in COPD patients is a self-perpetuating, abnormal immune and inflammatory response driven by factors including ageing, pollutant inhalation (including smoking) and infection. As many patients with COPD have multiple concurrent chronic conditions, which require an integrative management approach, there is a need to greater understand the shared disease mechanisms contributing to multimorbidity. The intercellular transfer of extracellular vesicles (EVs) has recently been proposed as an important method of local and distal cell-to-cell communication mediating both homeostatic and pathological conditions. EVs have been identified in many biological fluids and provide a stable capsule for the transfer of cargo including proteins, lipids and nucleic acids. Of these cargo, microRNAs (miRNAs), which are short 17-24 nucleotide non-coding RNA molecules, have been amongst the most extensively studied. There is evidence to support that miRNA are selectively packaged into EVs and can regulate recipient cell gene expression including major pathways involved in inflammation, apoptosis and fibrosis. Furthermore changes in EV cargo including miRNA have been reported in many chronic diseases and in response to risk factors including respiratory infections, noxious stimuli and ageing. In this review, we discuss the potential of EVs and

EV-associated miRNA to modulate shared pathological processes in chronic diseases. Further delineating these may lead to the identification of novel biomarkers and therapeutic targets for patients with COPD and multimorbidities.

Keywords: EV - extracellular vesicle, miRNA - microRNA, COPD - chronic obstructive pulmonary disease, multimorbidity, inflammation

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide due to its prevalence, severity and the absence of an effective treatment to reverse disease progression (1). Individuals with COPD experience progressive lung function decline with periods of acute exacerbation (AECOPD) that impact quality of life and present a high economic burden due to direct medical costs and loss of working days (2–5). The development of more sensitive diagnostics and personalised treatments for COPD remains challenging due to our limited understanding of the complex molecular mechanisms underlying the disease (6).

The main pathological driver of COPD is inhalation of noxious stimuli such as cigarette smoke (CS) and particulate matter (PM) air pollution. Repeated exposure to respiratory toxins compromises the function of the immune system, induces chronic inflammation and directly damages structural cells, leading to emphysema and vascular remodelling (7, 8). In addition, damage caused by noxious stimuli promotes features of accelerated ageing including a state of cell cycle arrest, known as cellular senescence, that increases the release of inflammatory factors and has been associated with COPD pathogenesis (9). Furthermore exposure to noxious stimuli and age-associated immune alterations contribute to a dysfunctional immune response and increased susceptibility to acute respiratory infections observed in COPD (10–12). Respiratory infections result in elevation of airway and systemic inflammation on top of the chronic inflammation present in stable COPD and are the predominant cause of AECOPD (13).

The occurrence of multiple chronic conditions termed "comorbidities" or "multimorbidities" are common in patients with COPD (14, 15) (**Figure 1**). Other conditions commonly observed in conjunction with COPD include cardiovascular disease (CVD), diabetes, osteoporosis and gastro-oesophageal reflux disease (15). While the term "comorbidity" refers to the combined effects of additional conditions in reference to an index chronic condition, "multimorbidity" indicates that no single condition holds priority over any of the co-occurring conditions (16). As many patients with COPD have multiple concurrent chronic conditions, which require an integrative management approach, there is a need to greater understand the shared disease mechanisms contributing to multimorbidity.

Our current understanding of the mechanistic drivers for multimorbidity in COPD, including the role of shared risk factors were recently reviewed in detail by Burke and Wilkinson (15). These authors suggested that disease co-occurrence may not be by chance but as a result of shared genetic predisposition and responses to biological and environmental stressors (15). They also highlighted current and novel management strategies that target these underlying mechanisms. In support of this suggestion, network analyses have identified shared genes, proteins and biological pathways common to COPD and its most prevalent coexisting diseases (17). In addition, there is accumulating evidence that exposure to shared risk factors including inhalation of noxious stimuli and accelerated ageing may act as a central mechanism contributing to the development of multiple chronic diseases (18, 19). Furthermore, Burke and Wilkinson introduced the concept that extracellular vesicles (EVs) may contribute to dissemination of inflammation and therefore multimorbidity in COPD patients (15).

Recently EVs have been reported as local and systemic immune and inflammatory mediators that may act to spread or alleviate disease (15, 20–22). During acute and chronic inflammation, vascular permeability is dramatically increased and the alveolar-capillary barrier is reduced allowing lung inflammatory mediators such as proteins and EVs to reach the systemic circulation and potentially distant organs (21, 23). In this review we appraise the latest evidence around the potential role of EVs as circulating inflammatory mediators which could propagate systemic inflammation and multimorbidity in response to shared risk factors including ageing, inhaled noxious stimuli and respiratory infections.

EXTRACELLULAR VESICLES

EVs are highly heterogeneous lipid bilayer particles that have been isolated from a variety of cell types and biological fluids including serum, plasma, urine and bronchoalveolar lavage fluid (BALF) (24–30). They are generated and released by cells *via* a range of mechanisms that have been used to categorise EVs into three distinct subgroups; exosomes, microvesicles and apoptotic bodies (**Figure 2**) (31). However, our understanding of the role of specific EV subgroups remains limited due to the technical challenges associated with isolating pure subgroups, including EV size overlap and the current lack of subgroup specific markers (32). Therefore, this review will consider the overall role of EVs rather than specific subgroups.

EVs were originally considered to be cell debris but have since been shown to transfer lipids, proteins and nucleic acids locally and systemically as a form of intercellular communication mediating both homeostatic and pathological conditions (33, 34). Furthermore distinct EV-associated cargo have been reported depending on the origin and the physiological

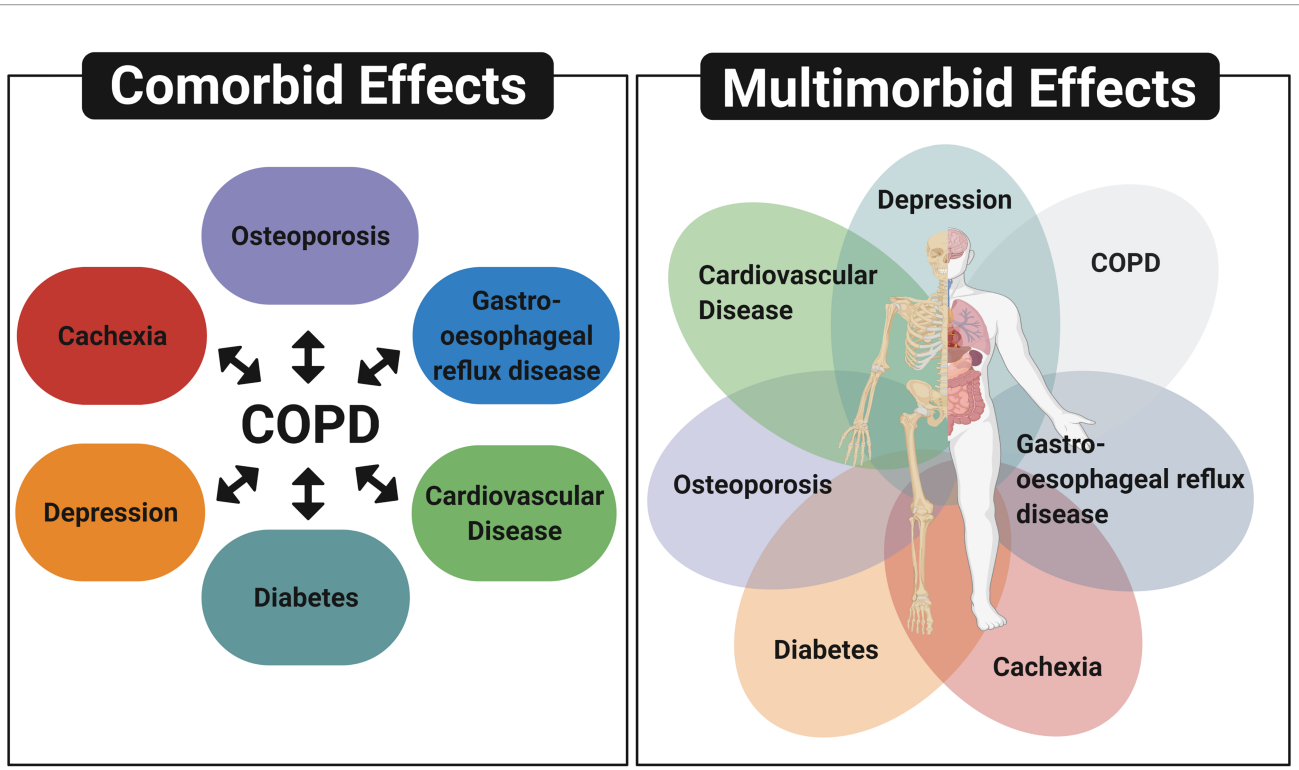

FIGURE 1 | Comorbidity and multimorbidity in patients with COPD. The term ''comorbidity'' refers to the combined effects of additional conditions in reference to COPD, whereas "multimorbidity" indicates that no single condition holds priority over any of the co-occurring conditions. Diagram depicts other conditions commonly observed in patients with COPD, which include cardiovascular disease, depression, cachexia, diabetes, osteoporosis and gastro-oesophageal reflux disease. Created with BioRender.com.

conditions including disease status (35, 36). EVs are enriched in surface proteins with immunoregulatory functions, such as the major histocompatibility molecules (MHC) class I and II (37). In addition, the transfer of immune and inflammatory mediators such as cytokines, chemokines and proteolytic enzymes *via* EVs has been shown to be altered in response to stimuli and to modulate recipient cell behaviour (38).

One of the most widely studied EV cargo are microRNAs (miRNAs). MiRNAs are short 17-24 nucleotide, non-coding RNA molecules that regulate gene expression by translational repression or degradation of mRNA (39). The miRNA content of EVs has been reported to be markedly different from that of the parent cell, suggesting that cells are capable of sorting miRNA into EVs (40). In support of this a number of sorting mechanisms have been reported and were recently discussed in detail in a review by Groot et al. (41). These mechanisms broadly include RNA-binding proteins such as hnRNPA2B1, membranous proteins involved in EV biogenesis such as Caveolin-1 and posttranscriptional RNA modifications such as 3'-end uridylation (42, 43). Furthermore there is evidence that the miRNA cargo of EVs can be taken up and alter gene expression and biological processes, such as immune response in the recipient cells (44, 45). The intercellular transfer of EV miRNA has therefore been implicated in mediating a range of pathophysiological processes including in the development of COPD (46).

Given the ability of EVs to transfer cargo it is possible they allow distal communication to contribute to or protect against pathological mechanisms underpinning the development of multiple chronic diseases. The following sections will discuss the current research around the potential role of EVs and EV-associated miRNA as a shared disease mechanism that could contribute to the development of COPD and multimorbidity.

AGEING, INFLAMMATION AND THE DEVELOPMENT OF CHRONIC DISEASES

The world's ageing population presents a major challenge for health care services globally, particularly as the prevalence of many chronic diseases increases with age (47, 48). There is accumulating evidence that ageing induces a state of chronic inflammation, that may simultaneously contribute to the development of multiple age-related chronic diseases such as COPD, CVD and osteoporosis (10, 49, 50). This process, known as inflammaging, is characterised by significantly higher levels of circulating pro-inflammatory markers, such as interleukin (IL)-1, IL-6, IL-8, IL-13, IL-18, C-reactive protein (CRP), transforming growth factor-β (TGFβ) and tumour necrosis factor (TNF) (49). Inflammaging is thought to be a consequence of the accumulation of cellular damage due to mitochondrial dysfunction, defective autophagy/mitophagy, an impaired ubiquitin/proteasome system and

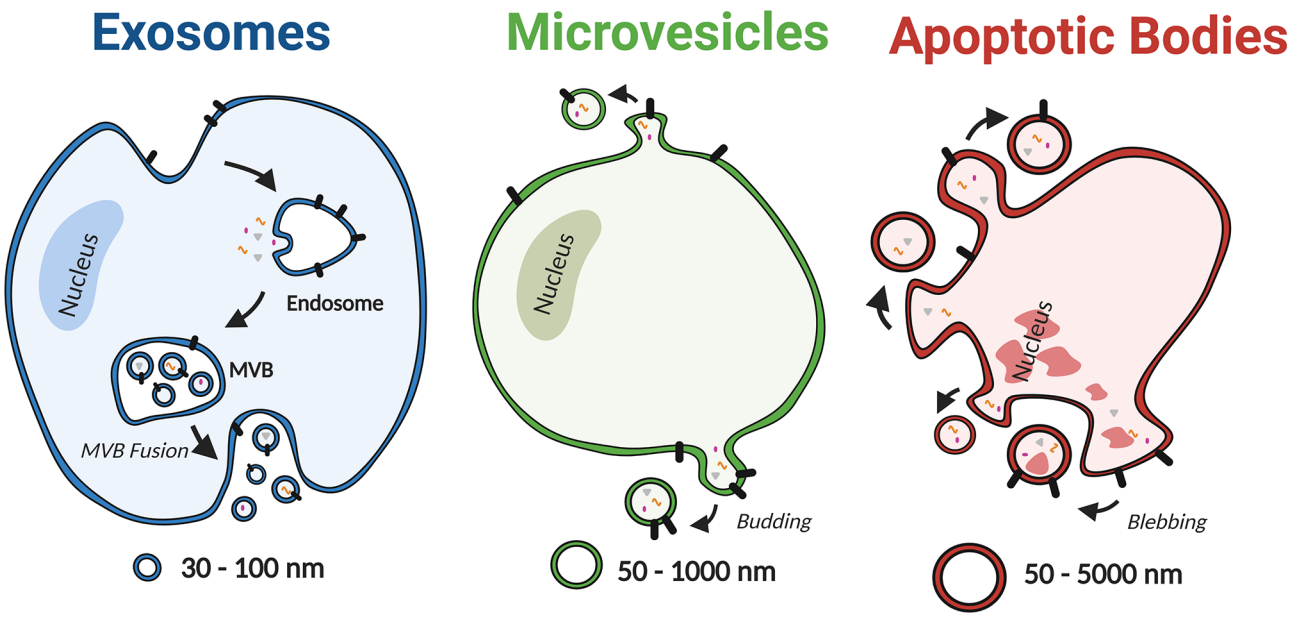

FIGURE 2 | Schematic depiction of EV subtypes, including exosomes, microvesicles, and apoptotic bodies. Based on the mechanism of biogenesis, EVs can be categorised into three distinct subgroups; exosomes, microvesicles and apoptotic bodies. Exosomes are the smallest subclass of EVs with a diameter ranging from 30 nm to 100 nm. They are produced as intraluminal vesicles by inward budding of the endosomal membrane to form multivesicular bodies (MVB), which release vesicles upon fusion with the plasma membrane. In contrast, MVs are 50 nm to 1000 nm in diameter and are formed by direct budding from the plasma membrane. Lastly apoptotic bodies have the broadest range of diameters (50–5000 nm) and are produced by cells undergoing apoptosis. Created with BioRender.com.

endoplasmic reticulum stress in combination with exhaustion of endogenous damage-associated molecular pattern (DAMP) clearance mechanism (51–54). Cellular senescence, defined as irreversible cell cycle arrest, has also been suggested to contribute to inflammaging. This process is driven by a variety of mechanisms including the DNA damage response and age-related telomere attrition that activate the senescence-associated secretory phenotype (SASP) wherein cells release high levels of pro-inflammatory factors (55). The number of senescent cells increases with age, generating low-grade inflammation which has widely been implicated in the pathogenesis of age-related diseases (56). Inflammaging is also associated with changes in immune cell function and subset composition with age, known as immunosenescence. The key changes occurring in immunosenescence were recently summarised by Feehan et al. and broadly include reduced phagocytosis, altered immune-modulatory cytokine expression, increased autoimmunity and diminished activation of the adaptive immune response (57). Furthermore there is increasing evidence that COPD and its coexistent conditions represent an acceleration of the ageing process (58, 59).

EVs as Immunomodulatory Factors in Ageing

Age related changes in the concentration of circulating EVs has been a topic of debate as it remains difficult to accurately measure the concentration of EVs due to the complexity of isolating pure EV populations, detecting EVs and enumerating them (60). Using nano-particle tracking analysis (NTA), Eitan et al. reported that the concentration of circulating EVs decreased with age in human plasma (61). These authors suggested this decrease was partially due to enhanced internalization by B cells, as determined by a FACS-based assay using fluorescently labelled EVs (61). It was also suggested that the reduction in EV concentration with age could be a consequence of an impaired clearance mechanism, given EVs may function to dispose of unwanted proteins and other molecules (61). Later studies using NTA have reported no significant difference in the concentration of plasma EVs from non-smokers, smokers and patients with COPD (62, 63). However using NTA to determine EV concentration has major limitations given it cannot differentiate other particles such as lipoproteins that are commonly co-isolated with EVs. Other studies have demonstrated changes in EV concentration based on common EV markers. For example, the concentration of CD9 positive EVs was shown to be significantly elevated in COPD patients and to correlate with physiological markers of multimorbidity including CRP and IL-6 (64, 65).

Inflammation is a major hall-mark of aging and age related diseases. A number of studies have suggested an age-related increase of EV miRNAs with an anti-inflammatory role, which may act as a compensatory mechanism to oppose the hyperinflammatory state that increases during normal aging and is accelerated in progression of aging-related diseases such as COPD. Plasma EVs from aged mice have been shown to be enriched in miR-192 that functions to suppress the inflammatory response in macrophages (66). This was demonstrated by a significant reduction of IL-6 and IL-1β expression in

stimulated murine macrophages following treatment with EVs isolated from miR-192 transfected RAW264.7 cells. Furthermore, exogenous intravenous administration of EVs, isolated from miR-192 transfected RAW264.7 cells, were shown to significantly reduce macrophage recruitment and expression of IL-6, IL-12, TNF, interferon (IFN) and CCL2 in lung tissue of mice following inoculation with inactivated influenza whole virus particles (WVP). Increased levels of miR-192 have also been reported in plasma derived EVs from COPD patients suggesting that the EV miR-192 response to hyperinflammatory state is exaggerated in COPD patents (63). Furthermore, EVs containing miR-192 have been shown to delay diabetic retinopathy and inflammatory responses in rheumatoid arthritis, supporting the role of miR-192 in mediating multiple disease pathways (67, 68). Other miRNA including miR-21, miR-146a and miR-223 have been identified to be elevated in EVs isolated from the plasma of old mice (69). These miRNA were shown to contribute to an anti-inflammatory phenotype, as demonstrated by increased expression of *Arg1*, *Il10* and *Mrc1* in LPS-stimulated macrophages and reduced endothelial cell response to VEGF (69). Similarly to miR-192, studies have shown miR-21, miR-146a and miR-223 to be increased in EVs isolated from patients with COPD or other chronic diseases, such as osteoporosis, CVD and diabetes (70–77). Therefore, these EV miRNA may provide a common link and potential biomarker for accelerated ageing and age-related diseases. However, although these studies suggest an increase in anti-inflammatory EV miRNA as a mechanism to decrease inflammation, other studies have suggested that removal of miRNA, such as miR-223, from alveolar macrophages and T cells *via* EVs releases suppression of the NLRP3 inflammasome thereby promoting activation (78, 79). Further studies using clinically relevant samples are required to establish if the increase in anti-inflammatory EV miRNA observed in ageing and age-related chronic diseases is a mechanism to counteract chronic inflammation or is a mechanism to release suppression of cellular immune and inflammatory pathways.

Mesenchymal stem cells (MSC) have been of particular scientific interest because of their potent immunomodulatory and anti-inflammatory properties. However stem cell exhaustion has been implicated in ageing and several chronic diseases including COPD (80). Huang et al. demonstrated that human MSC EVs from a young donor suppressed the activation of IL-6, IL-1β and TNF in the lung following LPS insult when injected into mice (81). On the other hand, this anti-inflammatory response was not observed for MSC EVs isolated from an old donor. Significantly higher levels of anti-inflammatory miR-223 and lower levels of pro-inflammatory miR-127 and miR-125b were observed in MSC-EVs from young vs old mice which may explain this phenomenon (81). In a separate study, MSC-EVs that originated from older rats were demonstrated to have a lower content of miR-133b-3p and miR-294, two miRNAs that inhibit TGF-β1-mediated epithelial-to-mesenchymal transition, which contributes to fibrosis (82). Reduced expression of miR-133b has also been identified in COPD patients, coronary artery disease and osteoporosis with a range of suggested functions including regulating vascular smooth muscle cells and osteoblast differentiation (83–85). These studies suggest EVs from aged MSC may be less capable of protecting against chronic inflammation and tissue damage, which in turn may promote accelerated ageing and the development of chronic diseases such as COPD.

Endothelial cells that line the lumen of blood vessels not only act as a physical barrier but play a pivotal role in regulating blood flow and immune cell recruitment (86). Ageing and exposure to noxious stimuli induces senescence of endothelial cells which contributes to endothelial dysfunction in both COPD and CVD (87, 88). Epigenetic regulation of DNA damage and senescence has been reported as a pathogenic mechanism linked to endothelial dysfunction in COPD patients (89). Mensà et al. demonstrated that EVs isolated from an *in vitro* model of endothelial replicative senescence enhanced the senescent associated profile in recipient endothelial cells including increased expression of cell cycle inhibitor p16 and SASP factors including IL-6 and IL-8 (90). The mechanism by which EVs from senescent cells can spread premature senescence was suggested to be due to inhibition of the epigenetic regulators DNMT1 and SIRT1 *via* increased levels of EV miR-21 and miR-217 (90). Furthermore, EVs from the plasma of elderly humans and senescent cultured endothelial cells, have been shown to promote calcification in vascular smooth muscle cells, a risk factor for the development of CVD (91). In addition, EVs released from senescent endothelial cells have been shown to be enriched in miR-31 that inhibits osteogenic differentiation of MSCs, providing a possible link between endothelial dysfunction and osteoporosis (92).

Research over recent years into characterising the age-related changes in EVs reveals diverse functional changes in EVs with age and suggest it is unlikely to be as simple as concluding they either promote or inhibit "inflammaging" and chronic disease. More likely, there are different subtypes of EVs with different functions depending on their origin and the mechanism by which they are released. Further research is required to characterise these distinct EV populations across the human lifespan. Increased susceptibility to infection and the multimorbidity observed in COPD may be partially attributed to the reduced immune function observed with ageing as discussed in the following section.

RESPIRATORY INFECTIONS, INFLAMMATION AND THE DEVELOPMENT OF CHRONIC DISEASE

Respiratory infections are the primary driver of COPD exacerbations, which lead to worsening of symptoms and increased risk of hospitalisation and mortality (93–96). Respiratory viruses, including rhinovirus, respiratory syncytial virus (RSV) and influenza, are commonly associated with COPD exacerbations whilst colonisation of bacteria such as non-typeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and *Moraxella catarrhalis* in the airways of COPD

patients is common during both stable disease and exacerbations (12, 13, 97–100). In particular, acquisition of new bacterial strains appears to be associated with an increased risk of COPD exacerbations (101, 102). Additionally, acute childhood infections are thought to play a role in initiating pathological mechanisms which could predispose individuals to chronic diseases in later life (103, 104). The potential long-term impact of respiratory infections has been exemplified by the recent SARS-CoV-2 pandemic with severe COVID-19 leading to multiple organ damage and a range of long-term systemic effects (105, 106). In addition, greater morbidity and increased mortality has been observed following infection with SARS-CoV-2 in individuals with existing chronic diseases such as COPD, CVD and diabetes (107–111). However, the long-term impact of COVID-19 disease on the body and resultant pathological mechanisms that could drive susceptibility to lung diseases, such as COPD and other systemic diseases, is still unravelling.

Immune and Inflammatory Role of EVs in Response to Respiratory Infection

Respiratory infections have been reported to trigger increased levels of lung-derived EVs released from alveolar macrophages and epithelial cells (112, 113). EVs released in response to respiratory infections, that are frequently detected in AECOPD, contribute to the production of immune and inflammatory mediators. A recent study reported significant differences in small RNA EV cargo released by the human alveolar epithelial A549 cell line when infected with RSV (114). These EVs were shown to activate the innate immune response as demonstrated by increased production of cytokines and chemokines including CXCL10, CCL5 and TNF in other A549 cells and human monocytes (114). On the other hand, proteomic characterisation of EVs released from human macrophages upon influenza infection revealed EVs may directly transfer pro-inflammatory cytokines (115). EVs have also been shown to upregulate type I IFNs, a key mediator of anti-viral responses. Liu et al. demonstrated that EVs produced by influenza infected A549 cells induced IFN production to inhibit viral replication through upregulation of miR-1975 (116). Similarly, BALF EVs from a murine model of highly pathogenic avian influenza have been reported to have an increased level of miR-483-3p and to potentiate IFN immune response (117). Furthermore, pattern-associated molecular patterns (PAMPs)-containing EVs have also been shown to stimulate a proinflammatory response in macrophages through TLR and enhance T cell activation (118, 119). In addition SARS-CoV-2 viral RNA within EVs derived from A549 epithelial cells has been shown to be internalised by cardiomyocytes and up-regulate inflammatory genes (120). The systemic dissemination of inflammation by macrophage-derived EVs has also been demonstrated using a LPS challenged murine model (121). This effect was proposed to be due to the interaction of histones on the outer surface of vesicles with TLR4 (121).

Endothelial dysfunction driven by respiratory infections has been associated with higher incidence of acute cardiovascular events following COPD exacerbations (122, 123). The concentration of endothelial EVs has been reported to be elevated in patients with COPD who have frequent exacerbations and may contribute to systemic effects by mediating coagulation, vascular tone and angiogenesis (124). Indeed coagulant proteins have been reported to be enriched in EVs in the plasma of COPD and cardiovascular patients (62, 125). EVs released in response to respiratory infections may be a contributing mechanism. For example, SARS-CoV-2 infection induces the release of tissue factor (TF) positive EVs into the circulation that was suggested to contribute to thrombosis and mortality in patients with COVID-19 (126–129).

Although there are a growing number of studies that report that EVs released in response to infection are pro-inflammatory and can induce systemic inflammation, there is currently no direct research into whether host EVs released in response to infection contribute to development and exacerbation of chronic diseases including COPD. However plasma EVs from COPD patients have been shown to contain higher levels of miR-125b that has previously been suggested to directly reduce antiviral signaling and cause exaggerated inflammation and impaired antiviral responses to IAV (63, 130). Therefore, further research is required to determine if dissemination and uptake of EVs contribute to the exacerbated or persistent inflammation observed in chronic disease in response to infection. In addition, given that EVs have been shown to modulate the IFN response, they may have useful anti-viral therapeutic applications. The potential of IFN treatment in the prevention of virally induced exacerbations in COPD is currently being investigated (131, 132).

Gram-negative bacteria associated with COPD, such as NTHi and *M. catarrhalis*, have also been shown to release EVs known as outer membrane vesicles (OMVs). OMVs are a similar size to host EVs, approximately 20 to 350 nm and can also export a range of cargo including proteins and small RNAs (133). Despite a wide number of studies demonstrating the effects of OMVs in host-microbe interactions, including their ability to enter the systemic circulation and induce a variety of immunological and metabolic responses, the exact mechanisms of bacterial vesicles and their content are still largely unknown (134, 135). Bacterial OMVs can deliver their cargo to a range of host cells including epithelial cells, neutrophils and macrophages and subsequently stimulate an inflammatory response (133, 136, 137). For example, OMVs of *M. catarrhalis* can bind to TLR2 on epithelial cells and are subsequently internalized, causing a pro-inflammatory response and increased levels of IL-8 (138). Furthermore, OMVs have been implicated in the formation of biofilms that increase tolerance to antimicrobial treatments and the immune system (99, 139). While the presence of NTHi and *M. catarrhalis* have been associated with a heightened risk of COPD exacerbation, the mechanisms for this remains unclear. Given that NTHi and *M. catarrhalis* have been shown to release OMVs, that can activate host immune responses as well as potentially support bacterial colonization, they may provide a novel mechanism contributing to the nature of chronic and recurrent bacterial infections in AECOPD and systemic disease. In support of this, a recent review highlighted the role of

Porphyromonas gingivalis in promoting the development of related systemic diseases including diabetes and cardiovascular disease through long distance transmission of OMVs (140, 141). Further research is, therefore, required to determine role of OMVs in COPD and other systemic diseases.

Our understanding of the role of EVs in activating the host immune response against respiratory pathogens, or facilitating infection, remains sparse. The release of pro-inflammatory EVs in response to infection may have the ability to induce inflammation, both locally in the lung and distally, contributing to the pathology of COPD and allied chronic diseases. Further studies are required to elucidate the mechanisms by which EV cargoes modulate the immune response of recipient cells over the course of infection and whether this is dysregulated in chronic diseases and could contribute to an impaired immune response. In contrast, other environmental risk factors such as CS have been shown to increase the incidence of multiple chronic diseases.

RESPIRATORY TOXINS, INFLAMMATION AND THE DEVELOPMENT OF CHRONIC DISEASE

Exposure to respiratory toxins, such as CS and air pollution, has been shown to be the primary risk factor for COPD as well as significantly increasing the risk of developing CVD and diabetes complications. Sustained exposure to harmful stimuli results in profound functional and structural changes to the airway epithelium including changes in mucous production, impairment of epithelium regeneration and reduction in cilia development (142). In addition, smoking contributes to immune dysregulation including an increase in pro-inflammatory effects such as increased immune cell recruitment as well as immunosuppressive effects including suppression of immune cell effector functions (143). Furthermore, the damage caused by respiratory toxins has been associated with accelerated ageing and increased susceptibility to infections that contribute to chronic disease pathology, as discussed previously. Studies in smokers with mild-moderate COPD have shown that the relationship between COPD and CVD is mediated through established cardiovascular risk factors such as tobacco smoking rather than through COPD itself (18). As with respiratory infections, a link between CS exposure in early life and the development of chronic disease during adulthood has also been demonstrated (144). However, while smoking cessation slows the rate of decline of pulmonary function in COPD patients it does not halt disease progression and systemic inflammation persists. Therefore, EVs may provide a mechanism contributing to the self-perpetuating spread of systemic inflammation.

Immune Modulating EVs Released in Response to Respiratory Toxins

Oxidative stress induced by the imbalance between oxidants and antioxidants from exposure to CS has been shown to increase the levels of EVs released by the airway epithelium. Benedikter et al. proposed that exposure of the BEAS-2B human bronchial epithelial cell line to cigarette smoke extract (CSE) increases the release of EVs due to oxidative depletion of surface thiols (145). In support of this proposal, antioxidants such as N-acetyl-L-cysteine (NAC) were shown to prevent CSE-associated increases in EVs (145). However, it is possible that the increased levels of EVs detected were due to reduced EV uptake, as another study reported reduced EV uptake following exposure to CSE that was reversed by the presence of NAC (146). While there is overwhelming evidence that oxidative stress and oxidative damage play a pivotal role in the pathogenesis of COPD and other systemic diseases, further research is required to determine if redox-dependent thiol modification is a potential mechanism contributing to the modulation of EVs in multimorbid patients' (147).

Exposure to respiratory toxins upregulates inflammatory pathways that result in increased immune cell recruitment and release of pro-inflammatory mediators commonly upregulated in COPD and other chronic diseases. EVs released in response to respiratory toxins have been shown to promote cytokine release in epithelial cells. Heliot et al. reported that EVs isolated from the BAL of smokers increased secretion of IL-6 by BEAS-2B cells (148). Similarly, Martin et al. reported that EVs released by THP-1 macrophages which were exposed to $PM_{2.5}$ promoted the release of IL-6 and TNF from BEAS-2B cells (149). The release of EVs from airway epithelial cells in response to respiratory toxins has been reported to induce recruitment of monocytes and activation of macrophages (30, 150). One study reported that EVs released from BEAS-2B cells under oxidative stress activate macrophages and promote expression of TNF, IL-1β, and IL-6 though increased EV levels of miR-320a and miR-221 (151). Increased levels of miR-221 have been reported to enhance smoking-induced inflammation in COPD (152). Furthermore, increased EV miR-320a has been reported in the plasma of osteoporosis patients and was suggested to impair osteoblast function and induces oxidative stress (77, 153). The manifestation of systemic disease has been suggested to be potentially caused by elevated levels of pro-inflammatory Wnt5a in circulating EVs in response to smoking and in COPD patients (154). Elevated levels of Wnt5a have also been demonstrated in other chronic diseases including heart failure (155). In addition, the production of circulating procoagulant EVs in response to CS has been demonstrated, further supporting the role of EVs as a mechanism contributing to the systemic effects of inhalation of noxious stimuli (156). Overall these studies suggest that EVs released in response to noxious stimuli contain cargo that have been associated with chronic disease and promote inflammation.

In COPD, airway epithelial cells and immune cells secrete an increased level of proteolytic enzymes resulting in chronic inflammation and destruction of lung parenchyma (7, 157, 158). Li et al. reported a 3-fold increase in EV matrix metalloproteinase (MMP)-14 released from macrophages following exposure to CSE (159). In addition, EVs have been found to be associated with neutrophil elastase that was

suggested to contribute to the ability of EVs to degrade extracellular matrix and promote alveolar destruction (160). These studies provide a mechanism by which EVs, released in response to noxious stimuli, may contribute towards tissue damage that promotes airway remodelling. EVs have also been reported to play a role in airway remodelling by mediating fibrosis through epithelial-mesenchymal transition (EMT) and myofibroblast differentiation. CSE has been shown to increase miR-210 and miR-21 in human bronchial epithelial cell EVs, leading to suppression of autophagy and an increase in myofibroblast differentiation (161, 162). On the other hand, He et al. reported a reduction in EV miR-21 released from CSE-treated BEAS-2B cells, which indirectly modulated EMT by alleviating the polarization of M2 macrophages (73). Furthermore, Coresello et al. reported no significant changes in the level of EV miR-21 released from human small airway epithelial cells in response to CSE (163). Variations in the type of airway epithelial cell models, CS sources and EV isolation methods could account for the differences in EV miR-21 levels reported between the studies (164). As mentioned previously, miR-21 has been identified in elevated levels in EVs in response to risk factors such as ageing, as well as from patients with chronic disease including COPD, CVD, diabetes and oseteoporosis. Furthermore, miR-21 has been widely reported as an inflammatory mediator and suggested as a key switch in the inflammatory response (165). Therefore it will be vital to complete further research with standardised EV isolation and characterisation techniques to fully understand the biologically relevant effects of EV miR-21 and its contribution to multimorbidity.

Exposure to respiratory toxins damages the epithelial and endothelial barrier, This contributes to the "overspill" of inflammatory mediators, including EVs produced in the lung, to the circulation. An increase in circulating EVs and alteration of their cargo in response to noxious stimuli may contribute to systemic inflammation and the development of chronic diseases such as COPD. Further studies are required to understand if a distinct EV population can be used to discern the smokers who will go on to develop multiple chronic diseases.

FUTURE PERSPECTIVES

The field of EV research is still relatively new and our understanding of the role of EVs is rapidly evolving. Given the proposed function of EVs in mediating pathways that are central to multiple chronic diseases they may pose as novel biomarkers or useful therapeutic targets (**Figure 3**). However, no definitive link has yet been established between circulating EVs and the development or exacerbation of chronic diseases. A critical question is whether EVs directly contribute to associated pathology or are simply a consequence of the disease. The impact of exposure to different risk factors in modulating disease relevant EVs needs to be determined, alongside their relative contribution and combinatory effects. Furthermore, while a number of studies have alluded to EVs as systemic immune modulators able to reach distant organs, further studies are required to validate this theory.

Current studies investigating the role of EVs in chronic diseases have been limited by the lack of a gold standard for EV isolation and functional characterisation. EV profile characterisation has been demonstrated to be dependent on the experimental models and techniques applied, with variable particle yield, genomic and proteomic EV profiles reported between different methodologies (25). Application of recently emerging technologies will allow better isolation and characterisation of distinct EV populations. For example, microfluidic technologies, such as asymmetric flow field-flow fractionation (AF4), have recently been demonstrated as an improved technique for isolating distinct nanoparticle subpopulations and therefore allow better characterisation of heterogeneous EV populations (166). Furthermore, there is growing interest in techniques that allow single-EV analysis and therefore can tease apart the distinct biophysical and molecular properties of individual EVs in a heterogeneous population. One recent example is single-particle interferometric reflectance imaging (SP-IRI), recently developed and sold as an automated platform called the ExoView system (NanoView Biosciences) (167, 168). This system can be used for multiplexed analysis and allows simultaneous sizing and protein profiling. Once techniques for EV isolation and characterisation are standardised, the use of multi-omics approaches, including transcriptomic, proteomic, metabolomic, and lipidomic would be beneficial to better understand the function and relationship of EV biomolecules (169). However, this will require more transparent reporting of methodological details and increased data availability in future studies.

Another issue is that our current understanding of the immunomodulatory role of EVs in response to biological and environmental risk factors is based largely on *in vitro* cell culture models that may oversimplify the *in vivo* functions of EVs due to the limited intercellular interactions. Moreover, many *in vivo* studies into the function of EVs have been based on murine studies that have previously shown to have limitations regarding recapitulation in the human biological system. Therefore, further work using relevant human *ex vivo* and co-culture models are required to obtain clinically relevant data. Additionally, while clinical samples provide the biological complexity necessary, they should include a well characterised patient cohort which is large enough to reduce bias due to patient heterogeneity. In clinical studies, analysing EVs across a range of chronic diseases using a standardised isolation protocol and with stratification of multimorbid patients will also be necessary to compare disease related changes in EV cargo.

CONCLUSION

On review of recent EV literature it is apparent that there is overlap in EV cargo shown to be altered across a range of chronic diseases and in response to disease risk factors such as ageing,

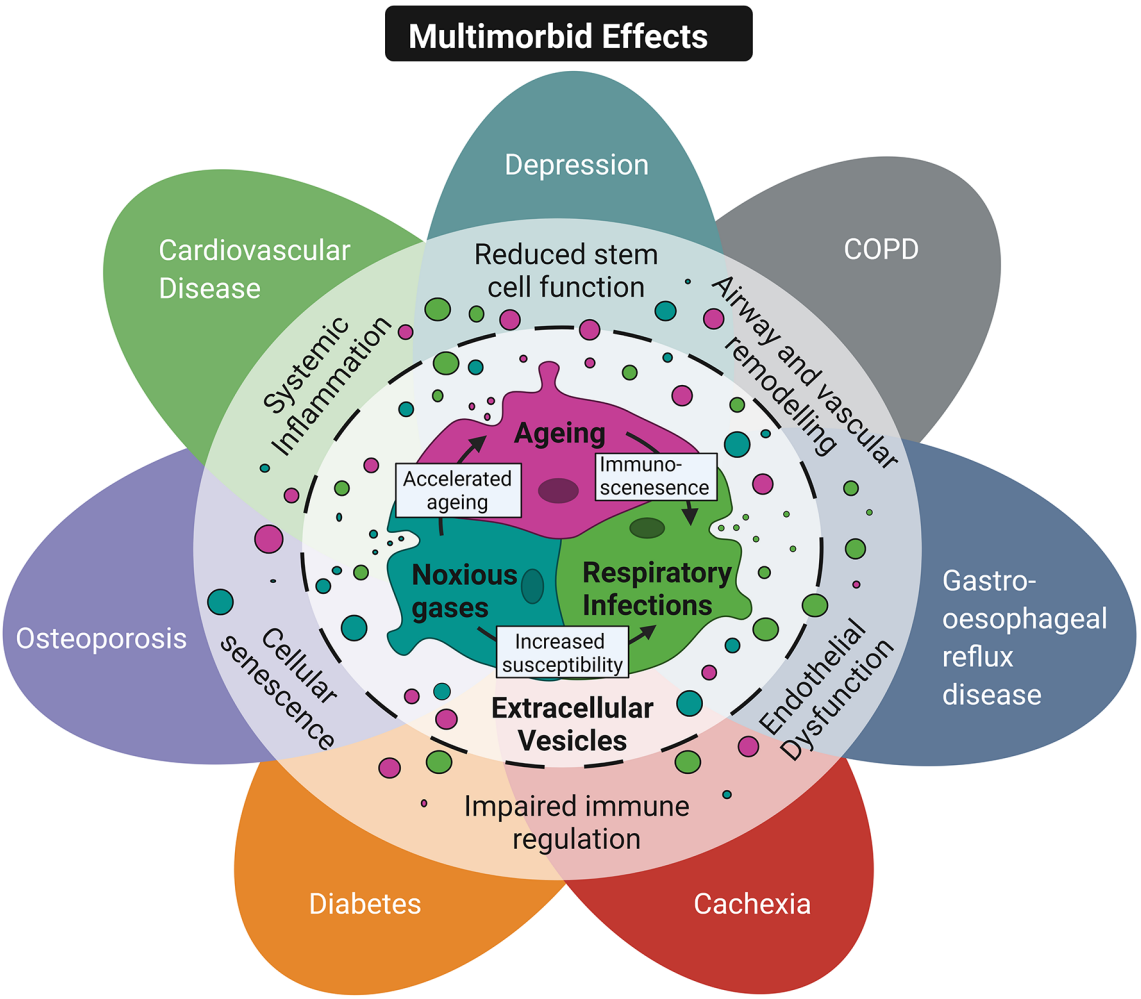

FIGURE 3 | Conceptual diagram of EVs as a common mechanism in multimorbidity. Shared risk factors including ageing, inhalation of noxious stimuli and respiratory infections are thought to contribute to multimorbidity in COPD patients. These risk factors can exacerbate each other. For example ageing promotes immunosenescence and reduces the ability of the immune response to neutralise infection. In addition, smoking increases susceptibility to infection and accelerates features of ageing. These risk factors have also been shown to promote the release of EVs that mediate key pathological features in multimorbid COPD patients' such as reduced stem cell function, airway and vascular remodeling, endothelial dysfunction, impaired immune regulation, cellular senescence and systemic inflammation. Created with BioRender.com.

infection and smoking. Furthermore many of these changes in EV cargo mediate pathological features, such as chronic inflammation, that are central to multimorbidity in COPD patients and therefore may provide novel diagnostics and therapeutics. However, further studies investigating the function of EVs in multimorbidity using physiologically relevant, disease specific, *ex vivo* models are required.

AUTHOR CONTRIBUTIONS

LR: conceptualization, investigation, literature searching, analysis, project administration, writing original draft, reviewing and editing. CS: supervision, conceptualization, reviewing & editing. AW: supervision, reviewing & editing. KS: supervision, conceptualization, reviewing & editing. TW: supervision, conceptualization, reviewing & editing. All authors contributed to the article and approved the submitted version.

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A Differential Signature of Circulating miRNAs and Cytokines Between COVID-19 and Community-Acquired Pneumonia Uncovers Novel Physiopathological Mechanisms of COVID-19

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Coronavirus Disease 2019 (COVID-19) pneumonia is a life-threatening infectious disease, especially for elderly patients with multiple comorbidities. Despite enormous efforts to understand its underlying etiopathogenic mechanisms, most of them remain elusive. In this study, we compared differential plasma miRNAs and cytokines profiles between COVID-19 and other community-acquired pneumonias (CAP). A first screening and subsequent validation assays in an independent cohort of patients revealed a signature of 15 dysregulated miRNAs between COVID-19 and CAP patients. Additionally, multivariate analysis displayed a combination of 4 miRNAs (miR-106b-5p, miR-221-3p, miR-25-3p and miR-30a-5p) that significantly discriminated between both pathologies. Search for targets of these miRNAs, combined with plasma protein measurements, identified a differential cytokine signature between COVID-19 and CAP that included EGFR, CXCL12 and IL-10. Significant differences were also detected in plasma levels of CXCL12, IL-17, TIMP-2 and IL-21R between mild and severe COVID-19 patients. These findings provide new insights into the etiopathological mechanisms underlying COVID-19.

Keywords: COVID-19, community-acquired pneumonia, microRNAs, plasma, soluble proteins

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is responsible for the disease known as coronavirus disease 2019 (COVID-19) (1). Although initially COVID-19 was defined as a low tract respiratory disease, it is now acknowledged as a complex disorder that compromises multiple organs and may cause long-lasting damage even in patients that overcome the acute phase (2). Major efforts have been devoted to identifying molecules able to predict the severity of COVID-19. These efforts were aimed to improve patient stratification and make a better use of the available resources to optimise health care. After several months of intensive research, some prognostic clinical biomarkers have been identified, including lymphocyte count, D-dimer, C reactive protein (CRP), ferritin, Interleukin (IL)-6 or viremia (3, 4). Moreover, dysregulation of other cytokines, including IL-2, IL-10, Interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1 or C-X-C Motif Chemokine Ligand (CXCL)10 in COVID-19 patients have also been reported (5).

Some of this research has also been focused on microRNAs (miRNAs), which are short (22 nucleotides in length on average) non-coding RNAs that function as post-transcriptional regulators by binding to mRNAs and preventing protein translation. Each miRNA can target multiple genes, which makes them important regulators of numerous cellular functions (6). Furthermore, miRNAs mediate cell to cell communication travelling either as free circulating molecules or inside exosomes, thereby modulating multiple immune system functions or inflammation in several diseases (7–9). These molecules can be used to predict the clinical course of patients with viral infections, since certain miRNAs are able to bind to distinct RNA viral genomes, blocking their replication (10). A specific miRNA signature has been determined for other viruses, e.g. influenza, which led researchers to find dysregulated pathways in critically ill patients (11).

In silico analyses of potentially relevant miRNAs in COVID-19 have been previously carried out. These studies focused on identifying host miRNAs specifically targeting the SARS-CoV-2 genome as a defence strategy (12) or viral miRNAs affecting expression of host genes that could be associated with the pathogenesis of the disease (13). Additionally, previous experimental studies have addressed the relationship of certain miRNAs (miR-2392, miR-146a-5p, miR-21-5p, miR142-3p, miR-15b-5p among others) with the pathogenesis of COVID-19 (14, 15).

However, to our knowledge, no studies have been published that identify miRNAs specifically dysregulated by SARS-CoV-2 infection compared to other types of pneumonia.

The aim of this work was to determine a profile of plasma miRNAs and soluble target molecules in COVID-19 compared to community-acquired pneumonia (CAP) as a specificity control group. CAP was selected because it comprises a group of low tract respiratory infections whose symptoms frequently resemble those of SARS-CoV-2 infection. Here, we identify circulating molecules, such as miRNAs and cytokines, which are differentially expressed in COVID-19 compared to CAP. This study provides novel insights into the molecular mechanisms of this pathology.

MATERIALS AND METHODS

Experimental Design and Patient Selection

To ensure an unbiased manipulation of the samples, a blinded study was carried out. A total of 123 COVID-19 patients were included in this retrospective study. They all had been diagnosed by a positive result of RT-qPCR for SARS-CoV-2 in nasopharyngeal swabs. Patients were admitted to the University Hospital de La Princesa from March 10th to April 21st, 2020 (first wave). Plasma samples were collected within 5 days upon admission.

COVID-19 patients were mainly treated with viral protease inhibitors (lopinavir/ritonavir), hydroxychloroquine and/or azithromycin, according to local therapeutic guidelines at the time. Patients treated with either corticosteroids or tocilizumab were excluded from the study. Demographic and clinical variables of the study populations were collected.

The CAP cohort consisted of 33 adult patients, presenting symptoms of lower respiratory tract infection together with the appearance of a new infiltrate on a chest radiograph and the absence of an alternative diagnosis during follow-up, according to the usual definition. All of them were diagnosed with CAP and admitted to the University Hospital de La Princesa between 2014 and 2015. This cohort has been previously used in other studies, in the context of research project on prognostic biomarkers in CAP (16, 17). CAP instead of healthy subjects was selected as the control group because its similarities in symptoms with COVID-19 and to rule out non-specific changes in miRNAs due to a general inflammatory/stress status. Plasma samples were collected at admission, according with ATS/IDSA guidelines in force (18), aliquoted and stored at -80°C until use. These samples were obtained from the Biobank of the University Hospital de La Princesa.

Our screening cohort was composed of 38 COVID-19 patients, 20 with mild disease and 18 with severe disease, classified according to symptoms severity (19). The validation cohort comprised 43 mild and 42 severe COVID-19 patients (**Supplementary Figure 1**).

Human Plasma Extraction and RNA Purification From COVID-19 Patients

To collect the plasma, EDTA tubes with 10 ml of venous peripheral blood were centrifuged 20 min at 2000 x g at 4°C. 1 ml of plasma was then aliquoted and stored at -80°C for RNA extraction.

Total RNA was isolated from 200 µl of plasma using miRNeasy Serum/Plasma Advanced Kit (QIAGEN), following the manufacturer's instructions. Plasma samples were centrifuged 5 minutes at 4°C and 3000 x g. 1.25 µg/ml MS2 bacteriophage RNA (Roche Diagnostics) and 1 µl of UniSp2, UniSp4 and UniSp5 RNA spike-in templates were added to each sample as a quality control of the RNA extraction process. The RNA was eluted in 20 µl of RNase-Free Water and then stored at -80°C until use.

To assess hemolysis, absorbance (Abs) at 414 nm of all plasma samples was measured using NanoDrop One/One^{C}

Spectrophotometer (Thermofisher Scientific) and those severely hemolysed samples (Abs > 5) were discarded.

Reverse Transcription (RT) and RT-qPCR Assays

The isolated RNA was reverse transcribed to complementary DNA (cDNA) using miRCURY LNA RT Kit (QIAGEN) following manufacturer's instructions and quality control of this process was carried out using cel-miR-39 and Unisp6 RNA spike-in templates.

The screening of the candidate miRNAs by qPCR was performed by means of miRCURY LNA miRNA Focus PCR Panels (QIAGEN), which comprises 179 commonly found miRNAs in human plasma.

For qPCR validation assays, miRCURY LNA miRNA Custom PCR Panels (QIAGEN) were used. Both assays were performed using miRCURY LNA SYBR® Green PCR Kit (QIAGEN). QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific) was used for qPCR plates reading.

qPCR Data Analysis

A quality control of the RNA extraction was performed by means of the spike-in previously added to the extraction and outlier samples were removed from the analysis.

Ct values above 36 were discarded for the global mean calculation. Interplate calibration (IPC) was performed using UniSp3 as described in the kit's Handbook. Then, Relative Quantities (RQ) were calculated as the log base 2 values of the Ct difference between miRNAs and UniSp2 RNA spike-in as described elsewhere (20). Normalisation factor (NF) was calculated as the geometric mean of the RQs of all expressed targets per sample. Normalised Relative Quantities (NRQ) were obtained by dividing the RQs by the sample specific NF.

Data from discovery cohort were normalized using the Global Mean normalization method, as previously described (21). For the miRNAs validation experiments, a group of stable miRNAs was selected from the screening panel using the software packages Normfinder and geNorm. miR-15b-5p, miR-30d-5p, let-7i-5p and miR-15a-5p were chosen to normalise data of the COVID-19 mild vs. severe analysis. Normalisation of CAP and COVID-19 analysis was performed using miR-103a-3p, miR-320a, miR-30e-5p and miR-15b-5p.

Target Validation by ELISA Arrays

Plasma levels of soluble cytokines and proteins were analysed using a custom ELISA multiplex array (RayBiotech Life, GA, USA) according to manufacturer's instructions.

Statistical Analysis

Categorical variables were represented by absolute value and percentage, and continuous variables were represented by median and interquartile range (IQR).

A one-way ANOVA was used to select potential candidate miRNAs with significant differences ($p<0.05$) across groups (CAP and mild and severe COVID-19 patients) in the screening analysis. Then, of those potential candidates, a multiple linear regression analysis for miRNAs following normal distribution or a logistic regression analysis for miRNAs which could not be transformed to normally-distributed variables were used. Quantitative variables following a non-normal distribution were transformed to normally distributed variables. These models were adjusted by confounding variables such as sex, age, days post-onset of symptoms (POS), ethnic group, or hemolysis. To search for possible confounding variables, Pearson or Spearman correlations were employed. False discovery rate (FDR) correction was assessed by the Benjamini-Hochberg method (22).

Logistic regression analysis for CAP and COVID-19 classification was characterised by using all the variables with a stepwise procedure, with both backward and forward search based on Akaike information criteria, to select the critical variables. The discrimination validity of the score, and also the main value, were assessed by AUC of the ROC curve along with the 95% confidence interval (95%CI).

A Youden's J statistic was used to determine the sensitivity and specificity of the model. These statistical analyses were performed using our own codes and base functions in R, version 3.5.1 (http://www.R-project.org).

Graphs were performed with GraphPad Prism 8. For outlier detection and removal, ROUT algorithm with an FDR of 1% was used. The rest of the analyses were performed with Stata 14.0 for Windows (Stata Corp LP, College Station, TX).

Ethics Approval

Transfer of samples from the Biobank and the study protocol was approved by the local Research Ethics Committee (register number 4070) and it was carried out following the ethical principles established in the Declaration of Helsinki. All recruited patients (or their representatives) were informed about the study and gave an oral informed consent as proposed by AEMPS due to COVID-19 emergency.

RESULTS

Cohort Selection and Clinical Features of Study Populations

miRNA profile was assessed in a discovery cohort that consisted of 38 COVID-19 patients (20 with mild and 18 with severe disease), collected during the first wave of the pandemic in Spain, from March to April 2020. Median age was 59.5, 15 were female and 23 were male. High blood pressure (HBP) and dyslipidemia (DL) were the most common comorbidities found in the COVID-19 cohort (34.2% and 29% respectively). As a specificity control group, 9 patients (5 female and 4 male) presenting with CAP were included, with a median age of 62.

The validation cohort comprised 85 patients with COVID-19 (43 with mild and 42 with severe disease), recruited during the first wave, and 24 CAP individuals. CAP patients were recruited before pandemic (2014–2015) and in most cases CAP were of unknown etiology. Median age was similar between both cohorts (66.5 and 64), whereas the CAP cohort had higher proportion of

males (58.3% versus 41.1% in COVID-19). Among comorbidities, DL (45.9%) and cardiovascular disease (CD) (10.6%) were most frequently found in COVID-19 individuals when compared with CAP (25% and 8.3% respectively). On the other hand, chronic obstructive pulmonary disease (COPD) was higher in CAP patients (20.8% vs 9.4%). The main demographic and clinical characteristics of the study population are described in **Table 1**.

Differentially Expressed miRNAs Between COVID-19 and CAP

A screening of 179 miRNAs commonly found in plasma revealed the existence of great differences in the miRNA profile expression between COVID-19 and CAP patients. More than 40 miRNAs showed a significant differential expression (**Figure 1A**). Multiple linear regression analyses were then performed, adjusting by possible confounding variables such as sex, age, hemolysis or POS. Using this approach, we found 35 miRNAs expressed at significantly different levels with a FDR corrected p value<0.05 (**Figure 1B** and **Table 2**). Data analysis revealed 19 upregulated and 16 downregulated miRNAs in COVID-19 vs. CAP patients. To confirm these findings, 21 candidate miRNAs were selected for further validation in an extended cohort of patients (**Supplementary Figure 1**). Candidate miRNAs were chosen based on their expression levels in plasma and their potential biological interest. To confirm the differences detected in the discovery cohort, individual RT-qPCR for every candidate miRNA was carried out in the validation cohort. Multivariate analyses adjusted by POS, age, sex, hemolysis or ethnic group was performed to probe statistical differences between miRNA levels in COVID-19 and CAP. A total of 15 miRNAs displayed differences between groups with a FDR corrected p value<0.05, as shown in **Figure 1C** and **Supplementary Table 1**.

We next interrogated the possibility that mild and severe COVID-19 patients also displayed specific miRNA signatures. Due to subtle differences found in miRNAs expression between these groups, we chose those miRNAs with an absolute fold change in expression (Ct) higher than 1.3 and a raw p value below 0.1 for further validation (**Supplementary Table 2**). Those miRNAs with a high number of non-detectable values in expression (above 50% of the samples) were not considered. Thereby, we found 10 potential candidate miRNAs (**Supplementary Figure 2**). Only miR-185-5p

TABLE 1 | Demographic and clinical characteristics of the study population.

	Discovery cohort COVID-19			Validation cohort COVID-19			Discovery cohort CAP	Validation cohort CAP
	Total n = 38	Mild n = 20	Severe n = 18	Total n = 85	Mild n = 43	Severe n = 42	n = 9[2] [22.2%]	n= 24
Age	59.5 (51–69)	59 (49.5-69.5)	59.5 (51–38)	64 (55–76)	58 (51–68)	72 (61–83)	62 (59–72)	66.5 (60.5-80)
Sex (male)	23 (60.52)	12 (60)	11 (61.11)	35 (41.18)	13 (30.23)	22 (52.38)	4 (44.44)	14 (58.33)
Days post onset of symptoms	9.5 (7–13)	10 (7.5-14)	8.5 (5–11)	9 (6–12)	9 (7–13)	8 (6–12)	10 (4–15)	6 (3-12.5)
Ethnicity (Caucasian)	26 (68.42)	13 (65)	13 (72.22)	63 (74.1)	31 (72.09)	32 (76.19)	6 (66.67)	22 (95.65)
CURB65	1 (0–2)	1 (0-1.5)	1 (0–2)	1 (0–2)	0 (0–1)	1 (0–2)	1 (1–2)	2.5 (1–4)
Comorbidities	29 (76.32)	15 (75)	14 (77.78)	59 (69.41)	25 (58.14)	34 (80.95)	5 (55.56)	19 (76)
HBP	13 (34.21)	8 (40)	5 (27.78)	36 (42.35)	13 (30.23)	23 (54.76)	2 (22.22)	11 (45.83)
DM	3 (7.89)	0 (0)	3 (16.67)	19 (22.35)	5 (11.63)	14 (33.33)	1 (11.11)	0 (0)
DL	11 (28.95)	7 (35)	4 (22.22)	39 (45.88)	17 (39.53)	22 (52.38)	2 (22.22)	6 (25)
CD	10 (26.32)	5 (25)	5 (27.78)	9 (10.59)	3 (6.98)	6 (14.29)	1 (11.11)	2 (8.33)
COPD	2 (5.26)	0 (0)	2 (11.11)	8 (9.41)	3 (6.98)	5 (11.90)	2 (22.22)	5 (20.83)
Asthma	3 (7.89)	1 (5)	2 (11.11)	2 (2.35)	2 (4.65)	0 (0)	1 (11.11)	1 (4.17)
Immuno -deficiency	4 (10.53)	3 (15)	1 (5.56)	1 (1.18)	0 (0)	1 (2.38)	0 (0)	2 (8.33)
Acute treatment	38 (100)	20 (100)	18 (100)	82 (96.47)	40 (93.02)	42 (100)	7 (77.78)	24 (100)
Lopinavir/ ritonavir	25 (65.78)	11 (55)	14 (82.35)	52 (61.18)	28 (65.12)	24 (57.14)	–	–
Hydroxy-chloroquine	33 (86.84)	18 (90)	15 (88.24)	81 (95.29)	40 (93.02)	41 (97.62)	–	–
Antibiotics*	26 (68.42)	15 (75)	11 (64.71)	69 (81.17)	37 (86.05)	32 (76.19)	7 (77.78)	24 (100)
Beta lactam	–	–	–	–	–	–	3 (33.33)	15 (62.5)
Quinolone	–	–	–	–	–	–	4 (44.44)	3 (12.5)
Macrolide (Clarithromycin/ Azithromycin)	26 (68.42)	15 (75)	11 (64.71)	69 (81.17)	37 (86.05)	32 (76.19)	2 (22.22)	20 (83.33)
Isolated pathogen								
SARS-CoV-2	38 (100)	20 (100)	18 (100)	85 (100)	43 (100)	42 (100)	–	–
Unknown	–	–	–	–	–	–	7 (77.78)	20 (80)
S. aureus	–	–	–	–	–	–	1 (11.11)	1 (4)
S. pneumoniae	–	–	–	–	–	–	1 (11.11)	3 (12)

*All categorical variables are expressed as absolute count (percentage) and quantitative variables as median (Interquartile range). Missing data in each group of patients are expressed as [number] [percentage]. *CAP patients could be treated simultaneously with more than one type of antibiotic.*

HBP, high blood pressure; DM, diabetes mellitus; DL, dyslipidemia; CD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; S. aureus, Staphylococcus aureus; S. pneumoniae, Streptococcus pneumoniae.

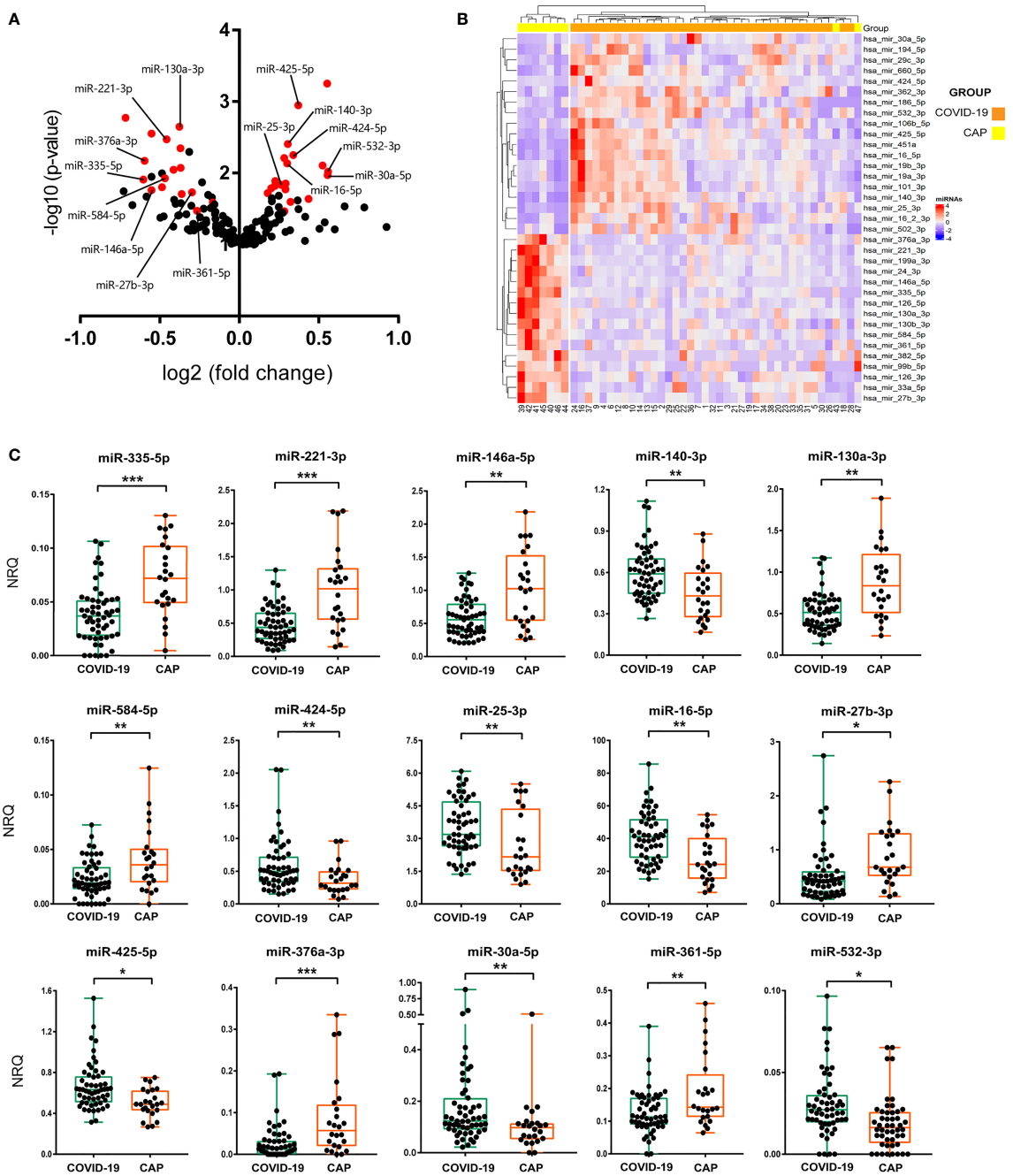

FIGURE 1 | miRNA signature in COVID-19 patients. **(A)** Volcano plot showing differential expression of 179 abundant miRNAs in human plasma between CAP and COVID-19 patients. Log_2 of fold change of normalised relative quantities (NRQ) and statistical significance ($-log_{10}$ of the p-value) from Mann-Whitney tests for each miRNA were assessed. In red, miRNAs with a corrected p value<0.05 assessed by multivariate statistical analysis. Names of the 15 miRNAs that were further validated are shown. **(B)** Hierarchical clustering heatmap of 35 differentially expressed miRNAs in plasma of 9 CAP and 38 COVID-19 individuals. Red and blue colour indicate upregulated and downregulated expression of COVID-19, respectively, as compared to CAP. **(C)** RT-qPCR of 15 validated miRNAs performed in the validation cohort. Box and whisker plots of NRQ for each miRNA are shown. A group of 4 highly stable miRNAs were used as normalisers. Statistical significance was assessed by means of multivariate statistical tests. *p<0.05, **p<0.01, ***p<0.001.

TABLE 2 | Differentially expressed miRNAs in COVID-19 vs. CAP patients found in discovery cohort.

miRNA	FDR corrected p-value[e]	Confidence Interval 95%		COVID-19/CAP
hsa-miR-140-3p	0.01790	0.5072	1.2578	↑
hsa-miR-335-5p[b]	0.00895	-0.4236	-0.1462	↓
hsa-miR-27b-3p[a]	0.00597	-1.1172	-0.3485	↓
hsa-miR-660-5p[a]	0.00448	0.3304	1.0587	↑
hsa-miR-130a-3p[a]	0.00358	-1.1136	-0.505	↓
hsa-miR-16-5p[a]	0.00298	0.5155	1.1372	↑
hsa-miR-146a-5p[a]	0.00256	-1.7077	-0.7462	↓
hsa-miR-362-3p[b]	0.00224	0.0898	0.2843	↑
hsa-miR-425-5p[b]	0.00199	0.2963	0.6154	↑
hsa-miR-101-3p[a]	0.00179	0.33	0.939	↑
hsa-miR-376a-3p[b]	0.00163	-0.4307	-0.1448	↓
hsa-miR-126-5p[a]	0.00149	-1.2407	-0.5048	↓
hsa-miR-382-5p[b]	0.00138	-0.2923	-0.1191	↓
hsa-miR-451a[b]	0.00128	7.8489	14.9547	↑
hsa-miR-19a-3p[a]	0.00119	0.2923	0.8424	↑
hsa-miR-19b-3p[a]	0.00112	0.2318	0.7337	↑
hsa-miR-24-3p[b]	0.00105	-1.3138	-0.612	↓
hsa-miR-199a-3p[d]	0.00099	–	–	↓
hsa-miR-584-5p[b]	0.00942	-0.3005	0.0837	↓
hsa-miR-16-2-3p	0.00895	0.1146	0.3954	↑
hsa-miR-532-3p	0.01705	0.0476	0.1899	↑
hsa-miR-424-5p[a]	0.01627	0.2387	1.0115	↑
hsa-miR-25-3p	0.01557	1.4206	6.1161	↑
hsa-miR-99b-5p[b]	0.01492	-0.2093	-0.5208	↓
hsa-miR-361-5p[b]	0.02065	-0.3181	-0.0672	↓
hsa-miR-221-3p[c]	0.01989	-1.1492	0.8271	↓
hsa-miR-194-5p[b]	0.03703	0.0689	0.3965	↑
hsa-miR-29c-3p	0.04042	0.5399	3.2983	↑
hsa-miR-33a-5p[b]	0.05034	-0.375	-0.0576	↓
hsa-miR-106b-5p[a]	0.05424	-3.4736	-0.2214	↑
hsa-miR-126-3p[a]	0.06137	-1.2407	-0.5048	↓
hsa-miR-502-3p	0.06289	0.0187	0.148	↑
hsa-miR-186-5p	0.07344	0.0166	0.1513	↑
hsa-miR-130b-3p[c]	0.08055	-4.882	-0.4597	↓
hsa-miR-30a-5p[c]	0.08098	0.4416	4.8825	↑

All miRNAs passing FDR correction (p-value<q-value) are shown. Multiple linear regression analyses were performed except for [c](logistic regression) and [d](Mann-Whitney tests). The arrows represent up or downregulation for each miRNA in COVID-19 with respect to CAP. miRNAs in bold were selected for validation assays. [a]log transformed miRNAs, [b]square root transformed miRNAs, [c]variable categorisation, [e]q-value threshold: 0.1.

was downregulated in severe cases of COVID-19. On the other hand, no statistically significant differences in the expression of the candidate miRNAs between COVID-19 mild and severe patients were observed in the validation cohort (**Supplementary Table 3**).

Logistic Regression Model Based on miRNAs for Classification of COVID-19/CAP Patients

Next, we developed a multivariate regression model able to differentiate and classify individuals with COVID-19 and CAP. For that purpose, the miRNAs selected for validation assays and other variables including age, sex, ethnic group, POS, IL-6, IgG, IgA, IgM, C3 and C4 were considered. Additional clinical variables shown in **Supplementary Table 4** and main comorbidities: HBP, diabetes mellitus (DM), DL, CD, COPD, asthma and secondary immunodeficiency were also included. Due to the high number of variables tested, a stepwise logistic regression procedure was employed that included the data corresponding to both discovery and validation cohorts. Among all these variables, we only found 4 miRNAs significantly contributing to the regression model: miR-106b-5p, miR-221-3p, miR-25-3p and miR-30a-5p. Hemolysis was included as confounding variable, showing no significant effect on the final model (p=0.12). To assess the performance of this model, a Receiver Operating Characteristic (ROC) analysis was carried out, which displayed a high area under the curve (AUC) value: 0.952 (0.895-1), with a sensitivity of 93.75% and a specificity of 89.09% (**Figure 2A**).

Analysis of Pathways and miRNA Targets

We next sought to identify the functional profiling of target genes of the 15 validated miRNAs. For that purpose, a Gene Ontology (GO) Enrichment Analysis was performed using Panther Classification System (http://pantherdb.org/). Only those target genes with strong evidence based on functional experiments, according to miRTarBase 8.0 (last accessed 09-22-2020), were considered. Interestingly, most of the enriched pathways were significantly associated with processes related to angiogenesis, regulation of endothelial cells or vasodilatation. Crucial pathways for cardiac muscle cell differentiation and proliferation were also present in the enrichment analysis. Other enriched target genes were related to immune response and inflammatory processes,

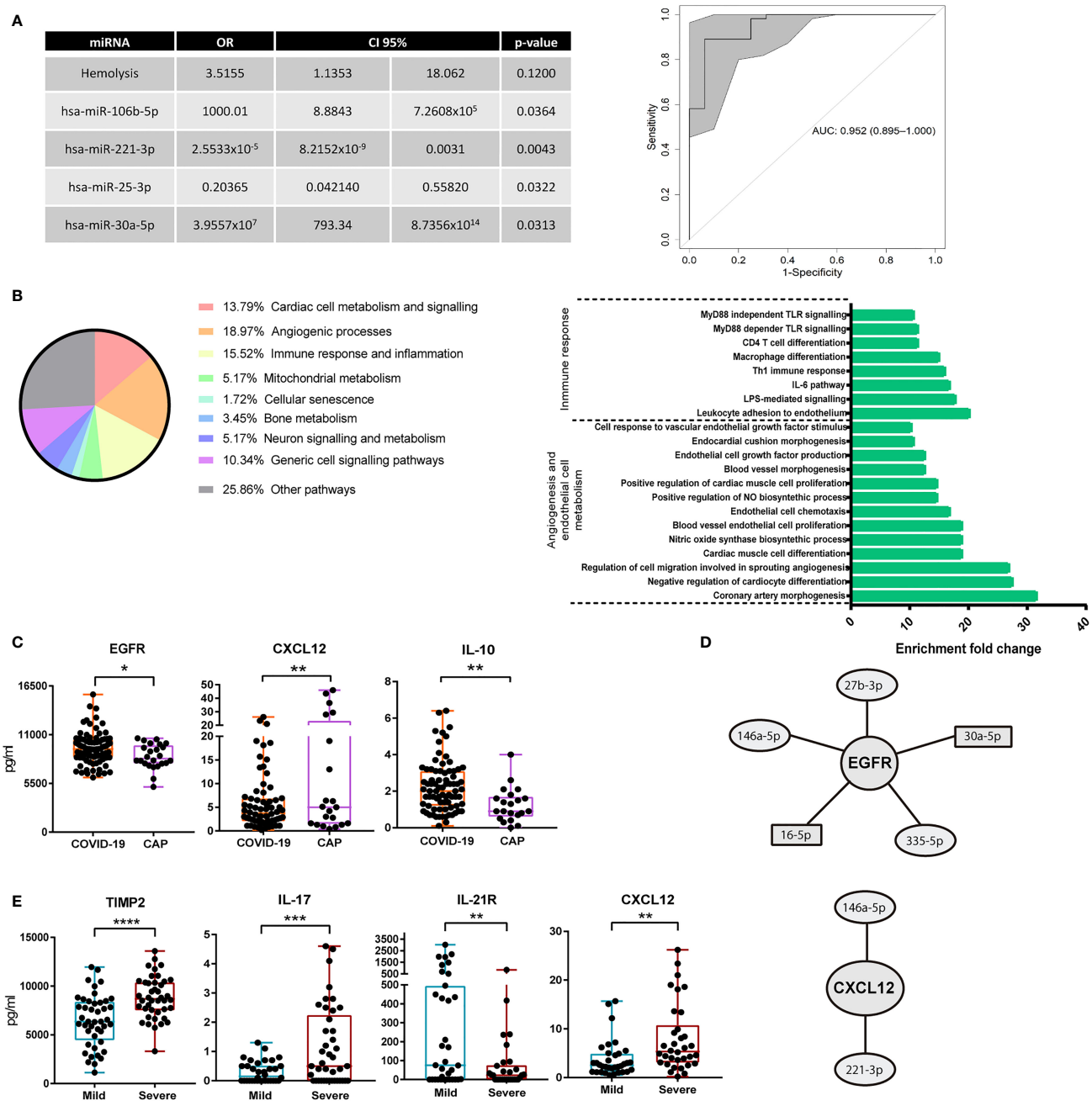

FIGURE 2 | Multivariate regression model for classification of COVID-19 pneumonia and CAP and differences in circulating proteins. **(A)** Left: final logistic regression model for patient classification based on miRNAs. Odds ratio (OR), confidence interval 95% (95%CI) and p-value for each variable were calculated. Stepwise procedure, with both backward and forward search based on Akaike information criteria, to select the critical variables were employed. Right: ROC curve showing the power of discrimination between CAP and COVID-19 of the variables combination shown in the table. Inside the graph, the AUC and its 95%CI. **(B)** Left: relative percentage of each biological process associated with the functional pathways with 10 fold enrichment or higher are shown. Pathway enrichment analysis was assessed using Panther Classification System. Right: enrichment of each individual functional pathway within the two most relevant biological processes. **(C)** Measurement of soluble cytokines in plasma of 24 CAP and 85 COVID-19 individuals. ELISA assays with plasma diluted 1/2 were carried out. Box and whisker plots are shown, statistical significance was assessed by Mann-Whitney tests. *p<0.05, **p<0.01. **(D)** miRNAs regulating EGFR and CXCL12 according to miRTarBase 8.0. Circles show miRNAs with lower expression in COVID-19 as compared to CAP. Rectangles show miRNAs with higher expression in COVID-19 as compared to CAP. **(E)** Box and whisker plots of soluble cytokines in plasma of 43 mild and 42 severe COVID-19 patients. ELISA assays with plasma diluted 1/2 were carried out. Statistical significance was assessed by Mann-Whitney tests. **p<0.01, ***p<0.001, ****p<0.0001.

particularly some controlling leukocyte adhesion to vascular endothelial cells, IL-6 mediated signalling, Th1 response, macrophage differentiation or MyD88 dependent Toll-Like Receptor signalling pathways (**Figure 2B**).

In order to find relevant molecules associated with COVID-19 pneumonia, we measured soluble plasma proteins whose mRNAs were targeted by the identified miRNAs, according to miRTarBase 8.0. We used GO enrichment analysis and Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to select relevant proteins regulated by several of the validated miRNAs. Thereby, potential targets of our candidate miRNAs associated with angiogenesis pathways (epidermal growth factor receptor, EGFR and vascular endothelial growth factor A, VEGFA), leukocyte adhesion to endothelium (intercellular adhesion molecule 1, ICAM-1), chemokines or cytokines crucial for immune surveillance and lymphocyte proliferation (CXCL12 and IL-21 receptor, IL-21R) or genes involved in cell growth and proliferation (insulin like growth factor receptor 1, IGF1R) were selected for quantification by ELISA. A previous set of cytokine quantification assays performed in a pilot screening of plasma soluble proteins with COVID-19 and CAP patients revealed differences in the plasma levels of IL-11, IL-17 and the tissue inhibitor of metalloproteinases 2 (TIMP-2) (**Supplementary Figure 3**). Hence, these molecules were also quantified in the validation cohort. Finally, IL-10 was also included due to its described relevance in COVID-19 (23).

ELISA assays revealed higher levels of EGFR and IL-10 in COVID-19 vs. CAP patients (**Figure 2C**). Conversely, CXCL12 was downregulated in COVID-19 patients. Both EGFR and CXCL12 were targets of 5 and 2 validated miRNAs, respectively (**Figure 2D**). Likewise, we found differences in the levels of several molecules depending on COVID-19 severity. TIMP-2 and IL-17 were upregulated, while IL-21R was downregulated, in severe cases (**Figure 2E**). The chemokine CXCL12 was also increased in severe vs. mild COVID-19 (**Figure 2E**). Interestingly, 3 out of 8 subjects with higher levels of CXCL12 (above 15 pg/ml) had bacterial superinfection or sepsis.

DISCUSSION

Growing interest on miRNAs as potential biomarkers or as therapeutic drugs has raised over the last few years, especially in cancer, cardiovascular or neurodegenerative diseases. In this regard, miRNAs may have greater relevance in the future, since they provide us with a better understanding of the pathogenic mechanisms involved in different diseases. Although numerous publications concerning infection by SARS-CoV-2 have been reported in the last year, very few studies addressed its relationship with miRNAs alterations. *In silico* analyses have focused on prediction of miRNAs targeting SARS-CoV-2 genome to find alternative therapies (12, 24). The present study describes a specific miRNA signature in the plasma of COVID-19 patients. A total of 15 miRNAs (7 upregulated and 8 downregulated in COVID-19), with common expression in human plasma and validated in an independent cohort appeared dysregulated in COVID-19 patients compared to CAP patients, revealing important differences in the pathophysiology of these two clinical entities despite their similarities in terms of respiratory symptoms. Due to these differences, we were able to develop a multivariate logistic regression model based on miRNAs that efficiently distinguishes patients with COVID-19 from patients with CAP.

Our study provides experimental evidence that confirms previous *in silico* bioinformatic analyses of possible miRNAs interacting with SARS-CoV-2 genome or playing a role in the host response to the virus. Particularly, dysregulation of miR-424-5p, miR-130a-3p, miR-25-3p, miR-27b-3p and miR-425-5p was predicted in other studies, but it was not verified experimentally (25). Among the miRNAs identified in our study, miR-335-5p was significantly downregulated in COVID-19 vs. CAP patients. This miRNA has been previously associated with suppression of inflammatory processes (26). Its repression during SARS-CoV-2 infection may contribute to the widely described general proinflammatory status (27). In line with these observations, we found low levels of plasma miR-146a-5p in COVID-19 vs. CAP patients. Previous studies have described downregulation of this miRNA during SARS-CoV-2 infection (14). miR-146a regulates inflammation by targeting TNF receptor associated factor 6 (TRAF6), therefore reducing expression of NF-kB (28, 29). Moreover, decreased levels of miR-146a have been linked to higher risk of thrombotic events and neutrophil NETosis (30).

GO enrichment analysis revealed a potential involvement of vascular system biology in this pathology, specifically angiogenesis and response to endothelial damage. This is consistent with several articles that report atherosclerotic plaques, prothrombotic changes in endothelium, increased intussusceptive angiogenesis and a subsequent enhanced risk of thrombosis (31, 32). EGFR is a protein involved in a great number of biological processes; some of them related to blood vessel growth, inflammation *via* NF-kB or profibrotic and atherosclerotic events. In this context, EGFR, a known target of miR-27b-3p, miR-146a-5p, miR-16-5p, miR-335-5p and miR-30a-5p, is found at higher levels in patients with COVID-19, likely enhancing these events (33–35).

Intriguingly, we observed increased levels of the chemokine CXCL12 in CAP vs. COVID-19 patients. CXCL12, which is the CXCR4 ligand, is necessary for effective hematopoiesis, T cell and memory B cell homing to the lymph nodes or monocyte recruitment. Inhibition of this axis is used by several viruses in order to increase their proliferation by reducing the number of circulating immune cells (36). Whether low levels of this chemokine in COVID-19 patients could be triggered by SARS-CoV-2 is a possibility that merits further exploration. miR-146a-5p, miR-221-3p and their target CXCL12 (according to miRTarBase), were downregulated in COVID-19 patients. This observation suggests either non-canonical regulation by these miRNAs or the prevalence of other regulatory mechanisms modulating the expression and secretion of CXCL12. Moreover, CXCL12 was found at higher levels in the plasma of severe vs. mild

COVID-19 patients. In our cohort, 3 severe COVID-19 patients, with higher levels of CXCL12, had sepsis or bacterial superinfection. Thus, we cannot rule out the relationship between higher levels of this chemokine and the presence of superinfection or sepsis in critically ill COVID-19 patients, since CXCL12 has a role in neutrophil recruitment from bone marrow (37). The study of the role of miR-221-3p and the interaction with its target, PARP-1, during COVID-19 pathogenesis would be of great interest, since emerging evidence highlights relevant pro and antiviral properties of this protein (38).

IL-17 was found at higher levels in patients with severe COVID-19 disease, in agreement with a prior study indicating its crucial role in the pathogenesis of acute respiratory distress syndrome (ARDS) (39). IL-17 induces recruitment of neutrophils to the lung, exacerbating proinflammatory cytokine release and leading to ARDS. Therefore, the IL-17 signalling pathway could be a potential target to treat the cytokine storm observed in the most severe COVID-19 cases (40). Another well-studied cytokine in the pathogenesis of this disease is IL-10. Several published works show an increase of IL-10 in critically ill COVID-19 patients despite its general anti-inflammatory nature, even proposing this protein as a prognostic biomarker (41, 42), together with other well-known inflammatory cytokines such as IL-6. Here, we report an increased amount of IL-10 in COVID-19 vs. CAP patients. Conversely, no differences were observed between mild and severe disease. Finally, IL-21 is involved in antiviral defence by enhancing Th1 and IFN-γ responses (43). Likewise, its role for a correct B cell memory differentiation and germinal centre reaction leading to effective antibody response could be decisive for viral clearance (44). Thus, low levels of its receptor, IL-21R, in severe COVID-19 could lead to an impaired IFN expression and facilitate the spread and replication of SARS-CoV-2. Receptor proteins like IL-21R or EGFR may be found in plasma by leakage from different cells and tissues. However, caution should be taken when interpreting these results, since soluble EGFR may act as a regulator of its signalling pathway, as previously described (45).

On the other hand, our data revealed clear differences in TIMP-2 levels between severe and mild cases of COVID-19. One of the functions of this inhibitor of matrix metalloproteinases (MMP) is to participate in the regulation of the renewal of the extracellular matrix. Besides this role, it was proposed as an inhibitor of angiogenesis by MMP-independent mechanisms (46). Therefore, this protein may be participating in the response to direct endothelial damage caused by SARS-CoV-2.

One of the limitations of this study is the low number of patients with mild disease, which preclude to identify differences in miRNAs between mild and severe COVID-19, as most of the studied subjects belong to moderate and severe subgroups of patients. Furthermore, sample collection days vary between COVID-19 (within first 5 days upon admission) and CAP (day right after the admission). However, no significant difference in days post-symptoms onset between CAP and COVID-19 in discovery cohort (p=0.98) and validation cohort (p=0.07) were found. Another limitation is the variety of treatments within COVID-19 and CAP patients, which makes hard the extrapolation of these results. On the other hand, the main challenge when using RT-qPCR for miRNA expression analysis and its implementation in clinical practice is the lack of a standard method of normalisation among different laboratories. Further studies on miRNAs derived from lung and other tissues are needed to delve into our knowledge of the pathogenesis of this disease.

Despite these limitations, this study conclusively proves the existence of a differential profile in circulating miRNAs between COVID-19 and CAP patients. Analysis of the identified miRNAs showed regulatory functions associated with angiogenesis and inflammation, indicating that endothelial damage and vascular compromise is definitively one of the main conditions driven by SARS-CoV-2. Here, we describe new miRNAs and soluble cytokines that may contribute to gain insight into COVID-19 pathogenic mechanisms and set the basis for the urgently needed design of novel therapeutic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Universitario de la Princesa. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: PM-F, PV-T, MJ-F, SR, CL-S, ML-P, HF, and FS-M. Acquisition of data and samples: PM-F, PV-T, ER-V, AV, AL-S, JMG-R, and JA. Statistical analyses: PM-F, PV-T, MJ-F, SR, and AS-G. Drafting the manuscript: PM-F and PV-T. Revising the manuscript: PM-F, PV-T, MJ-F, SR, ML-P, ER-V, AS-G, IG-Á, AA, CM-C, JMG-R, JA, IG-A, AA , FS-M, and EM-G. Obtaining financial support: IG-A, AA, and FS-M. All authors contributed to the article and approved the submitted version.

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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.2021.815651/full#supplementary-material

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Cell-Crossing Functional Network Driven by microRNA-125a Regulates Endothelial Permeability and Monocyte Trafficking in Acute Inflammation

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Opening of the endothelial barrier and targeted infiltration of leukocytes into the affected tissue are hallmarks of the inflammatory response. The molecular mechanisms regulating these processes are still widely elusive. In this study, we elucidate a novel regulatory network, in which miR-125a acts as a central hub that regulates and synchronizes both endothelial barrier permeability and monocyte migration. We found that inflammatory stimulation of endothelial cells induces miR-125a expression, which consecutively inhibits a regulatory network consisting of the two adhesion molecules VE-Cadherin (CDH5) and Claudin-5 (CLDN5), two regulatory tyrosine phosphatases (PTPN1, PPP1CA) and the transcription factor ETS1 eventually leading to the opening of the endothelial barrier. Moreover, under the influence of miR-125a, endothelial expression of the chemokine CCL2, the most predominant ligand for the monocytic chemokine receptor CCR2, was strongly enhanced. In monocytes, on the other hand, we detected markedly repressed expression levels of miR-125a upon inflammatory stimulation. This induced a forced expression of its direct target gene CCR2, entailing a strongly enhanced monocyte chemotaxis. Collectively, cell-type-specific differential expression of miR-125a forms a synergistic functional network controlling monocyte trafficking across the endothelial barrier towards the site of inflammation. In addition to the known mechanism of miRNAs being shuttled between cells *via* extracellular vesicles, our study uncovers a novel dimension of miRNA function: One miRNA, although disparately regulated in the cells involved, directs a biologic process in a synergistic and mutually reinforcing manner. These findings provide important new insights into the regulation of the inflammatory cascade and may be of great use for future clinical applications.

Keywords: microRNA network, inflammation, immune cell trafficking, monocytes, endothelial cells

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INTRODUCTION

Infections, trauma and many other insults evoke expression and secretion of an elaborate sequence of inflammatory mediators that lead to the efficient recruitment of targeted immune cells to the affected site. Activation of the inflammatory cascade aims at limiting the spread of pathogens and tissue damage, initiating tissue repair, and eventually ending up with resolution. There are various plasmatic and cellular systems involved in orchestrating these fine-tuned processes including not only immune cells but also the endothelial cell layer, which increases its permeability thereby opening the barrier for leukocytes to infiltrate the tissue and resolve the menacing noxa. However, dysregulation of these inflammatory reactions can lead to life-threatening diseases with massive organ damage, as seen in sepsis and SIRS (1, 2). Hallmark of these conditions is a hyperpermeable endothelial barrier leading to circulatory break-down and massive accumulation of undirected dysfunctional immune cells in the tissue. How these processes are regulated still remains elusive, and deeper insights into the underlying molecular mechanisms are urgently necessary to develop new targeted therapy concepts.

The role of microRNAs (miRNAs) as regulators of inflammation has been well defined during the last decade. Innumerable single miRNA-target interactions have been identified to exert regulatory effects in specific cell types (3–5). In the last few years, however, it has become increasingly clear that the full regulatory impact of miRNAs is displayed by complex cell-crossing regulatory networks, where individual miRNAs target multiple genes in different cell types, thereby potentiating their impact on cellular functions (6). MiRNA networks that regulate the inflammatory cascade on a cell-type-spanning level have not been identified, yet. We explored the question of whether a specific miRNA could fulfil such a function as a master regulator of the inflammatory cascade across cell types.

We here uncover a miR-125a-driven functional network that impacts two different cell entities, endothelial cells and monocytes, resulting in synergistic effects within the inflammatory cascade. Upon inflammatory stimuli, miR-125a up-regulation in endothelial cells decreases the expression of five target molecules regulating the endothelial barrier thereby increasing its permeability. Simultaneously, miR-125a down-regulation in monocytes leads to forced expression of chemokine receptor 2 (CCR2), which induces monocyte trafficking towards the site of inflammation.

The here presented cell-spanning network is a new way of functional regulation, which fundamentally differs from the known concept of miRNAs being shuttled between cells *via* extracellular vesicles. Based on disparate expression patterns, miR-125a synergistically orchestrates the inflammatory cascade in endothelial cells and monocytes in response to inflammatory cytokines.

MATERIALS AND METHODS

Cell Culture

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cords of healthy neonates directly after cesarean delivery at the Department of Gynecology and Obstetrics, University Hospital, LMU Munich, Germany. Written informed consent from the mother was obtained before donating umbilical cords in accordance with the Declaration of Helsinki. HUVEC were isolated from umbilical vein vascular wall by collagenase A (Roche) treatment and cultured in Endothelial Cell Basal Medium (ECGM; PromoCell) with SupplementMix (PromoCell), 10% FCS (Biochrom AG) and 1% Penicillin/Streptomycin (Pen/Strep, Gibco). Cells of one single cord at passage 2-4 were used for each of the independent experiments.

HEK-293 cells were purchased from the American Type Cell Culture Collection and cultured in Dulbecco's Modified Eagle Medium (Gibco) supplied with 10% FCS, 2% L-Glutamine, (Gibco) 1% Penicillin/Streptomycin (Gibco) and 1% MEM NEAA (Gibco). HEK-293 cells used for experiments were not cultured beyond passage 20. Monocytes were maintained in RPMI (Lonza) supplemented with 20% FCS, 1% Penicillin/Streptomycin/Glutamine, 1% HEPES (Gibco). All cells were cultured at 37°C and 5% CO2.

Isolation of Primary Human Monocytes, Inflammatory Stimulation and Differentiation

PBMCs were isolated from Lithium-heparinized freshly drawn blood of healthy volunteers with Histopaque 1077 (Sigma) according to manufacturer's instructions. Thereafter, monocytes were extracted using the Pan Monocyte Isolation Kit II, human (Miltenyi) according to the instructions. For inflammatory stimulation, monocytes were immediately seeded in RPMI media supplemented with 50ng/ml IL-6 (Miltenyi) and compared to monocytes incubated without IL-6. IL-6 was used to mimic early inflammatory conditions as IL-6 clinically precedes acute phase proteins occurring in the blood of patients with acute inflammatory diseases (7). Monocytes show potent endothelial adhesion and transmigration upon IL-6 stimulation (8).

For macrophage differentiation, primary monocytes were incubated in RPMI supplemented with 50ng/ml M- or GM-CSF (Miltenyi) for 6 days. Media was changed every 2 days.

For investigation of miR-125a induction in HUVEC, cells were cultivated until monolayers reached 80% confluency and serum-starved with ECGM containing 0.5% FCS for 12 hours at 37°C. The culture medium was removed and cells were incubated in ECGM with 25ng/ml TNF and 50ng/ml IFN-γ (both Miltenyi). The combination of TNF and IFN-γ is known as a potent inducer of proinflammatory micro-RNAs (9, 10).

To address the early phase of inflammation, stimulation time-points were chosen between 3-4 hours.

Transfections

Transfections were conducted using the NEON electroporation device (Life Technologies) according to the manufacturer's protocol. When HUVEC reached 80-90% confluency, cells were detached and transfected with Ambion® hsa-miR-125 premiR™, hsa-miR-125 mirVana™ miRNA inhibitor (miR-125 inhibitor; both Thermo Fisher) or respective siRNA (Dharmacon). Transfections were carried out at final concentrations of 50nM (premiR™) or 100nM (miR-125a

inhibitor, siRNA). Electroporation for HUVEC was carried out using 1 pulse of 1350 Volt and 30 ms.

For monocytes, transient transfections of miRNA mimic (premiR™) and siRNAs were performed using the NEON electroporation device at a cell density of $2x10^6$ cells and a final concentration of 50nM premiR™ or 100nM siRNA per transfection. Transfection efficiency of miRNAs was determined by flow cytometry using Cy3-labeled premiR™ negative control (Thermo Fisher). Knockdown efficiency of siRNAs was evaluated by qRT-PCR or SDS-PAGE. Cell viability was assessed after electroporation with 50 nM premiR™ negative control using flow cytometry of propidium iodide stained cells. Viability of monocytes was between 70 to 83% and viability of HUVEC was between 81 to 89%.

ECIS

For electric cell-substrate impedance sensing (ECIS) measurements, transfected HUVEC were seeded at a density of 100,000 cells/well into gelatin-coated electric cell-substrate impedance sensing arrays, each containing 8 wells with 40 gold electrodes per well (ECIS 8W10E+ PET; Ibidi). After 48 hours of incubation, cells were stimulated with TNF (25 ng/ml) for 24h. Impedance was measured directly after seeding for 72h with multiple frequencies mode using the ECIS®Z system (Applied BioPhysics). For analysis of barrier function, the area under the curve (AUC) of normalized impedance values over time at 4,000 Hz was calculated.

FITC-BSA Passage

Transendothelial passage of macromolecules was assessed by measuring the passage of Fluorescein isothiocyanate conjugate bovine serum albumin (FITC-BSA, 66 kDa; Sigma-Aldrich) across confluent HUVEC monolayers. HUVEC were cultured in 24-well plates containing cell culture inserts (pore size 0.4 µm, Greiner Bio-one) with 200 µl ECGM growth medium in the upper chamber and 800 µl ECGM growth medium in the lower chamber. 48 hours after transfection, HUVEC were stimulated with 25ng/ml TNF for 24 hours. Subsequently, 10µg FITC-BSA was added to the upper compartment of each insert and the medium of bottom wells was collected after 30 minutes. Fluorescence intensity was measured using a FilterMax F3 (Molecular Devices) using Fluorescence Intensity (FL) read mode with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Quantitative Real-Time PCR

Total RNA was isolated from HUVEC or primary human monocytes using the miRNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA amount and quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher) and reversely transcribed to cDNA using Oligo-dT Primers, Random Hexamers (Qiagen), dNTPs, RNAse OUT, and Superscript® III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using specially designed primers (Probe Finder Software: Roche; primers synthesized by Metabion) and UPL Probes (Roche). All analyses were performed in duplicates on a Light Cycler 480 instrument (Roche) with 10ng of cDNA/well. For HUVEC, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA Box Binding Protein (TBP) were used as reference genes. For monocytes, Beta-2 Microglobulin (B2M) and TBP served as reference genes. QRT-PCR was conducted as previously described (11). Quantification cycle values were calculated by the "second derivative maximum" method computed by the LightCycler® software. Primer sequences are supplied in **Supplementary Table S1**.

For quantifying miRNA expression, total RNA was reversely transcribed as previously described (12), using the specific primers of the TaqMan MicroRNA Assay for miR-125a-5p (Assay ID 002198, Applied Biosystems). Expression of miR-125a-5p was studied by qRT-PCR using the TaqMan MicroRNA Assay with U47 (Assay ID 001223, Applied Biosystems) as the reference RNA. Taqman qRT-PCR conditions comprised initial denaturation for 10 minutes (95°C), and 50 cycles of 95°C for 15 s, 60°C for 60 s; 40°C for 30 s.

Next Generation Sequencing and Bioinformatics Analysis

For transcriptome analysis, primary HUVEC from five different donors were transfected in five independent experiments using either hsa-miR125-5p Pre-miR miRNA or negative control and cultivated for 18 hours. After additional stimulation with TNF (25ng/ml) RNA was isolated and analyzed by IMGM Laboratories GmbH (Martinsried). Quality control for all total RNA samples was done on the 2100 Bioanalyzer (Agilent Technologies) using RNA 6000 Nano LabChip Kits (Agilent Technologies). Library preparation was performed with the TruSeq® Stranded mRNA HT technology (Illumina), according to the manufacturer's protocol, including fragmentation, a poly-T oligo pulldown and sequencing adapter ligation. RNA sequencing was performed on the Illumina NextSeq® 500 next generation sequencing system and its high output mode with 1 x 75 bp single-end read chemistry with >10 million reads per sample. For RNA Seq analysis and heatmap generation CLC Genomics Workbench and Bioconductor/R v3.0.2 was used. Differentially expressed genes were calculated by using non-corrected p-value < 0.01 and fold change +1.25 to -1.25 in order to identify potential targets which were further validated using qRT-PCR and western blots. RNA-Seq data is available on the Gene Expression Omnibus under the accession number: GSE196161 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196161).

Analysis of potential miR-mRNA interactions was performed using the public databases TargetScan7.2 (http://www.targetscan.org), and miRIAD (http://bmi.ana.med.uni-muenchen.de/miriad/). Gene Set Enrichment Analysis was performed with the investigating gene sets function on http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp. Briefly, we computed overlaps between the list of differentially expressed genes found in Next Generation Sequencing with previously mentioned filtering strategy and the gene sets canonical pathways_KEGG and gene ontology_biological processes from the Molecular Signatures Database v7.5. Gene sets with a false discovery rate corrected p-values < 0.01 were seen as significant.

SDS-PAGE

Cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing 1% protease and phosphatase inhibitors (Cell Signaling Technologies). Protein concentrations were assessed through BCA assays (Thermo Fisher) according to the manufacturer's instructions. Cell lysates were electrophoresed on SDS-PAGE gels and then electroblotted on polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Biorad). Nonspecific binding was blocked with 5% non-fat milk or 5% Bovine Serum Albumin (BSA) in TBS-Tween-20 (TBST; Sigma) for 1 hour. Primary antibodies for VE-Cadherin (sc-9989), PTP1B (sc-133259), Claudin-5 (sc-374221), PP1α (sc-271762), Ets-1(sc-55581), TGFβ RII (sc-17792), Rac-2 (sc-517424) and α-actinin-4 (sc-393495) (all Santa Cruz Biotechnology) were diluted in TBST with 5% non-fat milk, Phospho-VE-Cadherin antibody (Tyr658, Cat. No.44-1144G; Thermo Fisher) was diluted in TBST with 5% BSA. β-Actin (#4970; Cell Signaling Technologies) served as the loading control. Immunoreactive bands were visualized by using peroxidase-conjugated secondary antibodies (Anti-rabbit IgG, #7074; Anti-mouse IgG, #7076; Cell Signaling Technologies) and the ECL western blot detection system (BioRad).

Cloning of Reporter Constructs

The 3'UTRs of CDH5, PTPN1 and CCR2 were amplified from genomic DNA (50 ng) by PCR using the following cycling conditions and the primers listed in **Supplementary Table S2**. PCR products were ligated into the StrataClone Blunt Vector Arms (Agilent Technologies) according to manufacturer's instructions and then subcloned into the psiCHECKTM2 vector (Promega) using XhoI/NotI restriction enzymes (New England Biolabs) and T4 DNA Ligase (Roche). Site-directed mutagenesis of plasmid DNA was conducted using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. Sequences were verified by Sanger sequencing (Eurofins). Plasmids were purified using the Qiaprep Spin Plasmid Miniprep Kit (Qiagen) and the Pure Yield Plasmid Midiprep System (Promega). DNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher).

Reporter Gene Assays

Co-transfection of luciferase reporter plasmids and premiR™ was carried out using 100,000 HEK-293 cells, 50 nM hsa-miR125 premiR™ and 1 µg of Psi-CHECK™2 plasmid, followed by 40 hours of incubation. Dual-Glo Luciferase Assay system (Promega) was used according to the manufacturer's protocol and luminescence was measured on a FilterMax F3. All experiments were performed in triplicate.

Immunofluorescence

µ-Slides $I^{0.4}$ Luer (80176; Ibidi) were pre-coated with 0.2% gelatin. Pre-miR transfected HUVEC (1.5×10^6 cells/mL) were seeded in µ-Slides, incubated for 72 hours at 37°C and 5% CO_2 in antibiotics-free medium. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Next, cells were washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and incubated for 1 hour at room temperature in blocking solution: 5% normal goat serum, 5% bovine serum albumin in PBS. Primary antibody incubation (VE-Cadherin, sc-9989; claudin-5, sc-374221; Santa Cruz Biotech) was performed overnight at 4°C. Slides were washed and stained with secondary antibodies (Alexa Fluor 488; 1: 400; Invitrogen). Images of 10 randomly chosen microscopic fields per slide 200x magnification were acquired using a LEICA-TCS SP5 Confocal Microscope (Leica) with the Leica application suite AF software, version 2.7.

Chemotaxis Assays

One day prior to the experiment, chemotaxis slides (Ibidi) and RPMI media were equilibrated at 37°C and 5% CO_2 in a humidified incubator. $2x10^6$ Monocytes were resuspended in 175µl RPMI supplemented with 20% FCS, 1% Penicillin/Streptomycin/Glutamine and 1% HEPES. After adding 25µl of Bovine Collagen I pH=7 (Thermo Fisher), the suspension was thoroughly mixed and 6µl were introduced in the µ-Slide Chemotaxis (ibidi). The slide was placed in a cell culture incubator for 1h to ensure proper ECM matrix formation. Immediately before time-lapse microscopy, the reservoirs were loaded with RPMI media alone or RPMI media supplemented with 50ng/ml CCL2, respectively, to create a chemokine concentration gradient. Time-lapse microscopy was performed on a Zeiss Axio Observer Z1 equipped with a gas incubation system (Ibidi). For each channel, three representative fields of vision were chosen and pictures were obtained for 4h (1 picture/minute). Single-cell tracking analysis was performed using ImageJ and the Chemotaxis and Migration Tool (Ibidi).

Statistics

If not stated otherwise, values shown represent means ± standard error of the mean (SEM). N refers to the number of independent experiments with cells obtained from different donors. *p*-values were calculated using student's *t*-test or paired *t*-test for all data with normal distribution and Wilcoxon signed rank test otherwise. Statistical analyses were performed using GraphPad Prism 7. *p*-values below 0.05 were considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

RESULTS

Up-Regulation of miR-125a in Response to Inflammatory Stimuli Strongly Impacts the Transcriptome of Endothelial Cells

MiR-125a is derived from its precursor in two isoforms, miR-125a-3p and miR-125a-5p. While miR-125a-5p is abundantly expressed, its counterpart is barely detectable in most tissues (ratio 509:1) (13, 14). Accordingly, miR-125a-5p is further referred to as miR-125a.

MiR-125a expression is increased in the blood of patients with acute inflammatory conditions (15–17). Thus, we first investigated whether miR-125a expression in endothelial cells

is influenced by inflammatory stimulation. As shown in **Figure 1A**, we detected a sharp increase of miR-125a expression upon cytokine stimulation with tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) (+68.9% ± 22.3%, n=6, $p<0.05$).

To investigate the functional impact of this up-regulation, we analyzed the transcriptome of HUVEC after miR-125a overexpression (transfection efficiency: **Supplementary Figure S3A**) and TNF stimulation. Next Generation Sequencing (NGS) identified 468 genes as differentially regulated (**Figure 1B** and **Supplementary Tables S4**). *In-silico* analysis identified the pathways *GO_regulation of cell adhesion* and *KEGG_adherens junctions* to be significantly regulated by miR-125a (**Supplementary Tables S5, S6**), pointing to a crucial role of miR-125a in inflammation-induced changes of the endothelial barrier.

To identify direct targets of miR-125a possibly regulating endothelial permeability, we combined pathway analysis with *in-silico* target prediction for extracting mRNAs that a) are involved in pathways regulating cell adhesion and cellular junctions and b) contain miR-125a binding sites in their 3' untranslated region (3'UTR). We found seven potential targets meeting both criteria: VE-Cadherin (CDH5), Protein Tyrosine Phosphatase Non-Receptor Type 1 (PTPN1), Protein Phosphatase 1 Catalytic Subunit Alpha (PPP1CA) ETS proto-oncogene 1 (ETS-1), Transforming Growth Factor Beta Receptor 2 (TGFβR2), Rac Family Small GTPase 2 (RAC2) and Actinin Alpha 4 (ACTN4). Validation of NGS results in HUVEC transfected with miR-125a by qRT-PCR resulted in a significantly decreased mRNA expression of all investigated targets (**Figure 1C**): CDH5 (-39.9% ± 3.8%; n=6; $p<0.001$), PTPN1 (-31.6% ± 4.5%; n=7; $p<0.001$), PPP1CA (-24.6% ± 6.5%; n=8; $p<0.01$), ETS1 (-31.3% ± 7.2%; n=8; $p<0.05$), TGFβR2 (-32.4% ± 5.6%; n=6; $p<0.01$), RAC2 (-43.7% ± 4.9%; n=6; $p<0.01$), ACTN4 (-36.7% ± 4.2%; n=6; $p<0.01$). These results could be confirmed on protein level by western blot analysis for all targets (**Figure 1D**). Following TNF stimulation, endothelial barrier disruption depends on the phosphorylation and subsequent degradation of VE-Cadherin (18). Accordingly, we confirmed in western blot analysis that miR-125a overexpression increased expression of phosphorylated VE-Cadherin (pVE-Cadherin, Tyr658) after TNF stimulation (**Figure 1D**).

Moreover, NGS data showed a strong downregulation of Claudin 5 (CLDN5) expression, although its 3`-UTR does not contain potential miR-125a target sites. This finding was corroborated by qRT-PCR and western blot analysis (**Figure 2C**: qRT-PCR: -64.5% ± 1.5%; n=4; p<0.01; protein: -80.2% ± 7.7%; n=4). Accordingly, not only a reduced surface expression of CDH5 (**Figure 1E**) but also of CLDN5 (**Figure 1F**) was detected by immunofluorescence after transfection of miR-125a in TNF-stimulated HUVEC monolayers. We decided to pursue this hint as CLDN5 has been shown to play an important role in the TNF-mediated disruption of the endothelial barrier (19).

In the analysis of the transcriptome, it was particularly striking that the expression of the CC-chemokine ligand 2 (CCL2) was strongly enhanced upon miR-125a overexpression (1.48-fold). This finding could be validated both by qPCR and ELISA (**Figures 1G, H**: qRT-PCR: -65.6% ± 17.7%; n=6; p<0.01; protein: -89.0% ± 14.1%; n=6; p<0.05). Since CCL2 is one of the most prominent chemokines involved in migration and activation of monocytes, this data suggests miR-125a to be additionally involved in monocyte recruitment during the inflammatory cascade.

Collectively, we found 8 genes with particular importance for endothelial barrier function to be down-regulated, and one major monocyte-attracting chemokine to be up-regulated in response to endothelial miR-125a overexpression.

CDH5, PTPN1, PPP1CA and ETS1 Are Directly Regulated Targets of miR-125a While CLDN5 Expression Is Regulated Through Mutual CLDN5/CDH5 Regulatory Crosstalk

In order to assess whether the potential targets with in-silico predicted binding sites are indeed directly regulated by miR-125a, we performed luciferase reporter assays containing the *Renilla* luciferase gene fused to the 3'UTR of the respective target. While PPP1CA and ETS-1 were already known and confirmed targets of miR-125a (20, 21), direct interaction of miR-125a with the respective 3'UTR of CDH5, PTPN1, TGFβR2, RAC2 and ACTN4 had to be determined. Transient co-transfection of HEK-293 cells with the respective reporter vectors and miR-125a confirmed repression of the wild-type CDH5 plasmid by 50.9% ± 1.53% (n=5, $p<0.01$; **Figure 2A**). Site-directed mutagenesis of the two predicted binding sites reconstituted luciferase intensity, revealing that both are indeed functional binding sites of miR-125a. Co-transfection of the wild-type PTPN1 plasmid with miR-125a decreased luciferase activity by 16.3% ± 1.86% (n=6, $p<0.001$; **Figure 2B**), and mutation of the binding site restored luciferase activity. In contrast, luciferase activity of the vector with 3'UTR of TGFβR2, RAC2 and ACTN4 did not change after co-transfection with miR-125a (data not shown), indicating that they are not direct targets of miR-125a.

Direct regulation of CLDN5 by miR-125a was unlikely since the 3'UTR of CLDN5 does not contain any putative binding sites. This assumption was confirmed by luciferase reporter assays revealing no significant regulation after co-transfection with miR-125a (data not shown). Recent evidence suggests that expression of CLDN5 strongly depends on CDH5 expression indicating a transcriptional relationship (22, 23). Thus, we hypothesized that miR-125a affects CLDN5 expression indirectly *via* direct targeting of CDH5. To test this, we knocked-down either CDH5 or CLDN5 expression using siRNA and analyzed its effect on the expression of the respective molecule. We could show a reciprocal regulation for both adhesion molecules since knock-down of CDH5 caused a significant downregulation of CLDN5 and vice versa (**Figure 2D**: qRT-PCR: CLDN5 -19.3% ± 5.7%, n=6, $p<0.05$; CDH5 -22.8% ± 4.9%, n=5, $p < 0.05$. **Figure 2E**: protein: CLDN5 -32.4% ± 8.6%; CDH5 -27.8%± 0.3%).

Together, these findings indicate that CDH5 and PTPN1 are newly discovered *bona fide* targets of miR-125a, PPP1CA and ETS-

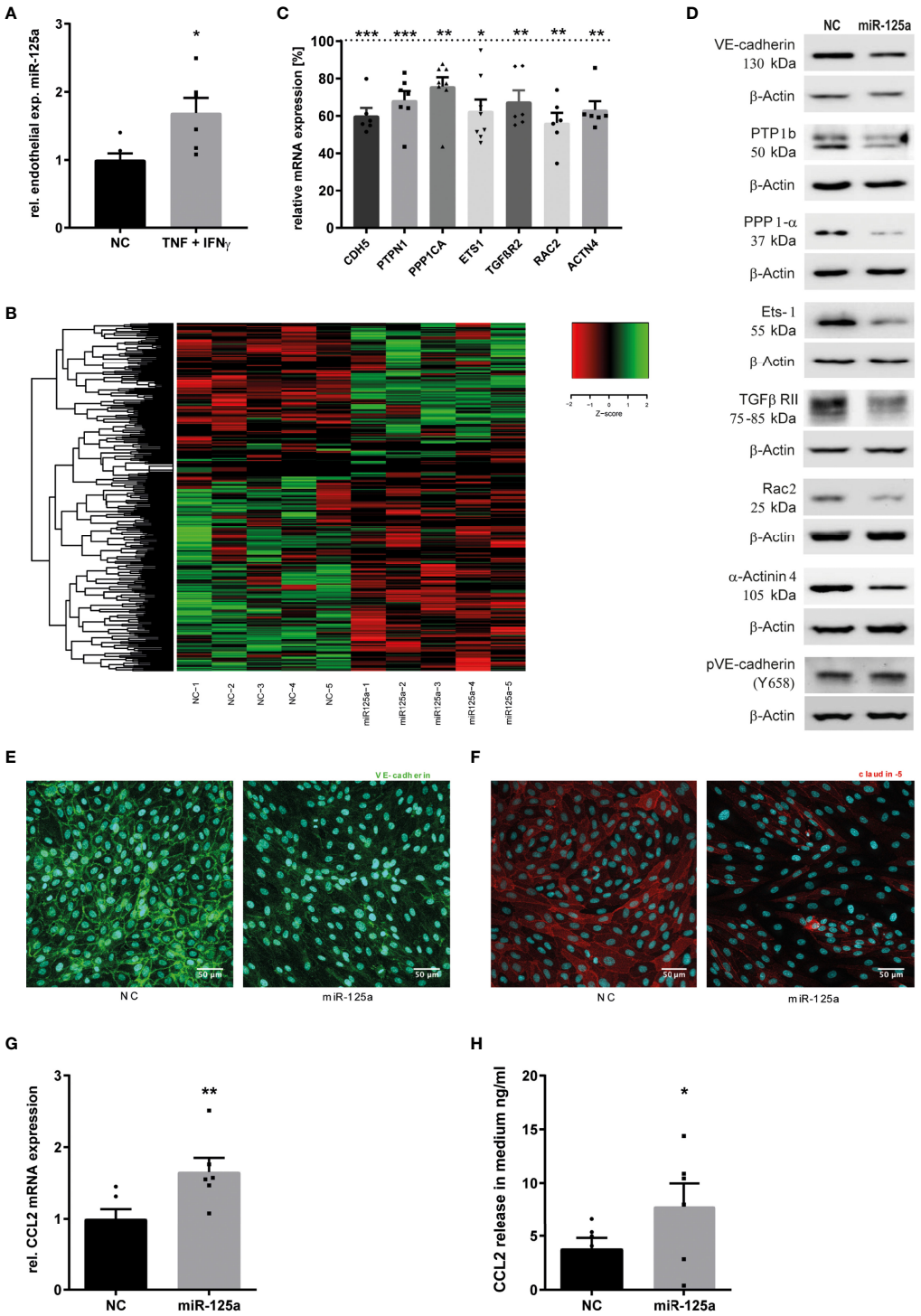

FIGURE 1 | MiR-125a expression upon inflammatory stimulation of HUVEC and its effect on endothelial transcriptome. **(A)** Endothelial expression of miR-125a after cytokine treatment with 25ng/ml TNF and 50 ng/ml IFN-γ for 4 hours (n=6, p<0.05). **(B)** Clustered heatmap of z-scaled expressions of significantly differentially expressed genes in HUVEC transfected with either miR-125a or scrambled control (NC) (Whole transcriptome RNAseq, n=5). Green color indicates enhanced expression, reduced expression is indicated in red color. **(C, D)** mRNA (qRT-PCR) and protein (SDS-PAGE) expression of differentially regulated genes extracted from RNAseq data (n=6-8, **p<0.01 ***p<0.001). **(E, F)** Immunofluorescent staining of VE-Cadherin and Claudin-5 in endothelial cells transfected with miR-125a or scrambled control (NC). One exemplary picture of three independent experiments is shown. **(G, H)** expression levels of CCL2 mRNA and protein in HUVEC and cell culture supernatant, respectively (n=6; **p<0.01, *p<0.05).

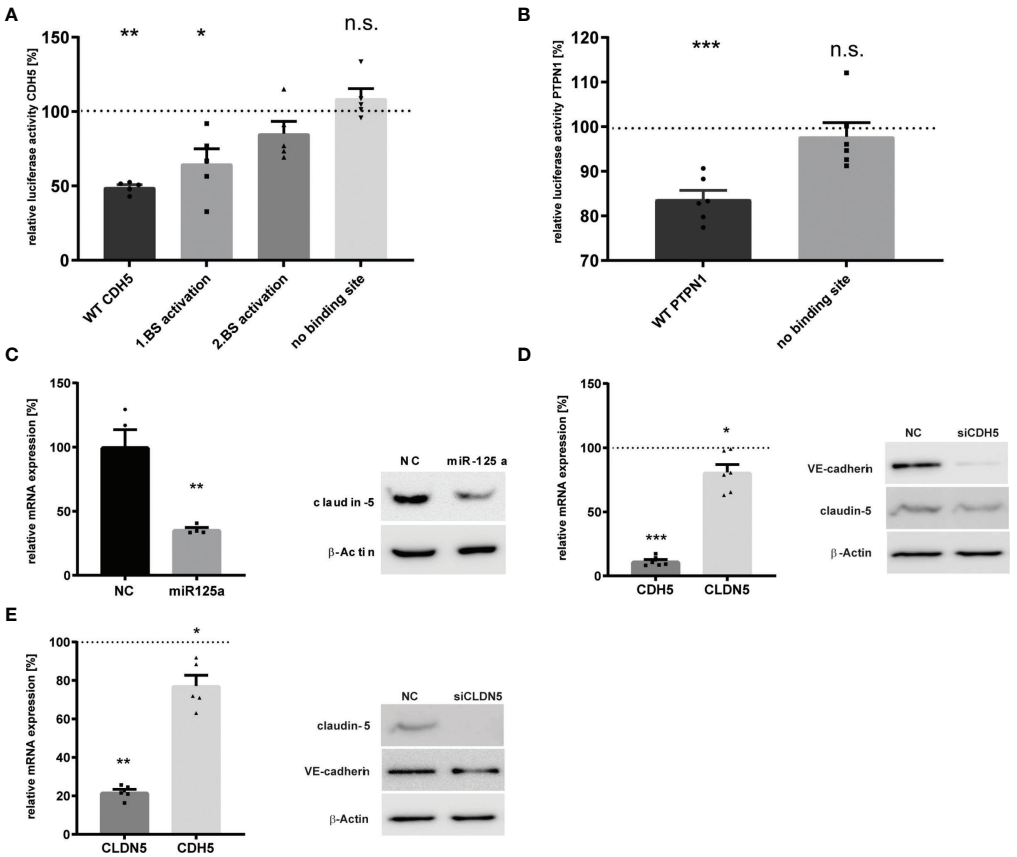

FIGURE 2 | Validation of the miR-125a-related network in HUVEC. **(A)** CDH5 3`UTR Luciferase reporter gene activity after co-transfection of the luciferase constructs with miR-125a or scrambled control (NC). Luciferase constructs: wt_CDH5: both binding sites of miR-125a in the CDH5 3`UTR are functional; mut1_miR-125a: site-directed mutagenesis of binding site 1; mut2_miR-125a: site-directed mutagenesis of binding site 2; mut1+2_miR-125a: site-directed mutagenesis of both binding sites (n=5, p<0.01). **(B)** PTPN1 3`UTR Luciferase reporter gene activity with functional miR-125a binding site (wt PTPN1) and site-directed mutagenesis of the miR-125a binding site (mut1_miR-125a). n=6 p<0.001). **(C)** mRNA (left) and protein (right) expression of Claudin-5 after transfection with miR-125a or scrambled control (NC). n=4, p<0.01. **(D, E)** Knock-down of CDH5 **(D)** and CLDN5 **(E)** by RNA interference. mRNA (left) and protein (right) levels as measured by qRT-PCR and SDS-PAGE, respectively. n=5-6. ***p<0.001, **p<0.01, *p<0.05, n.s., not significant.

1 are known direct targets, while CLDN5 represents an indirect component of the miR-125a-regulated functional network.

A miR-125a-Driven Functional Network Induces a Hyperpermeable Phenotype of Endothelial Cells

We next aimed at investigating the impact of the identified miR-125a-driven functional network on the endothelial barrier. To this end, we assessed the transendothelial passage of FITC-conjugated albumin and the electric impedance of the endothelial monolayer *via* Electric Cell-Substrate Impedance Sensing (ECIS) in real-time. As shown in **Figure 3A**, we could show that overexpression of miR-125a significantly impaired endothelial barrier function, as shown by an increase in basal and TNF-induced passage of macromolecules (**Figure 3A**: MiR *vs*. NC: 116.0% ± 29.1%, n=6, p<0.01; miR+TNF *vs*. NC+TNF: 128% ± 27.6%, n=6, p<0.001). These findings were corroborated by a decrease of the endothelial electrical impedance in HUVEC overexpressing miR-125a (**Figure 3B**: AUC -17.1% ± 4.5%, n=4, p<0.01).

The next step was to investigate whether the detected miR-125a-specific network members indeed account for the hyperpermeable phenotype observed after miR-125a overexpression. We performed siRNA knock-down of CDH5, PTPN1, PPP1CA, ETS1 and CLDN5 (knock-down efficiency: **Supplementary Figure S3C**). Knock-down of CDH5 or CLDN5 increased permeability as measured by FITC-albumin flux and decreased impedance measured by ECIS in basal as well as in TNF-stimulated HUVEC monolayers (**Figures 3C–F**: Permeability assay: siCDH5+TNF *vs*. NC+TNF: 82.4% ± 23.9%, n=5, *p*<0.05; siCLDN5+TNF *vs*. NC+TNF: 61.4% ± 16.2%, n=4, *p*<0.05; ECIS: CDH5: AUC -27.4 ± 4.5%, n=4, *p*<0.01, CLDN5: AUC -17.1% ± 4.5%, n=4, *p*<0.01). Monolayer impedance after TNF stimulation in ECIS was significantly decreased after PTPN1 knock-down. Permeability as measured by albumin-flux was also increased, however, without reaching statistical significance (**Figures 3G, H**: Permeability assay: siPTPN1+TNF *vs*. NC+TNF 16.7 ± 11.2%, n=8, p=0.149; ECIS: AUC after TNF -4.2 ± 1.7%,

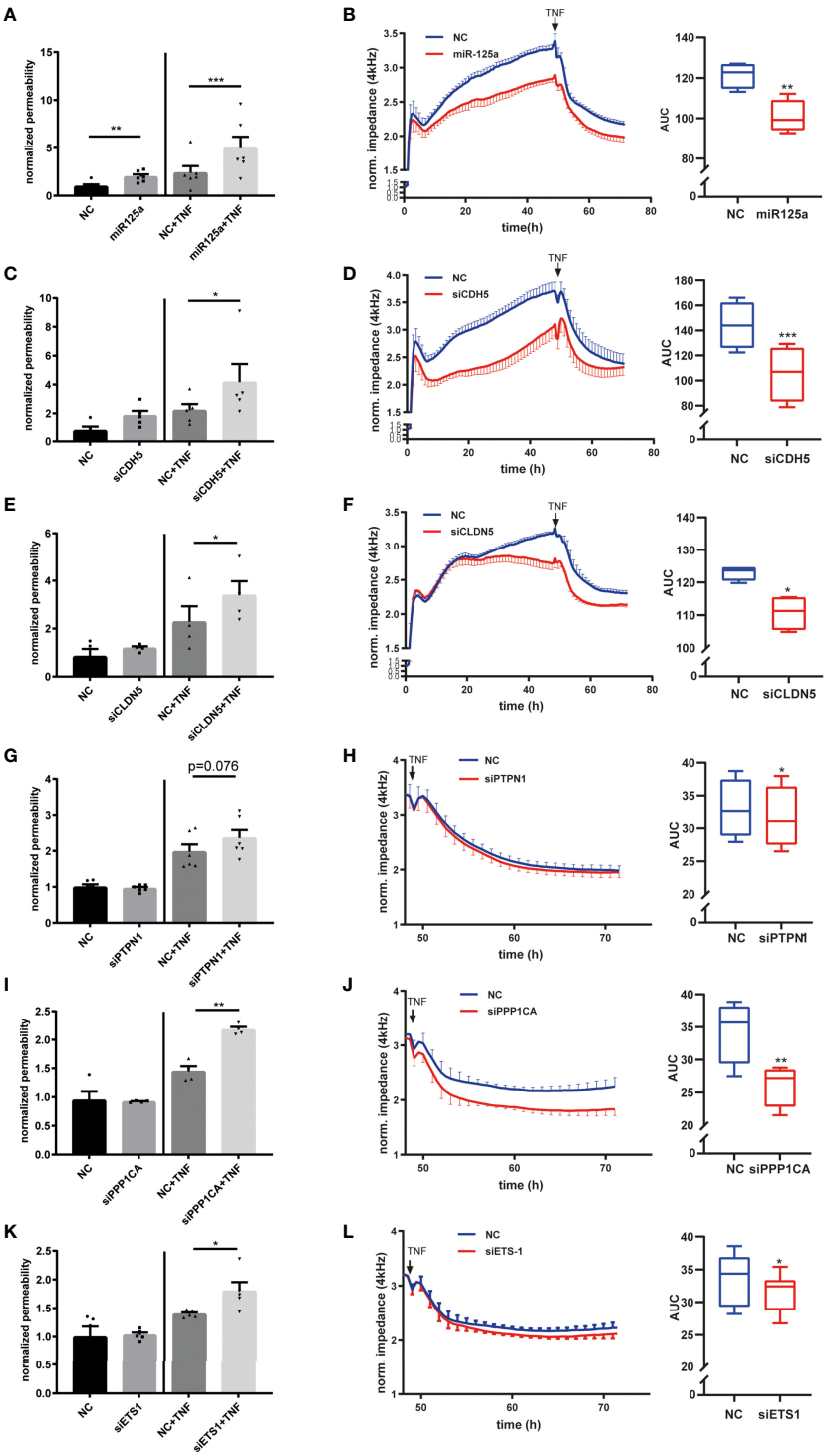

FIGURE 3 | Functional relevance of the miR–125a-driven network on endothelial permeability. **(A)** Transfection of HUVEC with miR-125a increased FITC-Albumin permeability across endothelial monolayers under basal and stimulated (TNF 25ng/ml, 24 h) conditions. FITC-Albumin passage was measured 72 hours after transfection (n=6, ***p<0.001, **p<0.01, *p<0.05). **(B)** Compared to scrambled control (NC, blue), miR-125a (red) impaired endothelial barrier function as indicated by decreased endothelial electrical impedance in both basal and inflammatory conditions mimicked by TNF stimulation (n=4, **p<0.01). **(C–L)** Individual knock-down of each of the functional network members by specific siRNA interference targeting the indicated gene. **(C, E, G, I, K)** Measurement of FITS-BSA-Passage in basal and TNF-stimulated conditions **(D, F, H, J, L)** Measurement of electrical impedance, basal and after TNF stimulation (n=4-6, ***p<0.001, **p<0.01, *p<0.05).

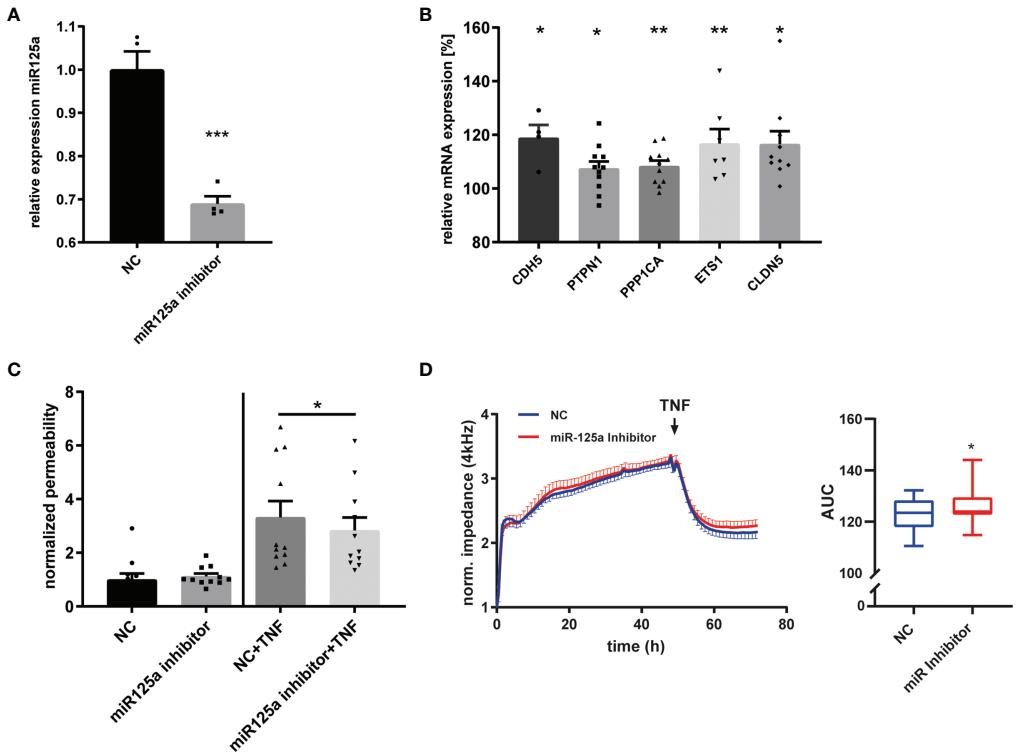

FIGURE 4 | MiR-125a inhibition and its impact on endothelial barrier function. **(A)** Expression of miR-125a in HUVEC after transfection of a miR-125a-inhibitor ("Antimir") as measured by qRT-PCR (n=4, ***p<0.001). **(B)** mRNA levels of CDH5, PTPN1, PPP1CA, ETS1, CLDN5 after transfection of a miR-125a-inhibitor compared to a scramble control (n=10-11, **p<0.01, *p<0.05). **(C)** Endothelial permeability as measured by FITC-Albumin passage across an endothelial monolayer in basal (left) and inflammatory (right) conditions after transfection of a miR-125a-inhibitor or a scrambled control (NC). n=11, *p<0.05. **(D)** Impedance measurement of the endothelial monolayer after miR-125a inhibition (red) compared to scrambled control (NC, blue). n=11, *p<0.05.

n= 4, $p<0.05$). Knock-down of PPP1CA or ETS1 did not affect the endothelial barrier function under basal conditions but significantly enhanced barrier breakdown upon 24 hours TNF stimulation in permeability assays and ECIS (**Figures 3I–L**: Permeability assay: PPP1CA+TNF *vs.* NC+TNF: 44.9% ± 10.3%, n=4, $p<0.01$; ETS1 +TNF *vs.* NC+TNF: 28.5% ± 9.3%, n=5, $p<0.05$; ECIS: PPP1CA: AUC after TNF -23.8 ± 2.1%, n=4, $p<0.01$; ETS1: AUC after TNF -5.0% ± 3.2%, n=6, $p<0.05$).

We next transfected HUVEC with a miR-125a-specific miRNA inhibitor (Antimir) which reduced miR-125a expression in qRT-PCR (-31.1% ± 1.5%, n=4, p<0.001; **Figure 4A**), and consecutively increased mRNA levels of the five functional network targets CDH5 (+18.9% ± 4.1%; n=4; $p<0.05$), PTPN1 (+7.5% ± 2.5%; n=11; $p<0.05$), PPP1CA (+8.4% ± 1.9%; n=11; $p<0.01$), ETS1 (+16.8% ± 5.0%; n=7; $p<0.01$), and CLDN5 (+16.5% ± 4.4%; n=10; $p<0.05$, **Figure 4B**). As expected, miR-125a knock-down was able to attenuate the TNF-induced barrier disruption (**Figure 4C**, albumin flux -10.42% ± 15.14%, n=11, p<0.05), and increased monolayer impedance in ECIS (**Figure 4D**; AUC +2.3% ± 0.9%, n=11, $p<0.05$).

In summary, we discovered a regulatory network driven by miR-125a that regulates the barrier function of human endothelial cells in response to inflammatory stimuli.

In Primary Human Monocytes, Chemokine Receptor Type 2 Is Regulated by miR-125a

In endothelial cells, we found the Chemokine CCL2 to be strongly induced by miR-125a (**Figures 1G, H**). CCL2 is a specific ligand of the C-C Chemokine Receptor Type 2 (CCR2). CCR2, in turn, is strongly expressed on monocytes and is the central mediator of monocyte chemotaxis (24, 25). Sebastiani et al. showed that miR-125a directly regulated CCR2 on regulatory T-cells (26). We hypothesized that miR-125a may also regulate CCR2 on monocytes thus orchestrating the interplay of the endothelium with monocytes during the onset of acute inflammation. To investigate our hypothesis, we transfected primary human monocytes with miR-125a (transfection efficiency: ~61%, **Supplementary Figure S3B**) and analyzed CCR2 expression. As shown in **Figure 5**, overexpression of miR-125a significantly reduced CCR2 mRNA (**Figure 5A**: -46.3% ± 21.5%, n=13, p<0.001) and protein expression (**Figure 5B**).

We next investigated whether miR-125a directly interacts with the 3`UTR of CCR2. *In-silico* analyses revealed two putative seed sequences of miR-125a at positions 113-119 and 169-176 of the 3`UTR of CCR2 (**Figure 5C**). To provide experimental proof that CCR2 is a direct target of miR-125a, we cloned the full

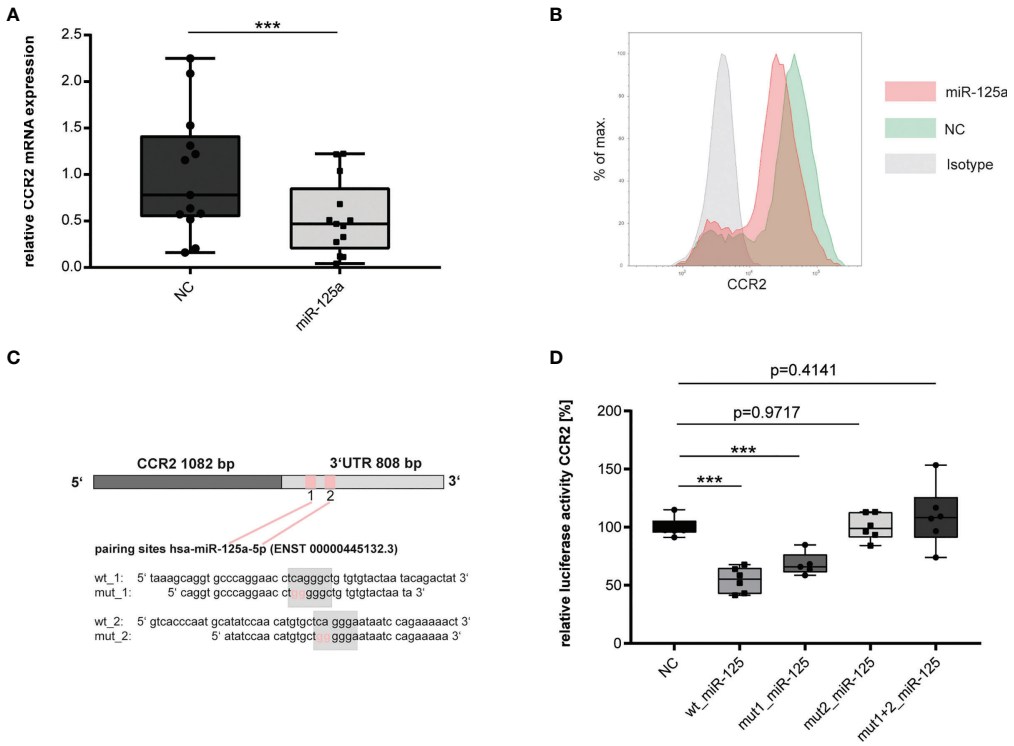

FIGURE 5 | Validation of miR-125a direct target CCR2. **(A)** mRNA (n=13,***p<0.001) and **(B)** protein (n=3, one exemplary experiment is shown) expression of CCR2 in primary human monocytes after tranfection of miR-125a or scrambled control (NC). **(C)** Schematic representation of the two predicted miR-125a binding sites within the 3`UTR of CCR2, including the site-directed oligonucleotide exchanges introduced for luciferase experiments. **(D)** CCR2 3`UTR Luciferase reporter gene activity after co-transfection of the luciferase constructs with miR-125a or scrambled control (NC). Luciferase constructs: wt_CCR2: both binding sites of miR-125a in the CCR2 3`UTR are functional; mut1_miR-125a: site-directed mutagenesis of binding site 1; mut2_miR-125a: site-directed mutagenesis of binding site 2; mut1+2_miR-125a: site-directed mutagenesis of both binding sites (n=6, ***p<0.0001).

3`UTR of the CCR2 gene into reporter constructs and performed luciferase reporter gene assays. Co-transfection of miR-125a and the reporter construct significantly decreased luciferase activity (**Figure 5D**, -45.6 ± 5.53, p<0.001, n=6). Site-directed mutagenesis of the miR-125a seed sequence 1 partially restored luciferase activity to 68% (± 5.42%, n=5, p>0.001), whereas both mutation of seed sequence 2 as well as mutation of seed sequences 1 + 2 completely restored luciferase activity, abolishing the effect of miR-125a in reporter gene quenching (n=6, p=0.818/0.414).

These results corroborate previous data from Sebastiani et al. (26) showing that miR-125a also regulates CCR2 expression in human monocytes by directly interacting with the seed sequences in the CCR2-3`UTR.

MiR-125a Regulates Monocyte Chemotaxis *via* CCR2

To corroborate the central role of CCR2 for monocyte chemotaxis, we first conducted gene-specific knockdown by RNA interference and combined time-lapse microscopy with subsequent cell tracking analyses. This revealed a clear impairment of CCL2-triggered chemotaxis after CCR2 knockdown (**Figure 6A, B**; -71.6% ± 16.2%, center of mass: Control 93.94 ± 29.64µm; siCCR2: 22.33 ± 13.45µm, p=0.0475, n=3). We next assessed if miR-125a induces a similar phenotype by performing Boyden chamber migration assays and detected a significant impairment of CCL2-directed migration after transfection of miR-125a (**Figure 6C**, -32.2% ± 9.9%, n=8, p<0.01). Accordingly, time-lapse microscopy experiments and subsequent cell-tracking analysis revealed a significant reduction of CCL2-induced directional migration (**Figures 6–F**, -48% ± 13.3%; Control: 0.25 ± 0.038µm; miR-125a: 0.13 ± 0.017µm, n=3, p<0.05) and velocity (**Figure 6G**, -27.5% ± 6.2%; Control: 3.63 ± 0.39µm miR-125a: 2.63 ± 0.28µm, n=3, p<0.05) after miR-125a overexpression without significantly reducing the overall accumulated distance (**Figure 6H**, n=3, p=0.4). These results show that miR-125a inhibits monocyte chemotaxis by specifically targeting CCR2.

Inflammation Represses miR-125a and Thereby Induces CCR2 in Primary Human Monocytes

We next analyzed whether acute inflammation may not only impact miR-125a expression in endothelial cells but also in human monocytes. To this end, we stimulated primary human monocytes with IL-6 (50ng/ml) and quantified the expressional

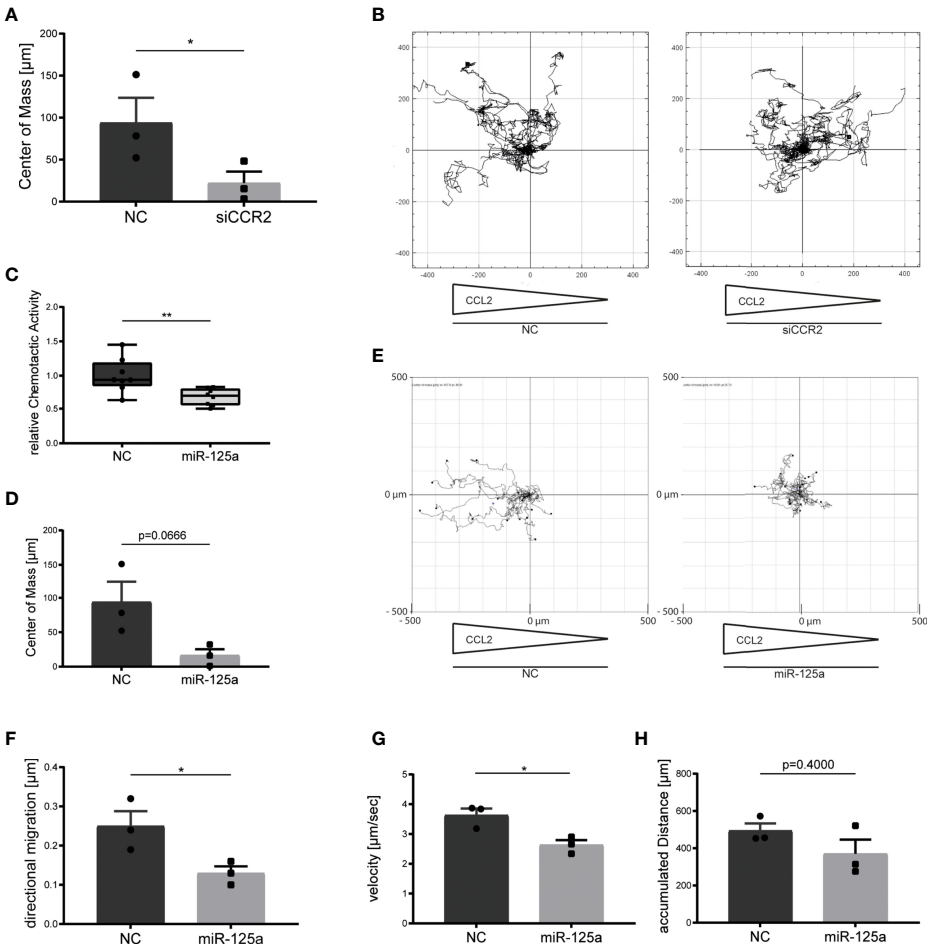

FIGURE 6 | Effect of miR-125a on monocyte chemotaxis. CCL2-specific monocyte chemotaxis after transfection of siRNA specifically targeting CCR2 **(A, B)**, miR-125a **(C–H)** or the respective scrambled controls (NC). Monocyte migration was recorded by time-lapse microscopy and subsequently analyzed by single-cell tracking analyses. (n=3, *p<0.05, **p<0.01). **(A)** Center of mass representing the average of all single cell endpoints. **(B)** Exemplary chemotaxis plot of three independent experiments. Arrows below the plot indicate the CCL2 concentration gradient. **(C, D, F–H)** miR-125a impacts chemotactic activity, center of mass, directional migration and velocity without affecting the accumulated distance. **(E)** Exemplary chemotaxis plot of three independent experiments. Arrows below the plot indicate the CCL2 concentration gradient.

changes of miR-125a and its target gene CCR2. We found miR-125a to be significantly reduced (**Figure 7A**, -12.7% ± 15,.%, n=7, p<0.05), whereas expression of CCR2 was induced as early as 3 hours after stimulation (**Figure 7B**, +164%, n=6, p<0.05). It thus can be assumed that the increase in migratory capacity of monocytes upon inflammatory stimuli is mediated by down-regulation of miR-125a.

Expression of miR-125a Strongly Increases During Monocyte-to-Macrophage Differentiation

After crossing the endothelial barrier and reaching the perivascular tissue, monocytes differentiate into macrophages and become resident. Previous studies have shown that the monocyte-to-macrophage differentiation is associated with a sharp decrease of CCR2 expression (27–29). The underlying mechanisms have remained elusive, so far. In accordance with these studies, we found CCR2 to have almost disappeared after inducing macrophage differentiation *in vitro* (**Figure 7C**, M-CSF/GM-CSF >-99% ± 0.2%, n=6, p<0.001). Simultaneously, a dramatic increase in miR-125a expression occurred (**Figure 7D**, M-CSF +11,700% ± 800%, n=5, p<0.001; GM-CSF +5,797% ± 1,665%, n=6, p<0.01), suggesting that induction of miR-125a mediates the loss of CCR2 during monocyte-to-macrophage differentiation, thus contributing to sedentariness.

DISCUSSION

Extravasation of leukocytes from the bloodstream to the inflamed tissue is a hallmark of the early inflammatory cascade. This dynamic process is characterized by complex

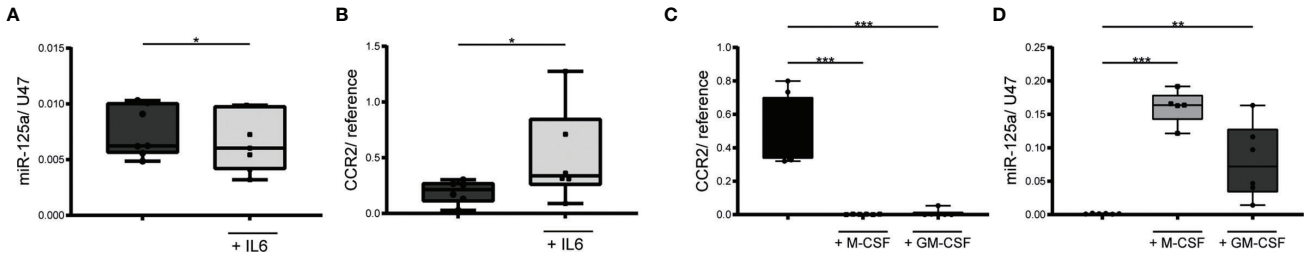

FIGURE 7 | MiR-125a and CCR2 expression in monocytes during inflammation and monocyte differentiation. Expression levels of miR-125a **(A)** and CCR2 **(B)** after incubation of primary human monocytes with IL6 for 3 hours (n=7, *p<0.05). Quantification of CCR2 **(C)** and miR-125a **(D)** expression in primary human monocytes immediately after differentiation with M-/GM-CSF for 6 days. (n=5-6, **p<0.01, ***p<0.001).

interactions between migrating leukocytes and the transiently passable endothelial barrier, which need to be precisely orchestrated to initiate an effective early immune response (30–32). Yet, the underlying regulatory mechanisms are widely unknown.

Increasing evidence proposes an impact of miRNAs on endothelial and immune cell function (33–37). While these studies focused on single miRNA/target interactions, we here aimed to identify a miRNA-driven circuitry that regulates the inflammatory cascade on a cell-type-spanning level. We focused on miR-125a, as recent studies detected elevated levels of miR-125a in the blood of patients with acute inflammatory conditions associated with endothelial dysfunction (15–17).

We first focused on the impact of miR-125a on endothelial cells. Using NGS-based transcriptome analysis after miR-125a overexpression of HUVEC cells, we could identify the pathways *GO_regulation of cell adhesion* and *KEGG_adherens junctions* to be specifically affected, which led us to assume miR-125a to be involved in the regulation of endothelial cell permeability. Indeed, functional analyses showed that miR-125a overexpression induced a hyperpermeable phenotype during the acute inflammatory response. Both NGS and functional data thus clearly indicated that miR-125a has barrier-disruptive properties. To find the underlying mechanism, we extracted genes from the NGS dataset that displayed a) a strong endothelial expression and b) differential regulation after overexpression of miR-125a. We were able to newly elucidate a complex regulatory network directed by miR-125a, consisting of the two adhesion molecules VE-Cadherin (CDH5) and Claudin-5 (CLDN5), of two regulatory tyrosine phosphatases (PTPN1, PPP1CA) and of the transcription factor ETS-1. Individual knockdown of each of the network targets independently increased endothelial permeability. Central effector element in this network is CDH5, the central gatekeeper of endothelial barrier function and regulator of paracellular permeability (38, 39). The other direct target genes evaluated in this study, ETS-1, PTPN1 and PPP1CA, exert their effects *via* CDH5 thus strongly multiplying the impact of miR-125a:

ETS-1 exhibits barrier-protective effects *via* transcriptional induction of VE-Cadherin (40, 41). MiR-125a-mediated repression of ETS-1 reduces VE-Cadherin levels and further increases endothelial barrier permeability during acute inflammation. The two phosphatases, on the other hand, act on a post-transcriptional level. Posttranslational phosphorylation of VE-Cadherin at different tyrosine residues and subsequent internalization is one of the main triggers for endothelial barrier hyperpermeability during acute inflammation and transendothelial leukocyte migration (18, 42). PTPN1, coding for protein phosphatase 1B, and PPP1CA, a subunit of the protein phosphatase 1, have been shown to prevent phosphorylation and subsequent internalization of VE-Cadherin, thus stabilizing adherens junctions and dampening endothelial barrier dysfunction during acute inflammation (43–46). As expected, targeting of these two phosphatases by miR-125a resulted in increased levels of phospho-VE-Cadherin (Tyr658) and increased permeability of the endothelial monolayer. Phosphorylation at this tyrosine residue Tyr658 is required for leukocyte transendothelial migration (47) and inflammatory triggered hyperpermeability (18). The second adhesion molecule significantly down-regulated by miR-125a, CLDN5, did not reveal as a direct target gene. In fact, we could show that it is indirectly regulated by CDH5. We could identify a secondary negative feedback loop: CDH5 down-regulation leads to CLDN5 repression, which then further decreases CDH5 expression, thereby amplifying the regulatory impact of miR-125a. The mechanisms underlying the CDH5-CLDN5 loop have already been described by Morini et al. (22).

Increased endothelial permeability is associated with enhanced leukocyte transmigration through the endothelial monolayer (42, 47). Guided by the surprising finding of our NGS analysis that expression levels of the chemokine CCL2 in stimulated endothelial cells after miR-125a transfection were strongly increased, we next turned our attention to monocytes as the master mediator of monocyte trafficking is CCL2. Produced by endothelia, it is recognized by the chemotactic receptor CCR2, abundantly expressed on nearly all classical monocytes, that subsequently prompts them to actively migrate towards the inflammatory focus (27, 48, 49). We hypothesized that miR-125a, which is also expressed in monocytes (33, 50), might modulate the CCL2/CCR2 axis in these immune cells thereby regulating their trafficking under inflammatory conditions. We identified two novel binding sites of miR-125a within the 3`UTR of CCR2. We further could show

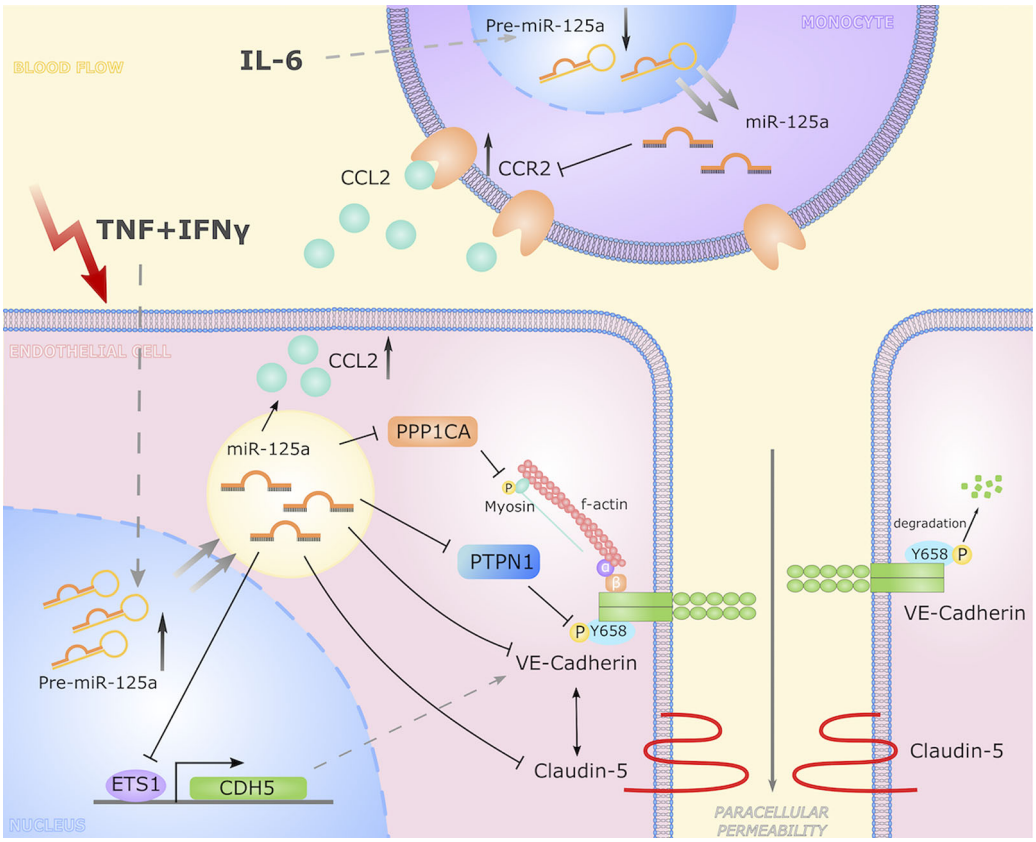

FIGURE 8 | Graphical representation of the miR-125a driven cell-crossing functional network. In response to inflammatory stimulation, the expression of miR-125a increases in endothelial cells. Here, miR-125a directly regulates CDH5, PTPN1, PPP1CA, and ETS-1 while CLDN5 levels are reciprocally linked to CDH5 expression. By reducing expression of ETS-1, miR-125a inhibits VE-Cadherin transcription. *Via* targeting PTPN1 and PPP1CA, miR-125a affects levels of phosphorylated VE-Cadherin, destabilizing adherens junctions and thus increasing paracellular permeability. In turn, monocytic expression of miR-125a is reduced upon inflammatory stimulation, leading to an induction of CCR2 expression, impacting monocyte chemotaxis.

that overexpression of miR-125a down-regulated CCR2 expression levels and strongly impaired CCL2-specific monocyte chemotaxis. Under inflammatory conditions, miR-125a expression in primary human monocytes was strongly diminished and CCR2 was concomitantly induced, enabling increased monocyte trafficking to the inflammatory site.

After entering the inflamed area, monocytes differentiate into macrophages, terminate CCR2 expression, and become stationary (51–53). The molecular mechanisms underlying this CCR2-specific downregulation, however, remain elusive so far. We here provide novel evidence that miR-125a is dramatically induced upon stimulation of monocytes with macrophage-inducing differentiation factors (M-CSF, GM-CSF), while the CCR2 almost completely disappears. We thus propose that direct interaction of miR-125a with the 3`UTR of CCR2 is a previously underestimated molecular mechanism that regulates macrophage trafficking and residence.

In summary, we discovered and characterized a novel regulatory network directed by miR-125a that regulates and synchronizes the process of endothelial barrier permeability and monocyte migration upon inflammatory stimulation. While previous studies already identified cell-type spanning effects of miRNAs *via* shuttling of extracellular vesicles between cell types (54–57), we here report that one miRNA, although disparately regulated in endothelia and monocytes, directs a biologic process in a synergistic and mutually reinforcing manner (illustrated in **Figure 8**). These findings expand the knowledge on regulation of inflammatory processes and may open up new roads in diagnosis and therapy of inflammation-driven diseases.

DATA AVAILABILITY STATEMENT

The datasets of RNA-Seq presented in this study can be found in online repositories: https://www.ncbi. nlm.nih.gov/geo/, GSE196161. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of the medical faculty of the Ludwig-Maximilians-University München (LMU), Germany.

The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MBM and MH designed and performed experiments, analyzed and interpreted data and wrote the manuscript. LL, ST, DE, VL, and KP performed experiments and analyzed and interpreted data. SH performed experiments, analyzed data and prepared figures. MS provided critical tools and edited the manuscript. SK designed experiments, interpreted data, wrote the manuscript and supervised the study. All authors contributed to the article and approved the submitted version.

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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.2022.826047/full#supplementary-material

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REVIEW
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Interaction Between Non-Coding RNAs and Interferons: With an Especial Focus on Type I Interferons

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Interferons (IFNs) are a group of cellular proteins with critical roles in the regulation of immune responses in the course of microbial infections. Moreover, expressions of IFNs are dysregulated in autoimmune disorders. IFNs are also a part of immune responses in malignant conditions. The expression of these proteins and activities of related signaling can be influenced by a number of non-coding RNAs. IFN regulatory factors (IRFs) are the most investigated molecules in the field of effects of non-coding RNAs on IFN signaling. These interactions have been best assessed in the context of cancer, revealing the importance of immune function in the pathoetiology of cancer. In addition, IFN-related non-coding RNAs may contribute to the pathogenesis of neuropsychiatric conditions, systemic sclerosis, Newcastle disease, Sjögren's syndrome, traumatic brain injury, lupus nephritis, systemic lupus erythematosus, diabetes mellitus, and myocardial ischemia/reperfusion injury. In the current review, we describe the role of microRNAs and long non-coding RNAs in the regulation of IFN signaling.

Keywords: lncRNA, miRNA, interferon, expression, biomarker

INTRODUCTION

Being firstly recognized as antiviral factors that interfere with viral replication (1), interferons (IFNs) are a group of cellular proteins classified in three families (2). Thirteen IFN-α variants, a single IFN-β and numerous other IFNs (IFN-ϵ, -k, -ω, and -δ) are classified as Type-I IFNs (3, 4). Type II IFN family only includes IFN-γ (5), a protein that potentiates proinflammatory signals through priming macrophages for antimicrobial functions and induction of nitric oxide synthesis and inhibition of the activity of NLRP3 inflammasome (6, 7).

Secretion of IFNs from infected cells can lead to induction of innate immune response resulting in cytokine release and induction of function of natural killer cells and antigen presentation (3, 8). These proteins have critical roles in the regulation of immune responses in the course of microbial infections. Moreover, expressions of IFNs are dysregulated in autoimmune disorders. Based on

these roles, the identification of cellular mechanisms of regulation of IFNs has practical significance. Regulation of IFNs expressions is accomplished by different mechanisms, including of binding of regulatory molecules to their 3′ untranslated regions (3′ UTRs). This region contains both AU-rich elements (AREs) and microRNA (miRNA) recognition elements (2). RNA-binding proteins can target AREs and either induce mRNA degradation or stabilize mRNA. Meanwhile, the binding of miRNAs with miRNA recognition elements is involved in the regulation of mRNA translation *via* the miRNA-induced silencing complex (2). In the current review, we describe the role of miRNAs and long non-coding RNAs (lncRNAs) in the regulation of IFNs.

Interactions Between miRNAs and IFNs

miR-301a has been found to contain a binding site in the 3′-UTR of the Interferon regulatory factor 1 (*IRF-1*) gene. Through the modulation of the expression of this gene, this miRNA participates in the proliferation of hepatocellular carcinoma cells. Expression of miR-301a has been increased in primary hepatocellular carcinoma tumors and cell lines, parallel with down-regulation of IRF-1. *In vitro* studies have shown the role of chronic hypoxia in the induction of miR-301a and down-regulation of IRF-1. Moreover, suppression of miR-301a induces cell apoptosis and reduces cell proliferation. Taken together, the regulation of miR-301a on IRF-1 expression is implicated in the pathogenesis of hepatocellular carcinoma (9). Another study in this malignancy has shown that IRF-1 can induce the expression of miR-195 to inhibit CHK1 expression. Up-regulation of IRF-1 or down-regulation of CHK1 induces cell apoptosis and increases PD-L1 expression in hepatocellular carcinoma cells (10).

In lung cancer cells, miR-19 has been shown to influence the expression of IFN-induced genes and MHC class I, signifying the impact of miR-19 in connecting inflammation and carcinogenesis (11). The IRF2-targeting miRNA miR-1290 has also been shown to be up-regulated in lung cancer. Over-expression of miR-1290 has been correlated with lymph node metastasis and advanced clinical stage. miR-1290 could enhance cell proliferation, colony formation, and invasive abilities in lung cancer cells. This miRNA could also promote the expression of cell proliferation-related proteins CDK2 and CDK4 and induce epithelial-mesenchymal transition (EMT) (12). Another study in lung cancer samples has shown up-regulation of IRF6 and down-regulation of miR-320, a miRNA that targets IRF6. IRF6 siRNA or miR-320 mimics could inhibit the growth and migration of lung cancer cells. Taken together, the miR-320/IRF6 axis has been suggested as a molecular axis involved in the pathogenesis of lung cancer (13).

Experiments in squamous cell carcinoma samples and cell liens have shown up-regulation of IRF2-targeting miRNA miR-664. This miRNA has been found to increase tumorigenic behaviors of cells both *in vitro* and *in vivo* (14).

Participation of IFN-related miRNAs has also been assessed in the pathoetiology of non-malignant conditions. For instance, the IRF2 targeting miRNA miR-221-3p has been found to be over-expressed in patients with the major depressive disorder compared with normal persons. Notably, serum miR-221-3p levels have been positively correlated with the level of depression. Mechanistically, miR-221-3p can enhance the expression of IFN-α in astrocytes through targeting IRF2. In fact, this miRNA participates in the induction of anti-neuroinflammatory signals by ketamine and paroxetine through the IRF2/IFN-α axis (15).

miR-126 and miR-139-5p are two miRNAs that participate in the dysregulation of plasmacytoid dendritic cells in systemic sclerosis. Expressions of these miRNAs have been correlated with the expression of type I IFN-responsive genes. TLR9 stimulation of plasmacytoid dendritic cells has induced expressions of miR-126 and miR-139-5p in cultures of normal cells as well as those obtained from patients with systemic sclerosis. USP24 has been identified as a target of miR-139-5p (16). **Table 1** summarizes the results of investigations that assessed the effects of miRNAs on IFN signaling.

miR-26a is an example of miRNAs that participate in the regulation of host immune responses during viral infections. Expression of this miRNA is increased upon infection with Feline Herpes Virus 1 (FHV-1). This virus could induce the expression of miR-26a through a cGAS-dependent route since down-regulation of cellular cGAS could result in blockage of poly (dA:dT) or FHV-1-induced expression of miR-26a. Functional studies have shown the impact of miR-26a in the induction of STAT1 phosphorylation and enhancement of type I IFN signals, which inhibit viral replication. In fact, miR-26a directly targets SOCS5 mRNA. SOCS5 silencing has led to an increase in STAT1 phosphorylation and induction of antiviral responses mediated by type I IFNs (37).

Another study has shown a time-dependent down-regulation of miR-155 upon infection with the dengue virus. Exogenous up-regulation of this miRNA could limit replication of the dengue virus *in vitro*, indicating that down-regulation of miR-155 has a beneficial effect for replication of this virus. The results of *in vivo* experiments have also confirmed the impact of miR-155 in protection against the life-threatening effect of dengue virus infection. This activity of miR-155 has been shown to be exerted through targeting Bach1, and subsequent activation of the HO-1-mediated suppression of NS2B/NS3 protease activity of dengue virus. Taken together, modulation of miR-155 expression has been suggested as a therapeutic option for the management of dengue virus infection (38). miR-218 is another miRNA that can regulate host responses to viral infections since its down-regulation by porcine reproductive and respiratory syndrome virus can facilitate replication of this virus through suppression of type I IFN responses (39). **Table 2** shows the effects of miRNA on IFN signaling in the context of viral infections. **Figure 1** illustrates the aberrant expression of various miRNAs, which adversely affect the IFN signaling pathway triggering several kinds of human diseases and malignancies as well as their role in the context of viral infections.

Interactions Between lncRNAs and IFNs

LncRNAs are a group of regulatory non-coding RNAs that share several characteristics with mRNAs, but lacking open reading frames. They participate in epigenetic regulation of

TABLE 1 | The effects of miRNAs on IFN signaling (ANT, adjacent normal tissue).

Type of diseases	miRNA	Sample	Cell Line	Target, Pathway	Discussion	Ref
Nasopharyngeal carcinoma	miR-9	–	CNE2, 5–8F	IFI44L, PSMB8, IRF5, PSMB10, IFI27, IFIT2, TRAIL, IFIT1	miR-9 modulates levels of IFN-induced genes and MHC class I.	(17)
Hepatocellular Carcinoma (HCC)	miR-301a (Up)	20 pairs of HCC and ANTs	Huh7, Hep3B, HepG2, Hepa1-6	IRF-1, Caspase-3	miR-301a *via* down-regulating IRF-1 could induce HCC.	(9)
HCC	miR-195 (–)	30 pairs of HCC and ANTs; WTB6 mice	Hepa1-6, Huh-7, Hep3B, HepG2	IRF-1, IFN-γ, CHK1, STAT3	IRF-1 *via* modulating miR-195 by down-regulating CHK1 could up-regulate apoptosis in HCC.	(10)
HCC	miR-146a (Up)	–	PLC/PRF/5	INF-α, SMAD4, STAT1/2	miR-146a could suppress the sensitivity to INF-α in HCC cells.	(18)
Lung Cancer (LC)	miR-19 (–)	–	CNE2, HONE1, A549, HCC827	IRF-1/7/9, IFI-6/27/35, HLA-B/F/G	miR-19 *via* regulating the expression of interferon could affect the expression of IFN-induced genes and MHC class I in human lung cancer cells.	(11)
Non-Small Cell Lung Cancer (NSCLC)	miR-1290 (Up)	41 pairs of NSCLC and ANTs	A549, H1299, SPC-A1, H1970, H460, BEAS-2B	IRF-2, CK2/4, E/N-cadherin	Overexpression of miR-1290 by targeting IRF2 could contribute to cell proliferation and invasion of NSCLC.	(12)
NSCLC	miR-320 (Down)	21 pairs of NSCLC and ANTs	A549, NCI-H2170	IRF-6	miR-320 *via* targeting IRF-6 could affect pathogenesis of NSCLC.	(13)
Cutaneous Squamous Cell Carcinoma (cSCC)	miR-664 (Up)	Athymic nude mice	HSC-1, A431, HSC-5, HaCaT	IRF-2	miR-664 *via* suppressing IRF-2 could function as an oncogene in cSCC.	(14)
Cervical Cancer (CC)	miR-587 (Up)	41 pairs of CC and ANTs, nude mice	Ect1/E6E7, HeLa, SiHa, CaSki, C-33A	IRF-6, Cyclin-D1, CDK4	miR-587 by repressing IRF6 could promote CC.	(19)
Gastric Cancer (GC)	miR-19a, miR-18a (–)	20 pairs of GC tissues and ANTs; BALB/c nude mice	MKN45, AGS, SGC7901, GES1	IFN-γ, IRF-1, Axin2, SMAD2, Wnt/β-catenin	IRF-6 by regulating MIR17HG-miR-18a/19a axis *via* Wnt/β-catenin signaling could promote GC metastasis.	(20)
Glioblastoma (GBM)	miR-203a (Down)	NSG mice	MT330, SJG2	IFN-α, IFN-β, IFN-λ1, IFI-1/6, IFT20, p65, NF-κB, STAT1-3	miR-203a *via* an ATM-dependent interferon response pathway could suppress GBM.	(21)
Osteosarcoma	miR-4295 (Up)	15 pairs of OS and ANTs	MG-63, Saos-2, hMSC	IRF-1	miR-4295 *via* targeting IRF1 could promote cell proliferation, migration and invasion.	(22)
Systemic Lupus Erythematosus (SLE)	miR-146	WBCs from patients with SLE	THP-1 cells		Type I IFN inhibits miR-146a maturation *via* increasing expression of MCPIP1.	(23)
Major Depressive Disorder (MDD)	miR-221-3p (Up)	(n=64) perioperative patients	Astrocytes	IRF-2, IFN-α, NF-κB	miR-221-3p *via* targeting IRF2 could up-regulate IFN-α expression in MDD patients.	(15)
Systemic Sclerosis	miR-126, miR-139-5p (Up)	Blood samples of SS patients (n=72) and healthy control (n=26)	pDCs	IFI-6, IFIT1, CXCL10, USP24, TLR-7/8/9	miR-126 and miR-139-5p *via* TLR9-mediated response and IFN signaling could regulate the activation of plasmacytoid dendritic cells.	(16)
Newcastle Disease (ND)	gga-miR-455-5p (Down)	–	293T, BHK-21	IFN-I, SOCS3	gga-miR-455-5p *via* targeting cellular suppressors of SOCS3 could suppress ND virus replication.	(24)
Sjögren's Syndrome (SS)	miR-1248 (–)	–	phSG	IFN-β, IRF-1/9, IFIT1, IFI-6/44, IFIH1, MX1, JAK-1/2, STAT-1/2/3	miR-1248 could activate IFN-β *via* the direct association with both AGO2 and RIG-I.	(25)
Traumatic Brain Injury (TBI)	miR-155 (-)	C57BL/6 mice		IFN-I, IFN-α2/4/5, IL-6, IFN-β1, IRF-1, TNF-α, SOCS1C	Up-regulation of miR-155 after brain injury promotes IFN-I to exert a neuroprotective function.	(26)
Edwardsiella Tarda TX1 (E. tarda TX1)	pol-miR-194a (Up)	Fish	FG-9307, 293T	IFN-I, IRF-7	pol-miR-194a *via* targeting IRF7 could participate in the regulation of flounder immune response and microbial infection.	(27)
–	miR-17 (-)	C57BL/6J mice	VSMCs, RAVSMCs	IRF-9	miR-17 knockdown *via* up-regulating IRF-9 expression could promote vascular smooth muscle cell phenotypic modulation.	(28)
–	miR-155 (-)	–	EPC, BHK-21	IFN-I, PIAS4a	Overexpression of miR-155 *via* targeting IFN-I could contribute to antiviral response in EPC.	(29)
–	miR-181a,	–	U937, 293T, monocytes, MDM, MDDC	IFN-I/II, IFN-α, IFN-β, IFN-γ, ERK, STAT-1	Interferons *via* down-regulating miR-181a and miR-30a could induce the expression of SAMHD1 in monocytes.	(30)

(Continued)

TABLE 1 | Continued

Type of diseases	miRNA	Sample	Cell Line	Target, Pathway	Discussion	Ref
	miR-30a (-)					
–	miR-155, miR-155* (–)	–	HeLa, PDC	IFN-I, IFN-α, IFN-β, NF-κB, PI3K, AKT, p38	miR-155 in cooperation with its star-form partner miR-155* could regulate IFN-I production.	(31)
–	Bta-miR-204 (Down)	bEEC	–	IFN-τ, BoLA, PD-L1/2	IFN-τ by down-regulating bta-miR-204 could enhance the expression and function of BoLA.	(32)
–	miR-30c-5p (–)		Vero E6,	IFN-I/III, IFN-λ, IFIT1, ISG-15, SOCS-1	The coronavirus PEDV *via* the miR-30c-5p/SOCS1 axis could evade type III interferon response.	(33)
–	miR-744 (–)	–	RMCs	PTP1B, INF-I, CCL2/5, CXCL10, IL6, ERK, p38, MX1, IFIT3, TYK2, STAT1/3, JAK1, NF-κB	miR-744 by targeting PTP1B could enhance the INF-I signaling pathway in primary human renal mesangial cells (RMCs).	(34)
–	miR-155 (Up)	C57BL/6 mice	293T, RAW264.7, BMMs	SOCS1, IFN-β, MITF, TRAP	INF-β-induced miR-155 by targeting SOCS1 and MITF could inhibit osteoclast differentiation.	(35)
–	miR-221 (–)	–	H69, HIBEpiC	ICAM-1, IFN-γ, PRRSV, p65	miR-221 *via* targeting ICAM-1 translation regulating IFN-γ could induce ICAM-1 expression in human cholangiocytes.	(36)

gene expression through modulation of histone or DNA marks as well as regulation of the stability of RNAs and interacting with regulatory proteins (59).

Several lines of evidence suggest that lncRNAs include an important subgroup of the IFN target genes. Additionally, the IFN response has been shown to be regulated by several lncRNAs encoded by host or pathogens (60). Kambara et al. have identified approximately 200 lncRNAs whose expressions are induced by IFN in primary human hepatocytes (61). Notably, among them has been lncRNA-CMPK2/NRIR which has exhibited an intense induction after IFN stimulation in various human and mouse cells. This lncRNA is located near the protein-coding IFN-stimulated gene CMPK2. Expression of this lncRNA has been shown to be induced in a JAK-STAT-dependent manner. Silencing of lncRNA-CMPK2/NRIR has resulted in a significant decrease in HCV replication in IFN-induced hepatocytes, implying the role of this lncRNA in the modulation of antiviral effects of IFN (61).

NRAV is another lncRNA whose expression is regulated by IFNs. Microarray analyses in cells overexpressing this lncRNA has shown down-regulation of several ISGs. NRAV has been shown to be able to partially preclude IFN-induced expression of its target ISGs, possibly *via* affecting its transcription or through epigenetic mechanisms (62).

IFNG-antisense-1 (IFNG-AS1) is another lncRNA that participates in the regulation of IFN responses. This lncRNA is located downstream of the *IFNG* locus. Expression of IFNG-AS1 is strongly correlated with expression of IFNG (63, 64). CD4+ and CD8+ T cells as well as NK cells express this lncRNA. IFNG-AS1 expression by CD4+ T cells depends on two transcription factors being involved in Th1 polarization, namely STAT4 and TBX21 (65).

AFAP1-AS1, lncMX1-215, linc00513, BANCR, IFITM4P, LUCAT1, NEAT1, MALAT1, DANCR, NRIR, and FIRRE are among lncRNAs whose interactions with IFN signaling have been assessed (**Table 3**). For instance, the up-regulated lncRNA AFAP1-AS1 can participate in the invasiveness of lung cancer cells through increased expression of IRF7 and induction of RIG-I-like receptor signals (66). On the other hand, lncMX1-215 is an IFNα-induced lncRNA that can affect the immunosuppressive responses through interfering with H3K27 acetylation (67).

LncRNAs can also affect response to protozoan parasites such as cryptosporidium. NR_033736 is a novel lncRNA that has been found to be up-regulated in intestinal epithelial cells upon infection with this protozoon. This lncRNA can suppress transcription of type I IFN-controlled genes in host cells infected with this microorganism. Notably, type I IFN signaling can trigger the expression of NR_033736. In fact, NR_033736 participation in the negative feedback regulatory mechanism of type I IFN signaling results in fine-tuning of innate defense mechanism against microorganisms in the epithelial cells (68).

Investigations in the context of lupus nephritis have shown that RP112B6.2 *via* targeting the IFN-I by epigenetically inhibiting the expression of SOCS1 could aggravate symptoms of this disease (69). Linc00513 is another lncRNA that participates in the pathogenesis of lupus through promoting IFN signaling (70).

The impact of lncRNAs on IFN signaling has also been assessed in the context of diabetes mellitus. Lnc10 contains a type I diabetes-associated single nucleotide polymorphism. This lncRNA can regulate the expression of the IRF7-driven inflammatory network regulating gene Ebi2 in immune cells. Expression of Lnc10 in pancreatic β-cells has been shown to be up-regulated by diabetogenic incitements, including pro-inflammatory cytokines and viral infections (71). **Figure 2** represents the role of several lncRNAs in various types of human cancers and immune-related disorders as well as their impact on viral infections *via* regulating the IFN signaling pathway.

TABLE 2 | The effects of miRNA on IFN signaling in the context of viral infections.

Virus	miRNA	Sample	Cell Line	Target	Discussion	Ref
Infectious stress	miR-22	Mir22-KO mice	–	–	miR-22 enhances the IFN response to viral infections.	(40)
Infection with influenza virus	miR-144	Wild-type mice	–	TRAF6-IRF7	miR-144 diminishes host responses to the influenza virus.	(41)
Feline Herpes Virus 1(FHV-1)	miR-26a (Up)	–	F81, 293T	IFN-α, IFN-β, ISG-15, SOCS5, STAT-1	miR-26a by targeting SOCS5 and promoting Type I IFN signals could inhibit FHV-1 replication.	(37)
Human Herpes Simplex Virus Type 1 (HSV-1)	miR-23a (–)	–	HeLa	IRF-1, RSAD2, EGFP, Myc	miR-23a *via* suppression of IRF-1 could facilitate the replication of HSV-1.	(42)
Dengue Virus (DENV)	miR-155 (Down)	Breeder ICR mice	Huh-7	HO-1, IFN-α-2/5/17, BACH1, Nrf2, OAS-1/2/3	miR-155 by inducing HO-1-mediated antiviral interferon responses could inhibit DENV replication.	(38)
Porcine Reproductive & Respiratory Syndrome Virus (PRRSV)	miR-218, miR339-5p, miR-99b, miR-365-5p, miR-378, miR-345, miR-27b-3p	SPF pig	PAMs, marc-145, Vero-E6, ST, 293T	IFN-I, IFN-β, SOCS3	Downregulation of miR-218 by PRRSV could facilitate viral replication *via* repressing of type I IFN responses.	(39)
PRRSV	miR-30c (–)	–	PAM, Marc-145, U4A	IFN-I, IFNAR-2, ISG-15, OAS-1, JAK-1	miR-30c *via* targeting IFNAR-2 could promote type 2 PRRSV infection.	(43)
PRRSV	miR-382-5p (Up)	–	MARC-145, 293T, BHK-21	IFN-I, IFN-β, HSP60, MAVS, IRF-3, TBK1	miR-382-5p by negatively regulating the induction of IFN-I could promote PRRSV replication.	(44)
PRRSV2	miR-541-3p (Up)		MARC-145, MA-104, 293T	IRF7,	miR-541-3p *via* IRF7 could promote the replication of PRRSV2.	(45)
Influenza A virus, TMEV	miR-673 (–)	Dgcr8$^{-/-}$ mouse, Dicer$^{+/+}$ mouse	NIH3T3, ESC	IFN-β1, MAVS	During pluripotency, an interaction between MAVS (mitochondrial antiviral signaling protein) and miR-673 could act as a switch to suppress the antiviral IFN.	(46)
Influenza Virus A/WSN/33 (H1N1)	miR-302a (–)	C57BL/6 mice	A549, THP-1, 293T, MLE-12, H9	IFN-β, TNF-α, IRF-5, CCL-2/5, IL-6/8, M1, NP, NF-κB	miR-302a *via* targeting IRF5 expression and cytokine storm induction could suppress IAV.	(47)
H1N1	miR-93 (Down)	C57BL/6 mice	AT2, MLE12, A549, 293T, Murine T-cells, Murine B-cells, B-cells, NK cells	IFN-I, IFN-β, IRF-3, IL-6/8/10, NF-κB, ISG15, OAS1, RIG-I, p38/65, ERK, JAK-1/2	Inhibition of miR-93 by up-regulating JAK-1 could promote interferon effector signaling to suppress influenza A infection.	(48)
Influenza A virus (IAV) H5N1	miR-21-3p (Down)	26 H5N1-infected patients serum samples and 13 serum samples from normal persons	A549	IFN-I, FGF2, IFN-β, IFN-α, MxA, OAS	miR-21-3p by refraining IFN-I response could modulate FGF2 to facilitate influenza A virus H5N1 replication.	(49)
Foot & Mouth Disease Virus (FMDV)	miR-103, miR-107 (Down)	20 pairs of blood samples from patients with enterovirus 71 (EV71) and normal blood samples	VERO, RD	IFN-I, IFN-α, IFN-β, SOCS3, STAT3	miR-103/miR-107 by regulating SOCS3/STAT3 pathway could inhibit EV71 replication and facilitate IFN-I response.	(50)
FMDV	miR-4334-5p (Up)	–	PK-15, BHK-21	IFN-β, TNF-α, OAS, ISG54, ID1, VP1	miR-4334-5p by suppressing IFN pathways *via* direct targeting ID1 could facilitate FMDV propagation.	(51)
HIV-1	miR-128 (–)	–	HeLa, 293T, THP-1, Jurkat	INF I, IFN-α, TNPO3,	IFN-I *via* enhancing miR-128 by targeting TNPO3 mRNA could modulate HIV-1 Replication.	(52)
Infectious Bursal Disease Virus (IBDV)	gga-miR-27b-3p (Up)	–	DF-1	IFN-I, IFN-β, IRF3, NF-κB, SOCS3, SOC6, STAT-1	gga-miR-27b-3p *via* targeting cellular suppressors of SOCS3 and SOCS6 could enhance type I IFN signals and inhibit replication of IBDV.	(53)
IBDV	gga-miR-155 (Up)	–	DF-1	TANK, SOCS1, IFN-I, chIRF3	gga-miR-155 *via* targeting SOCS1, and TANK could enhance IFN-I and suppress IBDV.	(54)
IBDV	gga-miR-9* (Up)	–	DF-1	IRF-2, INF-β	gga-miR-9* by targeting IRF-2 to promote IBDV replication could inhibit IFN production in antiviral innate immunity.	(55)
Hepatitis C Virus (HCV)	miR-122 (–)	–	Huh7	INF-α, INF-β, EGFP, SOCS1	miR-122 *via* blocking suppressor of SOCS1 could modulate INF-I expression.	(56)

(Continued)

TABLE 2 | Continued

Virus	miRNA	Sample	Cell Line	Target	Discussion	Ref
Human Papillomavirus 16 (HPV16)	miR-122 (–)	–	SiHa, CaSki, C33A	OAS-1, MxA, pmCherry-E6, IFN-α, IFN-β, STAT1, SOCS1	miR-122 *via* blocking suppressor of cytokine signaling 1 in SiHa cells could inhibit HPV E6 gene and enhance interferon signaling.	(57)
Human Cytomegalovirus (HCMV)	Hcmv-miR-UL112 (–)	–	PBMCs, K562	TNF-I, IFNAR, CD107	Hcmv-miR-UL112 activity by inhibiting INF-I secretion could attenuate NK cells.	(58)

DISCUSSION

Non-coding RNAs that regulate IFN signaling have been shown to participate in the pathogenesis of different types of cancer as well as immune-related disorders. IRFs are the most investigated molecules in the field of effects of non-coding RNAs on IFN signaling. For instance, IRF-1 has been shown to have functional interactions with miR-301a, miR-195, miR-19a, miR-18a, miR-4295, miR-124, and miR-155. Meanwhile, IRF-2 has interactions with miR-1290, miR-664, and miR-221-3p. Besides, IRF-6 interacts with miR-320, miR-587, miR-19, and miR-18a. These interactions have been best assessed in the context of cancer, revealing the importance of immune function in the pathoetiology of cancer.

In addition, IFN-related non-coding RNAs may contribute to the pathogenesis of neuropsychiatric conditions through modulation of immune responses in CNS-resident cells. Major depressive disorder is an example of these conditions in which the role of IRF-targeting miRNAs has been identified. Among non-malignant conditions, are systemic sclerosis, Newcastle disease, Sjögren's syndrome, traumatic brain injury, lupus nephritis, systemic lupus erythematosus, diabetes mellitus, and myocardial ischemia/reperfusion injury have been found to be associated with dysregulation of IFN-related non-coding RNAs.

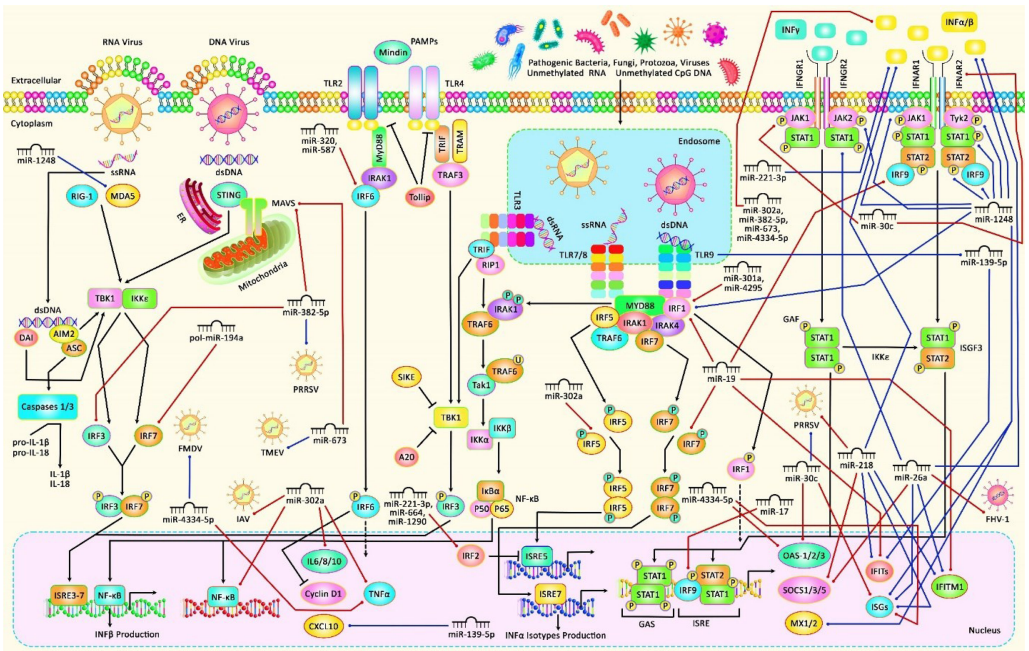

FIGURE 1 | A schematic diagram of the interaction between several miRNAs and interferons in causing various human diseases. Mounting evidence has demonstrated that miRNAs could have an important contribution to the regulation of expression of IFN-induced genes. Aberrant expression of such ncRNAs could lead to various human diseases such as major depressive disorder, Sjögren's Syndrome, Systemic Sclerosis as well as different kinds of cancers. As an illustration, a recent study has detected that overexpression of miR-301a could promote hepatocellular carcinoma *via* directly targeting IRF1 (9). Moreover, another research has figured out that miR-587 could play a key role in the progression of cervical cancer by down-regulating the expression of IRF6 (19). In addition, another finding has denoted that miR-1248 *via* activating the expression levels of IFN-β, IRF1/9, MX1, JAK-1/2, STAT-1/2, TYK2 as well as direct association with both AGO2 and RIG-I could have a crucial role in Sjögren's syndrome (25). Furthermore, miR-26a could suppress feline herpesvirus 1 (FHV-1) replication *via* targeting SOCS5 and up-regulating the expression levels of IFN-α, IFN-β, ISG-15, STAT-1, and IFITM1 in type I IFN signaling (37). Blue lines indicate the positive regulatory effect among miRNAs and their targets, and crimson lines depict negative effects among them. All information regarding the role of these miRNAs in the modulation of the IFN signaling cascade in various types of human diseases and cancers can be seen in **Tables 1** and **2**.

TABLE 3 | Interactions between lncRNAs and IFNs.

Type of Diseases	LncRNAs	Sample	Cell Line	Target	Discussion	Ref
NSCLC	AFAP1-AS1 (Up)	NSCLC (n=165), banging lung tumor patients (n=118), health control (n=173)	A549, H1975, H1650, H1395 H12994	IRF-7, IFN-γ, RIG-I, Th1/2, IL-10/12, Bcl-2, TNF-α, NF-κB	AFAP1-AS1 *via* upregulating IRF-7 and the RIG-I-like receptor signaling could promote migration and invasion of NSCLC.	(66)
Head & Neck Squamous Cell Carcinoma (HNSCC)	lncMX1-215	70 HNSCC and 18 normal oral mucosa tissues from patients; BALB/c nude mice	HN4, HN6, HN30, Cal27, SCC4, SCC25, Detroit 562, 293T	IFN-α, H3K27ac, H3k18ac, H3K9ac, Caspase-3 PARP, Snail, STAT-1	IFN-α-induced lncMX1-215 by interfering with H3K27 acetylation could decrease immunosuppression in HNSCC.	(67)
Cryptosporidium Infection	NR_033736	BV2 mice,	IEC4.1, HCT-8, BV2, RAW264.7	IFN-α, IFN-β1, IFN-α12/13, IFN-I, ISGF-3, IFI-44, IFIT-1, OAS2/3, IRF-9, H3K4me3, STAT-2	NR_033736 *via* regulating IFN-I-mediated gene transcription could induce intestinal epithelial anti-cryptosporidium defense.	(68)
Lupus Nephritis (LN)	RP11-2B6.2 (UP)	22 LN kidney biopsies and 7 control samples, PBMC	HeLa, HK2	IFN-I, IFI27, IFIT-1/3, ISG, Mx2, OASL, ASO1, CXCL10, JAK1, STAT-1, SOCS1	RP112B6.2 *via* targeting the IFN-I by epigenetically inhibiting the expression of SOCS1 could aggravate symptoms of LN disease.	(69)
Systemic Lupus Erythematosus (SLE)	linc00513 (–)	139 SLE patients	Hela, THP-1, PBMCs	IFN-I, IRF-9, OAS-1/2/3, IFI-27/44/44L, ISG-15/20, IFIT-1/3, Mx1/2, XAF1, NF-kB, STAT-1/2	Overexpression of linc00513 *via* promoting IFN signaling could play a role in lupus pathogenesis.	(70)
Diabetes Mellitus Type 1	Lnc10 (–)	–	EndoC-βH1	IFN-I, IFN-γ, IFITM1, IL-1β, STAT-1	Overexpression of lnc10 *via* IFN-I could enhance the immune response in pancreatic β-cells.	(71)
Myocardial I/R Injury	BANCR (–)	–	iPS cell-derived cardiomyocytes	IFN-β, IFNAR-1, STAT-1/2	BANCR by targeting STAT-1 could promote IFN-β-induced cardiomyocyte apoptosis.	(72)
Infectious Bursal Disease Virus (IBDV)	loc107051710 (–)	–	DF-1	IRF-8, IFI-1/6, IFN-α, IFN-β, Mx1, IFIT-5, STAT-1/2	loc107051710 by regulating IRF-8 could promote the production of IFN-α and IFN-β, thereby modulating the antiviral activity of ISGs.	(73)
Influenza A Virus (IAV)	IVRPIE (Up)	–	A549, BEAS-2B, MDCK, BHK21	IFN-β1, ISG, IRF-1, IFIT-1/3, Mx1, ISG-15, IFI44L	IVRPIE *via* regulating IFN-β1 and ISG expression could promote host antiviral immune responses.	(74)
Influenza A Virus (IAV); H1N1, IAV-PR8, IAV- CA04	ISR (–)	C57BL/6 mice	A549, 293T, NIH/3T3, 4T1, MDCK	IFN-β, IFNAR-1, RIG-I, MxA, ISG-15, OAS2	ISR could be regulated by RIG-I-dependent signaling; during IAV infection, it could also govern IFN-β production and inhibit viral replication.	(75)
Influenza Virus A/WSN/33 (H1N1)	IFITM4P (–)	–	A549, 293T, K562, HeLa, MDCK, Huh7, Mcf7, HepG2	IFITM-1/2/3, miR-24, Mx1, RIG-I, p65, IL-6	IFITM4P by acting as a competing endogenous RNA could regulate host antiviral responses.	(76)
Influenza Virus A/WSN/33 (H1N1), Sendai Virus (SeV)	Lnc-MxA (–)	–	MDCK, 293T, A549,	IFN-β, RIG-I, MAVS, IRF-3, INFAR-1, p65, ISG-15, MxA	Lnc-MxA by forming RNA-DNA triplexes could inhibit β interferon transcription.	(77)
Herpes Simplex Virus 1 (HSV-1), Influenza A Virus (IAV), LPS	LUCAT1 (–)	PBMCs	THP-1, THP-1 KO, hMDDC	IFN-I, IFN-α, IFN-β, IRF-3, IFI1-6, ISG, TNF-α, Mx2, JAK-1/3, STAT1	LUCAT1 by interacting with STAT1 in the nucleus could limit the transcription of ISGs.	(78)
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)	RP1-20B21.4, RP11-329L6.1, RP11-498C9.3, NEAT1, MALAT1 (–)	Dataset	–	miR-122, miR-122-5p, IRF-9, IFIT-1/2/3, MX1, OAS2/3, IFNL-1 IFNG, JAK, STAT-1	The SARS-CoV-2 infection could lead to differential expression of lncRNAs. Also, IFN response is involved in SARS-CoV-2 infection.	(79)
HIV-1-BAL-HSA	NRIR, MIR3945HG, C8orf3, AC053503.1, AL359551.1 (–)f	–	$CD14^+$ monocytes, MDMs	IFN-α, IFN-ε, IFN-γ, IFN-λ, Mx1, IFIT2	Interferons could mediate the Response of lncRNAs in macrophages in HIV.	(80)
Vesicular Stomatitis Virus (VSV), VSV-GFP	lncLrrc55-AS (–)	C57BL/6 mice	RAW264.7, NIH/3T3, 293T, MDCK, MLE12, 3LL, Hepa	IFN-α4, IFN-β, IRF-3, IFN-I, p65, p38, ERK, JAK, STAT-1	Interferon-inducible cytoplasmic lncLrrc55-AS by strengthening IRF3 phosphorylation could promote antiviral innate responses.	(81)

(Continued)

TABLE 3 | Continued

Type of Diseases	LncRNAs	Sample	Cell Line	Target	Discussion	Ref
–	GRASLND, NEAT1 (–)	–	ASCs	IFN-II, IFN-α, IFN-β, IFN-γ, IRF-1/2/6, IFI-44/44L, IFNGR-1/2, STAT-1/2	GRASLND *via* suppressing the IFN-II pathway could enhance chondrogenesis.	(82)
–	BANCR (–)	–	ARPE-19,	IFN-γ, IL-1β, TNF-α, JAK, STAT-1	IFN-γ by activating the JAK-STAT1 pathway could upregulate the expression of BANCR in retinal pigment epithelial cells.	(83)

These non-coding RNAs represent a novel group of biomarkers for these conditions since their expressions are dysregulated in the biofluids of patients with these disorders.

Consistent with the important role of IFN signaling in the response of the immune system to viral infections, non-coding RNAs that regulate these signals can also participate in the pathophysiology of these conditions. The interactions between non-coding RNAs and IFN signaling have been assessed in the context of SARS-CoV-2, HIV, and influenza infections. Particularly, some miRNAs have been reported to enhance antiviral responses through modulation of IFN signaling. Identification of the impact of these miRNAs in response to viral infections could facilitate the design of efficient therapeutic modalities for these disorders. The preliminary results of *in vitro* and *in vivo* studies have suggested modulation of expression of certain miRNAs as an efficient strategy for limiting viral infections.

Notably, single nucleotide polymorphisms in the seed region of IFN-interacting miRNAs can interfere with or induce their bindings with miRNA targets. These polymorphic regions can hypothetically affect IFN responses, thus participating in the pathogenesis of autoimmune disorders, malignancies, or viral infections.

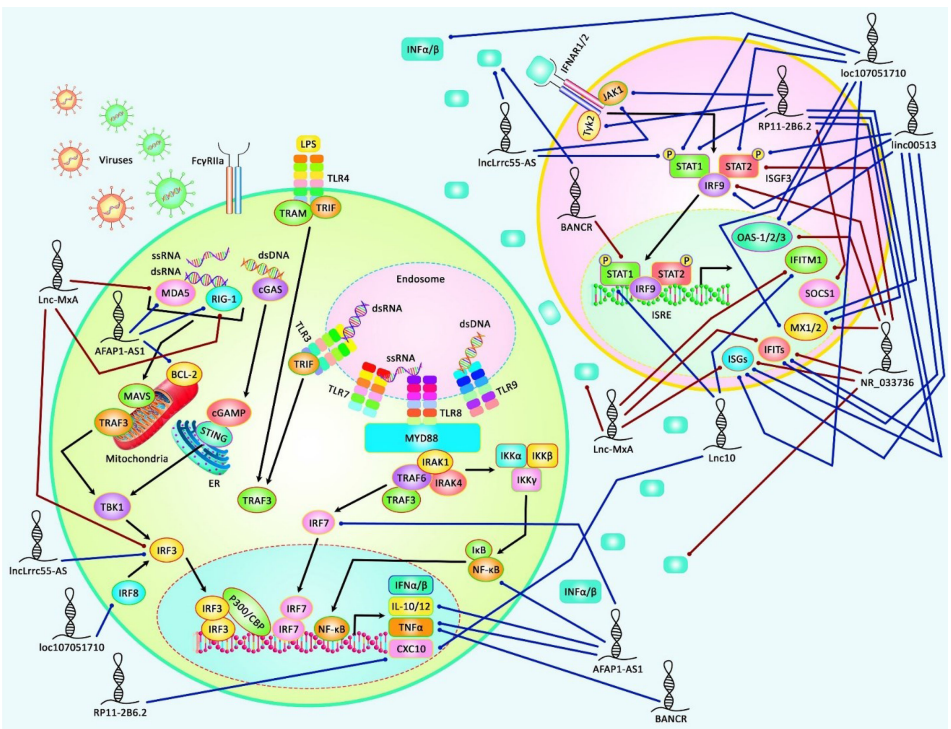

FIGURE 2 | A schematic illustration of the role of several lncRNAs in regulating the IFN signaling pathway in several human diseases, including autoimmune conditions and viral infections. Accumulating evidence has illustrated that lncRNAs modulating IFN signaling cascade could participate in the pathogenesis of various kinds of human cancers as well as immune-related disorders. It has been reported that lncRNA RP11-2B6.2 could play an important role as a positive regulator of type I INF signaling pathway in Lupus Nephritis *via* up-regulating the expression levels of IFIT-1/3, ISG, Mx2, CXCL10, JAK1, STAT-1, TYK2, and decreasing SOCS1 expression (69). Moreover, another research has revealed that lncRNA loc107051710 could elevate the expression levels of IFN-α, IFN-β, Mx1, STAT-1/2, OAS *via* modulating IRF8, thereby enhancing the antiviral activity of ISGs to prevent infectious bursal disease virus (IBDV) infection (73). Blue lines indicate the positive regulatory effect among lncRNAs and their targets, and brown lines depict a negative one among them. All the information regarding the role of these lncRNAs involved in the modulation of the IFN signaling cascade in various types of immune deficiency diseases and cancers can be seen in **Table 3**.

Identification of these variants within the human genome might facilitate the design of specific treatment modalities for these conditions in the context of personalized medicine.

Several dysregulated IFN-related miRNAs, particularly miR-9, miR-18, miR-301a, miR-195, miR-19, miR-1290, miR-320, miR-664, miR-587, miR-203a, and miR-4295 have been shown to participate in the pathogenesis of human cancers. These miRNAs represent appropriate targets for anti-cancer therapies since they can affect immune responses against cancer. Future studies are needed to evaluate the effects of these miRNAs-targeting therapies in xenograft models of cancer.

Taken together, non-coding RNAs that regulate IFN signaling can participate in a variety of malignant and non-malignant disorders, particularly those related to abnormal immune responses.

AUTHOR CONTRIBUTIONS

SG-F wrote the draft and revised it. MT designed and supervised the study. EJ, BH, YP and FD collected the data and designed the figures and tables. All the authors read and approved the submitted version.

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GLOSSARY

ESCs	Embryonic Stem Cells
FGF2	Growth Factor2
bEEC	Primary Bovine Endometrial Epithelial Cell
BoLA	Bovine Leukocyte Antigen
VSMCs	Vascular Smooth Muscle Cells
RAVSMCs	Rat Aortic VSMCs
RD	Rhabdomyosarcoma Cells
pDCs	Plasmacytoid Dendritic Cells
hMSC	Normal Human Mesenchymal Stem Cells
ATM	Ataxia-Telangiectasia Mutated
MDDCs	Monocyte-Derived Dendritic Cells
MDMs	MDM, Monocyte-Derived Macrophage
PBMC	Peripheral Blood Mononuclear Cell
GES1	Gastric Epithelial Cell Line
HSP60	Heat Shock Protein 60
MAVS	Mitochondrial Antiviral Signaling Protein
BHK	Baby Hamster Kidney
DENV	Dengue Virus
FHV-1	Feline Herpesvirus 1
CHK1	Checkpoint Kinase 1
INCR1	IFN-Stimulated Non-Coding RNA 1
ASCs	Adipose-Derived Stem Cells
MDCK	Madin-Darby Canine Kidney
mulNTEPI	Murine Intestinal Epithelial
BoLA	Bovine Leukocyte Antigen
PME-1	Phosphatase Methylesterase 1
PAMs	Porcine Alveolar Macrophages
VERO	African Green Monkey Kidney Cells
RD	Human Rhabdomyosarcoma Cells
EPC	Epithelioma Papulosum Cyprini
LPS	lipopolysacaridase
IRF-1	Interferon Regulatory Factor-1
ISGs	Interferon-Stimulated Genes
IFNAR	Heterodimeric Interferon Receptor
hMDDC	Primary Human Monocyte-Derived Dendritic Cells
MDMs	Monocyte-Derived Macrophages
ASCs	Adipose-Derived Stem Cells
LPS	Lipopolysaccharide
bEEC	Primary Bovine Endometrial Epithelial Cell
RIG-I	Retinoic Acid-Inducible Gene I
IFT	Intraflagellar Transport
IFIT1	Interferon Induced Protein with Tetratricopeptide Repeats 1
IFIH1	Interferon Induced with Helicase C Domain 1

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Changes in the Small Noncoding RNAome During M1 and M2 Macrophage Polarization

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Macrophages belong to a special phagocytic subgroup of human leukocytes and are one of the important cells of the human immune system. Small noncoding RNAs are a group of small RNA molecules that can be transcribed without the ability to encode proteins but could play a specific function in cells. SncRNAs mainly include microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and repeat RNAs. We used high-throughput sequencing analysis and qPCR to detect the expression changes of the small noncoding RNAome during macrophage polarization. Our results showed that 84 miRNAs and 47 miRNAs with were downregulated during M1 macrophage polarization and that 11 miRNAs were upregulated and 19 miRNAs were downregulated during M2 macrophage polarization. MiR-novel-3-nature and miR-27b-5p could promote expression of TNF-α which was marker gene of M1 macrophages. The piRNA analysis results showed that 69 piRNAs were upregulated and 61 piRNAs were downregulated during M1 macrophage polarization and that 3 piRNAs were upregulated and 10 piRNAs were downregulated during M2 macrophage polarization. DQ551351 and DQ551308 could promote the mRNA expression of TNF-α and DQ551351overexpression promoted the antitumor activity of M1 macrophages. SnoRNA results showed that 62 snoRNAs were upregulated and 59 snoRNAs were downregulated during M1 macrophage polarization, whereas 6 snoRNAs were upregulated and 10 snoRNAs were downregulated during M2 macrophage polarization. Overexpression of snoRNA ENSMUST00000158683.2 could inhibit expression of TNF-α. For snRNA, we found that 12 snRNAs were upregulated and 15 snRNAs were downregulated during M1 macrophage polarization and that 2 snRNAs were upregulated during M2 macrophage polarization. ENSMUSG00000096786 could promote expression of IL-1 and iNOS and ENSMUSG00000096786 overexpression promoted the antitumor activity of M1 macrophages. Analysis of repeat RNAs showed that 7 repeat RNAs were upregulated and 9 repeat RNAs were downregulated during M1 macrophage polarization and that 2 repeat RNAs were downregulated during M2 macrophage polarization. We first reported

the expression changes of piRNA, snoRNA, snRNA and repeat RNA during macrophage polarization, and preliminarily confirmed that piRNA, snoRNA and snRNA can regulate the function of macrophages.

Keywords: macrophage polarization, miRNA, piRNA, snoRNA, snRNA, repeat RNA

INTRODUCTION

Macrophages belong to a special phagocytic subgroup of human leukocytes and are one of the important cells of the human immune system (1). They participate in both specific and innate immune processes and play an important role in the host's resistance to pathogen invasion and inflammatory response (2). Macrophage polarization is a response to different degrees of activation in a specific time and space; that is, different macrophage subtypes form in different tissues, different microenvironments and different signal stimuli, and there are mainly M1-polarized macrophages with a proinflammatory phenotype and M2-polarized macrophages with an anti-inflammatory phenotype (3). M1 polarized macrophages overexpress CD80, CD86 and CD16/32c and can produce a proinflammatory response and proinflammatory-related factors such as interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor (TNF-α) (4). M1 macrophage polarization has been shown to be involved in the pathophysiology of many diseases, including arthritis, tumors, atherosclerosis, bacterial viral infection, diabetes, sebaceous gland disease and cryptococcal cheilitis (5). In the process of tumorigenesis and development, M1-polarized macrophages also show antitumor activity and the potential to induce tumor tissue destruction and are likely to participate in T cell-mediated tumor elimination and the balance stage. Macrophages with an M1 polarization phenotype promote the progression of RA by releasing various inflammatory cytokines (6). At the same time, M1 polarized macrophages have the potential to differentiate into mature osteoclasts, which can promote an increase in bone resorption by secreting proinflammatory cytokines and other mechanisms to mediate the occurrence of osteoporosis (7). Unlike classically activated M1 macrophages, M2 macrophages have immunomodulatory effects and highly express anti-inflammatory cytokines, including IL-10, IL-13 and transforming growth factor (TGF-β) (8). M2 macrophages could promote the regression of inflammation and immune escape of pathogens, inhibit parasites, and promote tissue repair and tumor development (9). Macrophages are a group of immune cells with high plasticity and heterogeneity that play an important role in maintaining the stability of the immune system. Under the action of different stimulating factors, macrophages can polarize into M1 and M2 types, and their polarization process is affected by a variety of signaling pathways.

Small noncoding RNAs are a group of small RNA molecules that can be transcribed without the ability to encode proteins but could play a specific function in cells (10). At present, researchers divide ncRNAs into two categories according to their length: short chain noncoding RNAs (sncRNAs) with a length of 18-200 nt and long chain noncoding RNAs (lncRNAs) with a length > 200 nt (11). Short chain noncoding RNAs are not long, but they play an important regulatory role in biological processes such as cell metabolism, proliferation, differentiation, apoptosis and immunity (12–14). The abnormal regulation of sncRNA will lead to the disorders of metabolic activities of life and the occurrence of a variety of diseases. SncRNAs mainly include microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and repeat RNAs (15, 16).

MiRNAs are single-stranded RNAs with a length of 20-25 nucleotides. They usually inhibit its protein synthesis and expression or leads to the degradation of mRNA by targeting the 3'-untranslated region (3'-UTR) of target mRNAs to play a posttranscriptional regulatory role (17). A miRNA can often target multiple mRNAs, and mRNAs can be regulated by a variety of miRNAs. Therefore, miRNAs can be involved in almost all aspects of biological processes, such as development, cell differentiation, intercellular communication, and immune regulation (18). miR-21 increased M2 macrophages phenotype and decreased M1 macrophages phenotype (19). miR-155 promoted M1 polarization by activating JNK pathway (20). Piwi-interacting RNA is a small RNA molecule with a length of approximately 26-31nt (21). It interacts with PIWI family proteins specifically expressed by germ cells and interacts with PIWI proteins to form the piRNA silencing complex (piRISC) (22). Compared with other known RNA family members, piRNAs showed a different set of nucleotide sequences. According to different sources, piRNAs can be divided into three categories: transposon sources, mRNA sources and lncRNA sources (23). The mature piRNA combined with PIWI can direct its complex to the transposon target of the target transcript in the nucleus and mobilize the silencing mechanism to organize the expression of transposable elements to maintain the integrity of the genome (24). To date, piRNAs have been shown to be involved in transposon silencing, epigenetic regulation, gene and protein regulation, genome rearrangement, spermatogenesis and germ cell maintenance (24, 25). Small nucleolar molecule RNA (snoRNA) is a kind of short stranded noncoding RNA related to the chemical modification of ribosomal RNA (rRNA) (26). They often serve as guides for posttranscriptional modification of rRNA. According to their sequence, structure and function, they can be divided into two categories: Box C/D snoRNA and Box H/ACA snoRNA (27). All snoRNAs are paired with specific proteins to form small nucleolar ribonucleoprotein complexes. After snoRNAs recognize and bind the complementary sequence on the target rRNA, and they send a signal to the paired protein for accurate base modification to play an important regulatory

function under a variety of physiological and pathological conditions (24). Nuclear small RNA (snRNA) is a kind of small molecular RNA with a length of 100-215 nt in the eukaryotic genome that exists in the nucleus (28). SnRNA is highly conserved, the content is small in cells, and the expression is spatiotemporally specific (29). After transcription according to the gene template, snRNA is transported out of the nucleus and combined with snRNA binding protein in the cytoplasm to form small nuclear ribonucleoproteins (snRNPs), which once again pass through the nuclear pore and enter the nucleus to become mature snRNA (30). The main function of snRNA is to participate in the splicing of RNA in cells, produce mature mRNA and rRNA, and regulate protein synthesis (31). Repeat RNA is a kind of RNA containing specific repeats (32). At present, there are relatively few reports on the function of these repeat RNAs.

In this study, we used high-throughput sequencing analysis and qPCR to detect the expression changes of the small noncoding RNAome during macrophage polarization to construct the changes of the small noncoding RNAome during macrophage polarization to identify the key small RNA regulating macrophage polarization to find a new target to intervene in macrophage polarization.

METHOD AND MATERIALS

M1 and M2 Macrophage Polarization Induction

According to previous studies, we used RAW264.7 cells to induce macrophage polarization (33, 34). For M1 macrophage polarization, we added LPS (100 ng/ml)- and IFN-γ (20 ng/ml)-stimulated RAW264.7 cells for 48 hours. For macrophage M2 polarization, we stimulated RAW264.7 cells with IL-4 (20 ng/ml) and IL-10 (20 ng/ml) for 48 hours.

Small RNA Sequencing

PCR product bands of about 140-150bp were used for small RNA sequencing. An Illumina NextSeq 550 was used for sequencing, and the sequenced original image data were converted into the original sequencing sequence. To comprehensively predict noncoding RNAs of different lengths, we first extracted the sequencing sequences of the reference genome in each sample, analyzed the sequences of all samples using cdhit, and screened the noncoding RNAs using the RFAM database. miRDeep2 were used for miRNA analysis. Blastn(2.9.0+) were used for piRNA, snoRNA and snRNA analysis. RepeatMasker were used for repeatRNA analysis. The sequencing data of each sample were compared, and the read count of small RNA of each sample was counted. The CPM of every non-coding RNAs were list in Supplementary material S1. The expression of all small RNAs in the two groups of samples was counted to judge whether there was a significant difference in the expression between the two groups of samples. For the experiments with biological repetition, we used 10 deseq for analysis. For experiments without biological duplication, we used edger for analysis. Finally, the genes with Padj less than 0.05 and a difference multiple greater than or equal to 2 or 1.5 were selected as small RNAs with significant differential expression.

RNA Isolation and qPCR Analysis

According to the reagent instructions, macrophage RNA was extracted by TRIzol reagent, and the quality and concentration of the extracted RNA were detected by a Nanodrop2000. For the detection of miRNA, piRNA and a small part of snoRNA and snRNA, 1 μg of total RNA from all samples were extracted and reverse transcribed with miRNA first strand cDNA synthesis (tailing reaction) according to the instructions into cDNA. For the detection of most of the snoRNAs and snRNAs, 1 μg of total RNA was extracted from all samples, and PrimeScript™ RT Master Mix was used for reverse transcription into cDNA according to the instructions. QPCR detection was performed according to the instructions of the SYBR premix ex Taq Kit (RR420a, Takara, Tokyo, Japan). The relative expression value of small RNA was calculated using the $2^{-\Delta\Delta CT}$ method and U6 as an internal parameter. Primers used are listed in the supplementary material S2.

Tumor Model Establishment

The animal model was constructed according to our previous research report (35). This study was conducted in accordance with guidelines approved by the Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (approval number: XHEC-NSFC-2021-232). 8 week old male C57BL/6 mouse purchased from Shanghai Jihui experimental animal breeding Co., Ltd. RM1 cells (1×10^6 cells) were subcutaneously injected into the right rear flanks of the mouse. There days after tumor cell inoculation, mouse treated with intratumoral injection of LPS-pretreated M1 macropahge (1×10^6 cells) every 3 d. On 14^{th} day, all mice were euthanized, and solid tumors were surgically removed for measurements.

Statistical Analysis

All data are shown as the mean ± S.D. of three independent tests ($n\geq3$), and the independent sample t-tests statistical analysis was performed using Excel 2007; graphs were generated by GraphPad Prism 9. P values less than 0.05 were considered significant.

RESULTS

Changes in miRNA Expression During Macrophage Polarization

MiRNAs play an important role in regulating cell function. Therefore, we first analyzed the expression changes of miRNAs during macrophage polarization. The results showed that 84 miRNAs were upregulated and 47 miRNAs were downregulated during M1 macrophage polarization (**Figures 1A–C** and **Figure S1**). qPCR analysis showed that 6 miRNAs, mmu-miR-155-5p, mmu-miR-155-3p, mmu-miR-692, mmu-novel-264-mature, mmu-novel-183-mature and mmu-miR-541-5p, were upregulated during M1 polarization (**Figure 1D**). On the other

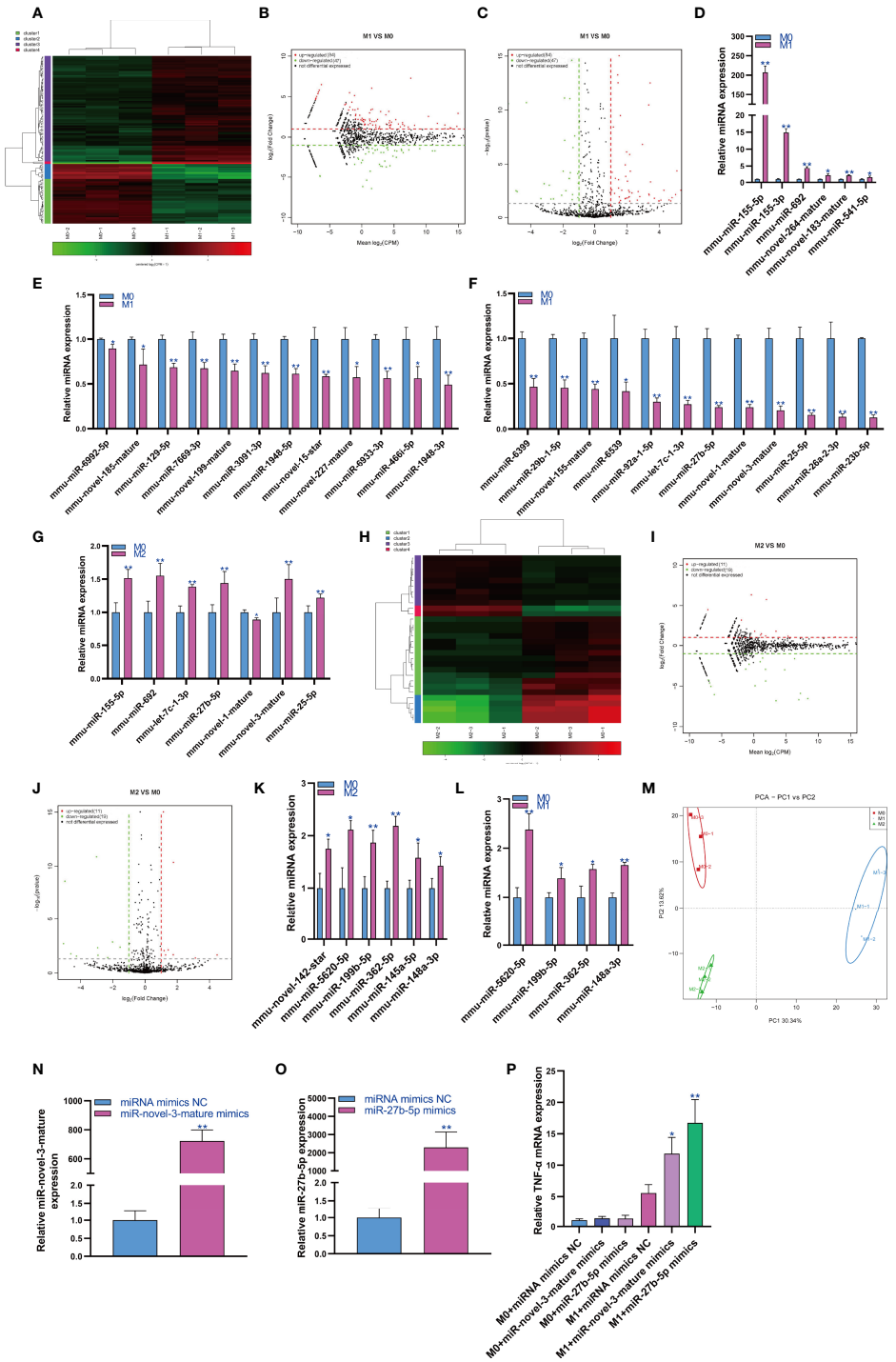

FIGURE 1 | Changes in miRNA expression during macrophage polarization. Hotmap **(A)**, MA **(B)** and volcano plot **(C)** show the small RNA sequencing results of the expression of miRNA in M0 and M1 macrophages. **(D)** The expression of miRNAs upregulated in M1 macrophages detected by qPCR. **(E, F)** The expression of miRNAs downregulated in M1 macrophages detected by qPCR. **(G)** The expression of miRNA in M2 macrophages detected by qPCR. Hotmap **(H)**, MA **(I)** and volcano plot **(J)** show the small RNA sequencing results of the expression of miRNA in M0 and M2 macrophages. **(K)** The expression of miRNA in M2 macrophages detected by qPCR. **(L)** The expression of miRNA in M1 macrophages detected by qPCR. **(M)** PCA of miRNA expression in M0, M1 and M2 macrophages. * indicates $p < 0.05$ compared to M0, and ** indicates $p < 0.01$ compared to M0. **(N)** The expression of miR-novel-3-mature in RAW264.7 cells transfected with miRNA mimics NC or miR-novel-3-mature mimics were detected by qPCR. **(O)** The expression of miR-27b-5p in RAW264.7 cells transfected with miRNA mimics NC or miR-27b-5p mimics were detected by qPCR. **(P)** The expression of TNF-α in RAW264.7 cells were detected by qPCR. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to M1+ miRNA mimics NC group.

hand, qPCR analysis showed that 24 miRNAs, including 7 novel predicted miRNAs, were downregulated during M1 polarization (**Figures 1E, F**). Then, we detected the expression of miRNAs with significant differentiation during M1 polarization and during M2 polarization. qPCR results showed that the expressions of mmu-miR-155-5p and mmu-miR-692 were upregulated and mmu-novel-264-mature was downregulated during M2 polarization, which was consistent with M1 polarization. However, the expressions of mmu-let-7c-1-3p, mmu-miR-27b-5p, mmu-novel-3-mature and mmu-miR-25-5p were upregulated during M2 polarization, which was opposite to that during M1 polarization (**Figure 1G**). The results showed that 11 miRNAs were upregulated and 19 miRNAs were downregulated during macrophage M2 polarization (**Figures 1H–J**). qPCR analysis showed that 6 miRNAs, mmu-novel-142-star, mmu-miR-5620-5p, mmu-miR-199b-5p, mmu-miR-362-5p, mmu-miR-145a-5p and mmu-miR-148a-3p, were upregulated during M2 polarization (**Figure 1K**). However, we did not find that miRNAs were downregulated during M2 polarization. Then, we detected the expression of miRNAs that were significantly upregulated during M2 polarization and during M1 polarization. qPCR results showed that the expressions of mmu-miR-5620-5p, mmu-miR-199b-5p, mmu-miR-362-5p and mmu-miR-148a-3p were upregulated during M1 polarization, which was consistent with M2 polarization (**Figure 1L**). All differentially expressed miRNAs were list in Supplementary material S3 and the differentially expressed miRNA were divided into four cluster according to the similar expression pattern (**Figure S2**). The target genes of differentially expressed miRNA analyzed by bioinformatics were listed in Supplementary material S4.PCA showed that the expression of miRNA could distinguish M0, M1 and M2 macrophages (**Figure 1M**). MiR-novel-3-mature mimics and miR-27b-5p mimics were transfected in RAW264.7 cells for overexpressed miR-novel-3-mature and miR-27b-5p in RAW264.7 cells (**Figures 1N, O**). At the same time, RAW264.7 cells were induced into M1 polarization, we found that overexpression of miR-novel-3-nature and miR-27b-5p could promote the mRNA expression of TNF-α which was marker gene of M1 macrophages (**Figure 1P**).

Changes in piRNA Expression During Macrophage Polarization

The regulatory role of piRNAs in the occurrence and development of diseases has attracted increasing attention. Therefore, we analyzed the expression changes of piRNA during macrophage polarization. The results showed that 69 piRNAs were upregulated and 61 piRNAs were downregulated during M1 macrophage polarization (**Figures 2A–C**). qPCR analysis showed that 11 piRNAs were upregulated and 19 piRNAs were downregulated during M1 polarization (**Figures 2D, E**). Then, we detected the expression of piRNAs with significant differentiation during M1 polarization and during M2 polarization. qPCR results showed that the expression of DQ709776 and DQ690018 was downregulated during M2 polarization, which was consistent with that during M1 polarization. However, the expression of DQ551351 was downregulated and the expressions of DQ554593 and DQ551308 were upregulated during M2 polarization, which was opposite to that during M1 polarization (**Figure 2F**). The results showed that 3 piRNAs were upregulated and 10 piRNAs were downregulated during macrophage M2 polarization (**Figures 2G–I**). We only found that the expression of DQ7138872 was upregulated during M2 polarization using qPCR analysis (**Figure 2J**). On the other hand, we found that DQ7138872 was also upregulated during M1 polarization (**Figure 2K**). The PCA showed that the expression of piRNA could distinguish M0 macrophages from M1 macrophages and M1 macrophages from M2 macrophages but could not distinguish M2 and M0 macrophages (**Figure 2L**). PiRNA DQ551351 mimics and DQ551308 mimics were transfected in RAW264.7 cells for overexpressed DQ551351 and DQ551308 in RAW264.7 cells (**Figures 2M, N**). At the same time, RAW264.7 cells were induced into M1 polarization, we found that overexpression of DQ551351 and DQ551308 could promote the mRNA expression of TNF-α which was marker gene of M1 macrophages (**Figure 2O**). As M1-polarized macrophages showed antitumor activity, we detected the effect of piRNA DQ551351 on the antitumor activity of M1 macrophages, and the result showed that piRNA DQ551351overexpression promoted the antitumor activity of M1 macrophages (**Figures 2P, Q**).

Changes in snoRNA Expression During Macrophage Polarization

SnoRNA is a class of highly conserved molecules among species. Therefore, we analyzed the expression changes of snoRNA during macrophage polarization. The results showed that 62 snoRNAs were upregulated and 59 snoRNAs were downregulated during M1 macrophage polarization (**Figures 3A–C**). qPCR analysis showed that snoRNA ENSMUST00000082493.3 was upregulated during M1 polarization (**Figure 3D**). On the other hand, qPCR analysis showed that 35 snoRNAs were downregulated during M1 polarization (**Figures 3E, F**). Then, we detected the expression of snoRNAs with significant differentiation during M1 polarization and during M2 polarization. qPCR results showed that the expressions of ENSMUST00000083419.3, ENSMUST00000158683.2, ENSMUST00000083752.3 and ENSMUST00000104514.3 were upregulated during M2 polarization, which was contrary to that during M1 polarization. However, the expression of ENSMUST00000082967.3 and ENSMUST00000175746.3 was downregulated during M2 polarization, which was consistent with that during M1 polarization (**Figure 3G**). The results showed that 6 snoRNAs were upregulated and 10 snoRNAs were downregulated during M2 macrophage polarization (**Figures 3H–J**). qPCR analysis showed that only ENSMUST00000104032 was upregulated during M2 polarization (**Figure 3K**). However, we did not find snoRNA downregulation during M2 polarization using qPCR. qPCR results showed that the expression of ENSMUST00000104032 was also upregulated during M1 polarization, which was consistent with that during M2 polarization (**Figure 3L**). PCA showed that the expression of snoRNA could distinguish M0, M1 and M2 macrophages (**Figure 3M**). In order to study the effect of snoRNA

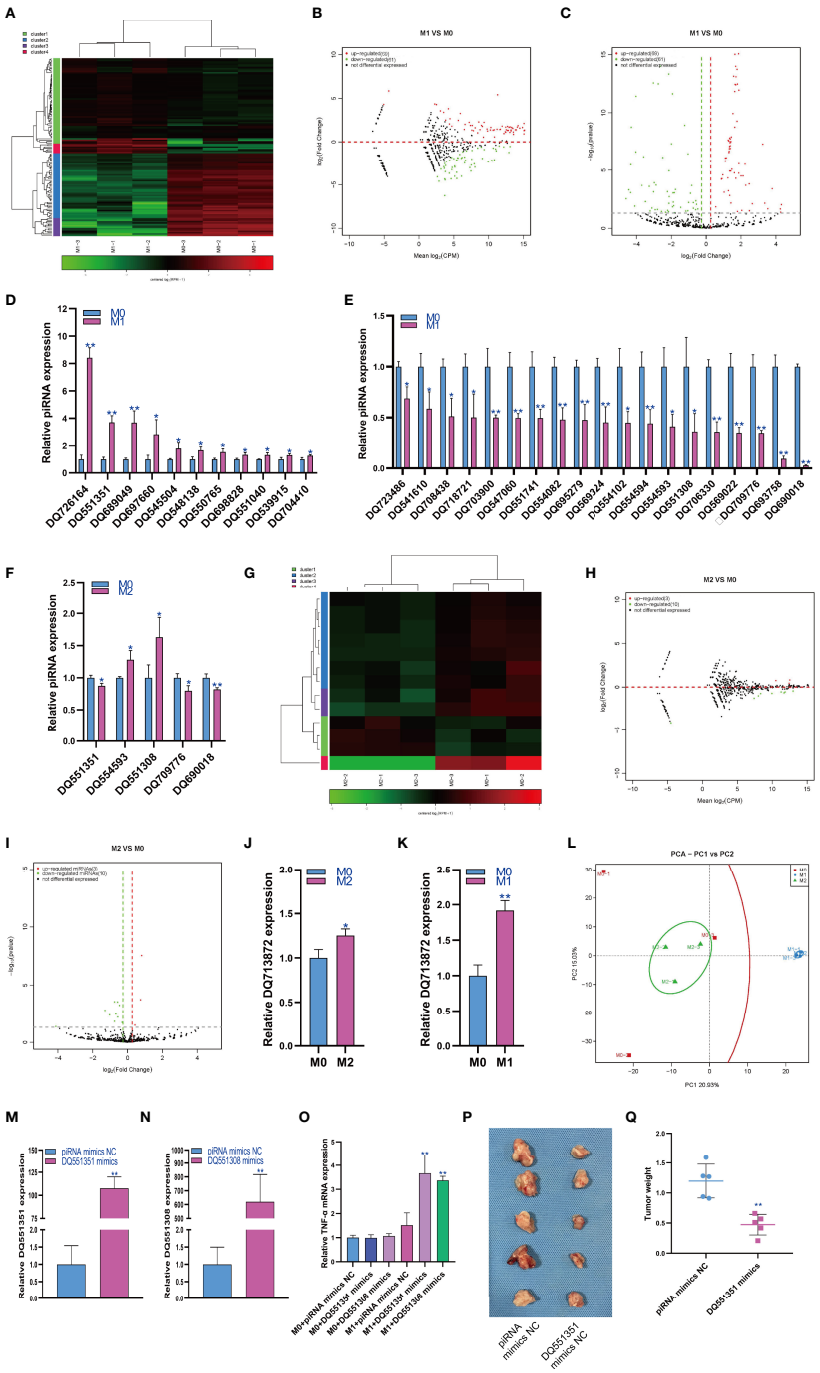

FIGURE 2 | Changes in piRNA expression during macrophage polarization. Hotmap **(A)**, MA **(B)** and volcano plot **(C)** show the small RNA sequencing results of the expression of piRNA in M0 and M1 macrophages. **(D)** The expression of piRNA upregulated in M1 macrophages as detected by qPCR. **(E)** The expression of piRNA was downregulated in M1 macrophages as detected by qPCR. **(F)** The expression of piRNA in M2 macrophages as detected by qPCR. Hotmap **(G)**, MA **(H)** and volcano plot **(I)** show the small RNA sequencing results of the expression of miRNA in M0 and M2 macrophages. **(J)** The expression of piRNA in M2 macrophages as detected by qPCR. **(K)** The expression of piRNA in M1 macrophages as detected by qPCR. **(L)** PCA of piRNA expression in M0, M1 and M2 macrophages. * indicates $p < 0.05$ compared to M0, and ** indicates $p < 0.01$ compared to M0. **(M)** The expression of piRNA DQ551351 in RAW264.7 cells transfected with piRNA mimics NC or piRNA DQ551351 mimics were detected by qPCR. **(N)** The expression of piRNA DQ551308 in RAW264.7 cells transfected with piRNA mimics NC or piRNA DQ551308 mimics were detected by qPCR. **(O)** The expression of TNF-α in RAW264.7 cells were detected by qPCR, * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to M1+ miRNA mimics NC group. **(P)** Photographs showed the size of the tumor in mouse injected with RAW264.7 cells with piRNA mimics NC or piRNA DQ551308 mimics. **(Q)** Tumor weight in mouse injected with RAW264.7 cells with piRNA mimics NC or piRNA DQ551308 mimics, ** indicates $p < 0.01$ compared to piRNA mimics NC group.

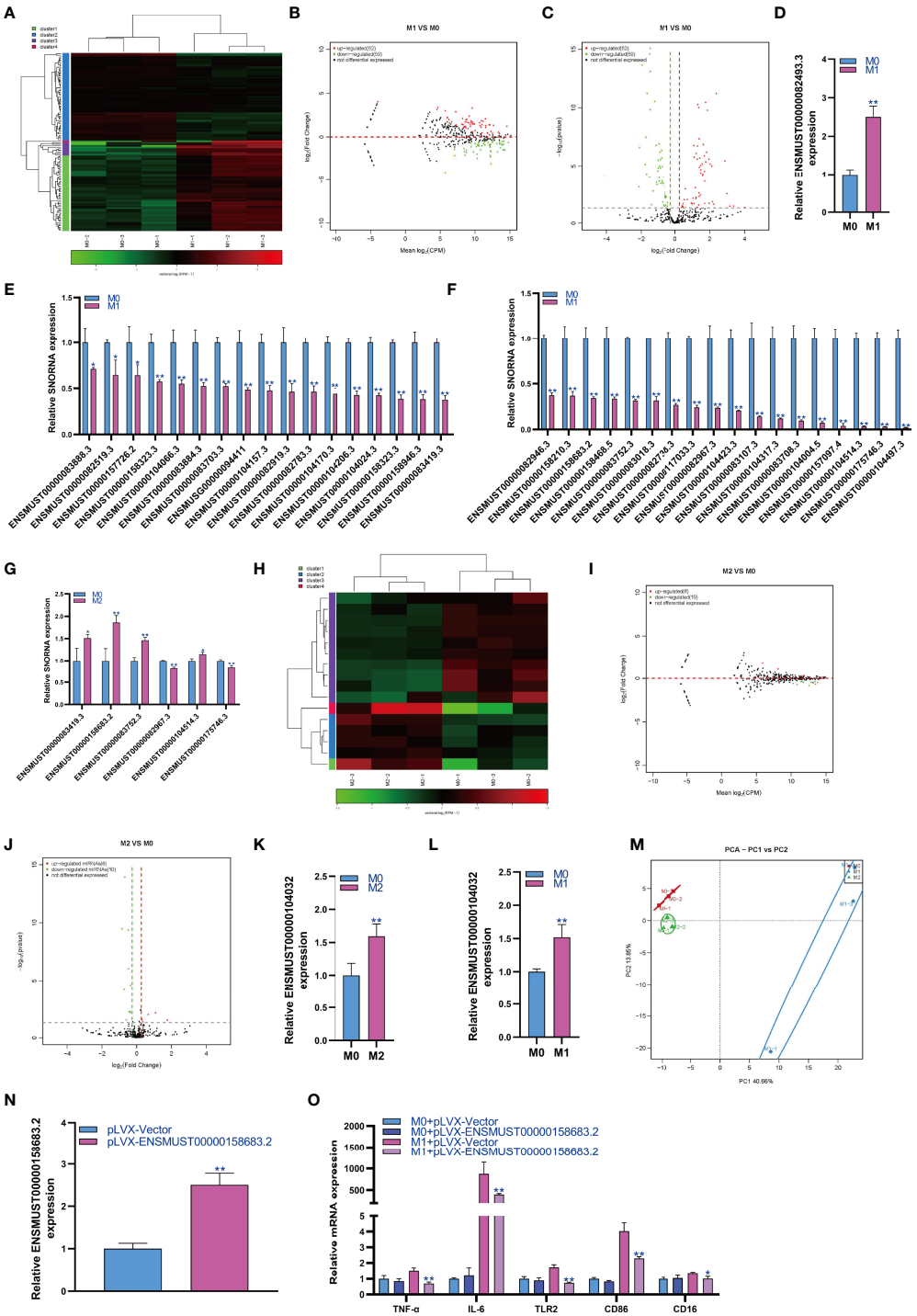

FIGURE 3 | Changes in snoRNA expression during macrophage polarization. Hotmap **(A)**, MA **(B)** and volcano plot **(C)** show the small RNA sequencing results of the expression of snoRNA in M0 and M1 macrophages. **(D)** The expression of snoRNA upregulated in M1 macrophages as detected by qPCR. **(E, F)** The expression of snoRNA was downregulated in M1 macrophages as detected by qPCR. **(G)** The expression of snoRNA in M2 macrophages as detected by qPCR. Hotmap **(H)**, MA **(I)** and volcano plot **(J)** show the small RNA sequencing results of the expression of snoRNA in M0 and M2 macrophages. **(K)** The expression of snoRNA in M2 macrophages as detected by qPCR. **(L)** The expression of piRNA in M1 macrophages as detected by qPCR. **(M)** PCA of snoRNA expression in M0, M1 and M2 macrophages. * indicates $p < 0.05$ compared to M0, and ** indicates $p < 0.01$ compared to M0. **(N)** The expression of snoRNA ENSMUST0000 0158683.2 in RAW264.7 cells transfected with pLVX-Vector or pLVX-ENSMUST00000158683.2 were detected by qPCR. **(O)** The expression of TNF-α, IL-6, TLR2, CD86 and CD16 in RAW264.7 cells were detected by qPCR, * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to M1+ pLVX-Vector group.

on M1 polarization of RAW264.7 cells, we overexpressed snoRNA ENSMUST00000158683.2 in RAW264.7 cells by lentivirus and the qPCR result showed that the expression of snoRNA ENSMUST00000158683.2 in pLVX-ENSMUST00000158683.2 group were higher than pLVX-Vector group (**Figure 3N**). At the same time, RAW264.7 cells were induced into M1 polarization, we found that overexpression of ENSMUST00000158683.2 could inhibit the mRNA expression of TNF-α, IL-6, TLR2, CD86 and CD16 which was marker gene of M1 macrophages (**Figure 3O**).

Changes in snRNA Expression During Macrophage Polarization

SnRNA can effectively regulate the physiological function of cells by regulating the alternative splicing of mRNA. Therefore, we analyzed the expression changes of snRNA during macrophage polarization. The results showed that 12 snRNAs were upregulated and 15 snRNAs were downregulated during M1 macrophage polarization (**Figures 4A–C**). qPCR analysis showed that 14 snRNAs were downregulated during M1 polarization (**Figure 4D**) and that snRNAs were not upregulated during M1 polarization. Then, we detected the expression of snRNAs that were downregulated during M1 polarization and during M2 polarization. qPCR results showed that the expression of ENSMUST00000104032 was downregulated during M2 polarization, which was consistent with M1 polarization (**Figure 4E**). The results showed that 2 snRNAs were upregulated during macrophage M2 polarization (**Figures 4F, G**). qPCR analysis did not find the differential expression of snRNA during M2 polarization. The PCA showed that the expression of snRNA could distinguish M0 macrophages from M1 macrophages and M1 macrophages from M2 macrophages but could not distinguish between M2 and M0 macrophages (**Figure 4H**). In order to study the effect of snRNA on M1 polarization of RAW264.7 cells, we overexpressed snRNA ENSMUST00000157129.4 and ENSMUSG00000096786 in RAW264.7 cells by lentivirus and the qPCR result showed that the expression of snRNA ENSMUST00000157129.4 and ENSMUSG00000096786 in pLVX-ENSMUST00000157129.4 and pLVX-ENSMUSG00000096786 group were higher than pLVX-Vector group (**Figures 4I, J**). At the same time, RAW264.7 cells were induced into M1 polarization, we found that overexpression of ENSMUST00000157129.4 and ENSMUSG00000096786 could promote the mRNA expression of IL-1 and iNOS which was marker gene of M1 macrophages (**Figure 4K**). We detected the effect of ENSMUSG00000096786 on the antitumor activity of M1 macrophages, and the result showed that ENSMUSG00000096786 overexpression promoted the antitumor activity of M1 macrophages (**Figures 4L, M**).

Changes in Repeat RNA Expression During Macrophage Polarization

Repeat RNA is a kind of very special RNA molecule with repetitive sequences. Therefore, we analyzed the expression changes of repeat RNA during macrophage polarization. The results showed that 7 repeat RNAs were upregulated and 9 repeat RNAs were downregulated during M1 macrophage polarization (**Figures 5A–C**). On the other hand, the results showed that 2 repeat RNAs were downregulated during macrophage M2 polarization (**Figures 5D, E**). PCA showed that the expression of repeat RNA could distinguish M0, M1 and M2 macrophages (**Figure 5F**).

DISCUSSION

As an important part of the innate immune system, macrophages have the functions of phagocytosis and killing pathogenic microorganisms and immune information transmission. They play an important role in inflammatory defense and tissue development and in maintaining the dynamic balance of the body. Resting macrophages can undergo morphological, functional and phenotypic differentiation under the action of different microenvironment signals *in vivo* and *in vitro*, that is, macrophage polarization (36). According to the difference in their immunological function, polarized macrophages can be divided into M1 macrophages with the classical activation pathway and into M2 macrophages with the alternative activation pathway (37). Macrophage polarization is controlled by many molecules, such as signaling pathways, transcription factors, epigenetic mechanisms and posttranscriptional regulatory factors, which play a corresponding role in the process of changes in the body's microenvironment and affect disease progression and outcome (38). This functional transformation is closely related to the selective gene expression regulation of signaling pathways.

The small noncoding RNAome has received increasing attention in the regulation of cell function. However, the changes in the small noncoding RNAome under normal physiological and pathological conditions need to be further discussed. Panagiotis and his colleagues reported expression changes in the small noncoding RNAome in equine and human chondrocytes during aging (39). Michelle reported the expression changes of the small noncoding RNAome in the plasma of patients with rheumatoid arthritis and found that the contents of miRNA and TDRS in the plasma of RA patients increased significantly, while the contents of yDRS decreased significantly (40). Their research mainly focused on the expression changes of miRNAs, snoRNAs, snRNAs and tRNAs. In our study, we mainly focused on the expression changes of miRNAs, piRNAs, snoRNAs, snRNAs and repeat RNAs during macrophage polarization.

MiRNAs have been considered important regulators of macrophage function, and part of their role is achieved by regulating the polarization of macrophages. Cho et al. found that the production of miRNAs in macrophages is necessary for the anti-inflammatory polarization of macrophages by using macrophage-specific Disher gene knockout (41). Banerjee et al. found that the expression of miR-125a-5p in M2 macrophages was higher than that in M1 macrophages and confirmed that miR-125a-5p could reduce the expression of the M1 phenotype induced by LPS and promote the expression of M2 induced by IL-4 by targeting KLF13 to regulate the phagocytosis and bactericidal activity of macrophages (42). Li et al. found that exosomes derived from bone marrow mesenchymal stem cells containing miR-124-3p can mediate the expression of Ern1 in macrophages and induce M2 phenotypic polarization of macrophages (43). In addition, a large number of studies have confirmed that many kinds of miRNAs can affect the polarization process of macrophages to M1 or M2. MiR-27a, miR-27b, miR-130a, miR-130b, miR-155, miR-21 and miR-26a

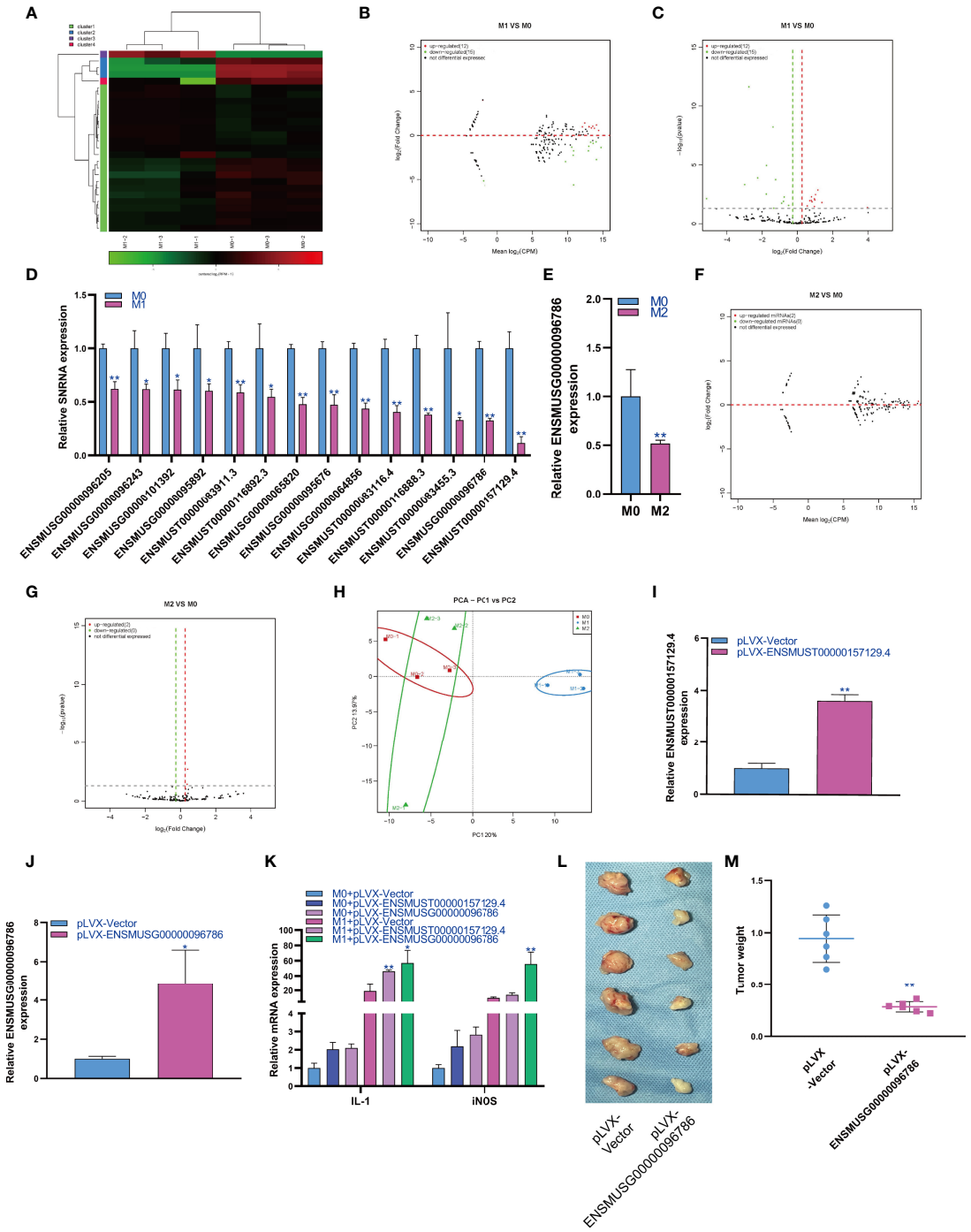

FIGURE 4 | Changes in snRNA expression during macrophage polarization. Hotmap **(A)**, MA **(B)** and volcano plot **(C)** show the small RNA sequencing results of the expression of snRNA in M0 and M1 macrophages. **(D)** The expression of snRNA in M1 macrophages as detected by qPCR. **(E)** The expression of snRNA in M2 macrophages as detected by qPCR. MA **(F)** and volcano **(G)** show the small RNA sequencing results of the expression of snRNA in M0 and M2 macrophages. **(H)** PCA of snRNA expression in M0, M1 and M2 macrophages. * indicates $p < 0.05$ compared to M0, and ** indicates $p < 0.01$ compared to M0. **(I)** The expression of snRNA ENSMUST00000157129.4 in RAW264.7 cells transfected with pLVX-Vector or pLVX- ENSMUST00000157129.4 were detected by qPCR. **(J)** The expression of snRNA ENSMUSG00000096786 in RAW264.7 cells transfected with pLVX-Vector or pLVX- ENSMUSG00000096786 were detected by qPCR. **(K)** The expression of IL-1 and iNOS in RAW264.7 cells were detected by qPCR, * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to M1+ pLVX-Vector group. **(L)** Photographs showed the size of the tumor in mouse injected with RAW264.7 cells with pLVX-Vector or pLVX- ENSMUSG00000096786. **(M)** Tumor weight in mouse injected with RAW264.7 cells with pLVX-Vector or pLVX- ENSMUSG00000096786, ** indicates $p < 0.01$ compared to pLVX-Vector group.

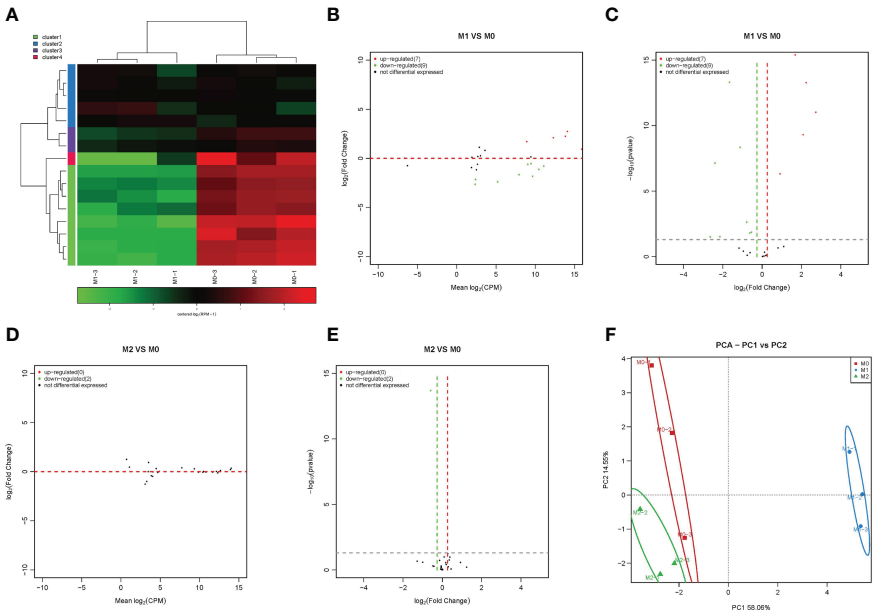

FIGURE 5 | Changes in repeat RNA expression during macrophage polarization. Hotmap **(A)**, MA **(B)** and volcano plot **(C)** show the small RNA sequencing results for the expression of repeat RNA in M0 and M1 macrophages. MA **(D)** and volcano **(E)** show the small RNA sequencing results for the expression of repeat RNA in M0 and M2 macrophages. **(F)** PCA of repeat RNA expression in the M0, M1 and M2 macrophages.

could promote M1 polarization and inhibit M2 polarization to promote inflammation (44–47). MiR-9, miR-146a, miR-146b, miR-124, Let-7c and miR-210 could inhibit M1 polarization and promote M2 polarization to inhibit inflammation (38, 48–50). In our study, we also found that the expression of miR-155 increased during macrophage polarization to M1. At the same time, we also found that multiple new miRNAs may be involved in regulating macrophage polarization.

PiRNAs are a class of ncRNAs first found and identified in Drosophila testes (51). Its structure, biosynthetic process and partial regulation have gradually become clear in recent years. At the same time, it has been proven to play an important role in some pathophysiological processes, especially in tumorigenesis. Yin et al. reported that the expression of piR-823, piR-18849 and piR-19521 increased in the serum and tissues of patients with colorectal cancer, suggesting that they may be related to the pathogenesis of this kind of cancer and can be used as diagnostic markers of colorectal cancer (52, 53). Stohr et al. found that the expression of PIWIL1, PIWIL2 and PIWIL4 decreased in patients with renal cell carcinoma and considered that they could be used as prognostic biomarkers of this kind of cancer (54). Han et al. confirmed that piRNA-30473 can trigger the downstream signal cascade by regulating m6A RNA methylation, resulting in the occurrence and adverse prognosis of diffuse large B-cell lymphoma (55). We found that the expression of piRNA changed significantly during macrophage polarization. However, no study has reported the regulatory effect of piRNAs on macrophage polarization, so we will further study the regulatory effect of piRNAs on macrophage polarization.

SnoRNA is a kind of noncoding RNA mainly derived from introns, with a length of approximately 50-250 nucleotides (56). SnoRNA plays an important role in cell development, homeostasis and many diseases. Ellen et al. demonstrated for the first time that snoRNA is involved in the biology of articular chondrocytes. Under different arthritis conditions, the expression level of U3 snoRNA in articular chondrocytes is reduced, significantly reducing the protein translation ability of the cell (57). Mandy et al. found abnormal expression of the snoRNA genome in aging cartilage and diseased tendons, in which the expression of SNORD26 increased in osteoarthritis, while SNORD44 and SNORD78 decreased with age (58). At present, the regulatory effect of snoRNA on macrophage polarization has not been reported. In this study, we also reported the expression changes of snoRNA during macrophage polarization for the first time. Our results show that the expression of snoRNA is significantly different in the process of macrophage polarization, which may be involved in the regulation of macrophage polarization.

Small nuclear RNAs are a class of noncoding RNAs with a length of 100-215 nucleotides that widely exist in the nucleus of eukaryotes and mainly include the U1, U2, U3, U4, U5, U6 and U11 genes (29). Increasing evidence has shown that snRNA is related to the regulation of gene expression in different types of tumors. Wang et al. found that U11 may be involved in the regulation of gene expression in bladder cancer cells, which can alter gene expression by affecting the PI3K-Akt signaling pathway and may be a potential biomarker for the clinical diagnosis and treatment of bladder cancer (28). In other systems, Cheng et al. found that abnormalities in U11 snRNA in neuronal cells significantly interfere with RNA splicing,

thus damaging nerve cell function and affecting hippocampal synaptic plasticity and spatial learning and memory ability (59). This study also reported the expression changes of snRNA during macrophage polarization for the first time. Moreover, we found that the expression of multiple snRNAs decreased during macrophage polarization to M1 but did not find that the expression of snRNAs increased during macrophage polarization to M1 and M2.

Sequencing results showed that repeat RNA was differentially expressed during M1 and M2 macrophage polarization. However, due to the rare research on repeat RNA and its short length, it has been difficult to verify the sequencing results by conventional real-time PCR. More studies are needed to explore the role of repeat RNA in chondrocyte aging. This study also reported the expression changes of repeat RNA during macrophage polarization for the first time, and the function of repeat RNA needs further study.

CONCLUSION

Our results show that the expression of miRNA, piRNA, snoRNA, snRNA and repeat RNA changes during the polarization of macrophages to M1 or M2. At the same time, there are no reports on the role of piRNA, snoRNA and snRNA in the process of macrophage polarization, but our results preliminarily show that the expression of these sncRNAs is significantly upregulated or downregulated during macrophage polarization, suggesting that they may be regulated and changed in this process, which may play an important role in the process of macrophage polarization. Overexpression of miRNA miR-novel-3-nature and miR-27b-5p and piRNA DQ551351 and DQ551308 could promote the mRNA expression of TNF-α which was marker gene of M1 macrophages. Overexpression of snoRNA ENSMUST00000158683.2 could inhibit the mRNA expression of TNF-α. Overexpression of snRNA ENSMUST00000157129.4 and ENSMUSG00000096786 could promote the mRNA expression of IL-1 and iNOS which was marker gene of M1 macrophages. piRNA DQ551351 and snRNA ENSMUSG00000096786 overexpression promoted the antitumor activity of M1 macrophages. In view of these significant differences in sncRNA, further research is still needed in the future to explore its action targets and mechanisms to better analyze the process of macrophage polarization and deepen the understanding of various pathophysiological changes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO - GSE198744.

ETHICS STATEMENT

The animal study was reviewed and approved by Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

Conceptualization: XiaZ and CW; methodology: DM, JY, and XZ; software: YW, JP, and CW; data curation: LD and CW; writing, review, and editing: CW. All authors contributed to the article and approved the submitted version.

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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.2022.799733/full#supplementary-material

Supplementary Figure 1 | Macrophage polarization. **(A)** The iNOS mRNA expression during M1 polarization. **(B)** The CD206 mRNA expression during M2 polarization. ** indicates $p < 0.01$ compared to M0 group.

Supplementary Figure 2 | The cluster of different expressed miRNA during macrophage polarization. The different expressed miRNA were divided into four cluster according to the similar expression pattern among M1 **(A)** and M2 **(B)** macrophage polarization.

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REVIEW
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Differentially Expressed miRNAs in Ulcerative Colitis and Crohn's Disease

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Differential microRNA (miRNA or miR) regulation is linked to the development and progress of many diseases, including inflammatory bowel disease (IBD). It is well-established that miRNAs are involved in the differentiation, maturation, and functional control of immune cells. miRNAs modulate inflammatory cascades and affect the extracellular matrix, tight junctions, cellular hemostasis, and microbiota. This review summarizes current knowledge of differentially expressed miRNAs in mucosal tissues and peripheral blood of patients with ulcerative colitis and Crohn's disease. We combined comprehensive literature curation with computational meta-analysis of publicly available high-throughput datasets to obtain a consensus set of miRNAs consistently differentially expressed in mucosal tissues. We further describe the role of the most relevant differentially expressed miRNAs in IBD, extract their potential targets involved in IBD, and highlight their diagnostic and therapeutic potential for future investigations.

Keywords: miRNA, ulcerative colitis, Crohn's disease, inflammatory bowel disase, Transcriptomics

INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic, chronic inflammation that primarily affects the gastrointestinal tract. IBD patients experience frequent hospital admissions, many operations, and poor quality of life due to the disease complications (1, 2). Like many other immune-related diseases, the etiology of IBD is not well understood. However, it is generally believed to be a multifactorial disease where environmental factors, genetics, immune dysregulation, and microbiome dysbiosis trigger an inappropriate immune response in lamina-propria, which challenges mucosal homeostasis (3).

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major types of IBD. While CD shows a patchy transmural inflammatory pattern, UC is mainly limited to the innermost layers and

rarely affects other layers of the intestine wall (1, 2). CD is associated with many pathophysiological complications, and its clinical symptoms vary according to the disease location (4). UC is more prevalent and mainly affects the colon (rectum) and generally has a milder course, with patients less prone to disease complications (5, 6). Genome-wide association studies (GWAS) identified 245 unique IBD loci. These susceptible loci are crucial in defining the disrupted intestinal immune system and disease pathways and constitute a solid genetic component of IBD (7–9).

Advances in IBD genetics, high-throughput sequencing technologies, and transcriptome studies provide new insights associated with noncoding RNAs, including long noncoding RNAs (lncRNAs) (10) and microRNAs (miRs or miRNAs) in various diseases (11, 12). Differentially expressed miRNAs are highly correlated with inflammatory and autoimmune disorders, including psoriasis (13), rheumatoid arthritis (14), multiple sclerosis (15), and IBD (16, 17). Mature miRNAs are short (~22 nt long) single-stranded noncoding RNAs derived from pre-miRNA hairpins (typically ~80 nt), and many of these are further processed from primary miRNA transcripts (pri-miRNA) of several hundred nucleotides when multiple pre-miRNAs are contained. The pri-miRNA can be intergenic or of intronic origin nucleotides and can be evolutionarily conserved. MiRNAs are involved in regulating gene expression post-transcriptionally (17–19), where the mature miRNA binds to its target typically with a seed sequence of 6 nucleotides from position 2-7 and with the remaining part binding often with a few nucleotide bulges.

Various studies indicate that differentially expressed miRNAs affect mRNA at several levels of regulation: transcriptional, post-transcriptional, chromatin modification, and genomic imprinting. miRNAs can affect biological processes through endogenous RNA competition, regulation of RNA transcription, protein sponges, and translation regulation. These regulations can cause decreased stability and translational repression that affects various biological functions, including proliferation, migration, cell signaling, autophagy, and apoptosis (3, 17, 20, 21). It is estimated that miRNAs regulate more than 60% of the mRNA through complementary pairing at 3′ untranslated regions (UTRs) (20). miRNAs are not only acting as local regulators within the cells; they also can be found in places far from their origin and are directly or indirectly involved in virtually all types of regulation of biological processes in living organisms (17, 21).

Furthermore, some miRNAs are stable in body fluids such as serum, plasma, urine, saliva, and other tissues (22–25). Many efforts are currently ongoing to identify differentially expressed miRNAs in IBD as biomarkers. Since the expression of differentially expressed miRNA in IBD and many other diseases seems to happen early in the disease, the evaluation of circulating miRNA or tissue-specific levels could be helpful for early diagnosis and successful treatments. Thus, it is highly important to study miRNA-expression profiles and their target genes as biomarkers for diagnosis, prognosis, progression, and treatment response.

This review presents an overview of current knowledge on differentially expressed miRNAs in IBD patients' mucosal tissues and peripheral blood. To enrich the findings from the literature, we combined the literature curation with a meta-analysis of publicly available miRNA high-throughput datasets in mucosal tissues. We further discuss the importance of the most relevant miRNAs in the disease based on the available knowledge and suggest the miRNA participation role in developing chronic inflammation that characterizes pathogenesis. Finally, we discuss the relevance of miRNA differential expression for prediction/early diagnosis, disease progression and treatment responses, and the obstacles in the way.

Differentially Expressed miRNAs in IBD Patient's Mucosal Tissues

Ulcerative Colitis

The first miRNA profiling study in IBD was performed in 2008 and compared biopsy samples from patients with active UC (aUC), inactive UC (iUC), chronic active CD (aCD), microscopic colitis, infectious colitis, and irritable bowel syndrome with healthy controls (26). Eleven miRNAs were differentially expressed in patients with aUC compared to the controls. miR-192-5p, miR-375-3p, and miR-422b-5p were significantly downregulated, and miR-16-5p, miR-21-5p, miR-23a-5p, miR-24-3p, miR-29a-3p, miR-126-3p, miR-195-5p, and let-7f-5p were significantly upregulated (26).

Following this pioneering observation, subsequent studies have identified many new miRNAs while reconfirming already identified ones. It is not surprising that the findings are not consistent as many variables differ between studies, including treatment, inflammatory status, disease duration, anatomical biopsy locations, different healthy control cohorts, and miRNAs profiling platforms. Regardless of these differences, several miRNAs are frequently reported as being differentially expressed. miR-21-5p (26–32), miR-155-5p (27, 29, 33–35), miR-31-5p (27, 31, 33, 36), miR-146a-5p (27, 30, 32, 34), miR-126-3p (26, 28, 32), miR-29a-3p (26, 36), miR-16-5p (26, 28), miR-223-3p (32, 35) and miR-24-3p (26, 30) showed to be constantly upregulated while miR-192-5p (26, 28, 30), miR-141-3p (32, 37), and miR-375-3p (26, 30) were downregulated in UC biopsies when compared to control biopsies (in at least two independent studies). Also, many miRNAs showed differential regulation when inactive UC is compared with active UC and control (**Table 1**).

Crohn's Disease

In another pioneering study in 2010, Fasseu et al. identified 14 and 23 miRNAs differentially expressed ($0.001 < p < 0.05$) in iUC and inactive CD (iCD) patients, respectively (**Tables 1**, **2**). Among them, 8 were commonly differentially expressed in iUC and iCD (miR-26a-5p, miR-29a-3p, miR-29b-5p, miR-30c-5p, miR-126-3p, miR-127-3p, miR-196a-5p, miR-324-3p). Further analysis showed that miR-26a-5p, miR-29b-5p, miR-126-3p, miR-127-3p, and miR-324-3p had coordinated differential regulation in the non-inflamed and inflamed colonic mucosa of IBD patients. On the other hand, miR-196b-5p, miR-199a-3p, miR-199b-5p, miR-320a-5p, miR-150-5p, and miR-223-3p demonstrated significant difference when non-inflamed UC and CD colonic biopsies were compared. Based on

TABLE 1 | Differentially expressed miRNAs in human UC colonic tissue based on literature review.

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
miR-16-5p, -21-5p, -23a-5p, -24-3p, -29a-3p, -126-3p, -195-5p, let-7f-5p	miR-192-5p, -375-3p, and -422b-5p	Macrophage inflammatory peptide (MIP)-2 alpha which is a chemokine expressed by epithelial cells showed to be the target of miR-192-5p.	Microarray qRT-PCR	Sigmoid and colon	aUC/15 iUC/15 CO/15	USA	(26)
miR-21-5p, -203-5p, -126-3p and 16-5p	miR-320-5p, -192-5p	miR-16, -143, and -145 are expressed in response to DNA damage	qRT-PCR	Colon	UC/5 CO/5	USA	(28)
Non-inflamed: miR-15a-5p, -26a-5p, -29a-3p, -29b-5p, -30c-5p, -126-3p, -127-3p, -324-3p Inflamed: miR-7-5p, -26a-5p, -29a-5p, -29b-5p, -31-5p, -126-3p, -127-3p, 135b-5p, 324-3p	miR-199b-5p, -370-5p in both aUC and iUC	Commonly dysregulated in UC and CD: miR-26a-5p,-29a-3p,-29b-5p,-30c-5p,-126-3p,-127-3p,-196a-5p,-324-3p	qRT-PCR	Colon	iUC/8 CO/10	France	(36)
miR-21-5p, -155-5p	–	Other up-regulated miRNAs are (not significant): let-7a-5p,l et-7c-5p, let-7d-5p, let-7g-5p, miR-923-5p	Microarray qRT-PCR	Colon	Microarray: UC/2 qRT-PCR: UC/12 CO/12	Japan	(29)
aUC vs iUC: miR-650-5p, -548a-3p	aUC vs iUC: miR-630-5p, -489-5p, -196b-5p	–	Microarray qRT-PCR	Sigmoid and colon	aUC/9 iUC/9 CO/ 33	Italy	(38)
aUC vs CO: miR-21-5p, -31-5p, -146a-5p, -155-5p, -650-5p aUC and iUC vs CO: miR-675-5p	aUC vs CO: miR-196b-5p, -196b-3p, -200c-3p aUC and iUC vs CO: miR-378a-5p, -196b-5p, -10b-5p	miR-200c-3p directly regulates IL8 and CDH11 expression (regulators of immune and barrier integrity) and can be used for therapeutic purposes.	Affymetrix qRT-PCR	Colon	UC/17 CO /10	Belgium	(27)
miR-24-3p, -142-3p, -146a-5p, -21-5p, let-7i	miR-192-5p, -194-5p, -200b-5p, -375-3p	Rectal miR-24-3p was increased 1.47-fold in UC compared to CD samples.	qRT-PCR	Rectum	UC/18 CO/20	Netherlands	(30)
miR-19a-3p, -21-5p, -31-5p, -101-5p	–	miR-21-5p, -31-5p, and -142-3p were significantly upregulated and miR-142-5p was significantly downregulated in saliva of UC patients.	Microarray qRT-PCR	Colon	UC /41 CO/35	USA	(31)
miR-155-5p, -146a-5p	miR-122-5p	–	qRT-PCR	Colon	UC/10 CO/23	Hungary	(34)
miR-18a-5p, -21-5p, -31-5p, -99a-5p, -99b-5p, -125a-5p, -126-3p, -142-5p, -146a-5p, -223-3p	miR-141-3p, -204-5p	Upregulation of miR-31-5p, -125a-5p, -146a-5p and -223-3p, and downregulation of miR-142-3p in the inflamed mucosa of pediatric UC compared to children with CD was observed	qRT-PCR	colon biopsies	UC/32 CO/11	Hungary	(32)
–	miR-141-3p	miR-141-3p is important in inflammation by inducing CXCL5 upregulation in UC patients	qRT-PCR Western Blot	sigmoid and colon biopsies	aUC /15 CO/ 13	china	(37)
miR-125b-5p, -155-5p, -223-3p, -138-5p	miR-378d-5p	miR-200a-5p did not change significantly in the inflamed samples when compared with non-inflamed and controls.	Microarray qRT-PCR	colon biopsies	UC/8 CO/8	India	(35)
miR-31-5p, -155-5p	–	IL13Rα1 is downregulated in the inflamed UC mucosa and both miRNAs are targeting its 3UTR	qRT-PCR Western Blot	sigmoid and colon biopsies	aUC/11 CO/11	UK	(33)

All miRNAs are from comparison between the disease and healthy individual, unless otherwise stated.
aUC, active UC; iUC, inactive UC; CO, Control; N, Numbers per Group.

TABLE 2 | Differentially expressed miRNAs in human CD colonic tissue based on literature review.

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
Non-inflamed miR-7-5p, -26a-5p, -30b-5p, -30c-5p, -155-5p, -127-3p, -223-3p, -324-3p Inflamed: miR-26a-5p, -29b-5p, -126-3p, -155-5p, -127-3p, -185-5p, -196a-5p, -324-3p, -378-5p	miR-130b-5p in inflamed CD	Commonly dysregulated in UC and CD: miR-26a-5p, -29a-3p, -29b-5p, -30c-5p, -126-3p, -127-3p, -196a-5p,-324-3p	qRT-PCR	Colon	iCD/8 CO/10	France	(36)
colonic CD vs CO: miR-23b-3p, -106a-5p, and -191-5p active ileal CD vs CO: miR-16-5p, -21-5p, -223-3p and -594-5p	colonic CD vs CO: miR-19b-3p, -629-5p	Ten intestine region-specific miRNAs were identified. miR-22-5p, -31-5p, and -215-5p were significantly increased in the terminal ileum as compared to all four colonic regions	Microarray qRT-PCR	Terminal ileum, cecum, transverse colon, sigmoid, and rectum	Sigmoid CD/5 Terminal ileum CD/6 CO/13	USA	(39)
aCD versus iCD: miR-18a-3p, -629-3p, , let-7b, -140-3p	aCD versus iCD: miR-422a-5p, -885-5p, -328-5p		Microarray qRT-PCR	Colon	aCD/9 iCD/9	Italy	(38)
miR-19b-3p, -23b-3p, -106a-5p, -629-5p	–	CD vs UC: Significant differential expression of miR-19b-3p, -106a-5p, -629-5p Average expression of these three miRNAs and miR-23b-3p and -191-5p was significantly different between intermediate colitis and CD No significant difference was detected between UC, intermediate colitis and controls	qRT-PCR	Colon	CD/14 UC/12 Intermediate/16 CO/11	USA	(40)
miR-142-3p, -146a-5p, -21-5p, let-7i	miR-194-5p, -200b-5p, -192-5p and -375-3p	Rectal miR-24-3p correctly classified 84.2% of patients, with a sensitivity of 83.3% and specificity of 85.7%.	qRT-PCR	Colon	CD/12 CO/20	USA	(30)
Non-inflamed vs CO: miR-495-5p Inflamed vs non-inflamed: miR-361-3p	Inflamed vs CO: miR-192-5p Non-inflamed vs CO: let-7b-5p Inflamed vs non-inflamed: miR-124-3p		Microarray qRT-PCR	Terminal ileum	CD/16 CO/10	China	(41)
In B2 and/or B3: miR-31-5p, -215-5p, -223-3p	In B1: miR-150-5p In B2 and/or B3: miR-149-5p, -196b-5p, -203-5p	B1: nonstricturing and nonpenetrating (n=8) B2: structuring (n=6) B3: penetrating/fistulizing (n=7) The expression level of miR-31-5p was the most significant in both B2 and B3	RNA-Seq qRT-PCR	Colon	Sequencing: CD/21 CO/14 Validation: CD/20 CO/15	USA	(42)
miR-31-5p, -101-5p and -146a-5p	miR-375-3p	miR-101 in CD patients' saliva was significantly upregulated. ATG16L1 as a regulatory target of miR-142-3p and miR-93-5p	Microarray qRT-PCR	Colon	CD /42 CO/35	USA	(31)
miR-146a-5p and -155-5p Inflamed CD vs CO: miR-122-5p (not significant)	Inflamed CD vs intact CD: miR-122-5p	miR-146a and -155 have also been connected to TLR pattern recognition receptor family. TNF-α treatment in HT-29 cells increased the expression of miR-146a-5p and -155-5p, but not miR-122-5p.	RNA-Seq qRT-PCR	Colon	Intact pCD/14 Inflamed pCD/24 CO/23	Hungary	(34)

(Continued)

TABLE 2 | Continued

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
inflamed vs intact duodenal mucosa: miR-146a-5p inflamed CD vs CO duodenal mucosa: miR -155-5p		TGF-β treatment had no effect on miR-146a-5p miR-122-5p expression in duodenal epithelial cells, while significant downregulation was detected for miR-155-5p.	qRT-PCR	Duodenal	intact CD/ 10 inflamed CD/ 10 CO/10	Hungary	(43)
Inflamed vs CO: miR-18a-5p, -21-5p, -31-5p, -99a-5p, -99b-5p, -100-5p, -125a-5p, -126-3p, -142-5p, -142-3p, -146a-5p, -150-5p, -185-5p, and -223-3p Non-inflamed vs CO: miR-18a-5p, -20a-5p, -21-5p, -31-5p, -99a-5p, -99b-5p, -100-5p, -125a-5p, -126-3p, -142-5p, -146a-5p, -185-5p, -204-5p, -221-5p, and -223-3p	Inflamed vs CO: miR-20a, -141-3p, -204-5p Inflamed vs non-inflamed and CO: miR-141-3p, miR-204-5p	miR-31-5p, -125a-5p, -142-3p-5p, and -146a-5p showed alter expression between the inflamed mucosa of CD and UC	qRT-PCR	Colon	RNA-Seq: CD/4 CO/4 Validation: CD/15 CO/11	Hungary	(34)
miR-193b-3p, -19a-3p, let-7l, let-7l-3p, -1273D-5p, -886-5P, -668-5p, -720-5p, -455-3P, -3138-5p, -612-5p, -551B-5p, -4264-5p, -24-3p	miR-3194-5p, -196A-5p, -192-5p, -200A-5p, -192-3p, -1913-5p, -378b-5p, -323b-3P, -3150-5p, -422A-5p, -611-5p, -3184-5p, -4284-5p, -129-3p	miR-4284-5p, -3194-5p and -21-5p interact with JAK-STAT signaling and innate immune system	Microarray qRT-PCR	Colon	CD/15 CO/15	Italy	(44)
miR-144-5p, -451-5p, -31-5p and -142-3p iCD vs CO: miRplus-F1195 and -150-5p	miR-1973-5p, -1205-5p, -5481-5p, -491-5p -3p CD and iCD vs CO: miR-1205-5p downregulation	Inhibition of C10orf54 expression by miR-16-1-5p is one of the main causes of CD	Microarray qRT-PCR	Ascending colon	CD/7 iCD/7 CO/7	USA	(45)
miR-31-5p		a dramatic and highly significant upregulation (~60-fold) of miR-31-5p in IL patients compared with control	RNA-Seq qRT-PCR	Ascending colon	CD/76 CO/51	USA	(46)
miR-21-5p, -223-5p, -1246-5p	miR-30c-5p, -378-3p		Microarray qRT-PCR	Ileal colon	CD/18 CO/12	Belgium	(47)
miR-223-3p	miR-194-5p, -10b-5p, -215-5p, -192-5p, -10a-5p, -582-5p	miR-31-5p expression was location driven suggest a CD location subtypes	NanoString	Ileal Colon	CD/23 CO/ 38	Canada	(48)

All miRNAs are from comparison between the disease and healthy individual, unless otherwise stated.
aCD, active CD; iCD, inactive CD; CO, Control; N, Numbers per Group.

this screening, the authors suggested an important role of miRNAs in the inflammation at onset and/or relapse of IBD patients with quiescent mucosal tissues (36).

Succeeding studies have identified several miRNAs consistently shown to be differentially expressed between CD and control biopsies, including always upregulated miR-146a-5p (30–32, 34, 43), miR-21-5p (30–32, 39, 47), miR-31-5p (31, 32, 42, 45, 46), miR-223-3p (32, 39, 42, 48), miR-142-3p (30, 32, 45), let-7i-5p (30, 44), miR-23b-3p (39, 40), miR-106a-5p (39, 40) and constantly downregulated miR-192-5p (41, 44, 48), miR-194-5p (30, 48) and miR-375-3p (30, 31). There are also miRNAs with conflicting results including miR-150-5p (up in (32, 45), down in (42)), miR-19b-3p (up in (40), down in (39)), miR-215-5p (up in (42), down in (48)), and miR-629-5p (up in (40), down in (39)). Moreover, several miRNAs showed differential regulation when iCD compared with aCD and control (**Table 2**).

Differentially Expressed miRNAs in IBD Patient's Peripheral Blood

Ulcerative Colitis

Similar to the findings in tissue biopsies, miRNAs are also differentially expressed in the peripheral blood of UC patients. In a first study, Wu et al. compared the circulating miRNA profile of whole blood of aUC and iUC patients and healthy individuals (49). Their microarray investigation showed a significant increase in the expression level of twelve miRNAs, while one, miR-505-3p, showed a significant decrease when comparing patients with aUC with healthy controls. miR-505-3p expression was decreased around 7-fold in active outpatient blood. In contrast, 3.1- and 5.2-fold expression increases were demonstrated in the blood of the active UC patients for miR-103-2-3p and miR-362-3p, respectively. Furthermore, a comparison between the circulating miRNA in the peripheral blood of UC patients with healthy

TABLE 3 | Differentially expressed miRNAs in human UC peripheral blood based on literature review.

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
miR-28-5p, -151a-5p, -199a-5p, -340-3p, and miRplus-E1271 aUC and iUC: miR-103-2-3p, -362-3p, and -532-3p	aUC and iUC: miR-505-3p UC vs CD: miR-505-3p	UC specific: miRplus-E1153 miRs-28-5p, -103-2-3p, -149-3p, -151a-5p, -340-3p, -505-3p, -532-3p, and miR-plus-E1153, were able to distinguish aCD from aUC	Microarray RT-qPCR	Peripheral blood	aUC/13 iUC/10 CO/13	USA	(49)
miR-188-5p, -422a-5p, -378-5p, -500-5p, -501-5p, -769-5p, -874-5p		Classifier measurements demonstrated a predictive score of 92.8% accuracy, 96.2% specificity and 89.5% sensitivity in stratifying UC patients from controls using these miRNAs panel.	Microarray RT-qPCR	Peripheral blood	UC/20 CO/20	USA	(57)
miR-16-5p, -21-5p, -28-5p, -151a-5p, -155-5p and -199a-5p			RT-qPCR	Peripheral blood	UC/88 CO/162	Greece	(54)
miR-760-5p, -423-5p, -128-5p, -196b-5p, -103-5p, -221-5p, -532-5p, -15b-5p, -27a-5p, let-7g-5p, -93-5p, let-7d-5p, -598-5p, -142-5p, let-7e, -223-3p, -374b-5p, -19a-3p, -345-5p, -199a-3p, -24-3p, -30e-5p, -29a-3p, -28-3p aUC vs iUC: miR-650-5p and -548a-3p	miR-150-5p aUC vs iUC: miR-630-5p, -489-5p, and -196b-5p	miR-127-3p, -491-5p, -18a-5p, -145-5p, let-7b-5p, -185-5p, -29c-5p, -19b-3p, -20b-5p, -106a-5p, -17-5p, -222-5p, -135a-3p were common between CD and UC	TaqMan human miRNA array RT-qPCR	Serum	aUC/9 iUC/9 CO/33	Italy	(38)
miR-16-5p, -34b-3p UC vs CD: miR-377-3p, -1247-5p miR-34b-3p, -484-5p, -574-5p in both CD and UC	miR-99b-5p	miR16-5p regulates HMGA1/2 and ACVR2a while miR-34b regulates HNF4A, NOTCH1, c-MET/HGFR and CAV1 and miR-99b-5p regulates RAVER2 and mTOR which are all IBD-risk genes	Microarray	Peripheral blood	UC/36 CO/38	Germany	(53)
miR-595-5p, -1246-5p, -142-5p, -143-5p, -24-3p aUC vs iUC: miR-1246-5p and miR-595-5p		NCAM-1 and FGFR2 are two potential targets of miR-595 miR-1246 indirectly activates the proinflammatory nuclear factor of activated T cells	Microarray RT-qPCR	Serum	UC/62 CO/58	New Zealand	(52)
miR-223-3p, -23a-3p, -302-3p, -191-5p, -22-3p, -17-5p, -30e-5p, -148b-3p, -320e-5p	miR-1827-5p, -612-5p, -188-5p UC vs iUC: miR-4454-5p, -223-3p, -23a-3p, -148b-3p, -320e-5p, -4516-5p	Positive disease severity correlation of miR-223-3p, -4454, -23a-3p, -148b-3p, -320e-5p, and -4516-5p miR-4454-5p, -223-3p, -23a-3p, and-320e-5p showed higher sensitivity and specificity values (70% and 68%, 79% and 72%, 79% and 68%, and 67% and 67%, respectively) than C-reactive protein (37% and 95%)	Nanostring Analysis	Peripheral blood and serum	UC/24 iUC/22 CO/21	USA	(51)
miR-19a-3p, -101, -142-5p, -223-3p, -375-3p, and -494-5p	miR-21-5p, -31-5p, and -146a-5p	miR-21-5p, -31-5p, and miR-142-3p were significantly upregulated and miR-142-5p was significantly downregulated in saliva of UC patients.	Microarray qRT-PCR	Peripheral blood	UC /41 CO/35	USA	(31)
miR-223-3p		miR-223-3p demonstrated high Spearman r value in detecting the disease activity	RT-qPCR FC: 2.8	Serum	UC/50 CO/50	China	(3)
miR-29b-3p, -122-5p, -150-5p, -192-5p, -194-5p, -146a-5p, -375-3p	miR-199a-3p, -148a-3p	miRNA used in this study were discovered in IL10−/− mice model of UC and tested for orthologues in human. UC stratified from CO with 83.3% prediction rate	miRCURY LNA RT-qPCR Prediction	Serum	UC/12 CO/12	USA	(56)

(Continued)

TABLE 3 | Continued

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
aUC vs CO: miR-106a-5p iUC vs CO: miR-106a-5p and -362-3p		The expression level of miR-362-3p showed to be higher in UC vs CO but not significant.	RT-qPCR	Peripheral blood	aUC/20 iUC/12 CO/32	Iran	(55)
miR-16-5p, -21-5p and -223-3p	–	miR-155 expressed higher in CD than UC In remission group expression of miRNAs was dependent on disease activity	RT-qPCR	Serum	UC/15 CO/20 Remission UC/8	Germany	(50)

All miRNAs are from comparison between the disease and healthy individual, unless otherwise stated.
aUC, active UC; iUC, inactive UC; aCD, active CD; iCD, inactive CD; CO, Control; N, Numbers per Group.

individuals revealed a significant increase in the expression level of the miR-28-5p, miR-151a-5p, miR-199a-5p, miR-340-3p, and miRplus-E1271 in patients with aUC but not in iUC. Wu et al. further demonstrated that miRs-103-2-3p, miR-362-3p, and miR-532-3p are upregulated in both aUC and iUC. Following this initial study, in attempts to identify circulating miRNAs that contribute to UC development and to find proper biomarker candidates, many studies have been performed. From these studies miR-223-3p (3, 31, 38, 50, 51), miR-142-5p (31, 38, 52), miR-16-5p (50, 53, 54), miR-151a-5p (49, 54), miR-199a-5p (49, 54), miR-19a-3p (31, 38), miR-24-3p (38, 52), miR-28-5p (49, 54), miR-30e-5p (38, 51), miR-362-3p (49, 55) showed consistent upregulation in at least two independent studies, whereas none of the downregulated miRNAs had been validated in more than one study (possibly due to biases in which miRNAs are picked for validation). Moreover, miR-21-5p (up in (49, 50), down in (31)), miR-146a-5p (up in (56), down in (31)), miR-150-5p (up in (56), down in (38)), miR-188-5p (up in (57), down in (51)), miR-199a-3p (Up in (38), down in (56)) showed inconsistent differential regulation between different studies. miRNAs differential regulation was also detected when iUC was compared with aUC and control. miR-362-3p is the only miRNA that shows upregulation in two independent studies when iUC was compared with healthy control (49, 55) (**Table 3**).

Crohn's Disease

One of the first studies using whole blood for distinguishing CD patients from normal healthy individuals using miRNA profile was done by Wu et al. (49). Comparing the circulating miRNA of the aCD patients with healthy controls showed a significant increase in the expression of five miRNAs and a significant decrease in two others. Among them, miR-362-3p showed the most significant difference in expression of a 4.7-fold increase. Interestingly the expression of miR-340-3p showed a significant increase, and miR-149-3p showed a significant decrease in both active and inactive CD patients compared to the healthy controls.

Subsequent studies found miR-16-5p (38, 50, 54, 58), miR-484-5p (53, 58, 59), miR-362-3p (49, 54, 55), miR-106a-5p (54, 55, 58), miR-532-3p (49, 54), miR-30e-5p (58, 60), miR-223-3p (3, 50), miR-21-5p (50, 58), miR-200c-3p (54, 61), miR-199a-5p (49, 54), miR-195-5p (38, 58), miR-142-5p (52, 53), miR-140-5p (38, 58) to be consistently upregulated in CD patients in comparison with healthy controls (in at least two independent studies). However, similar to the UC studies, based on the lists manually curated from literature, no circulating miRNA is always downregulated when CD is compared to healthy controls (in more than one study). This could be because the main focus for blood-based biomarker discovery is on the upregulated miRNAs, not the downregulated ones. There are also miR-574-5p (up in (53), down in (60)) and miR-192-5p (up in (58), down in (60)) that were shown to be differentially expressed inconsistently between studies. Moreover, several circulating miRNAs showed differential regulation when iCD compared with aCD and control (**Table 4**).

Computational Meta-Analysis of Publicly Available High Throughput Studies

In addition to the literature curation, we also performed a meta-analysis of publicly available high throughput studies (microarray and RNA-Seq), including 3 UC (27, 62, 63) and 4 CD (42, 47, 62, 64) patient cohorts (**Table 1**). All included studies contained expression profiling at the level of the intestinal mucosa (colon or ileum). We combined the results of differential expression analysis between the UC or CD and the control group from each study as described in the supplementary section. The three UC datasets are consistent with each other, with most differentially expressed miRNAs being changed in the same direction, in contrast to the CD datasets, where many miRNAs are differentially expressed in opposite directions between the datasets (**Supplementary Figure 1**). The higher heterogeneity observed in the expression profiles from CD patients might be consistent with the more heterogeneous nature of CD compared to UC. There might also be other explanations, including different patients' demographics, different sample handling, and data generation in different labs.

We obtained a final set of 158 miRNAs consistently differentially expressed between UC patients and controls and 69 miRNAs between CD patients and controls (p-value < 0.05 in at least two datasets and a global adjusted combined logit p-value < 0.05) and consistent in the direction of regulation across all datasets (**Supplementary Files 1, 2** and **Supplementary Figure 2**).

The meta-analysis confirms most of the literature-curated miRNAs and at the same time provides dozens of other miRNAs

TABLE 4 | Differentially expressed miRNAs in human CD peripheral blood based on literature review.

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
miR-199a-5p, -362-3p, -532-3p, miRplus-E1271 aCD and iCD: miR-340-3p	miRplus-F1065 aCD and iCD: miR-149-3p	miR-199a-5p, -362-3p, -340-3p, -532-3p and miRplus-E1271 common in both CD and UC	Microarray RT-qPCR	Peripheral blood	aCD/14 iCD/5 CO/13	USA	(49)
miR-16-5p, -195-5p, -106a-5p, -20a-5p, -30e-5p, -140-5p, -484-5p, -93-5p, -192-5p, -21-5p and let-7b-5p		Area under the ROC curve values of 0.82 to 0.92 sensitivities of 70% to 83% and specificities of 75% to 100%	TaqMan Human MicroRNA Arrays RT-qPCR	Serum	CD/46 CO/32	USA	(58)
miR-16-5p, -23a-5p, -29a-3p, -106a-5p, -107-5p, -126-3p, -191-5p, -199a-5p, -200c-3p, -362-3p and -532-3p			RT-qPCR	Peripheral blood	CD/128 CO/162	Greece	(54)
miR-27a-5p, -140-3p, -140-5p, -16-5p, -195-5p aCD vs iCD: miR-188-5p, -877-5p	miR-877-5p aCD vs iCD: miR-140-5p, miR-145-5p, -18a-5p, -128-5p	miR-127-3p, -491-5p, -18a-5p, -145-5p, let-7b, -185-5p, -29c-5p, -19b-3p, -20b-5p, -106a-5p, -17-5p, -222-5p, -135a-3p are common in CD and UC miR-877-5p has role in disease remission	TaqMan human miRNA array RT-qPCR	Peripheral blood and Serum	aCD/9 iCD/9 CO/33	Italy	(38)
miR-34b-3p, -142-5p, -205-5p, -424-5p, -885-5p CD vs UC: miR-656-3p, -744-5p, -1908-5p miR-34b-3p, -484-5p, -574-5p common in both CD and UC	miR-570-3p, -1301-3p	miR-205-5p targets LRRK2, SHIP2/INPPL1, ZEB1, E2F1, ERBB3 and miR-142-5p targets NFE2L2/NRF2 and miR-424-5p targets MYB, CUL2, PU.1 which are either IBD-risk loci or IBD-related known genes	Microarray	Peripheral blood	CD/40 CO/38	Germany	(53)
miR-200c-3p, -181a-2-3p, and -125a-5p	miR-369-3p, -376a-5p, -376c-5p, -411-3p, -411-5p, and mmu-miR-379-5p	Validation cohort: Only miR-16 was significantly downregulated in patients (fold change 0.83, P=0.02).	OpenArray miRNA profiling RT-qPCR	Plasma	CD/6 CO/6 Validation CD/102	Denmark	(61)
miR-595-5p, -1246-5p, -142-5p, -143-5p aCD vs iCD: miR-1246-5p, -595-5p and -142-5p		Validation cohort: Only miR-1246-5p, -142-5p and -143-5p were upregulated and only miR-143-5p is significant. UC vs CD: miR-16-5p	Microarray RT-qPCR	Serum	CD/57 CO/58 Validation CD/10 CO/10	New Zealand	(52)
miR-101-5p and -375-3p	miR-21-5p, -31-5p, -146a-5p, and -155-5p	miR-101-5p in CD patients' saliva was significantly upregulated.	Microarray RT-qPCR	Peripheral blood	CD /42 CO/35	USA	(31)
miR-30e-5p	miR-1183-5p, -1827-5p, -1286-5p, -504-5p, -188-5p, -574-5p, -192-5p, -149-5p, and -378e-5p	Downregulated miR-1286 and miR-1273d-5p correlated with CD disease activity higher than C-reactive protein and calprotectin	Nanostring nCounter	Serum	aCD/21 iCD/24 CO/21	USA	(60)
miR-223-3p		2.2-fold upregulation in CD 2.8-fold upregulation in UC miR-223-3p has higher Spearman r value in IBD detection than hCRP and ESR.	RT-qPCR	Serum	CD/50 CO/50	China	(3)
miR-631-5p, -4521-5p, -562-5p, -766-3p, -302b-3p, -423-3p, -484-5p, -4707-3p, -483-3p, -4516-5p, -665-5p, -1260b-5p, -2117-5p, -216b-5p, -296-5p, -27b-3p, -188-3p, -770-5p, -1233-3p, -4755-5p, -627-3p, -767-3p, -339-5p		miR-874-3p targets ATG16L1 and reduces its expression and dysregulates autophagy by a reduction of LC3 in vitro Upregulated in iCD vs CO: miR-548g-3p, -4536-5p, -4448-5p,	NanoString	Peripheral blood mononuclear cells	aCD/35 iCD/10 UC/46 CO/39	Canada	(59)

(Continued)

TABLE 4 | Continued

Up-regulated	Down-regulated	Other finding/Comments	Method	Source	Group/N	Country	Ref
aCD and iCD vs CO: miR-1268a-5p, -1297-5p, -1909-3p, -197-3p, -197-5p, -410-3p, -936-5p, -542-5p, -549a-5p, -603-5p, -874-3p, -92a-3p, -933-5p, -941-5p		-30a-3p, -548q-5p, -4461-5p, -133a-3p, -597-5p, -619-3p, -644a-5p					
aCD and iCD vs CO: miR-106a-5p and -362-3p			RT-qPCR	Peripheral blood	aCD/22 iCD/10 CO/32	Iran	(55)
miR-16-5p, -21-5p and -223-3p		Upregulated miRs were detected in both IBD type, but were higher in CD No significant miR-155-5p expression In remission group miRNAs expression is disease activity dependent	RT-qPCR	Serum	CD/35 CO/20 Remission: CD/15	Germany	(50)

All miRNAs are from comparison between the disease and healthy individual, unless otherwise stated.
aCD, active CD; iCD, inactive CD; CO, Control; N, Numbers per Group.

not previously reported in UC or CD mucosa (e.g., miR-378a-3p, miR-191-5p, miR-92a-3p in UC; miR-30e-5p, miR-26b-5p, let-7f-5p, let-7g-5p, in CD; miR-146b-5p, miR-30d-5p, miR-148a-3p, miR-151a-5p in both UC and CD). In addition, few miRNAs showed different or no significant differential regulation compared to what was found in the literature, including miR-142-3p (30, 32, 45) in CD, which in literature curation showed to be constantly upregulated, while in the meta-analysis, it was constantly downregulated.

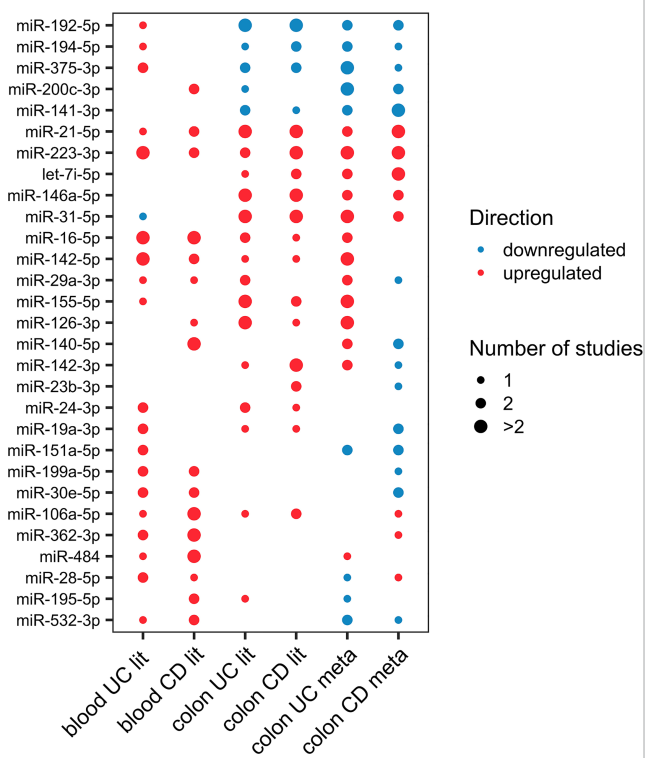

FIGURE 1 | Dot-plot of the 29 differentially expressed miRNAs (at least two studies) in either colon or blood of UC or CD from literature. The node size represents the number of studies, and the node color corresponds to the expression statues, where red means upregulation and blue means downregulation.

Moreover, miRNAs reported in the literature are predominantly upregulated (specifically for UC); however, the meta-analysis indicates an almost equal number of up- and downregulated miRNAs. This might be ascribed to the ease/bias of validation for the upregulated miRNAs for diagnostic purposes with available techniques. Furthermore, the downregulated miRNAs showed a higher average expression, possibly indicating a more substantial functional role of these miRNAs (65) (**Supplementary Figure 3**).

One of the studies (GSE89667) contained both UC and CD cohorts (62), and we used the UC versus CD comparison (adjusted p-value < 0.05), in conjunction with the results of the meta-analysis, to find a set of 18 miRNAs differentially expressed between UC and CD. Among these, e.g., miR-29a-3p, miR-155-5p, or miR-454-3p are upregulated in UC compared to CD, while miR-28-3p, miR-378a-5p or miR-422a are downregulated in UC compared to CD (**Supplementary Data, Sheet 3**).

Overlap of Colon and Blood miRNAs in UC and CD

There is great potential in identifying disease-specific miRNAs for diagnosis, progression, and therapeutic response. Consistently differentially expressed miRNAs in the colon and blood may have the highest clinical potential. From literature curation, 29 miRNAs were consistently differentially expressed in at least two studies in colon or blood of UC or CD (**Figure 1**).

Ulcerative Colitis

From the miRNAs with consistent differential regulation in at least two independent studies, miR-223-3p, miR-16-5p, and miR-24-3p showed upregulation in both mucosa and blood of UC patients compared with healthy individuals. miR-21-5p and miR-146a-5p

were also shown to be differentially expressed in both tissues. However, the blood data for these two miRNAs were inconsistent. Considering only one study, 18 miRNAs were commonly differentially expressed in both tissues (**Supplementary Data, Sheet 4**).

Crohn's Disease

From the miRNAs with consistent differential regulation in at least two independent studies in CD miR-223-3p and miR-21-5p showed upregulation in both mucosa and blood of patients compared with healthy individuals. miR-192-5p was also common and frequently downregulated in the mucosa; however, since the data for this miRNA in the blood is inconsistent, it was excluded. Finally, considering miRNAs differentially expressed in only one study, 13 miRNAs were shown to be commonly differentially expressed in both tissues (**Supplementary Data, Sheet 5**).

UC and CD miRNA Profile Similarities and Differences

Even the most experienced clinicians have problems in the initial diagnosis of IBD and stratifying its subtypes. Stratifying UC and CD has always been a challenge ascribed to their overlapping features. Although these IBD subtypes have common characteristics, significant genetic and clinical differences exist. Consequently, different transcriptome profiles, specifically distinct miRNAs signatures, might improve IBD subtype classification.

Colon

Many studies compared individuals with and without the diseases to stratify UC and CD based on mucosa biopsy miRNA signature (30–32, 34, 36, 38). Considering miRNAs validated to be differentially expressed in at least two studies in both UC and CD mucosa, miR-21-5p, miR-31-5p, miR-146a-5p, miR-223-3p showed to be commonly up- and miR-192-5p and miR-375-3p downregulated in both phenotypes.

Furthermore, considering miRNAs with consistent differential regulation in at least two independent studies, miR-155-5p, miR-126-3p, miR-29a-3p, miR-141-3p, miR-16-5p and miR-24-3p showed to be differentially expressed mainly in UC mucosa, while miR-142-3p, miR-150-5p, let-7i-5p, miR-23b-3p, miR-19b-3p, miR-215-5p, miR-629-5p, miR-194-5p and miR-106a-5p showed to be more frequently differentially expressed in CD mucosa.

To confirm the above observation, these miRNAs (from at least two studies) were more intersected against the literature miRNA lists, this time one study and more. The comparison showed that miR-29a-3p is only reported as significantly differentially expressed (SDE) in UC, and miR-23b-3p is only reported as SDE in CD. Moreover, the results for miR-150-5p and miR-215-5p were inconsistent.

Blood

Similar attempts to stratify UC and CD based on the blood miRNA profile of patients versus healthy individuals were made (3, 31, 38, 49, 50, 52–55). Considering frequently differentially expressed miRNAs in UC and CD blood, miR-223-3p, miR-142-5p, miR-16-5p, miR-199a-5p, miR-30e-5p, miR-362-3p were significantly differentially upregulated and were common between both phenotypes and thus could be considered as IBD biomarkers.

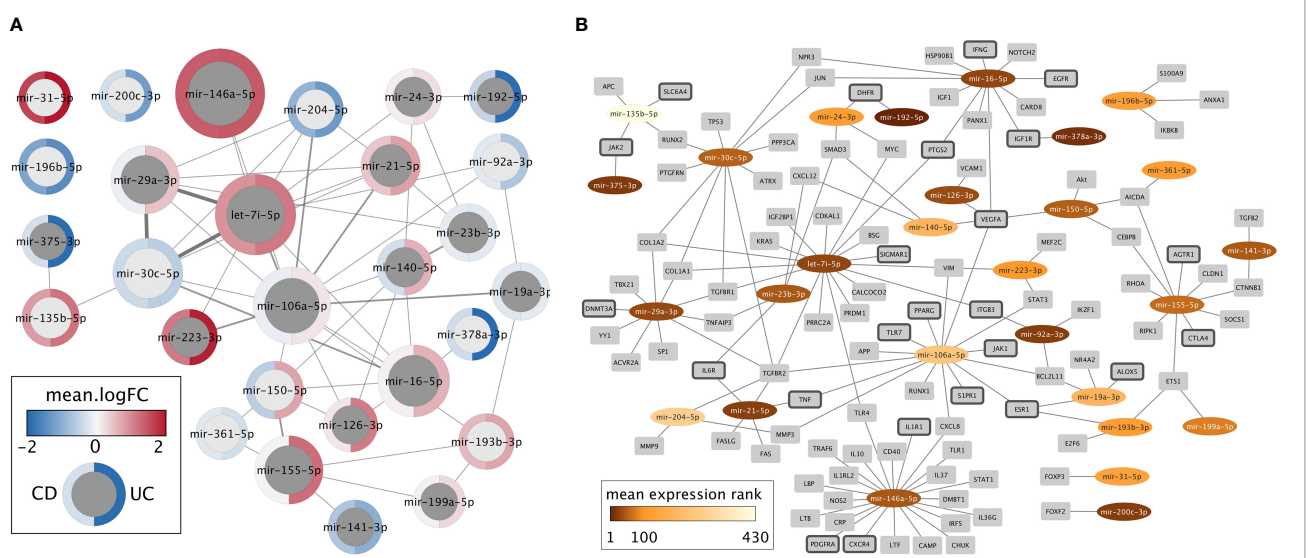

FIGURE 2 | Network representations of the 28 miRNAs with at least one experimentally determined target known to be related to IBD. **(A)** Network of miRNAs only. Dark gray nodes represent miRNAs detected by literature curation, while light gray nodes were not identified in the literature, but only in the meta-analysis. The size of each miRNA node corresponds to the number of IBD targets this miRNA has, and the width of the edges represents the number of shared IBD targets. The mean logFC of each miRNA, according to the meta-analysis, is shown for CD (left) and UC (right) using a blue-white-red gradient on the node border. **(B)** Network of miRNAs (oval nodes) and their target genes (rectangle nodes). miRNAs are colored based on their mean expression rank. Target genes that code for proteins with a clinically approved drug according to the Pharos database are highlighted by dark gray node border color.

Furthermore, considering miRNAs with consistent differential regulation in at least two independent studies, miR-146a-5p, miR-150-5p, miR-151a-5p, miR-188-5p, miR-199a-3p, miR-19a-3p, miR-24-3p, miR-28-5p showed to be mainly differentially expressed in UC. miR-484-5p, miR-106a-5p, miR-574-5p, miR-532-3p, miR-200c-3p, miR-195-5p, miR-192-5p, miR-140-5p showed to be more frequently differentially expressed in CD blood.

To confirm this observation, these differentially expressed miRNAs (from at least two studies) in each phenotype were once more intersected against the literature miRNA lists, this time one study and more. The results showed miR-146a-5p, miR-150-5p, miR-151a-5p, miR-199a-3p, miR-19a-3p, miR-24-3p were only SDE in UC. The results for miR-150-5p and miR-199a-3p were inconsistent. Furthermore, miR-200c-3p, miR-195-5p, and miR-140-5p showed only SDE in CD.

Most Relevant Differentially Expressed miRNAs

To develop miRNA-based novel diagnostics and therapeutics for IBD, it is vital to understand the miRNAs expression changes in correlation with the disease phenotype, underlying mechanisms that regulate miRNAs, the target genes, and their interplay. Despite the heterogeneity of differentially expressed miRNAs in IBD, 66 miRNAs were identified from literature curation and meta-analysis as relevant candidates for diagnostic or therapeutic purposes that might also represent causative agents in disease development (Supplementary data, Sheet 6). For this set of miRNAs, we extracted "experimentally observed targets" from QIAGEN Ingenuity Pathway Analysis (IPA) software program v70750971 (66) and intersected these targets with genes related to IBD extracted from IPA and literature (Supplementary data, Sheet 7). This resulting list of 28 miRNAs with at least one IBD target was visualized in Cytoscape (67) (**Figure 2**). In the following, we discuss most of these miRNAs in more detail.

Let-7i-5p: Let-7i-5p is the regulator of TLR4, which is important in cytokine-mediated responses and a regulator of IL-6 (68). In THP-1 cells transfected with let-7i-5p mimics, both mRNA and protein levels of TLR4 showed downregulation (69). Let-7i-5p seems to assist cells in resetting their protein profile in response to external stimuli in allergic inflammation; the exact mechanism is not yet clear (70). Let-7i-5p regulates collagens, IL-6, TGF-βR1, IGF-1, and caspase-3 as primary regulators of inflammation, fibrosis, hypertrophy, and apoptosis (68).

miR-16-5p: miR-16-5p in the colonic UC mucosa partly regulates the inflammatory responses through negative regulation of A2aAR (NF-κB inhibitor) expression. miR-16-5p mimics transfection in colonic epithelial cells, demonstrated to increase nuclear translocation of NF-κB p65 protein and thus increase the expression of IFN-γ and IL-8 as important pro-inflammatory cytokines (71).

miR-19a-3p: Serum miRNA profiling of CD patients with and without strictures showed miR-19a-3p and miR-19b-3p as potential pathogenic markers (72). Low levels of miR-19a-3p and miR-19b-3p were strongly correlated with stricturing CD and independent of site, gender, age, disease duration, and activity (72). Moreover, it has been reported that miR-19a-3p decreases the SOCS3 expression, which consequently enhances IFN-α and IL-6 signal transduction (73).

miR-21-5p: miR-21-5p showed an essential role in colon epithelial cell hemostasis (74), adaptive immune responses (75), cytokine regulation (76), and IBD-related complications (77). It has been demonstrated that in response to epithelial damage, miR-21-5p causes more intestinal permeability. Transfection of miR-21-5p mimics resulted in the loss of tight junction proteins, increased barrier permeability (74), and decreased CD3 and CD68 positive cells in the UC mouse model (78). The miR-21-5p knockout mice model also showed high resistance to dextran sulfate sodium (DSS) induced colitis, suggesting the pro-apoptotic effect of this miRNA. miR-21-5p also demonstrated an essential role in adaptive immune responses in T-cell function, with the highest detected expression in effector T cells, memory T cells, and the lowest in naive T cells (75). miR-21-5p has a regulatory role in innate immunity and is involved in TLR4 activation and monocyte differentiation. It is also induced by danger signals, such as activators of NF-kB in a negative feedback loop, to prevent damage (79). miR-21-5p is associated with disease activity in UC patients (80). Moreover, this miRNA regulates IL-12 release from dendritic cells and macrophages by targeting the IL-12p35 receptor (76). On the other hand, the association of this miRNA with irreversible IBD fibrosis and its increased level was observed in serum of humans with significant fibrosis (77) and development of dysplasia (81). It is noteworthy that several cellular injury models have shown to be TNF-α dependent with subsequent miR-21-5p induction (77, 82).

miR-23b-3p: miR-23b-3p represses autoimmune inflammation by suppressing (IL-17, TNF-α, IL-1β)-induced NF-κB activation, inflammatory cytokine expression by targeting TGF-β-activated kinase 1/MAP3K7 binding protein 2 (TAB2), TAB3 and inhibitor of NF-κB kinase subunit α. Conversely, IL-17 contributes to autoimmune pathogenesis by suppressing miR-23b-3p expression and promoting proinflammatory cytokine expression (83).

miR-24-3p: miR-24-3p is reported to be involved in T cells proliferation, differentiation, and immune response (84). It is also reported that miR-24-3p targets Bcl-2 and PAK4 as prosurvival genes, thus, inducing cell death (85). Overexpression of PMS2L2 prompts miR-24-3p gene methylation, resulting in its inhibition. PMS2L2 overexpression, stimulated by LPS, is shown to promote Bcl-2 expression and to inhibit Bax, cleaved-caspase-3, and cleaved-caspase-9 expressions (86). Furthermore, miR-24-3p regulates the processing of latent TGF-β1 release by furin targeting (87). miR-24-3p is reported to downregulate not only TGF-β1, furin, and TNFAIP3 (88).

miR-28-5p: miR-28-5p are shown to be involved in cell proliferation, migration, invasion, and epithelial to mesenchymal transition (EMT) (89). miR-28-5p can silence PD1 genes and regulate the PD1, Foxp3 positive and TIM3, Foxp3 positive, exhaustive Treg cells (90).

miR-29a-3p: miR-29a-3p has a seven-nucleotide wide binding site on the 3'UTR of the MCL-1 gene and could be involved in the UC pathogenesis through regulating this gene. Mcl-1 gene knockout is shown to cause apoptosis in the colonic epithelial HT29 cells (91). Increased expression of miR-29a-3p in the colon tissues of patients with irritable bowel syndrome increased intestinal membrane permeability, regulating the GLUL gene (92). Moreover, miR-29a-39 is reported to regulate pro-inflammatory cytokine secretion and scavenger receptor expression *via* LPL targeting in ox LDL-stimulated dendritic cells (93).

miR-30d-5p and miR-30c-5p: Oral administration of miR-30d-5p mimic ameliorates experimental autoimmune encephalomyelitis (EAE) through expansion of Tregs. In *Akkermansia muciniphila*, miR-30d-5p regulates lactase expression and increases *Akkermansia* abundance in the gut. Consequently, *Akkermansia* increases Tregs to suppress EAE symptoms (94). miR-30c-5p regulates ATG5 expression by targeting the 3'UTR (95). The inverse correlation between miR-30c-5p and ATG5 is not only observed in CD patients and intestinal epithelial T84 cells infected with the adherent-invasive *Escherichia coli* (AIEC) (95). The NF-κB pathway was shown to be activated in AIEC infected T84 cells, which induced the up-regulation of miR-30c-5p and consequently inhibited the ATG5 expression (95). It has further been reported that the autophagic activity inhibition by miR-30c-5p increased AIEC persistence within T84 cells and increased pro-inflammatory cytokines production (95). miR-30c-5p is also believed to regulate Th17 cells differentiation by targeting its negative regulators such as SMAD2, SMAD4, TGFβR2, SOCS3, FOXO3, and TSC1 (96). Thus, their differential regulation might cause an increase or decrease in Th17 cell numbers. ETS1, BCL6, and STAT1 are also among the important targets of miR-30c-5p (96).

miR-31-5p: miR-31-5p showed a gradual upregulation from normal to IBD conditions and seemed to target FIH-1, the inhibitor of HIF-1α protein (97). Also, in psoriasis, miR-31-5p inhibition in keratinocytes was shown to suppress NF-kB–driven promoter-luciferase activity and production of IL-1β, CXCL1, and CXCL5. miR-31-5p regulates these cytokine and chemokine expressions in endothelial cells and attracts leukocytes *via* STK40 as its primary target (98). miR-31-5p also targets Gprc5a, which is shown to be a critical regulator for peripherally derived regulatory T cells generation. miR-31-5p conditional deletion enhances induction of these regulatory T cells and decreases the severity of experimental autoimmune encephalomyelitis (99). IL-13 is a necessary type-2 T-helper cytokine, controlling epithelium function through the IL-13 receptor -A1. It has been shown that the transfection of miR-31-5p and miR-155-5p mimics reduces the expression of the IL-13 receptor, increases and blocks the phosphorylation of STAT6, and the expression of SOCS1 and CCL26 in the gut epithelium cell line, and therefore may contribute to disease aggravation (33). Furthermore, miR-31-5p is differentially expressed in post-ablation epithelium with increased barrier permeability (100).

miR-106a-5p: Serum level of miR-106a-5p in both CD and UC patients correlates with disease severity (55). Upon T cell activation, while most miRNAs are downregulated, miR-106a-5p is upregulated (101). In addition, in macrophages, miR-106a-5p can regulate SIRPα synthesis and, therefore, SIRPα-mediated inflammatory responses (102). miR-106a-5p deficiency showed to promote Treg induction IL-10 production and attenuate adoptive transfer colitis in T cell restricted deficiency (103). In non-colonic cell lines, miR-106a-5p regulates IL-10 expression (103). Moreover, in CD4+ T cells, miR-106a-5p miRNA family deletion also attenuated the inflammation in lymphopenic recipients. Global knock-out of miR-106a-5p was also shown to attenuate chronic murine ileitis (104). TGFβ appears to suppress miR-106a under physiological conditions to aid Treg induction. TNFα, on the other hand, appears to drive upregulation of miR-106a-5p under inflammatory conditions through NF-κB-dependent induction of the miR-106a-5p promoter, resulting in temporary suppression of normal immune regulation (104).

miR-126-3p: IκBα as the inhibitor of NF-κB was shown to be markedly decreased in active UC tissues (105). miR-126-3p and IκBα expression are inversely correlated in patients with active UC. miR-126-3p is shown to contribute to UC pathogenesis through binding to the 3′- UTR of IκBα and inhibiting the NF-κB signaling pathway (105). Anti-inflammatory activities of the red wine polyphenols were partly mediated through miR-126-3p induction (106). Polyphenolic red wine extract (WE) inhibited inflammation in LPS-stimulated human colon-derived CCD-18Co cells by inhibiting NF-κB and down-regulating pro-inflammatory agents, including TNF-α, IL-6, and CAMs. miR-126-3p was upregulated upon WE treatment in these cells, and NF-κB and VCAM-1 showed downregulation (107). VCAM-1 is one of the miR-126-3p targets (108). miR-126-3p knockdown is reported to up-regulate the PIK3R2 in CD8+ T cells (109) and alter the PI3K/Akt pathway activation responsible for regulatory T cells reduced induction and suppressive function (109). Moreover, IκB, an inhibitor of NFκB, is another target of miR-126-3p (109).

miR-140-5p: miR-140-5p is shown to downregulate TLR4 by being directly bound to its 3′UTR, which inhibits inflammatory cytokines secretion (110). Moreover, it has been demonstrated that miR-140-5p inhibited IL-6 and IL-8 secretion by regulating TLR4 expression (110).

miR-141-3p: miR-141-3p is aberrantly expressed in IBD and other autoimmune diseases, including lupus and psoriasis (111, 112). miR-141-3p targets CXCL12β (113), an epithelial cell-expressed chemokine whose inverse correlation with miR-141 is shown in the inflammation. Therefore, it is suggested that targeting CXCL12β by miR-141-3p might influence inflammatory cell trafficking into the inflamed sites. Thus, inhibiting colonic CXCL12β expression and blocking immune cell recruitment might be valuable for the CD treatment (113). miR-141-3p is also reported to suppress STAT4, thus, inhibiting inflammatory factors (114). miR-141-3p upregulation reduces the IL-1β, TNF-α, and IL-6 levels, consequently attenuating the chronic inflammatory pain severity (115). Furthermore, during Th17 cell induction, miR-141-3p expression is reported to be significantly upregulated (116). miR-141-3p can also exert

protective effects on cell damage (114). It is also reported that miR-141-3p alleviates LPS-induced intestinal epithelial cell injury by inhibiting RIPK1-mediated necroptosis and inflammation (117).

miR-142-5p(-3p): In thymically derived Tregs, miR-142-5p is the predominant isoform. Tregs limit the development of autoimmunity by suppressing self-reactive peripheral T effector cell responses (118). miR-142-5p is shown to target SMAD3, CYR61 (119), and PD-L1 (120). Regulation of PD-L1 expression is through binding to its UTR and inversely correlated with miR-142-5p (121). TNF-α, IFN-γ, and IL-10, as prominent players in the immune response, are related to the PD-L1/PD-1 pathway. It has been shown that miR-142-5p overexpression results in TNF-α and IFN-γ upregulation and IL-10 downregulation (121). ATG16L1, as one of the most commonly detected genetic variations in CD patients, is predicted to be the target of miR-142-3p (122, 123). miR-142-3p negatively regulates ATG16L1 in CD colon epithelial cells. Upregulation of miR-142-3p reduced the autophagic activity of thymic-derived regulatory T cells by decreasing the expression of ATG16L1 (124). miR-142-3p binds directly to KDM6A (a lysine demethylase), resulting in the demethylation of H3K27me3, an epigenetic modification to the DNA packaging protein Histone H3. This, in turn, upregulates the expression of the anti-apoptotic protein Bcl-2. It has also been shown that antagomir-mediated knockdown of miR-142-3p can affect the induced regulatory T cells regulatory function, cytokine expression, and apoptosis through Foxp3 expression (125). Moreover, downregulation of miR-142-3p in macrophages of aged mice contributed to IL-6-associated aging disorders and consequently age-related inflammatory diseases (126).

miR-146a-5p and miR-146b-5p(-3p): miR-146a-5p has previously been shown to regulate the innate immune responses and TNF-α pathway in skin inflammation (127). miR-146a-5p deficient mice also develop immune disorders (128). In IBD, this miRNA regulates NOD2 derived gut inflammation and promotes proinflammatory cytokines released from activated macrophages (129). Moreover, upregulation of miR-146a-5p in monocytes in response to LPS resulted in the downregulation of TLR4 signaling pathway downstream genes (130). On the other hand, in mouse colitis, miR-146b-5p overexpression was shown to alleviate intestinal injury *via* NF-κB activation, epithelial barrier function improvement, and increased survival rate (131). miR-146b-5p seems to up-regulate NFκB *via* siah2 suppressing. Siah2 prompts TRAF proteins ubiquitination which is upstream of NFκB (131). miR-146b-3p, another member of the miR-146 family, is shown to negatively regulate lipid kinase PI3Kγ in (132), suppress proinflammatory ADA2, and block TNF-α secretion (133). Furthermore, miRNA-146b-3p expression is significantly downregulated by increased STAT3 activation (134).

miR-149-5p: Through targeting MyD88, miR-149-5p negatively regulates TLR triggered inflammatory cytokine production (135). MyD88 is involved in the TLR/NF-κB pathway. miR-149-5p is also associated with an increased IBD risk in the Chinese population (136).

miR-150-5p: miR-150-5p is proposed as one of the primary regulators of immune diseases (137), mainly through inhibiting inflammatory cytokines including IL-6, IL-1β, and TNF-α (138). It is also reported that the miR-150-5p upregulation in immune cells promotes the proliferation and maturation of myeloid cells and lymphocytes (139). c-Myb, a target of miR-150-5p, is reported to be significantly downregulated in UC patients' colon and DSS-treated mice. miR-150-5p overexpression is reported to enhance apoptosis through targeting c-Myb, which damages the intestinal epithelial barrier (140).

miR-155-5p: miR-155-5p has shown a central regulatory role in innate and acquired immune systems. miR-155-5p is expressed in response to inflammatory mediators such as LPS, TLR ligands, and IFN-β and is induced in antigen-presenting cells, including plasmacytoid dendritic cells and macrophages. It has also been found that antigen-stimulated B and T cells induce miR-155-5p expression (141). Moreover, SOCS1, a negative regulator for activation of LPS-induced macrophage, JAK/STAT signal pathway, and antigen presentation by dendritic cells, is one of the main targets of miR-155-5p (142). In addition, Anti-miR-155-5p has been reported to suppress G-CSF, a regulator of granulopoiesis produced by macrophages during acute inflammation (143). Increasing expression of the level of this miRNA has also been shown in other inflammatory disorders, such as rheumatoid arthritis (144), atopic dermatitis (145), and multiple sclerosis (146). In addition, it has been reported that miR-155-5p is an oncogene (147).

miR-192-5p: miR-192-5p is shown to target MIP-2α (CXCL2), a CXC chemokine expressed by epithelial cells and essential in murine and human IBD. miR-192-5p is downregulated in inactive UC and demonstrated an inverse correlation with MIP2-α. miR-192-5p mimic was reported to inhibit MIP2-α induced MIP-2a expression (26). miR-192-5p is induced by TGF-β and TNF-α (26, 39) and regulates the collagen and chemokine expression, which are critical in inflammation and fibrosis (148). miR-192-5p is also identified as a tumor suppressor that can induce cell cycle arrest (149).

miR-193b-3p: miR-193b-3p differential regulation has been detected in several autoimmune diseases (150), mainly through inflammatory chemokines regulation (151). miR-193b-3p has been shown to target TGF-β2 and TGFBR3 3′-untranslated regions (152) and contribute to Th17 cells differentiation by inhibiting the negative regulators of Th17 differentiation expression and possibly through regulating TLR and Notch signaling pathways. Thus, suggesting the possible involvement of miR-193b-3p in the inflammatory response and Th17 function (153).

miR-194-5p: miR-194-5p is abundant in intestinal epithelial cells (39) and is shown to regulate the MAP4K4/c-Jun/MDM2 signaling pathway (154). Overexpression of miR-194-5p in the liver mesenchymal cells reduced the N-cadherin (155). In the Caco-2 intestinal epithelial cell model, HNF-1α induced miR-194-5p suggesting the influence on epithelial cell differentiation (156).

miR-195-5p: miR-195-5p is shown to correlate with IBD severity. An increase in miR-195-5p level can decrease c-Jun and p65 expression. Instead, miR-195-5p decreased expression increases Smad7 expression and consequently p65 and the AP-

1 upregulation, which might explain the steroid resistance mechanism in some UC patients (157). miR-195-5p overexpression was shown to reduce M1-like macrophage polarization. miR-195-5p levels are reported as upregulated in M2c macrophages. LPS and IFN-γ stimulated THP-1 macrophages had reduced TLR2 levels following miR-195-5p overexpression. miR-195-5p also significantly decreased IL-1β, IL-6, and TNF-α levels in M1-stimulated macrophage supernatant cultures. In addition, levels of phosphorylated forms of p54 JNK, p46 JNK and p38 MAPK were shown to decrease by adding miR-195-5p in M1 macrophages upon stimulation. Altogether it seems like miR-195-5p is involved in macrophage polarization by inhibiting TLR2 inflammatory pathway mediators (158).

miR-199a-5p: miR-199a-5p showed significant upregulation in blood from UC patients compared with healthy controls (54). miR-199a-5p seems to suppress HIF-1α and SIRT1 and play a role in Treg cell differentiation by inhibiting genes involved in Th17 differentiation while activating others in Treg development (159, 160). RORγt is a lineage-specific transcription factor for Th17 differentiation. In multiple sclerosis, RORγt expression, a predicted target for miR-199a-5p (using miRWalk, miRTarBase, DIANA miRPath, UniGene), showed a significantly higher level in the relapsing phase versus remitting phase. This is consistent with the upregulation of miR-199a-5p, which correlates with lower Th17 cells and lower expression of RORγt in remitting phase (96). It has also been reported that miR-199-5p targets the activin A receptor type 1B gene that causes decreased CCAAT/enhancer-binding protein α expression and eventually monocyte/macrophage differentiation inhibition (161).

miR-200c-3p: miR-200c-3p plays a role in the FN1 post-transcriptional regulation; hence, EMT triggers by their downregulation (162, 163) most probably by regulating the E-cadherin transcriptional repressors ZEB1 and SIP1 (164). miR-200c-3p is reported to suppress the IL-6, CXCL9, and TNF-α expression (165). IL-6 intensifies inflammation through miR-200c-3p downregulation (166). In a macrophage-like human monocytic cell line exposed to the TLR4 ligand LPS, miR-200c-3p inhibits NF-κB activation in response to a TLR4 agonist. miR-200c-3p is known to regulate the TLR4 signaling efficiency through the MyD88-dependent pathway (167).

miR-223-3p: miR-223-3p is shown to be involved in the activation of granulocytes and is overexpressed in naive CD4+ T-lymphocytes (168). Furthermore, the downregulation of miR-223-3p in primary macrophages increased TLR4 and STAT3 basal expression and LPS-stimulated TLR4, STAT3, and NOS2 expression. On the contrary, miR-223-3p mimics treatment in primary macrophages has decreased TLR4 expression while negatively regulating FBXW7 expression, a well-known suppressor of TLR4 signaling. Based on these outcomes, it is concluded that miR-223-3p abundance in macrophages can change macrophage activation and modulate the response to stimuli *via* effects on the TLR4/FBXW7 axis (169). It has also been shown that miR-223-3p mediates the cross-talk between the intestinal barrier and the IL-23 pathway by targeting CLDN8, a claudin protein that constitutes the backbone of the intestinal barrier (170). miR-223-3p has also been used as a biomarker in IBD (3). Thus, the evidence suggests its proinflammatory role and highlights its potential as a RNA biomarker that seems to be conserved between different species. miR-223-3p is also produced by neutrophils and monocytes and acts as a controller of NLRP3 inflammasome activity, regulating the intestine inflammatory process by affecting IL-1β production (171).

miR-375-3p: miR-375-3p is reported to be downregulated in the intestinal mucosa of UC and CD patients. TLR4 is one of the main targets of miR-375-3p with inverse correlation. miR-375-3p mediated upregulation of TLR4 induces NF-κB activation, which leads to an increase in pro-inflammatory factors (172). Intestines show a high level of miR-375-3p expression. Cell death, including apoptosis and/or necrosis, results in the miR-375-3p leak from cellular to extracellular space, eventually ending in the blood. Therefore, it is suggested that elevated miR-375-3p in serum may be a predictor of tissue damage (173).

miR-378a-3p: miR-378a-3p expression is reported to be inversely correlated with IL-33 expression; IL-33 is a predicted target of miR-378a-3p (174). miR-378a-3p is highly conserved between species, but not IL-33 (175). The miR-378a-3p is located in intron 1 of the PPARGC1B gene that is differentially regulated in UC patients' intestinal mucosa [26]. PPARGC1B protein is highly expressed in the intestinal epithelium (176) and is involved in the control of mitogenesis and mitochondrial metabolism (177), energy production, and biogenesis (178). Therefore, it can be concluded that in inflamed mucosa, the miR-378a-3p decrease might reflect a metabolic shift, possibly related to the increment of energy expenditure and ROS overproduction (179).

miR-424-5p: miR-424-5p is shown to control monocyte/macrophage differentiation. miR-424-5p expression upregulation is regulated by transcription factor PU.1. When upregulated, miR-424-5p induces monocyte differentiation *via* NFI-A inhibition (180) as its main target.

miR-532-3p: miR-532-3p acts as an antagonist for LPS/TNF-α stimulated macrophages by targeting the ASK1/p38 MAPK signaling pathway, thus suppressing the inflammation, which is mediated through this pathway. Thus, it has been suggested as a potential target for treating autoimmune inflammatory diseases (181).

CONCLUDING REMARKS

Early diagnosis and treatment are vital in IBD, as induction of early remission and maintenance can prevent long-term complications and eliminate the need for surgery. However, due to insufficient clinical sensitivity and specificity of current biomarkers and a large population of patients with functional bowel disorders, there is often a delay in the confident diagnosis of IBD and its sub-classification into either UC or CD (182). At the same time, the primary way to overcome IBD is to induce and maintain early remission. Most current IBD diagnostic tests reflect generalized inflammation and do not discriminate between IBD subtypes (182).

Since their discovery, thousands of miRNAs have been identified. Accumulating evidence suggests that specific miRNA expression signatures contribute to the IBD development and progression. Most studies reveal correlations between IBD and differentially expressed miRNAs instead of causal relationships. As discussed above, only a few studies investigate the underlying molecular mechanisms of the disease; thus, the precise function of most miRNAs in IBD has yet to be clarified. Furthermore, there has been a lack of reproducibility between studies, partly ascribed to a lack of standardized study designs and different approaches.

Moreover, many variables differ between studies, including age, sex, various treatment regimens, disease activity level and duration, having different control groups, sampling from different anatomic locations, sampling method, preservation and processing of the samples, and the different criteria for measuring expression fold change and significances (e.g., different FC, log FC, p-value and p-adj criteria). Thus, it is essential to understand the conditions under which a differentially expressed miRNA was discovered. For instance, epigenetic regulations are among the primary factor stimulated by the environment. Stimuli such as diet, lifestyle, work condition, and stress are elements as important as the clinical and technical manifestations of signs of disease. Regardless of these differences, while being aware of them, in this review, we attempted to identify and give an overview of the most frequently differentially expressed miRNAs in colon and blood of both UC and CD across multiple studies from literature and meta-analysis and further described the roles of selected miRNAs in the disease pathogenesis and their connection to IBD.

For biomarker studies, circulating miRNAs (of saliva, serum, urine, plasma, and other body fluids) attracted great interest as non- or semi-invasive clinical biomarkers mainly due to ease of access, stability, conserved structure, and ease of detection by quantitative approaches like real-time PCR. The need for endoscopic examination and invasive sampling of biopsies limit the use of colonic miRNAs as biomarkers. Thus, if a miRNA demonstrates a similar consistent differential regulation in colonic biopsies and blood of the IBD patients compared with healthy control, it can be used as a proper disease biomarker signature. miR-223-3p, in this case, might be an excellent example of such miRNAs. This miRNA is significantly differentially expressed in both UC and CD in blood and tissue biopsies and thus can be considered a reliable IBD biomarker candidate.

Anti-cytokines therapies have been relatively successful; however, not all patients respond to these treatments. As important post-transcriptional gene regulators, miRNAs were shown to contribute to disease aggravation through immune responses, inflammation, mucus barrier, and epithelium function dysregulation; thus, miRNA-based therapy might be developed as a potential therapeutic approach. In this case, miRNAs complementary antisense oligonucleotides or miRNA mimics can be potential therapeutics that abolish or mimic miRNA's function and, therefore, block inflammatory progression, modulate cytokines or chemokine hemostasis and increase the treatment sensitivity of conventional therapies. As such, miRNAs are used for modulating hypoxia (183, 184) and the inflammatory response by targeting major inflammatory pathways (185–189) and essential molecules, including tight junction proteins that maintain the integrity of the membrane (74, 190, 191).

FUTURE PERSPECTIVES

Although progress has been made towards understanding the role of miRNAs in IBD pathophysiology, many conditions and many more miRNAs remain insufficiently characterized for diagnostic and therapeutic applications, partly as it is still a relatively young field. Also, as a chronic disease with flare-ups and remission, besides comparing disease versus control, it is relevant to look at disease subgroups, e.g., the differences between active/inactive and inflamed/not inflamed intestinal regions. While some studies grouped patients into active UC, inactive UC, inflamed UC, and non-inflamed UC, still further studies are needed to improve our understanding. In addition, it remains to be determined how associations with IBD risk loci might affect miRNA's expression and the disease phenotype. Moreover, although it has been less focused on, the disease activity index can also be assessed by profiling miRNA specifically at different disease stages while maintaining that miRNA expression is often tissue or pathology specific.

Due to the IBD complexity and the lack of consistency between miRNA signatures, it is difficult to diagnose the disease, identify the subtypes, and monitor the disease status or location using a single or even a panel of miRNAs. Although there is an imperious need for faster ways to validate miRNAs as biomarkers, the sensitivity and specificity of miRNA candidates should be checked in large-scale studies to avoid false positive or false negative diagnosis.

Differentially expressed miRNAs profiling can be a valuable indication of phenotypic changes in IBD, showing an obvious correlation with disease evolution. However, differential expression *per se* does not indicate the ultimate role of the identified miRNAs in disease pathophysiology, as there are complex networks of interaction between miRNAs and their targets that also depend on the cell type, location, and tissue condition. It is noteworthy that many miRNAs might have the same target. Thus, when it comes to the therapeutic interventions using the miRNAs, the main issue is the side effects of miRNA-based drugs that need to be considered in extensive validation studies before miRNAs can enter the market and be incorporated into clinical practice. Also, miRNA expression as measured on high-throughput platforms, e.g., RNA-sequencing, has limitations. For example, if a highly expressed target is downregulated, the expression of the miRNA will appear as increased despite the miRNA being processed at the same rate, i.e., miRNA itself is not directly regulated. Extending miRNA analysis to be "target context-aware" rather than looking at miRNA solely from small RNA-sequencing will likely shed more nuances on to cause and effect of regulated miRNAs and

thereby pave the way for considering miRNAs in diseases. Despite the present limitations, we anticipate that miRNAs application and targeting will become routine diagnostic and therapeutic approaches in clinical settings as current techniques evolve rapidly.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. All authors contributed to the article and approved the submitted version.

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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.2022.865777/full#supplementary-material

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Immune Dysfunction Mediated by the ceRNA Regulatory Network in Human Placenta Tissue of Intrahepatic Cholestasis Pregnancy

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Pregnancy-related intrahepatic cholestasis (ICP) is a serious complication with adverse perinatal outcomes of preterm labor, fetal distress, or stillbirth. As a result, it is important to investigate and identify the potential critical pathogenic mechanisms of ICP. First, we collected the placental tissues from the ICP with placental weight and fetal birth weight loss for the whole transcriptome sequencing. Then we analyzed the differentially expressed (DE) circRNAs (DEcircRNAs) by SRPBM, DElncRNAs by FRKM, DEmiRNAs by TPM, and DEmRNAs by TPM and RSEM. Based on differential expression of term pregnancy placental tissues from pregnancies impacted by ICP (n=7) as compared to gestational aged matched control tissues (n=5), the circ/lncRNA-miRNA-mRNA competitive endogenous RNA (ceRNA) regulatory networks were constructed. The ceRNA regulatory networks covered 3,714 events, including 21 DEmiRNAs, 36 DEcircRNAs, 146 DElncRNAs, and 169 DEmRNAs. According to the functional analysis, ICP complications were linked to the immune system, signal transduction, endocrine system, cell growth and death, and transport and catabolism. Further evidence suggested that the expression of immune-related genes *KLRD1*, *BRAF*, and *NFATC4* might have a potential ceRNA mechanism by individual lncRNA sponging miR372-3p, miR-371a-3p, miR-7851-3p, and miR-449a to control downstream the level of TNF-α, IFN-γ, and IL-10, thereby regulating the pathophysiology of ICP. Furthermore, our results were validated by the qRT-PCR, western blotting and ELISA assays. In conclusion, this study is the first to evaluate placental ceRNA networks in pregnancies affected by ICP, showing alterations in immune regulatory networks which may impact fetal and placental growth. Overall our these data suggest that the ceRNA regulatory network may refine biomarker predictions for developing novel therapeutic approaches in ICP.

Keywords: intrahepatic cholestasis of pregnancy, ceRNA, immunity-related molecules, birth weight, placenta weight, bioinformatics analysis

INTRODUCTION

The competitive endogenous RNA (ceRNA) hypothesis that protein-coding messenger RNAs (mRNAs) and noncoding RNAs (ncRNAs) crosstalk with and regulate each other by microRNA response elements (MREs) competing for binding to common miRNAs (1, 2). MREs were known as the guide strand, retained within miRNA-induced silencing complex (miRISC), targeted to mRNA with partially complementary sequences (3, 4).ceRNAs serve as endogenous sponges,the competitive inhibitors of miRNA function, showing another novel layer of posttranscriptional regulation (1, 5). Recently, many researchers have focused on the ceRNA interactions which uncover a novel mechanism and play important roles in differentiation (2, 6, 7), cancer (8–11), immune-related diseases (12), cardiovascular diseases (13, 14), neurological diseases (15, 16), etc. Linc-MD1 "sponges" miR-133 and miR-135 to regulate the expression of transcription factors MAML1 and MEF2C, then to governs the time of muscle differentiation in mouse and human myoblasts (2).The lncRNA H19 functions as a ceRNA to sponge miRNA let-7 family leading to an increase in expression of let-7 targets in breast cancer,ovarian cancer and pancreatic cancer (8).Notably, ceRNA also acts crucial role in reproductive health (17), such as fetal and organ development (18), spontaneous abortion (19–21) and eclampsia in pregnancy (22). In pre-eclampsia, circVRK1 acts as a ceRNA to miR-221-3p to regulate PTEN, and further inhibit PI3K/Akt activation, thereby suppressing trophoblast cell migration, invasion and EMT (22). Therefore, most ceRNA interactions between mRNAs are linked to various disease states, but few have been linked to pregnancy-related diseases and pregnancy complications, ICP in particular.

Intrahepatic cholestasis of pregnancy (ICP) is a complication in 0.2-2% of pregnancies, characterized by maternal pruritus and elevated serum bile acids, transaminases, and occasionally, bilirubin (23–25). Its causative mechanism remains unknown, and the studies available are associated with hormonal, immunological, genetic, and environmental factors during pregnancy (26, 27). Ursodeoxycholic acid is controversial as a treatment, though it improves biochemical parameters (23). As a result, there is no effective treatment for ICP (23). ICP has been considered a benign and reversible disease for mothers, but perinatal babies, suffering the severe adverse pregnancy outcomes of fetal distress, spontaneous and iatrogenic preterm birth, and stillbirth (23, 28). Interestingly, recent studies have suggested that women with ICP increased the risk of later hepatobiliary cancer and immune-mediated and cardiovascular diseases (29).

The placenta is a temporary mammalian organ that connects the maternal and fetal circulatory systems. Molecules produced by the placenta contribute to fetal developmental programming and support the maternal organism to cope with the response of pregnancy (30). The placental-associated gene expression alterations may lead to its aberrant function and pregnancy complications (31–34). Therefore, a comprehensive, in-depth, and systematic understanding of the alterations and their associated actions in placental tissues is of great significance for making out the pathogenesis and adverse perinatal outcomes of ICP. Based on the principles of ceRNA regulation in pregnancy-related diseases, the whole transcriptome sequencing of placental tissues of ICP was done at the first time to profile the DEcircRNAs, DElncRNAs, DEmiRNAs, and DEmRNAs, construct the ceRNA regulatory networks, and explore the capability of ceRNA in the process of ICP.

MATERIAL AND METHODS

Data Resource

From January 2021 to June 2021, seven ICP pregnant women and five women as normal control (NC) were enrolled and delivered by cesarean section at the Maternity Center of the Third Affiliated Hospital of Chongqing Medical University. The inclusion criteria for the ICP group were: fasting serum total bile acid (TBA) level ≥10 μmol/L; with or without the presence of pruritus; elevated glutamate transaminase (ALT) and alanine transaminase (AST) with unknown causes; and the above symptoms and laboratory parameters disappeared after delivery. The Inclusion criteria for the control group were: no complications or comorbidities of pregnancy; no previous history of preterm birth, macrosomia, or low birth weight babies. Exclusion criteria: the existence of pre-pregnancy liver, biliary and pancreatic diseases, autoimmune diseases, combined hypertension during pregnancy, gestational diabetes or other pregnancy complications, and medical and surgical comorbidities. The indications for this cesarean section were: patients and family's request or scarred uterus. The study was approved by the Hospital Medical Ethics Committee (202107), and informed consent was followed for each pregnant woman participating in the experiment.

Sample Collection

Samples were obtained from the villous placenta, mid-way between the chorionic and basal plates, at four different positions within 5 minutes after placental separation during cesarean delivery. These placental tissues were washed with DEPC water to remove residual blood as possible, weighed and then placed into an RNA later solution or empty centrifugal tube and stored at -80°C or in liquid nitrogen.

RNA-Seq

According to the manufacturer's instructions, total RNAs were isolated using the RNeasy Plus Universal Mini Kit (Qiagen). High-quality RNA samples (OD260/280 = 1.8~2.2, OD260/230≥2.0, RIN≥8, 28S:18S≥1.0, >10 μg), verified by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the ND-2000 (NanoDrop Technologies), were constructed the sequencing library. Total RNAs (5 μg) were obtained following TruSeqTM stranded total RNA Kit from Illumina (San Diego, CA) to prepare for the transcriptome strand library. Firstly, ribosomal RNA (rRNA) was depleted with Ribo-Zero Magnetic kit and then fragmented by fragmentation buffer. Next, the first-stranded cDNAs were synthesized by random hexamer primers. After removing RNA templates, the ds cDNAs were generated

with dUTP in place of dTTP. Those ds cDNAs were isolated by AMPure XP beads with a single 'A' nucleotide added at 3' ends of the blunt fragments. Finally, multiple indexing adapters were ligated to the ends of the ds cDNAs. The 200–300 bp cDNA target fragments were selected, amplified, and quantified by TBS380. The RNA-seq library was sequenced by the Illumina HiSeq xten//NovaSeq6000 (2 × 150 bp read length). Additionally, sequencing adapters were ligated to total RNAs (3 μg) with TruseqTM Small RNA sample prep Kit (Illumina, San Diego, CA, USA). The ligated RNAs were transcribed to cDNA, then amplified (12 cycles) for libraries, quantified, and constructed by deep sequencing using Shanghai Majorbio Bio-Pharm Biotechnology Co., Ltd. (Shanghai, China).

Read Mapping and Transcriptome Assembly

The raw paired-end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then clean reads of RNA-seq were aligned to the human reference genome with orientation mode using HIASAT (https://ccb.jhu.edu/software/hisat2/index.shtml) software. StringTie (https://ccb.jhu.edu/software/stringtie/index.shtml?t=example) was used to assemble transcripts. Raw counts for annotated genes (protein-coding genes, rRNA, microRNA, LncRNA) in the General Transfer Format (GTF) annotation file was obtained.

Principal Component Analysis

To reveal the RNA-seq profile of placenta from ICP and normal pregnant women, we performed principal component analysis, and PC1-PC3 was used to correct and distinguish those samples.

Identification of Differentially Expressed (DE) RNAs

We analyzed differential expressed genes (DEGs) between the ICP and normal pregnant women (as a reference). TPM method was calculated the expression level of each transcript, and RSEM was quantified for gene abundances (http://deweylab.biostat.wisc.edu/rsem/). The *DESeq2/DEGseq/EdgeR* with adjusted *P*-value were used together to determine whether a gene is differentially expressed. If adjusted *P*-value ≤ 0.05 (*DESeq2 or EdgeR*), differential expressed mRNAs (DEmRNAs) with fold change > 2 or < -2, the gene was considered differentially expressed between two groups of samples.

The Coding Potential Calculator (CPC), Coding-Non-Coding index (CNCI), and Coding Potential Assessment Tool (CPAT) were applied to filter transcripts with coding potential. Then according to Pfam HMM, those transcripts with known protein domains were excluded by Pfam Scan. FRKM method was used to calculated the expression level of each lncRNA, and RNAs with |log2FC| >1 and FDR < 0.05 by EdgeR were thought to be significant differently expressed lncRNAs (DElncRNAs).

The CIRI (CircRNA Identifier) tools were used to identified circRNA and eliminate false positive candidates resulting from incorrectly mapped reads of homologous genes or repetitive sequences. Each circRNA's expression level was calculated by Spliced Reads per Billion Mapping (SRPBM) method. CircRNAs were extracted with |log2FC| >1 and *P*-value < 0.05 by DEseq and to construct the significant differently expressed circRNA set (DEcircRNAs).

Low-quality bases (Sanger base quality of < 20) of the 3' end and sequencing adapters were removed with the in-house perl scripts and the fastx toolkit software, respectively. All sizes ranging from 18 to 32 nt were eliminated from the initial data set. The non-miRNA sequences (rRNA, tRNA, snoRNA, etc.) were removed by a BLAST search of the Rfam database, version 10.1 (http://rfam.sanger.ac.uk/). The perfectly matched sequences from the BLAST search of the miRbase (version 21.0), were used to count and analyze the known miRNA expression profile. The hairpin structure of miRNA precursor can predict novel miRNA. Each miRNA's expression was calculated according to the transcripts per million reads (TPM) method. If $|\log_2 FC| > 1$ and FDR < 0.05 by DEseq2, the miRNAs were defined as differently expressed miRNAs (DEmiRNAs).

Gene Ontology (GO) and KEGG Annotation Analysis

To profile gene functions, we performed Gene Ontology Annotation for Gene lists. We performed annotation analysis for GO and KEGG pathways Annotation for Gene lists.

CeRNA Network

The psRobot was used to predict the lncRNA-miRNA-gene pairs and circRNA-miRNA-gene pairs. The Pearson correlation analysis was used to determine any positive correlations between DEcircRNAs, DEmiRNA, DElncRNAs, and DEmRNA in the ceRNA regulatory network. DElncRNAs targeted DEmRNAs, and interacted miRNAs were deleted from the ceRNA network in the opposite expression pattern between DElncRNAs and the targeted DEmRNAs. Hmisc and complot packages were used to compute and visualize the correlations. Those RNAs with Pearson correlation coefficients greater than 0.5 and $P < 0.01$ were employed. The ceRNA network was constructed by Cytoscape and presented by Sankey plot using the galluvial R package.

qRT-PCR Verification

According to the manufacturer's instructions, total RNAs were extracted by TRIzol reagent (Invitrogen) from individuals subjected to ICP patients and controls. The RNA was purified and reverse transcribed to cDNA by PrimeScript RT Reagent Kit (Takara). Finally, qRT-PCR was done with specific primers (**Table S10**) by TB Green Fast qPCR Mix (Takara): 95°C for 30 s, 40 cycles, 95°C for 10 s, 60°C for 30 s. Statistical analyses were carried out using GraphPad Prism software (version 7.0). All *P*-values are two-sided. $P < 0.05$ was considered statistically significant.

Western Blotting

The placenta tissues were taken from the liquid nitrogen tank and homogenized by grinding the tissue in liquid nitrogen. The homogenate was collected, added to the tissue lysis buffer

(P0013, Beyotime) with PMSF and cocktail, and sonicated (10s, 30s, 5-10 cycles) to make the tissue fully lysed. The supernatant was collected by centrifugation (12000 rpm/min, 10-15 min) and set aside at -80°C. The proteins were boiled with 5×SDS loading buffer, resolved by SDS-PAGE, and measured by indicated antibodies and anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase. Specific bands were visualized by enhanced chemiluminescence (ECL). Antibodies against the following epitopes or proteins were obtained from the indicated suppliers: NFATC4 (ab3447, Abcam), BRAF (20899-1-AP, Proteintech) and GAPDH (60004-1-Ig, Proteintech).

ELISA

The placenta tissues (removed residual blood) were taken from the liquid nitrogen and were mashed by tissue mashers (10000-15000r/min). And the pre-chilled PBS (0.01M, pH=7.4) with PMSF and cocktail were added to the homogenizer. Then the homogenate was centrifuged at 5000×g for 5-10 min and the supernatant was collected for ELISA. The levels of secreted IL-2, IL-10, IFN-γ, and TNF-α in placenta tissue were detected by ELISA kits IL-2 (mlbio, ml058063, China), IL-10 (mlbio, ml064299, China), IFN-γ (mlbio, ml077386, China), and TNF-α (mlbio, ml077385, China), according to the manufacturer's instruction.

RESULTS

Clinical Data on ICP Placenta

As shown in **Figure 1**, the TBA of ICP was significantly higher than that of NC ($P < 0.001$) (**Figure 1A** and **Table S1**). ALT ($P < 0.045$) and AST ($P < 0.079$) were slightly elevated in the ICP group compared with that in the control group, although there is no statistical significance in AST (**Figures 1B, C** and **Table S1**). No significant statistical difference has been found in the maternal age (y24-30 vs. y25-32), gestational age at birth/abortion (weeks, 37-39.3 vs. 38.3-39.3), and pre-pregnancy BMI (kg/m^2, 19.5-24.0 vs. 19.2-24.2) between the ICP and NC group (**Figures 1D–F** and **Table S1**). Of note, the birth weight ($P < 0.036$) and placenta weight ($P < 0.039$) of ICP losses significantly compared to that of the NC group (**Figures 1G,H** and **Table S1**). In summary, clinical data showed that the weight loss of the fetus and placenta arose in ICP compared to that in the NC groups.

RNA-Seq of Human Placenta Tissue

RNA-Seq results for twelve tissues (including seven ICP and five normal placenta tissues) were used for the comprehensive analysis. Information and quality of sequencing data are shown in **Table S2**. The reads distribution of 12 samples is shown in **Figures S1-4**. The datasets for the long RNA (circRNAs/mRNAs/mRNAs) and small RNA (miRNA) from ICP and NC groups were distinguished after normalization (**Figures 2A, B**). Total 1447 significant differentially expressed mRNAs (DEmRNAs) (794 upregulated and 653 downregulated), 157 significant DEcircRNAs (91 upregulated and 66 downregulated), 675 DElncRNAs (575 upregulated and 100 downregulated), and 27 significant DEmiRNAs (13 upregulated and 14 downregulated) were displayed in detail on **Tables S3-6**. In addition, DEmRNAs, DElncRNAs, DEcircRNAs, and DEmiRNAs were presented by volcano plots (**Figure 2C**) and heatmaps (**Figure 2D**), respectively.

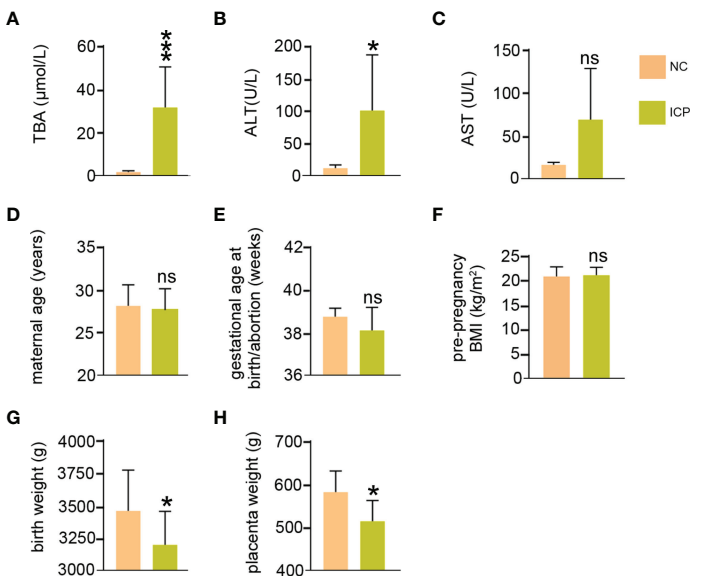

FIGURE 1 | The clinical data of pregnant women with ICP. **(A)** Maximum TBA levels during pregnancy of ICP group and normal group. **(B, C)** Maximum ALT and AST levels during pregnancy. **(D)** Maternal age at the time of termination of pregnancy. **(E)** Gestational age at birth. **(F)** Pre-pregnancy body mass index. **(G)** Fetal weight at birth. **(H)** Placenta weight at the time of termination of pregnancy. ns means not statistically significant *$P < 0.05$; vs. normal group.

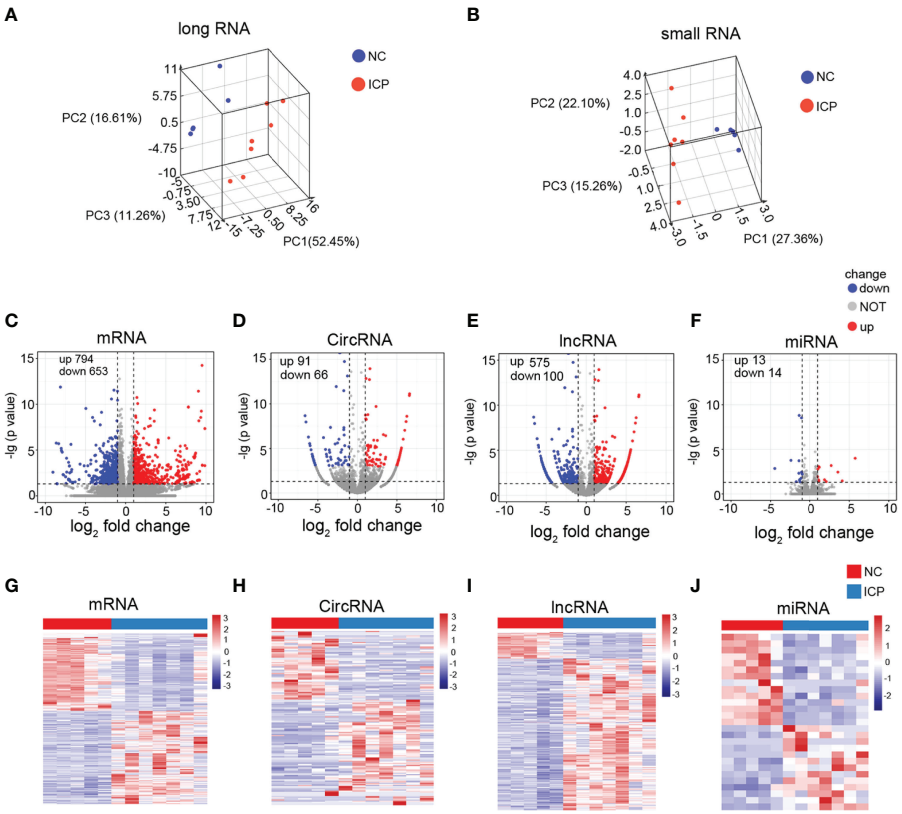

FIGURE 2 | Identification of the DERNAs in placenta tissue of ICP. **(A, B)** Box plots: the distributions of the datasets for long RNAs **(A)** and small RNAs **(B)** of twelve samples. **(C-F)** Volcano plots of the DEmRNAs **(C)**, DEcricRNAs **(D)**, DElncRNAs **(E)**, and DEmiRNAs **(F)** (red, up-regulated; blue, down-regulated). **(G-J)** Heatmaps for DEmRNAs **(G)**, DEcricRNAs **(H)**, DElncRNAs **(I)**, and DEmiRNAs **(J)** (red, up-regulated; green, down-regulated).

Construction of the ceRNA Regulatory Network

Long non-coding RNA (lncRNA), circular RNA (circRNA), and microRNA (miRNA) play prominent roles in pregnancy-related diseases (35–38). The ceRNA networks, composed of the lnc/circ/miR/mRNA, have been rarely reported in pregnancy-related diseases and pregnancy complications, especially in ICP. As a result, 157 DEcircRNAs, 675 DElncRNAs, 27 DEmiRNAs, and 1447 DEmRNAs were used to construct ceRNA networks to identify and investigate their roles in ICP. The ceRNA events occurred 3714, involving 21DEmiRNAs, 36 DEcircRNAs, 146 DElncRNAs, and 169 DEmRNAs (**Figure 3** and **Table S7**). The candidate ceRNAs might provide a comprehensive and illuminating insight into the molecular mechanisms of ICP.

Functional Analysis of the DEmRNAs in ceRNA Regulatory Network

Those 169 DEmRNAs (57 upregulated and 112 downregulated) from the circRNA/lncRNA-miRNA-mRNA ceRNA regulatory networks were analyzed and conducted with KEGG and GO annotation analysis (**Tables S8-9**). GO annotations showed that the DEmRNAs were mainly involved in cell part, binding, and cellular process to regulate cellular component, molecular function, and biological function (**Figure 4A**). Notably, these mRNAs of KEGG analysis were mostly enriched in signal transduction, endocrine system, immune system, cell growth, and death, as well as transport and catabolism (**Figure 4B**). The signal transduction pathways included MAPK, PI3K-AKT, mTOR, and Wnt signaling pathways, participating in immunomodulation, protein synthesis, and survival (**Table S9**). The endocrine system involved the estrogen signaling pathway, affecting apoptosis, cell adhesion, cell membrane components, and cytoplasmic signaling cascade response (**Table S9**). The immune system caused the changes of cytokines and chemokines (**Table S9**). Cell growth and death mainly caused cellular apoptosis, necroptosis, and ferroptosis (**Table S9**). Moreover, it was of great interest that both *BRAF* and *NFATC4* shared the top 5 transcript collections in GO and KEGG analysis (**Tables S8-9**, colored yellow). BRAF promotes the release of cytokines such as TNFα, GM-CSF, and IFN-γ (map04650), while NFATC4 alters the level of IL2 and IL10 (map04625) (**Figure 4C**). Previous studies have established that elevated TNFα and IFN-γ can damage the fetus and placenta (39, 40). As shown in **Figure 4C**, KLRD1 is the component of the multiple immune complexes, which trigger the immune response. Therefore, the immune dysfunctions mediated by the ceRNA regulatory network (BRAF- and its upstream KLRD1-

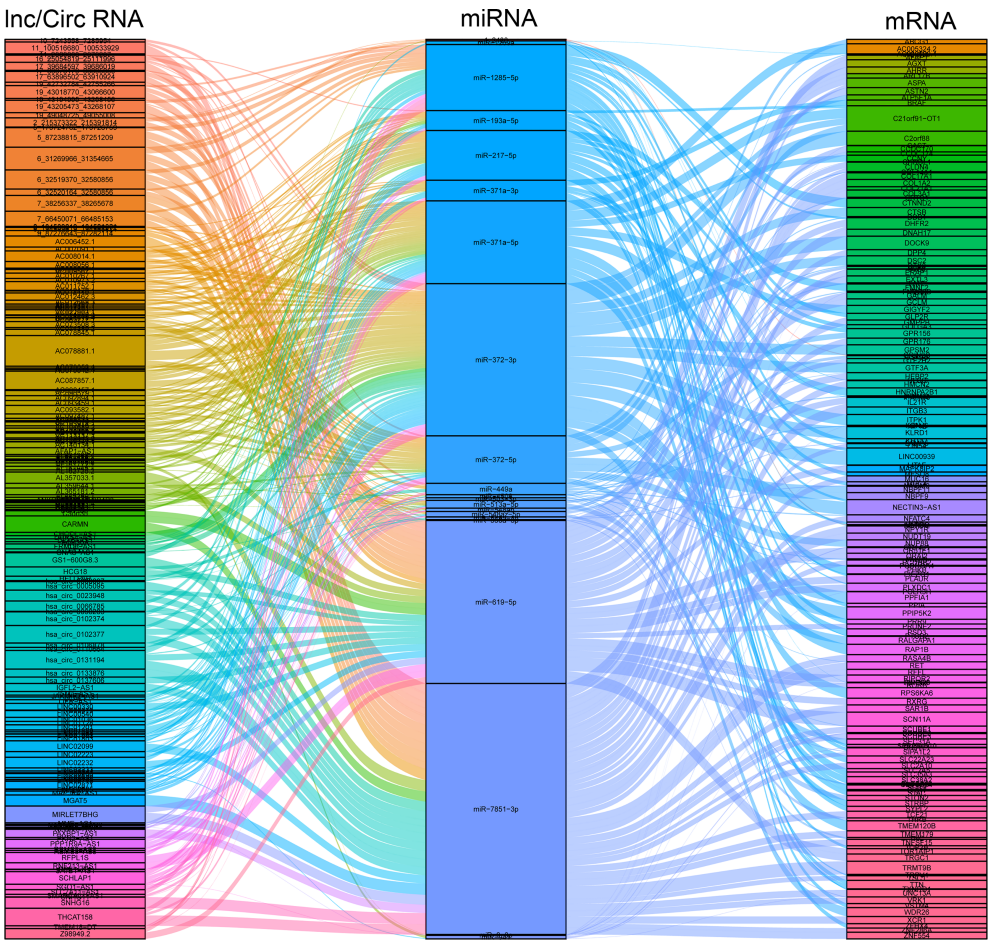

FIGURE 3 | Construction of the circ/lncRNA-miRNA-mRNA ceRNA regulatory network. The ceRNA regulatory network included 21DEmiRNAs, 36 DEcircRNAs, 146 DElncRNAs, and 169 DEmRNAs. ceRNA, competing endogenous RNA; circRNAs, circular RNAs; miRNAs, microRNAs; DE, differentially expressed.

and NFATC4-dependent ceRNA) offered novel sight and approaches for the progression of ICP.

The Validation of Differentially Expressed Genes in the ceRNA Network

The represented ceRNA events were chosen for qRT-PCR validation of placental tissues in clinical specimens. The ceRNA regulatory networks of KLRD1, BRAF, and NFATC4 were illustrated in **Figure 5A**, and their expressions obtained from RNA-seq were shown in **Figure 5B**. The results of qRT-PCR that the expression of lncRNA (XR_923862.2, XR_001740591.2, XR_001745862.1), miRNA (miR372-3p, miR-371a-3p, miR-7851-3p, and miR-449a), mRNA (KLRD1, BRAF, and NFATC4), and the downstream cytokines and chemokines (TNFα, IFN-γ, and IL-10) in the placental tissues (NC n=3, ICP n=3) were consistent with the sequencing data (**Figures 5C**). To further solidify our conclusions, we examined the protein expression levels of NFATC4 and BRAF in placental tissues and found that, consistent with mRNA levels, NFATC4 expression was decreased and BRAF was elevated in ICP placental tissues (n=7) (**Figure 5D**). Besides, the inflammatory factors IL-10 decreased, IFN-γ and TNF-α increased. Also, IL-10 and IFN-γ showed significantly statistical differences in ICP placental tissues (n=7) compared to that in healthy controls (n=5) (**Figure 5E**). Altogether, ceRNA networks were involved in the process and adverse perinatal outcomes of ICP.

DISCUSSION

Our clinical data provided evidence of the weight loss of the placenta and fetus in ICP compared to normal pregnant women, suggesting that ICP retrained placental development and fetal growth (**Figure 1**). The whole transcriptome sequencing of placental specimens from ICP patients was performed to better investigate the mechanisms of placental growth and fetal development during ICP. A novel aspect of our study was construction of ceRNA regulatory networks from analysis of differential expression libraries of mRNA, circRNA, lncRNA and miRNA in placental tissues from pregnancies affected by maternal

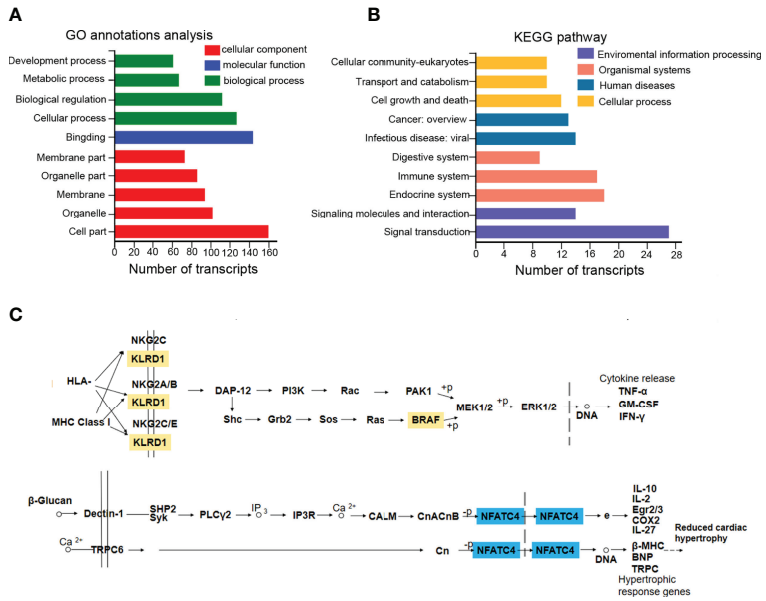

FIGURE 4 | The functional analysis of DEmRNAs in the ceRNA regulatory network. **(A, B)** 169 DEmRNAs were presented in GO and KEGG annotation analysis. **(C)** KEGG annotated diagram of the signaling pathways. Up-regulated labeled in orange and down-regulated labeled in blue.

ICP (**Figures 2**, **3**). These data supported that the role of ceRNAs (lnc/circRNA-miRNA-mRNA) was one of the major contributors to ICP. Futher, ceRNA serving as the competitive inhibitors of miRNA function, has been well established among various diseases including pregnancy-related disorders *via* celluar models and annimal models. In polycystic ovary syndrome (PCOS), lncRNA MALAT1 reduction could suppress TGFβ signaling through sponging miR-125b and miR-203a in granulosa cells (41). In unexplained recurrent spontaneous abortion, circRNA-DURSA/miR-760/HIST1H2BE axis, lncRNA-HZ04/miR-hz04/BPDE axis, and lncHZ05/miR-hz05/BPDE axis were proved to affect human trophoblast cell proliferation and apoptosis (42–44). In preeclampsia, lnc00511 was functioned as a molecular sponge for miR-29b-3p, antagonizing its ability to repress Cyr61 protein translation (45).

Further, the GO and KEGG analysis evidenced that BRAF and NFATC4 shared the top 5 transcript collections (**Tables S8-9**, colored yellow) and regulated the cytokines mediated immune dysfunction (map04650 and map04625). In the course of normal pregnancy, helper T (TH) cell type 1 cytokines are downregulated and TH type 2 cytokines are upregulated at the maternal-fetal interface, aimed at protecting the fetus from cytotoxic T cell responses which are associated with fetal rejection and pregnancy loss (46, 47). It has been confirmed that ceRNA involved in the differentiation of T cell subtypes, which was a side argument to our conclusion. LncRNA SNHG16/miR-16-5p/SMAD5-regulatory axis potentiates TGF-β1/SMAD5 pathway activation, thus inducing CD73 expression in Vδ1 T cells in breast cancer-derived exosomal (48). LncITSN1-2 has been demonstrated that it promotes IBD CD4 + T cell activation, proliferation, and Th1/Th17 cell differentiation by serving as a ceRNA for IL-23R *via* sponging miR-125a in inflammatory bowel disease (IBD) (49). Additionally, KLRD1 and BRAF in our study ascend the TH1-type cytokines such as TNF-α and IFN-γ (**Figures 4**, **5**), and NFATC4 rose TH2-type cytokine IL-10 (**Figures 4**, **5**). That would upset the balance between TH2 and TH1, tending to evolve into TH1 cytokine profiles, which may be potentially harmful in pregnancy. The inability of the mother to switch from TH1 to TH2 cytokine profiles at the fetal-maternal interface has been proposed as one of the primary causes of miscarriage, intrauterine growth restriction and preeclampsia. The TH1 (IFN-γ, TNF-α, and IL-12) cytokines are detrimental to pregnancy, may even cause fetal loss, and whereas TH2 (IL-4 and IL-10) cytokines are protective to pregnancy (50–52). Excess TNF-α promotes trophoblast apoptosis and damages the placenta directly (39). IFN-γ has been rendered bile acid secretion decrease, trophoblast apoptosis, and placental damage (40). IL-10 were identified involvement of the transplacental immune regulation during pregnancy. It has been demonstrated that IL-10 may influence Treg cell homeostasis through its effect on Treg cell Bcl-2 expression both in humans and mice and support the homeostatic and "uterine tolerance" (53, 54). IL-10 contributes to placental growth and remodeling since IL-$10^{-/-}$ mice exhibited placental damage and maternal blood sinus increase (55). Besides, treatment with IL-4 and IL-10 could rescue the adverse effects on placental dysplasia and fetal loss of targeting Tim-3 and CTLA-4 on the pregnancy outcome (56). All above suggested that the effects of ceRNA-induced inflammatory and immune factors were consistent with our clinical profile of placenta and fetus weight loss (**Figure 1**) and the ceRNA causative network (**Figure 5**). In a word, the ceRNA regulatory network mediated the immune dysfunction in human placenta tissue of ICP may

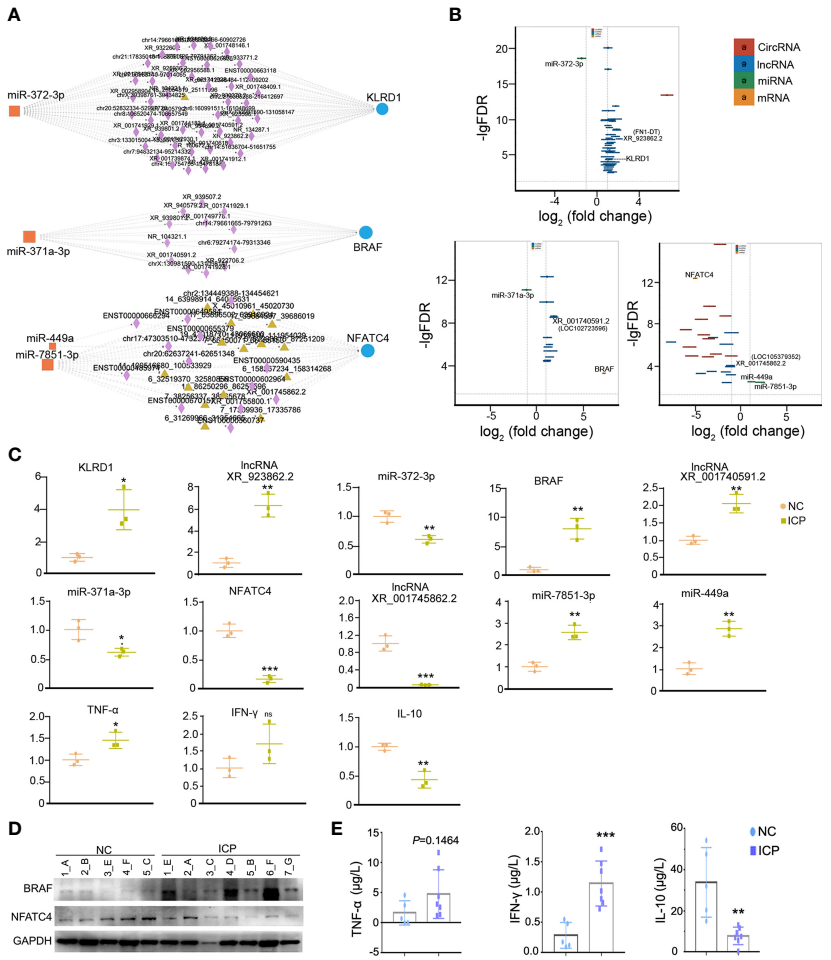

FIGURE 5 | The validation of obviously differentially expressed RNAs in the ceRNA network. **(A)** The ceRNA network of KLRD1, BRAF, and NFATC4 (rectangles, DEmiRNAs; triangles, DEcircRNAs; diamonds, DElncRNAs; Circles, DEmRNAs). **(B)** The fold changes of KLRD1, BRAF, and NFATC4 were involved in ceRNA regulatory network in the ICP group compared with the control group. **(C)** The validation on RNA expression in the ICP group (n=3) compared with the control group (n=3) by qRT-PCR. Each dot represents the average value of one sample in three experimental replicates. **(D)** The protein level of NFATC4 and BRAF in the ICP group compared with the control group. The letters represented the different placenta tissues. **(E)** Comparison of cytokines levels in placenta tissue between ICP and healthy controls. Cytokines concentrations of TNF-α, IFN-γ and IL-10 were compared between ICP patients (n=7) and healthy controls (n=5). Data were shown as mean ± SD. Differences were analyzed by unpaired *t* test. All P-values are two-tailed and significantly different when *P*-value is <0.05. **P*<0.05, ***P*<0.01, ****P*<0.001.

restrain fetal development and placental growth and refine biomarker predictions for developing novel therapeutic approaches in ICP.

In addition, a recent study that ICP pregnant women easily take place liver cancer and immune-mediated cardiovascular disease complications (29) made the concept controversial that ICP is a reversible and benign disease for pregnant women. Notably, our result provides new evidence for possible cardiovascular complications in pregnant women with ICP, that NFATC4 may inhibit the expression on downstream molecules of lower cardiac hypertrophy such as β-MHC, BNP, and TRPC (**Figure 4C**). Some studies have reported that ceRNA networks participate in human dilated cardiomyopathy (57).

The limitation of this study was the inability to confirm the ceRNA mechanism because of a lack of cellular models. The ceRNA is known that circRNA and lncRNA can compete to sponge miRNA *via* miRNA response elements (MREs), reversing the gene silencing. It's hard to identify the MRE that lnc/circRNAs (XR_923862.2, XR_001740591.2, XR_001745862.1) binding to miR-372-3p, miR-371a-3p, miR-7851-3p, and miR-449a. A further limitation of this study was the small samples, the bias that might occur during enrolment into the case series. More samples and experimental validations of these results were needed for more comprehensive analysis and in-depth studies.

In summary, we first evaluated that placental ceRNA networks in pregnancies affected by ICP, showing alterations in immune regulatory networks which may impact fetal and placental growth. In our study, it was found that lncRNA XR_001740591.2/miR-371a-3p/BRAF axis and lncRNA XR_001745862.1/miR-7851-3p, miR-449a/NFATC4 axises

most probably caused the restriction of placental and fetal growth.Overall the ceRNA regulatory network may refine biomarker predictions for developing novel therapeutic approaches in ICP.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited and accessible through "https://www.ncbi.nlm.nih.gov/sra/PRJNA846869", accession number PRJNA846869.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Chongqing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YW and YT conducted the data mining and drafted the manuscript, YT and XY prepared the clinical samples, YW and JX conducted bioinformatics analyses, YC did the PCR validation, SH and PY integrated all efforts. JX, YW and YT performed the assays according to the reviewers' suggestions, revised and responsed the comments. All authors contributed to the article and approved the submitted version.

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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.2022.883971/full#supplementary-material

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Modes of action and diagnostic value of miRNAs in sepsis

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Sepsis is a clinical syndrome defined as a dysregulated host response to infection resulting in life-threatening organ dysfunction. Sepsis is a major public health concern associated with one in five deaths worldwide. Sepsis is characterized by unbalanced inflammation and profound and sustained immunosuppression, increasing patient susceptibility to secondary infections and mortality. microRNAs (miRNAs) play a central role in the control of many biological processes, and deregulation of their expression has been linked to the development of oncological, cardiovascular, neurodegenerative and metabolic diseases. In this review, we discuss the role of miRNAs in sepsis pathophysiology. Overall, miRNAs are seen as promising biomarkers, and it has been proposed to develop miRNA-based therapies for sepsis. Yet, the picture is not so straightforward because of the versatile and dynamic features of miRNAs. Clearly, more research is needed to clarify the expression and role of miRNAs in sepsis, and to promote the use of miRNAs for sepsis management.

KEYWORDS

miRNA, sepsis, infection, innate immunity, biomarkers, critically ill

Abbreviations: AKI, acute kidney injury; ALI, acute lung injury; APACHE, acute physiology and chronic health evaluation; ARDS, acute respiratory distress syndrome; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; DAMP, damage or danger-associated molecular pattern; DC, dendritic cell; HMGB1, high mobility group box-1; HSP, heat shock protein; ICU, intensive care unit; IFN, interferon; IL, interleukin; IRF, IFN response factor; IRAK, IL-1 receptor-associated kinase; lncRNA, long non-coding RNA; LPS, lipopolysaccharide; MAMP, microbial-associated molecular pattern; MAPK, mitogen-activated protein kinase; miRNA, microRNA; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; PCT, procalcitonin; PRR, pattern-recognition receptor; SIRS, systemic inflammatory response syndrome; SOCS, suppressor of cytokine signaling; SOFA, sequential organ failure assessment; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, tumor necrosis factor.

1 Introduction

1.1 Innate immune sensing

Innate immune cells sense signals of microbial origin (microbial-associated molecular patterns or MAMPs, also known as pathogen-associated molecular patterns or PAMPs) or endogenous components released by injured or stressed cells (damage or danger-associated molecular patterns or DAMPs) through pattern-recognition receptors (PRRs). Lipopolysaccharide (LPS), peptidoglycan, flagellin, β-glucan, lipoproteins, glycoproteins, double-stranded and single-stranded RNA, and unmethylated CpG motif containing DNA from bacteria, mycoplasma, mycobacteria, fungi, parasites and viruses are MAMPs/PAMPs. The best described DAMPs are high mobility group box-1 (HMGB1), fibrinogen, fibronectin, nucleic acids, histones, heat shock proteins (HSPs), uric acid, ATP, cytochrome c, S100 molecules and serum amyloid A. The main families of PRRs comprise Toll-like receptors (TLRs), NOD-like receptors (NLRs), c-type lectin receptors, RIG-I-like receptors, cytosolic DNA sensors and scavenger receptors (1–4). The triggering of PRRs by MAMPs/DAMPs activates intracellular signal transduction pathways such as the nuclear factor-κB (NF-κB), interferon (IFN) response factor (IRF), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathways regulating the expression of cytokines, acute phase proteins, and adhesion, co-stimulatory and major histocompatibility complex molecules as well as metabolism. A fine control of these pathways is essential to restore homeostasis following injury.

1.2 Sepsis

Sepsis-3 alliance redefined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (5). Sepsis remains one of the leading causes of mortality worldwide. Recent estimations indicate that sepsis affects around 50 million people and is responsible of at least 11 million deaths annually worldwide (6). These numbers increased during the COVID-19 pandemic. Indeed, most patients dying from COVID-19 present respiratory failure (mostly acute respiratory distress syndrome, ARDS) and multi-organ failure, which are manifestations of sepsis (5). Despite progresses in basic, clinical and translational research, the pathophysiology of sepsis remains not fully understood. Sepsis-specific targeting strategies tested in clinical trials failed to show benefit for patients (7–19).

Sepsis is characterized by an exacerbation of antimicrobial defense mechanisms responsible for collateral tissue injury, organ dysfunctions and early mortality involved in around 10% of all fatal cases (Figure 1). The hyper-inflammatory response is associated with a concurrent shift towards inflammation resolution and tissue repair involved in immuno-paralysis or immunosuppression. The suppressive phase is related to the depletion of dendritic cells (DCs), T cells and B cells through apoptosis, a reduced expression of proinflammatory cytokines,

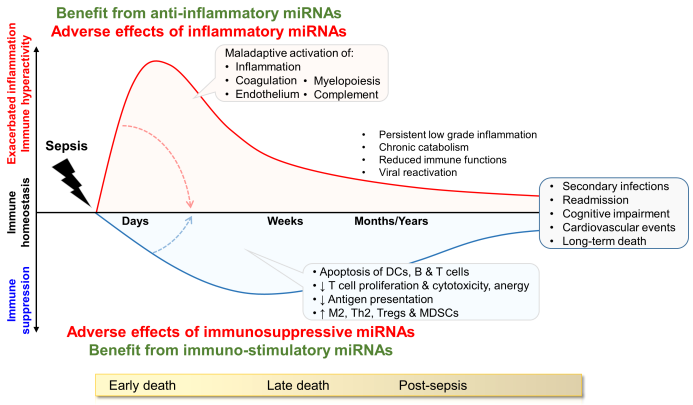

FIGURE 1
Model of immune status during sepsis and potential impact of miRNAs. The drawing shows the dysregulation of immune homeostasis over time, and lists pathophysiological consequences. The inflammatory and immunosuppressive responses are represented concurrently. Early deaths are mainly attributed to organ failure due to overwhelming inflammation. Late deaths are associated to immunosuppression causing increased susceptibility to (nosocomial) infections, viral reactivation and cardiovascular diseases. The influence of miRNAs may fluctuate over time. During the hyper-inflammatory phase of early sepsis, anti-inflammatory miRNAs can provide benefit to the host by dampening excessive immune reactions. In the immunosuppressive late phase of sepsis, inflammatory/immuno-stimulatory miRNAs can be beneficial by sustaining immune activity and protecting from nosocomial infections and reinfections. DCs: dendritic cells, MDSCs: myeloid derived suppressor cells; Th2: T helper 2, Tregs: regulatory T cells. M2 are pro-resolving/anti-inflammatory M2 macrophages.

costimulatory and antigen-presenting molecules, and an increased expression of anti-inflammatory cytokines and inhibitory checkpoint molecules. Immunosuppression can persist for months to years (Figure 1). A subset of patients with prolonged stay in intensive care units (ICUs) suffer from persistent inflammation, immunosuppression and catabolism syndrome (PICS) (20). Dysregulated immune responses favor the development of secondary infections, viral reactivation and long-term immune disabilities accounting for late morbidity and mortality (7–13, 20). Delayed mortality associated with viral reactivation and nosocomial infections represent 20-40% and long-term mortality 50-70% of total fatal sepsis cases. Twenty percent of sepsis survivors develop secondary infections within 30 days, and nearly half of sepsis survivors are re-hospitalized within a year.

The identification of biomarkers and targets is one the most burning areas of research in the sepsis field. A biomarker is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (21). The identification of diagnostic, prognostic and theragnostic biomarkers to distinguish sepsis, identify patients who may benefit from host-targeted therapies, predict responsiveness and monitor the effectiveness of treatment holds great promise for improving patient management (10, 12, 22–29). In the last years, microRNAs (miRNAs) have been suggested to be potential biomarkers and targets for sepsis.

In this review we aim to shed light on the role of miRNAs involved in the pathogenesis of severe infections and sepsis. We will start by briefly summarizing the biogenesis, modes of action, circulation and delivery of miRNAs, which are described comprehensively elsewhere (30–35).

2 miRNAs

2.1 Identification

Non-coding RNAs (ncRNAs) comprise a growing list of RNA species, including miRNAs, small interfering RNAs, long non-coding RNAs (lncRNA), Piwi-interacting RNAs, small nuclear RNAs, small nucleolar RNAs, extracellular RNAs and small Cajal body-specific RNAs. ncRNAs regulate numerous biological and pathological processes such as cancer and autoimmune, cardiovascular and metabolic diseases.

In 1993, Lee et al. and Wightman et al. described a small RNA of 22 nucleotides, *lin-4*, with antisense complementarity to the heterochronic gene *lin-14* in *Caenorhabditis elegans* (36, 37). In 2000, the description of *let-7, a* small RNA conserved in diverse species and with silencing abilities, highlighted the critical role of this category of RNA molecules (38–40). The following year, the term microRNA was coined by Tuschl et al. (41). Along with other groups, they paved the way for the discovery of numerous miRNAs. About 38'600 miRNAs have been identified in 271 species (http://www.mirbase.org). Around 2'600 human mature miRNAs are encoded in the human genome, with half annotated in miRBase V22 (42). The expression atlas of miRNAs generated by the Functional Annotation of the Mammalian Genome (FANTOM5) consortium revealed that the five most expressed miRNAs represent around 50% of the miRNA pool in a given human cell type (43). About half of miRNAs are cell type-enriched, a quarter are broadly expressed, and a quarter are expressed at small levels regardless the cell type.

2.2 Biogenesis

miRNAs can be encoded in non-coding (intergenic miRNAs) and intronic regions of genes. miRNAs are generated through canonical and non-canonical pathways (32, 44, 45) (Figure 2). In the canonical pathway, a long primary transcript (pri-miRNA) of hundreds to thousands nucleotides is generated by RNA polymerase II (Pol-II) or Pol-III and cleaved through the action of the RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) and the nuclear RNase III enzyme Drosha into a precursor-miRNA (pre-miRNA) of approximately 70 nucleotides (46–49). Intronic pri-miRNAs are generated from host RNA transcripts (pre-mRNAs) by RNA splicing and excised into pre-miRNAs by spliceosomal components. Their expression relies on transcription factors and Pol-II (50). pre-miRNAs are exported into the cytoplasm in an exportin-5/RanGTP-dependent manner. Pre-miRNAs are converted into active miRNAs of approximatively 22 nucleotides by a complex composed of the cytoplasmic RNase III Dicer and cofactors including transactivation response (TAR) RNA binding protein (TRBP) and the protein kinase RNA activator (PACT) (49, 51, 52). Of note, miRNAs (-5p and -3p) can be generated from the 5' and 3' arms of a pre-miRNA precursor, and co-expression of miRNA-5p and -3p species have been repeatedly reported. Non-canonical miRNA biogenesis pathways use different combinations of proteins, and are grouped into DGCR8/Drosha-independent and Dicer-independent pathways (Figure 2). Small hairpin RNA (shRNA) are cleaved by the DGCR8/Drosha complex and exported into the cytoplasm as in the canonical pathway, while pre-miRNA can be exported into the cytoplasm through exportin-1. A more detailed description of miRNA biogenesis pathways is beyond the scope of this review, but available in excellent reviews (32–35).

2.3 Modes of action

miRNAs interact with the 3′-untranslated region (3'-UTR) of mRNAs to induce mRNA degradation and translational

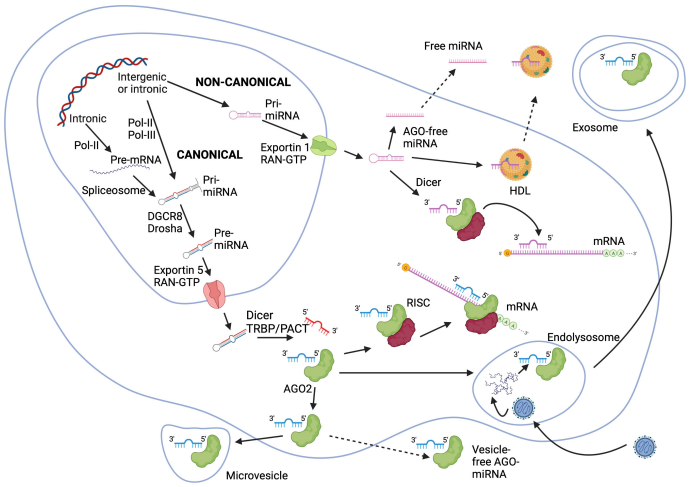

FIGURE 2
miRNA biogenesis *via* canonical and non-canonical pathways. In the canonical pathway, pri-miRNAs are turned into pre-miRNAs by the action of DGCR8 and Drosha within the nucleus. Intronic miRNAs can originate from host mRNA transcripts and processed into pre-miRNA by the spliceosome. Pre-miRNAs are exported into the cytoplasm through an exportin-5/RanGTP-dependent way, and are processed into mature miRNAs by Dicer with eventually RNA binding protein cofactors TRBP or PACT. In non-canonical pathways, shRNAs are cleaved by the DGCR8/Drosha complex and exported into the cytoplasm by exportin-1 before Dicer processing. Mature miRNAs bind to AGO proteins forming RISCs, which in turn silence or cleave mRNAs. Alternatively, miRNA-AGO complexes are exported out of the cell *via* vesicles (exosomes or microvesicles) or as vesicle-free complexes. miRNAs binding to HDLs are actively secreted. AGO-free miRNAs can be exported out of the cell as well. AGO, Argonaute; DGCR8, DiGeorge syndrome critical region gene 8; HDL, high density lipoproteins; miRNA, microRNA; mRNA, messenger RNA; PACT, protein kinase RNA activator; pre-miRNA, precursor-miRNA; Ran, Ras-related nuclear protein; RISC, RNA induced silencing complex; shRNA, small hairpin RNA; TRBP, transactivation response RNA binding protein. The Figure was created on BioRender.com.

repression. Additionally, miRNAs can interact with gene promoter, 5′-untranslated region (5'-UTR) and coding sequence, and can activate transcription in a phenomenon known as RNA activation (53). Finally, miRNA can interact with proteins to modify their activity.

Crosslinking and immunoprecipitation analyses revealed that most miRNA binding events have little functional consequences (54). miRNAs do not possess catalytic functions, but form effector ribonucleoprotein complexes known as RNA induced silencing complexes (RISCs) (55). Mature miRNA molecules bind with proteins of the Argonaute (AGO) family in an ATP-dependent manner. Four AGO proteins (AGO1-4) playing a key role in the formation of RISCs are expressed in humans (56). RISCs bind to target mRNA molecules based on complementarity of miRNA (Figure 2). The result can be translational inhibition by interfering with the eukaryotic initiation factor 4F (eIF4F) followed by the decay of the target mRNA. Moreover, AGO2 initiates mRNA deadenylation by poly (A)-deadenylases, uncapping and 5′−3′ degradation by an exoribonuclease (32, 55, 57). While full complementarity with the target mRNA triggers AGO2 and mRNA degradation, partial complementarity results in transient binding to RISC. It induces the unloading of miRNA from AGO2 (57, 58). AGO-free miRNA molecules and endogenous miRNA-mRNA duplexes have been studied during the past years (59, 60). Mature miRNAs may adopt secondary structures like hairpin and homoduplex that may increase their half-life, affinity and specificity for targets (61).

Free miRNAs interact with proteins, but the prevalence and outcome of such interactions are poorly described. For instance, miR-130b-3p binds to extracellular cold-inducible RNA binding protein (eCIRP) (62). miR-130b-3p and eCIRP are increased in the blood of septic mice and sepsis patient. eCIRP acts as a DAMP sensed through TLR4, promoting the release of inflammatory mediators. Upon binding to eCIRP, miR-130b-3p inhibits eCIRP/TLR4 interaction and cytokine release by immune cells. Injection of a miR-130b-3p mimic reduces cecal ligation and puncture (CLP)-induced inflammation and acute lung injury (ALI) in mice (62). Moreover, miR-130b-3p has been shown to inhibit M1 macrophage polarization (63). A single miRNA can thus interfere with immune responses through multiple ways.

2.4 Circulation and delivery

miRNA secretion and release are intrinsic to cell response to hypoxia, starvation, heat, triggering of PRRs and cytokine/growth factor receptors, and other environmental factors (32, 59, 64, 65). miRNAs are present in biological fluids like blood, plasma, serum, urine, tears, saliva, semen, cerebrospinal fluid, bronchial and peritoneal fluids, and breast milk (32, 59, 64). An

important feature of extracellular miRNAs is their stability and resistance to RNaseA-mediated degradation (64, 66). miRNAs in fluids exert paracrine or endocrine effects as signal transducers of intracellular communication (32, 65).

miRNAs are released passively accompanying apoptotic bodies or cell debris (from one to few μm) or secreted actively (59, 65). Secretion occurs through microvesicles of 100 to 1000 nm usually containing a RISC or a miRNA-AGO complex, and through exosomes (59). MAMPs or DAMPs trigger the release of exosomes containing miRNAs as well as DAMPs such as HMGB1, HSPs and histones, and cytokines, interleukins (ILs), chemokines and IFNγ. Early endosomes gradually turn to multivesicular bodies that integrate miRNAs and a RISC or similar complexes through mechanisms regulated by ceramide synthesis and neutral sphingomyelinase 2 (65, 67). Multivesicular bodies merge with lysosomes inducing the degradation of trapped material, or fuse with the cell membrane expelling exosomes containing miRNAs (59, 68). Exosomes can carry oncogenic miRNAs promoting tumor invasiveness (69), or anti-oncogenic and anti-angiogenic miRNAs inhibiting the growth of malignant cells (70). Similarly, exosomes can carry miRNAs that enhance or decrease cellular responses to MAMPs as reported for miR-155 and miR-146a in LPS-stimulated DCs (71). High-density lipoproteins (HDL) act as alternative carriers of miRNAs in the blood (Figure 2). This is an active and energy dependent procedure to differentiate from the passive release of miRNAs upon cell death (65).

The mechanisms of uptake of miRNAs by recipient cells is not fully deciphered (65). The uptake of microvesicles and exosomes occurs by endocytosis, phagocytosis or fusion with the plasma membrane. Endocytosis of microvesicles requires a docking step mediated by specific or non-specific molecules (72, 73). Because of their small size, microvesicles are also taken-up by micropinocytosis, which does not require a docking step (74). Exosomes and smaller extracellular vesicles are engulfed by phagocytosis mediated by TLRs and complement receptors. Exosomes released during sepsis impact on organs including lungs, kidneys, liver, heart and brain (75).

3 miRNAs in sepsis

Sepsis shows features of early immune hyper-activation and late immunosuppression. Accordingly, we may suggest that miRNAs having anti-inflammatory activities may be beneficial during early sepsis but detrimental during late sepsis. On the contrary, miRNAs having proinflammatory activities may be detrimental during early sepsis but beneficial during late sepsis (Figure 1).

Given that infection and stress modulate the expression of miRNAs, it is not surprising that miRNAs have been the focus of much interest. The stability, simple structure and expression of miRNAs in blood and other biological fluids represent an opportunity to stem new sepsis biomarkers (76). We will focus on promising miRNAs in sepsis. We will summarize observations about the modulation and the role of miRNAs *in vitro* and *in vivo* in models of sepsis (Table 1), and miRNAs as potential biomarkers in human sepsis (Table 2).

3.1 miRNAs and sepsis pathophysiology

3.1.1 miRNAs and innate immune cells

First, it should be recalled that miRNAs are not acting only as brakes, but also as promoters of inflammatory and innate immune responses. Cues to how miRNAs weight the inflammatory response have been obtained in studies using Dicer 1-deficient mouse macrophages depleted of miRNAs. Contrary to expectations, Dicer 1-deficient macrophages produce reduced levels of tumor necrosis factor (TNF), IL-6 and IL-12 in response to TLR1/2, TLR4, and TLR9 stimulation (84). It has been proposed that miRNAs expressed constitutively repress innate immune genes to preserve homeostasis, while stimulus-induced miRNAs fine-tune inflammatory responses and return to homeostasis (266).

miRNAs modulate immune signals by targeting positive or negative players of immune signaling pathways. This process is highly dynamic for several reasons. First, miRNAs are differentially expressed in innate and non-innate immune cell types. Second, miRNA expression is upregulated or downregulated in response to MAMPs/DAMPs/cytokines, and subjected to circadian rhythm (267). For example, miR-146a and miR-155 are upregulated while miR-27a and miR-532-5p are downregulated in macrophages exposed to LPS. Third, miRNAs regulate their own expression. The proinflammatory miR-375 inhibits the expression of the anti-inflammatory miR-21 by targeting the Janus kinase (JAK) 2-signal transducer and activator of transcription protein (STAT) 3 signaling pathway (185). Fourth, one miRNA targets many mRNAs, and one mRNA is regulated by various miRNAs. Consequently, miRNAs have additive or antagonistic effects on their targets. Fifth, one miRNA either inhibits or activates immune signaling, participating to feedback loop mechanisms controlling gene expression. Sixth, miRNAs circulate in fluids and act at a distance (268–272).

miRNAs target transcription factors, signaling proteins and growth factors to influence hematopoiesis and modulate the development of innate and adaptive immune cells. miRNAs regulate the functions of mature innate immune cells, including migration, phagocytosis, efferocytosis, production of cytokines, tolerance, tissue remodeling and promotion of tumor development (273–278). Macrophages display a continuum of functional states, ranging from proinflammatory M1 macrophages to pro-resolving/anti-inflammatory M2 macrophages. miR-155 is up-regulated in M1 macrophages. The knockout of *mir155* and miR-155 antagomir

TABLE 1 Selection of miRNAs related to sepsis.

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-15a/16	Increased in BMDMs exposed to LPS	↓ PU.1 & TLR4	Decreased phagocytic and bactericidal activities of BMDMs Decreased inflammatory response Increased survival of miR-15a/16 knockout mice with sepsis (CLP and *E. coli* peritonitis)	(77)
miR-15a/16	RAW 264.7 mouse macrophages exposed to LPS	↓ TLR4 & IRAK1		(78)
miR-15a-5p	Increased in RAW 264.7 mouse macrophages exposed to LPS	↓ TNIP2 ↑ NF-κB pathway	Increased expression of IL-1β, IL-6 and TNF miR-15a-5p inhibitor reduced cytokines and inflammation in mice challenged with LPS	(79)
miR-15-5p, miR378a-3p	Expressed in platelet-derived exosomes from sepsis patients	↓ PDK1	Modulate Akt/mTOR-related autophagy pathway and induced NETs formation involved in organ injury	(80)
miR-16	Increased in H69 human biliary epithelial cells and U-937 human monocytic cells exposed to LPS	↑ NF-κB pathway by suppressing SMRT	Increased expression of IL-1β, IL-6 and TNF	(81)
miR-17	Decreased in RAW 264.7 macrophages exposed to LPS	↓ BRD4	Inhibition of BRD4 /EZH2/TRAIL pathway Decreased inflammatory response of RAW 264.7 macrophages	(82)
miR-19a	Increased in B cells of sepsis patients	CD22 ↑ 2 days after LPS stimulation CD22 ↓ 4 days after LPS stimulation	Positive feedback loop of B cell response	(83)
miR19b	HEK293T and HeLa cells, MEFs, human synovial fibroblasts	↑ NF-κB pathway by suppressing A20/ Tnfaip3, Rnf11, Fbxl11/ Kdm2a and Zbtb16	Increased production of IL-6 and IL-8	(84)
miR-21	Increased in serum of pediatric sepsis patients	↑ NF-κB pathway & NLRP3 inflammasome	Induction of pyroptosis in mouse macrophages, human THP-1 monocytic cells & primary PBMCs via activation of the NLRP3 inflammasome	(85)
miR-21	Increased in bone marrow of sepsis mice (CLP)	↑ NFI-A protein	Increased number of MDSCs by arresting myeloid progenitor differentiation and maturation miR-21 antagomiR improves late-sepsis survival	(86)
miR-21	High levels in MDSCs from sepsis mice (CLP)	–	miR-21 up-regulated by STAT3 and C/EBPβ in MDSCs Involved in the expansion of MDSCs	(87)
miR-23a	Decreased in bone marrow mononuclear cells of sepsis mice	↓ lncRNA MALAT1 & MCEMP1	Decreased proliferation of monocytes Increased apoptosis of monocytes	(88)
miR-23a	Decreased in RAW 264.7 mouse macrophages exposed to LPS	↓ ATG12	Decreased autophagy, increased production of IL-6 and TNF	(89)
miR-23a-3p	Decreased in RAW 264.7 mouse macrophages exposed to LPS	↓ PLK1	Increased STAT1/STAT3 activation, TNF, IL-1β, IL-6 production and M1 polarization by macrophages Promotes ALI in mice challenged intratracheally with LPS	(90)
miR-26a	Decreased in serum and mononuclear of sepsis neonates	↓ IL-6		(91)
miR-26b	Decreased in MEG-01 human megakaryocyte cells exposed to LPS or TNF	–	Associated with ↑ expression of SLEP Downregulation of Dicer1 reduced miR-26b and increased SELP	(92)
miR-27a	Down-regulation by TUG1 (possible "sponge" action) in human cardiomyocyte cell line AC16	↓ TNF	LPS up-regulates miR-27a and down-regulates TUG1 TUG1 overexpression inhibits TNF and apoptosis of AC16 cells	(93)
miR-27a	Increased in lung tissues of sepsis mice	↑ TNF, IL-6	miR-27a neutralization decreases pulmonary inflammation and increases survival of sepsis mice	(94)
miR-27b	Increased in MSCs-derived exosomes of sepsis mice	↓ JMJD3 & JMJD3/NF-κB/p65 axis	Inhibition of pro-inflammatory response of BMDMs after LPS stimulation and in CLP-induced sepsis model	(95)
miR-30a	Increased in liver cells of sepsis rats	↓ SOCS-1	Increased apoptosis of liver cells via JAK/STAT pathway	(96)
miR-30e	Decreased in liver cells and tissues of sepsis rats	↓ FOSL2	Decreased apoptosis of liver cells via inhibition of JAK/STAT pathway	(97)

(Continued)

TABLE 1 Continued

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-34a	Increased in lung tissues of sepsis mice (CLP)	↓ SIRT1 & ATG4B	Increased oxidative stress, inflammatory response and sepsis-induced ALI	(98)
miR-34a	Increased in lung macrophages of suckling rats after LPS stimulation	↑ iNOS, phospho-STAT3/STAT3	Increased sepsis-induced ALI Inhibition of miR-34a decreases sepsis-induced ALI	(99)
miR-92a-3p	Increased in the BALF of the sepsis rats	↓ PTEN	Increased activation of alveolar macrophages Increased ALI via PI3K/AKT pathway	(100)
miR-98	Decreased in myocardial tissues of sepsis mice (CLP)	↓ HMGA2, TNF, IL-6	Decreased sepsis-induced cardiac dysfunction, liver and lung injury	(101)
miR-103a-3p	Decreased in sepsis patients	↓ HMGB1	Decreased HMGB1 expression, systemic inflammation and multi organ failure, and increased survival in mice with endotoxemia	(102)
miR-122	Huh7 hepatocellular carcinoma cell line	↓ SOCS1	Increased expression of IFNα & IFNβ	(103)
miR-122	Huh7 cells	↓ SOCS3	Increased expression of IFNα & IFNβ Decreased HBV replication	(104)
miR-122	HepG2, Huh7 and Huh7.5.1 hepatocellular carcinoma cell lines	↓ FGFR1, IGF1R, MERTK ↑ IFN signaling pathway	Decreased STAT3 Tyr705 phosphorylation, increased IRF1 signaling and IFNs expression in response to HCV and poly (I:C)	(105)
miR-122	Huh-7 and HepG2 cells	↓ HO-1	Inhibit HBV expression (HBsAg and HBeAg expression)	(106)
miR-122	Increased in HepG2 and Huh7 cells	↓ TLR4	Decreased the proliferation and the production of TNF and IL-6 by HepG2 and Huh7 cells	(107)
miR-122-5p	Increased by LPS in the heart of rats and in H9c2 rat cardiomyocytes	–	Inhibition of miR-122-5p reduced myocardial injury through inhibiting inflammation, oxidative stress and apoptosis in endotoxemic rats	(108)
miR-124	Decreased in organs of mice with LPS-induced acute lung injury (ALI)	↓ MAPK14 (p38-α)	Overexpression decreases IL-1β, IL-6, IL-10 and TNF in blood, and MAPK signaling and lung cell apoptosis and lung injury in ALI mice	(109)
miR-125b	Decreased in PBMCs exposed to LPS, and in PBMCs and serum of sepsis patients	↓ STAT3	Inhibition in peripheral blood monocytes increases STAT3 phosphorylation and the expression of PCT and NO Possibly acts downstream the *NED25* gene intergenic lncRNA	(110)
miR-125b	Decreased in PBMCs exposed to LPS, and in PBMCs of sepsis patients	↓ STAT3	Decreased PCT	(111)
miR-125-5p	Decreased in mice with CLP and ALI	↓ TOP2A	Endothelial cell–derived exosomal miRNA–125b–5p ↑ VEGF, protected from sepsis–induced ALI	(112)
miR-126-3p in platelet microparticles	Increased in primary macrophages exposed to miR-126-3p-containing platelet microparticles		Decrease of 367 RNAs & reduced expression of CCL4, CSF1 and TNF, increase phagocytic capacity by macrophages	(113)
miR-126-5p	Increased in hepatic cells of mice with sepsis induced AHI	↓ BCL2L2	Overexpression inhibits anti-apoptotic function of BCL2L2 in AHI LncRNA-CRNDE binds miR-126-5p and restores BCL2L2 function	(114)
miR-128	Increased in kidneys of mice with sepsis induced AKI	↓ NRP1	NRP1 downregulates TNF, IL-6, and IL-1β NRP1 inhibition by miR-128 increases inflammatory response	(115)
miR-128-3p	Decreased in HK2 cells exposed to LPS and serum of sepsis patients	↓ TGFBR2	Decreased TGFBR2 mediated apoptosis	(116)
miR-129	Decreased in lungs of mice with LPS-induced ALI	↓ TAK1	Inhibits TAK1/NF-κB pathway Decreased apoptosis and inflammation in sepsis-induced ALI	(117)
miR-129-5p	Decreased in kidneys of mice with LPS-induced AKI	↓ HMGB1	Inhibits HMGB1/TLRs/NF-κB pathway Decreased apoptosis of kidney podocytes	(118)
miR-129-5p	Overexpression (use of agonists) in sepsis mice (CLP)	↓ HMGB1	Decreased HMGB1, apoptosis and inflammation in sepsis-induced ALI	(119)
miR-130a	Decreased in sepsis patients with thrombocytopenia	↓ IL-18	–	(120)
miR-130b-3p	Increased in serum of sepsis mice (CLP) and in sepsis patients	Binds to and inhibit CIRP	Decreased CIRP-induced cytokine production by macrophages Delivery of miR-130b-3p in mice reduces CLP-induced inflammation and acute lung injury	(62)

(Continued)

TABLE 1 Continued

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-130b-3p	Increased in RAW 264.7 mouse macrophages exposed to IFNγ+LPS	↓ IRF1	Inhibits M1 macrophage polarization and production of CCL5, CXCL-10, iNOS & TNF Overexpression decreases lung inflammation in LPS-treated mice	(63)
miR-132	Increased in alveolar macrophages of sepsis rats	↓ AChE	Decreased ACh-mediated cholinergic anti-inflammatory reaction Inhibits NF-κB and STAT3	(121)
miR-133a	Decreased in TCMK-1 mouse kidney cell line exposed to LPS	↓ BNIP3L	Inhibits NF-κB pathway, apoptosis and TNF and IL-6 expression	(122)
miR-133a	Increased in the blood of sepsis mice (CLP) and sepsis patients	↓ SIRT1	Inhibition of miR-133a decreased CLP-induced inflammation and lung, liver and kidney injuries	(123)
miR-135a	Increased in serum of sepsis patients	↑ p38 MAPK	Activation of p38 MAPK and NF-κB pathways Aggravation of sepsis-induced inflammation and myocardial dysfunction	(124)
miR-139-5p	Decreased in lung tissues of sepsis mice (CLP)	↓ MyD88	Decreased inflammation, oxidative stress and ALI	(125)
miR-141	Decreased in serum of pediatric sepsis patients and in monocytes exposed to LPS	↓ TLR4	Decreased inflammatory response in neonatal sepsis	(126)
miR-142	Decreased in blood of sepsis patients and in macrophages of sepsis mice	↓ PD-L1	Decreased inflammation mediated by PD-L1	(127)
miR-143	Increased in the blood of healthy volunteers infused with LPS	–	Associated with strong reduction of BCL2 and silencing of inflammation related targets	(128)
miR-143 miR-150	Increased in mouse macrophages exposed to mycobacterial cell wall glycolipid and muramyl dipeptide	↓ TAK1 (miR-143) ↓ RIP2 (miR-150)	Negatively regulate the NOD2 pathway Suppress MDP-induced PI3K-PKC-MAPK-βcatenin-mediated expression of COX-2, SOCS3 and MMP-9	(129)
miR-143	Decreased in nasal mucosal tissues from patients with allergic rhinitis	↓ IL-13αR1	Decreased expression of GM-CSF, eotaxin and mucin 5AC of cells exposed to IL-13	(130)
miR-143	Decreased in HUVECs exposed to IL-1β	↓ ADAR1	Promotes the activation of HUVECs by IL-1β	(131)
miR-143	Increased in BEAS-2B human bronchial epithelium cells exposed to AngII and LPS	↓ ACE2	miR-143-3p inhibitor increased ACE2 and decreased IL-1β, IL-6 and TNF and apoptosis in cells exposed to AngII and LPS	(132)
miR-143	Decreased in lung tissues of mice with mycoplasmal pneumonia	↓ MyD88	miR-143 increased IL-10 and decreased IL-2, TNF and alveolar epithelial cell apoptosis through Bax and Bcl-2	(133)
miR-143	Human umbilical cord MSCs exposed to poly (I:C)	↓ TAK1 and COX-2	Infusion of TLR3-activated MSCs improved survival of sepsis mice (CLP); the co-infusion of miR-143 reduced survival benefit	(134)
miR-145	Decreased in blood samples of sepsis patients and in lung tissues of sepsis mice	↓ TGFBR2	Decreased LPS-induced inflammation and sepsis-induced ALI	(135)
miR-145	Decreased in HUVECs exposed to LPS	↓ TGFBR2	Decreased TGFBR2/SMAD2/DNMT1 pathway Decreased LPS-induced injury	(136)
miR-146	Decreased in EA.hy926 human vascular endothelial cells exposed to LPS	↓ NF-κB pathway	Decreased LPS-induced expression of inflammatory cytokines	(137)
miR-146a	Increased uptake of miR-146a-expressing plasmid by splenic macrophages of sepsis mice	↓ IRAK-1, TRAF6	Decreased sepsis-induced inflammation and organ failure Splenectomy abolishes these effects	(138)
miR-146a	Increased in peritoneal macrophages of sepsis mice after GSKJ4 treatment	–	Decreased expression of pro-inflammatory cytokines by JMJD3 inhibition after GSKJ4 treatment	(139)
miR-146a	Increased in heart-derived H9c2 cardiomyocytes exposed to LPS	↑ ErbB4 ↓ IRAK1, TRAF6, caspase-3	Decreased sepsis-induced inflammation and myocardial dysfunction	(140)
miR-146a	Increased in mouse peritoneal macrophages exposed to LPS	↓ Notch-1	Decreased NF-κB signaling Decreased sepsis-induced organ failure	(141)
miR-146a	Decreased in T cells of sepsis patients	↓ PRKCε	Decreased STAT4 activation via PRKCε downregulation Decreased Th1 differentiation	(142)
miR-146a/b	Increased in human pulmonary microvascular endothelial cells exposed to TNF	↑ IL-6, IL-8	Increased expression of HSP10	(143)

(Continued)

TABLE 1 Continued

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-146b	Decreased in the blood on healthy volunteers infused with LPS	–	Associated with rapid transcriptional activation of IRAK2	(128)
miR-146a-5p	Increased in plasma of sepsis mice (CLP) and sepsis patients	↓ IRAK1	Interacts with TLR7 and activates proteasome Knockout decreases inflammation, improves cardiac function, and survival of sepsis mice	(144)
miR-146a-5p	–	–	Activates TLR7 to induce TNF release, pulmonary inflammation, endothelial barrier disruption and ARDS in sepsis mice	(145)
miR-150	Decreased in MDSCs of sepsis mice (CLP) and in serum of sepsis patients	↓ ARG1	Decreased proliferation and immunosuppressive functions of MDSCs from sepsis mice (CLP)	(146)
miR-150	Decreased in the blood on healthy volunteers infused with LPS	–	Associated with rapid transcriptional activation of IRAK2	(128)
miR-150	Increased in the serum of sepsis mice (CLP)	–	–	(147)
miR-150	Increased in the serum of rats challenged with LPS	–	–	(148)
miR-150	Increased during recovery from LPS-induced injury in mice	↓ EGR2	miR-150$^{-/-}$ mice show increased mortality from LPS and CLP Rescuing miR-150 in lung endothelial cells decreased EGR2-dependent Ang2 expression, restored endothelial barrier function, and reduced mortality	(149)
miR-150	Decreased in the serum of mice challenged with LPS and in sepsis mice (CLP)	↓ NF-κB	Protects HUVECs from LPS-induced apoptosis, decreased TNF and IL-6, ICAM-1, VCAM-1 and E-selectin expression	(150)
miR-150-5p	Decreased in H9c2 cardiomyocytes exposed to LPS	↓ MALAT1	Decreased IL-6 and TNF production	(151)
miR-150-5p	Decreased in the heart of rats challenged with LPS	–	Decreased myocardial apoptosis associated with a reduced expression of Akt2, cleaved caspase 3 and Bax, and increased expression of Bcl-2 in rat heart and H9c2 cardiomyocytes	(152)
miR-150	Decreased in HUVECs exposed to LPS	↓ MALAT1	Decreased TNF and IL-6, ER stress-related proteins, cleaved caspase 3, Bax, apoptosis and increased IL-10 and Bcl-2 in LPS-stimulated HUVECs and PAECs from sepsis mice (CLP)	(153)
miR-150-5p	Decreased in RAW 264.7 macrophages exposed to LPS	↓ Notch1	Inhibits LPS-induced apoptosis and TNF, IL-1β, IL-6 production	(154)
miR-150	Decreased in THP-1 cells exposed to LPS	↓ STAT1	Decreased IL-1β, IL-6 and TNF secretion	(155)
miR-150-5p	Decreased in HK-2 human proximal renal tubular epithelial cells and in mice exposed to LPS	↓ MEKK3	Inhibits LPS-induced JNK pathway, apoptosis, inflammation (IL-1β, IL-6, TNF, BUN, Scr), and outcome of sepsis mice with AKI	(156)
miR-150-5p	Decreased in H9C2 cardiomyocytes and myocardial tissues of mice exposed to LPS	↓ XIST	Decreased c-Fos axis, TXNIP-mediated pyroptosis and sepsis-induced myocardial injury	(157)
miR-155	Comparison of miR-155-deficient and wild-type sepsis mice (CLP)	↑ Neutrophil extracellular traps	Increased neutrophil recruitment Increased sepsis-induced ALI	(158)
miR-155	Increased in pulmonary endothelial cells of sepsis mice and in HUVECs exposed to TNF	↓ Claudin-1	Increased vascular barrier breakdown and sepsis-related capillary leakage	(159)
miR-155	Increased in intestinal tissue of sepsis mice (CLP)	↑ NF-κB	Increased intestinal barrier dysfunction	(160)
miR-155	Increased in plasma and myocardial tissue of sepsis mice and patients	↑ NO, cGMP ↓ Angiotensin type 1 receptor	Increased sepsis-associated cardiovascular dysfunction	(161)
miR-155	Increased in HPMECs exposed to TNF	↑ IL-6, IL-8	Increased HSP10	(143)
miR-155	Increased in myocardial tissue of sepsis mice	↓ JNK phosphorylation, β-arrestin 2	Decreased sepsis-induced myocardial dysfunction	(162)
miR-155	Increased in liver tissue of sepsis mice	↑ JAK/STAT pathway ↓ SOCS1	Increased sepsis-induced AHI	(163)
miR-155	Increased in myocardial tissue of mice exposed to LPS	↓ Pea15a	Increased sepsis-induced myocardial dysfunction	(164)
miR-181-5p	Decreased in kidneys of sepsis mice (CLP)	↓ HMGB1	Decreased inflammatory response Decreased renal and hepatic dysfunction	(165)

(Continued)

TABLE 1 Continued

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-181a	Increased in mouse DCs exposed to HMGB1	↓ TNF mRNA	Dual influence of HMGB1 on maturation and cytokine expression in DCs (↑ at low but ↓ at high concentrations)	(166)
miR-181a	Increased in lung tissues of mice exposed to LPS	↓ Bcl-2	Increased apoptosis on ALI by down-regulation of Bcl-2	(167)
miR-181b	Decreased in myocardial tissue of sepsis rats (CLP)	↓ HMGB1	Decreased apoptosis of myocardial cells Decreased sepsis-induced myocardial injury	(168)
miR-181b	Decreased in HUVECs exposed to TNF	↓ NF-κB pathway, VCAM-1, importin-α3	Decreased sepsis-induced vascular inflammation and ALI	(169)
miR-181b	Increased in bone marrow of sepsis mice (CLP)	↑ NFI-A	Increased number of MDSCs by arresting myeloid progenitor differentiation and maturation miR-181b antagomiR improves late-sepsis survival	(86)
miR-181b	High levels in MDSCs from sepsis mice (CLP)	–	miR-181b up-regulated by phospho-STAT3 and C/EBPβ binding to miR-181b promoter in MDSCs, leading to MDSCs expansion	(87)
miR-181	Increased by ouabain in airway epithelial cells A549 and by LPS in THP-1 monocytic cells	↓ TNF mRNA stability	miR-181d agomir increases bacterial burden and decreases survival of sepsis mice (CLP)	(170)
miR-186	–	↓ PTEN ↑ PI3K/ARG (PTEN targets)	miR-186 administration in sepsis rats (CLP) decreases p53 via increased PI3K/AKT in kidney cells and decreases AKI	(171)
miR-186-5p	Decreased in sepsis patients	↓ NAMPT	miR-186-5p inhibited sepsis-induced coagulation disorders via targeting NAMPT and deactivating the NF-κB pathway	(172)
miR-194	Increased in rat H9c2 cardiomyocytes exposed to LPS	↓ Slc7a5 gene ↑ β-catenin, cyclin D1 (hypothesis)	Increased sepsis related myocardial injury	(173)
miR-195	Increased in lung and liver tissues of sepsis mice (abdominal sepsis)	↓ Bcl-2, Sirt1, Pim-1	Increased apoptosis Increased sepsis-induced ALI and AHI	(174)
miR-195-5p	Decreased in LPS-treated cardiomyocytes and sepsis mice (CLP)	↓ ATF6	Decreased inflammation, apoptosis, oxidative stress and endoplasmic reticulum stress in CLP mice	(175)
miR-199a	Increased in intestinal tissues of sepsis mice	↓ Surfactant protein D ↑ NF-κB pathway	Increased apoptosis in epithelial cells of intestinal tissues Increased intestinal barrier dysfunction	(176)
miR-200c-3p	Increased in A549 cells infected with H1N1 or H5N1 influenza virus	↓ ACE2 protein	Increased ALI and ARDS following viral infection (via NF-κB pathway)	(177)
miR-212-3p	Increased in RAW 264.7 macrophages exposed to LPS	↓ HMGB1	Decreased TNF and IL-6 production Decreased p38 and ERK1/2 phosphorylation (via HMGB1 inhibition)	(178)
miR-214-3p	Increased in myocardiac tissues of sepsis mice	↑ p-AKT, p-mTOR ↓ PTEN	Decreased sepsis-induced myocardiac dysfunction Decreased autophagy via AKT/mTOR pathway	(179)
miR-221	Increased in RAW 264.7 mouse macrophages exposed to LPS Sepsis mice (CLP)	↓ JNK2	Increased MCP-1 and CXCL1 levels, lung inflammation and injury in sepsis mice	(180)
miR-223	Increased in lungs of mice exposed to cigarette smoke and LPS and human in pulmonary cells and monocytes exposed to cytokines	↓ HDAC2	miR-223 levels negatively correlate with the HDAC2 expression in lungs from COPD patients Reduced HDAC2 increased fractalkine (CX3CL1) expression	(181)
miR-223	Increased in white blood cells of sepsis patients (especially survivors)	↓ FOXO1	Decreased lymphocytes apoptosis Negative correlation with SOFA score and clinical severity	(182)
miR-223	Decreased in HCAECs exposed to TNF	–	Platelet-derived miR-223 decreased ICAM1 expression in endothelial cells and reduced the binding of PBMCs to HCAECs	(183)
miR-326	Decreased in lung tissues and macrophages of mice exposed to LPS and sepsis mice (CLP)	↓ TLR4	Decreased sepsis-induced ALI	(184)
miR-375	Decreased in whole blood of sepsis patients	↓ miR-21, JAK2, STAT3	Decreased MDSCs in sepsis mice (CLP) miR-375 agomir promotes survival of sepsis mice	(185)
miR-376b	Decreased in renal tubular cells in sepsis mice with AKI	↓ NF-κB inhibitor ζ	Increased sepsis-induced AKI	(186)
miR-494	Increased in human lung cancer cells	↓ NQO1, Nrf2	Increased sepsis-induced ALI	(187)

(Continued)

TABLE 1 Continued

miRNA	Expression/model	Target and effect	Observation/impact of miRNA	Reference
miR-494-3p	Decreased in plasma of sepsis patients and in RAW 264.7 macrophages	↓ TLR6	Decreased sepsis-induced inflammatory response	(188)
miR-499a	Decreased in HUVECs exposed to LPS	↓ STAT1	Decreased LPS-induced inflammatory injury and apoptosis	(189)
miR-574-5p	Increased in serum of sepsis patients (especially survivors)		Increased viability of renal cell culture line (HK-2) Decreased sepsis-induced AKI	(190)
miR-1184	Decreased in THP-1 cells exposed to LPS and serum of pediatric sepsis patients	↓ TRADD	Decreased expression of TRADD, p65, IL-1β, IL-6 and TNF when overexpressed in THP-1 monocytic cells exposed to LPS	(191)
miR-1184	Decreased in monocytes exposed to LPS and in pediatric sepsis patients	↓ IL-16	Negatively correlates with IL-1β, IL-6, IL-16 and TNF in pediatric sepsis patients Overexpression of IL-16 reverses miR-1184-mediated inhibition of IL-1β, IL-6 and TNF in human monocytes	(192)
miR-1298	Increased in exosomes of sepsis patients	↓ SOCS6	Increased bronchial epithelial cell injury via SOCS6/STAT3 pathway Reverse effects by miR-1298 inhibition	(193)
miR-2055b	Increased in serum and organ tissues (lung, liver, spleen, colon) of sepsis mice	↓ HMGB1	Increased cholinergic anti-inflammatory activity in late sepsis via HMGB1 suppression	(194)

ACE2, angiotensin-converting enzyme 2; AChE, acetylcholinesterase; ADAR1, adenosine deaminase acting on RNA 1; AHI, acute hepatic injury; AKI, acute kidney injury; ALI, acute lung injury; Ang2, angiopoetin-2; ARDS, acute respiratory distress syndrome; ARG1, arginase 1; ATG, autophagy related; BALF, bronchoalveolar lavage fluid; BCL2, B-cell lymphoma 2; BCL2L2, BCL2-like 2; BMDM, bone marrow-derived macrophage; BNIP3L, BCL2 interacting protein 3 Like; BRD4, bromodomain containing 4; CCL, C-C motif chemokine ligand; BUN, blood urea nitrogen; C/EBP, CCAAT enhancer binding protein; cGMP, cyclic guanosine monophosphate; CIRP, cold-inducible RNA binding protein; CLP, cecal ligation and puncture; COPD, chronic obstructive pulmonary disease; COX, cyclooxygenase; CRNDE, colorectal neoplasia differentially expressed; CXCL, C-X-C motif chemokine ligand; DC, dendritic cell; DNMT1, DNA methyltransferase 1; EGR2, early growth response 2; ErbB4, Erb-B2 receptor tyrosine kinase 4; ERK, extracellular signal-regulated kinase; EZH2, Enhancer of zeste homolog 2; FGFR, fibroblast growth factor receptor; FOSL2, fos-like 2; FOXO1, forkhead box O1; GSKJ4, small-molecule inhibitor of JMJD3; HCAEC, human coronary artery endothelial cell; HK2, human kidney 2; HBV, hepatitis B virus; HMGA2, high-mobility group AT-hook 2; HMGB1, high mobility group box 1; HO-1, heme oxygenase-1; HPMEC, human pulmonary microvascular endothelial cell; HSP, heat shock protein; HUVEC, human umbilical endothelial cell; ICAM, intercellular adhesion molecule; IGF1R, insulin like growth factor 1 receptor; IL, interleukin; IL-13αR1, IL-13 receptor α1; iNOS, inducible nitric oxide synthase; IRAK, IL-1 receptor-associated kinase; JAK, janus kinase; JMJD3, jumonji domain-containing protein D3; JNK, c-Jun N-terminal kinase; lncRNA, long non-coding RNA; LPS, lipopolysaccharide; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MAPK, mitogen-activated protein kinase; MCEMP1, mast cell-expressed membrane protein 1; MDP, muramyl dipeptide; MEF, mouse embryonic fibroblast; MDSC, myeloid-derived suppressor cell; MEKK2, mitogen-activated protein kinase kinase; MERTK, myeloid-epithelial-reproductive tyrosine kinase; MOF, multiple organ failure; MSC, mesenchymal stem cell; mTOR, mammalian target of rapamycin; MyD88, myeloid differentiation primary response 88; NAMPT, nicotinamide phosphoribosyltransferase; NET, neutrophil extracellular trap; NFI-A, nuclear factor I A; NF-κB, nuclear factor kappa B; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; NO, nitric oxide; NQO1, NAD(P)H quinone oxidoreductase 1; Notch1, notch receptor 1; Nrf2, nuclear factor E2 p45-related factor 2; NRP1, neuropilin 1; PAEC, pulmonary arterial endothelial cell; PBMC, peripheral blood mononuclear cell; PCT, procalcitonin; PDK1, phosphoinositide-dependent protein kinase 1; PD-L1, programmed death-ligand 1; PI3K, phosphoinositide 3-kinase; PLK1, Polo-like kinase 1; PRKCε, protein kinase C epsilon; PTEN, phosphatase and tensin homologous protein; RIP2, receptor-interacting protein kinase 2; Scr, serum creatinine; SIRT1, silent information regulator T1; SLEP, P-selectin; SMAD2, Sma- and mad-related protein 2; SMRT, silencing mediator for retinoid and thyroid hormone receptor; SOCS, suppressor of cytokine signaling; SOFA, sequential organ failure assessment; STAT, signal transducer and activator of transcription; TAK1, transforming growth factor activated kinase 1; TCMK-1, transformed C3H mouse kidney-1; TGFBR2, transforming growth factor beta receptor II; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNIP2, TNFAIP3 interacting protein 2; TOP2A, topoisomerase II alpha; TRADD, TNF receptor type 1-associated DEATH domain protein; TRAF6, TNF receptor-associated factor 6; TRAIL, TNF related apoptosis-inducing ligand; TUG1, taurine-upregulated gene 1; TXNIP, thioredoxin-interacting protein; XIST, X-inactive specific transcript; VCAM-1, vascular cell adhesion molecule 1. ↑ means upregulated, and ↓ means downregulated.

reduces the expression of *Inos*, *Il1b*, *Il6*, *Il12*, and *Tnf* in M1 macrophages. In fact, around half of the 650 genes that make up the M1 signature rely on miR-155 (279). miR-130b-3p inhibits IRF1 expression, M1 macrophage polarization and the production of C-C motif chemokine ligand 5 (CCL5), C-X-C motif chemokine ligand 10 (CXCL10), inducible nitric oxide (NO) synthase (iNOS) and TNF (63). miR-223 regulates peroxisome proliferator-activated receptor-γ mediated M2 macrophage activation (280). Overall, many miRNAs have been associated with the polarization/activity of M1 macrophages (miR-9, miR-26a-2, miR-125a-3p, miR-125b, miR-127, miR-155-5p, miR-181a, miR-204-5p, miR-451) and M2 macrophages (miR-27a, miR-29b-1, miR-34a, miR-124, miR-125a-5p, miR-132, miR-143-3p, miR-145-5p miR-146a-3p, miR-193b, miR-222, miR-223, let-7c) (278, 281–283). miRNAs influence the differentiation, expansion and biological activities of myeloid-derived suppressor cells (MDSCs) that are associated with sepsis morbidity and mortality (17, 28, 284, 285). Thus, miRNAs shape both inflammation-associated antimicrobial defenses, anti-inflammatory and pro-resolving immune reactions and immunosuppression. The two facets can be driven by a single miRNA entity. miR-466l expression in polymorphonuclear neutrophils (PMNs) induces inflammation and precedes miR-466l expression in macrophages acquiring pre-resolving functions (286).

3.1.2 miRNAs and endothelium and coagulation activation in sepsis

DAMPs and MAMPs released during sepsis activate the complement and coagulation systems. Disseminated intravascular coagulation (DIC) affects around 35% of sepsis patients. Beside thrombosis, DIC is associated with bleeding due to the consumption of clotting factors, anticoagulant proteins, and platelets (9, 11). Thrombocytopenia develops in about 50% of sepsis patients. Signaling through PRRs and cytokine receptors triggers endothelial cells, increasing the expression of adhesion molecules, vascular permeability, transcellular

TABLE 2 miRNAs as biomarkers in human sepsis.

miRNA	Subjects (sepsis/controls [n] unless detailed) Sample type	Observations	Reference
miRNome (RNA-Seq)	Adults (117 sepsis survivors and 97 sepsis non-survivors based on 28-day mortality) Serum	Less than 200 miRNAs detected by sequencing **Increased** miR-15a, miR122, miR-193b*, miR483-5p & **decreased** miR-16 ,miR-223 in non-survivors vs survivors miR-15a, miR-16, miR-193b and miR-483-5p are associated with death in logistic regression analysis	(195)
miRNome (RNA-seq)	Adults (21 severe & 8 non-severe sepsis, 23 severe & 21 non-severe non-infective SIRS, 16 no SIRS) Plasma	116 detectable blood miRNAs generally up-regulated in SIRS vs no-SIRS patients and higher in non-infective SIRS than sepsis. Inversely correlate with IL-1, IL-6, IL-8 and CRP levels Top 5 miRNAs (miR-23a-5p, miR-26a-5p, miR-30a-5p, miR-30d-5p & miR-192-5p) discriminate severe sepsis from severe SIRS miRNA levels inversely correlate with IL-1, IL-6, IL-8, CRP, PSP levels, but not SOFA score	(196)
miRNome (RNA-seq)	Adults (22/23) Cells, serum & serum exosomes	77 miRNAs **decreased** & 103 miRNAs **increased** in patients (all compartments) 11 miRNAs in at least one compartment correlate with disease severity **Increased** cellular miR-199b-5p, potential early indicator for sepsis and septic shock **Decreased** exosomal miR-30a-5p & miR-125b-5p, and serum miR-193a-5p in non-survivors	(197)
miRNome (microarray, 3'100 probes)	Adults (6 sepsis with AKI, 6 sepsis without AKI, 3 healthy controls) Serum	37 miRNAs differentially expressed among the groups **Increased** miR-3165, miR-4270, miR-4321 & **decreased** miR-22-3p, miR-23a-3p, miR-142-5p, miR-191-5p, miR-4456 in sepsis vs controls miR-4321 upregulated in sepsis with AKI vs sepsis without AKI	(198)
miRNome (microarray, 2'661 probes)	Adults(31 pneumonia, 34 sepsis secondary to pneumonia, 21 healthy controls) Plasma	**Decreased** miR-940 & **increased** miR-765, miR-4800-5p, miR-6510-5p, miR-6740-5p, miR-7110-5p (microarray on 5 pneumonia & 5 sepsis) **Increased** miR-223-3p & miR-7110-5p in sepsis secondary to pneumonia compared to pneumonia and control groups	(199)
miRNome (microarray, 2'578 miRNAs)	Neonates (36 NEC & 101 sepsis patients, 164 controls) Plasma	16 miRNAs **decreased** & 230 miRNAs **increased** and in NEC vs non-NEC (microarray) **Increased** miR-1290 in NEC vs sepsis and controls 7 of 36 infants with NEC diagnosed 13.3 hours earlier using miR-1290 measurement	(200)
miRNome (microarray)	Adults (60/30) Blood	11 differentially expressed miRNAs **Increased** miR-155 confirmed by RT-qPCR miR-155 positively correlated with SOFA score, predictor of 28-day survival miR-155 levels proportional to of CD39+ Tregs %	(201)
miRNome (TaqMan OpenArray, 754 miRNAs)	Adults. Discovery and independent validation cohort with 530 ARDS patients and critically ill at-risk controls Blood	miR-92a & miR-181a are risk biomarkers for ARDS miR-424 is a protective biomarker for ARDS	(202)
miRNome (TaqMan Open Array, 754 miRNAs)	Adults (21/21) Platelets	121 **decreased** & 61 **increased** in patients **Decreased** miR-26b in patients, associated with increased SELP mRNA expression Lower levels associated with sepsis severity & mortality	(92)
miRNome (microarray, 470 miRNAs)	Adults (17/32) PBMCs and plasma	17 miRNAs differentiate sepsis from controls (microarray) **Decreased** miR-150 & miR-342-5p and **increased** miR-182 & miR-486 in sepsis miR-150 positively correlates with SOFA score & inversely correlates with IL-10, IL-18, TNF	(203)
Microarray (n probes?) & RT-qPCR	Adults (31/34) WBCs and T-cells	35 miRNAs differentially expressed in sepsis vs controls (microarray, 7 patients/group) **Decreased** miR-150, miR-342 & **Increased** miR-15a, miR-16, miR-93, miR-143, miR-223 and miR-424 miR-143 **increased** in T-cells, positive correlation with immunoparalysis miR-150 **decreased** in T-cells, negative correlation with immunoparalysis	(204)
miR-10a	Adults (62/20) PBMCs	**Decreased** in patients Negative correlation with disease severity	(205)

(Continued)

TABLE 2 Continued

miRNA	Subjects (sepsis/controls [n] unless detailed) Sample type	Observations	Reference
miR-15a miR-15b miR-16 miR-206 miR-223 miR-378 miR-451	Neonates (46/41) Serum	**Increased** miR-15a, miR-16 & **decreased** miR-378 and miR-451 in sepsis neonates	(78)
miR-15a miR-16	Adults (166 sepsis, 32 SIRS, 24 healthy controls) Serum	**Increased** in patients vs controls Higher levels of miR-15a in SIRS than in sepsis patients	(206)
miR-15b miR-122 miR-193b* miR-223 miR-483-5p miR-499-5p	Adults (166/24) Serum	**Increased** miR-223 & **decreased** miR-122, miR-193b*, miR-499-5p in patients	(207)
miR-15a miR-16 miR-122 miR-193b* miR-223 miR-483-5p	Adults(123 on day of admission, and 45 on days 1, 3, 5, 7, 10 and 14 of ICU admission)	**Increased** miR-122 in patients with coagulation abnormalities at days 1, 3, 7 and 10	(208)
miR-15a miR-16	Neonates (32 sepsis/30 controls with respiratory infection/pneumonia) Serum	**Increased** in sepsis neonates	(209)
miR-15a-5p miR-155-5p miR-192-5p miR-423-5p	Adult sepsis patients treated with gentamicin, vancomycin or non-nephrotoxic antibiotics (20/7/19) Blood at day 1, 4 & 7	Minor time-dependent changes of miR-15-5p and miR-423-5p miR-15a-5p at day 7 of gentamicin discriminates AKI and non-AKI miR-155-5p & miR-192-5p positively correlate with creatinine and NGAL in vancomycin miR-192-5p & miR-423-5p positively correlate with PCT and IL-6 in non-nephrotoxic antibiotics	(210)
miR-15b miR-378a	Neonates (25/25) Serum	**Increased** miR-15b in sepsis neonates **Decreased** miR-378a in sepsis neonates	(211)
miR-16a miR-451	Neonates (25/25) Serum	**Increased** in sepsis neonates	(212)
miR-19b-3p	Adults (103/98) Serum	**Decreased** in patients Independent prognostic factor for 28-day survival Negative association with IL-6 and TNF	(213)
miR-21	Adults (219/219) Plasma	**Decreased** in patients Poor predictive value for 28-day mortality risk	(214)
miR-21	Children (88/26) Blood	**Increased** in sepsis child/teens (0.2-19 years old)	(85)
miR-21 miR-29a miR-31 miR-146a miR-155	Neonates (42/42) Plasma	**Decreased** miR-29a & miR-146a & **increased** miR-21 & miR-155 in patients (late onset neonatal sepsis) Lower levels of miR-146a in the non-survivors	(215)
miR-22-3p	Adults (69/89) Serum and urine	Negative correlation with acute kidney injury (AKI)	(216)
miR-23a	Adults (27 sepsis, 22 non-infectious SIRS)	**Decreased** in sepsis vs SIRS patients	(89)
miR-23b	Neonates (27 early onset & 21 late onset sepsis) Serum	**Increased** in neonates with early onset sepsis	(217)
miR-25	Adults (70/30) Serum	**Decreased** in patients Negative correlation with severity	(218)
miR-26b	Adults (68 AKI and 87 non-AKI sepsis patients and 57 patients with non-infectious SIRS) Urine	**Increased** in patients with sepsis-associated AKI	(219)
miR-34a miR-199a-3p	Neonates (90/90) Serum	**Decreased** in sepsis neonates Associated with severity	(220)

(Continued)

TABLE 2 Continued

miRNA	Subjects (sepsis/controls [n] unless detailed) Sample type	Observations	Reference
miR-96	Neonates (66/58*) Serum	**Decreased** in sepsis neonates Targets IL-16	(221)
miR-101-3p	Neonates -	**Increased** in sepsis neonates Associated with PCT, CRP, IL-8 and TNF levels	(222)
miR-103	Adults (108/89) Serum	**Decreased** in patients Negative correlation with IL-1β, IL-6 and TNF levels	(223)
miR-103 miR-107	Adults (196/196) Plasma	**Decreased** in patients **Decreased** in ARDS when compared to non-ARDS patients Negative correlation with 28-days mortality	(224)
miR-122	Adults (25/25) Serum	**Decreased** in patients Limited predictive value for determination of outcome	(225)
miR-122	Adults (108/20) Serum	**Increased** in patients Independent risk factor for 30-day mortality	(226)
miR-122	Adults (232/24) Serum	**Decreased** in patients **Decreased** in ARDS when compared to non-ARDS patients Negative correlation with 28-days mortality	(227)
miR-122 miR-133a miR-143 miR-150 miR-155 miR-192 miR-223	Adults (204 ICU patients, among which 127 with sepsis) Serum	**Increased** miR-133a and **decreased** miR-143 & miR-223 in patients who did not survive the ICU stay A combination of 2 and 3 miRNAs predicts patients' long-term prognosis and survival in ICUs	(228)
miR-124	Adults (82/82) Plasma	**Decreased** in patients Negative correlation with lncRNA NEAT1 Negative correlation with 28-days mortality	(229)
miR-125a	Adults (196/196) Plasma	**Decreased** in patients Negative correlation with lncRNA MALAT1	(230)
miR-125a/b	Adults (150/150) Plasma	**Increased** in patients Positive correlation of miR-125b with 28-days mortality	(231)
miR-125b	Adults (120/120) Plasma	**Increased** in patients Positive correlation with 28-days mortality	(232)
miR-125	Adults (126/125) Plasma	**Decreased** in patients Negatively correlation with Scr, CRP, APACHE II score, SOFA score, TNF, IL-6, IL-8, IL-17	(233)
miR-126	Children (60/46) Serum	**Decreased** in patients Negative correlation with sepsis severity	(234)
miR-126	Adults (208/210) Plasma	**Increased** in patients Positive correlation with 28-days mortality	(235)
miR-127 miR-191 miR-320a	Adults (200 ICU among whom 140 sepsis/100) Platelets	**Increased** miR-320a/miR-127 ratio in sepsis patients	(236)
miR-130b-3p	Adults (15/7) Serum	**Increased** in patients	(62)
miR-132 miR-146a miR-155 miR-223	Neonates (25/25) Plasma	**Decreased** miR-132 & miR-223 in sepsis patients	(237)
miR-132 miR-223	Adults (80 sepsis-induced cardiomyopathy, 60 controls) Serum	Decreased in patients Negative correlation with CK, TNF & IL-6	(238)
miR-133a	Adults (223/76) Serum	**Increased** in patients Positive correlation with sepsis severity and 28-days mortality	(147)
miR-133	Adults (30/30) Serum	**Increased** in patients	(123)
miR-143	Adults (218 critically ill patients among which 135 sepsis/76 healthy controls) Serum	Trend for **decreased** levels in critically ill patients vs healthy controls No correlation with inflammatory markers Negative correlation with 28-days mortality	(239)

(Continued)

TABLE 2 Continued

miRNA	Subjects (sepsis/controls [n] unless detailed) Sample type	Observations	Reference
miR-143	Adults (103 sepsis, 95 SIRS, 40 healthy controls) Serum	**Increased** in patients, higher in sepsis than in SIRS Correlation with disease severity	(240)
miR-146a miR-155	Adults (146/19) Plasma	**Increased** in severe patients Positive correlation with sepsis-induced ALI	(241)
miR-146a	Adults (50 sepsis, 30 SIRS, 20 healthy controls) Serum	**Decreased** in sepsis vs SIRS patients and healthy controls	(242)
miR-146a	Adults (14/14) Plasma	**Decreased** in patients	(243)
miR-146a	Children (55/60) Serum	**Decreased** in sepsis children	(244)
miR-146a-5p	Adults (11/12) Plasma	**Increased** in patients Positive correlation with lactate and partial thromboplastin time	(144)
miR-146a/b	Adults (180/180) Plasma	**Increased** in patients Positive correlation of miR-146b with 28-days mortality	(245)
miR-146b	Adults (104/100) Plasma	**Decreased** in patients Negative correlation with ARDS	(246)
miR-147b	Adults (130 bacterial sepsis, 69 dengue hemorrhagic fever and 82 healthy controls) Plasma	**Increased** miR-146-3p, miR-147b, miR-155, miR-223 in sepsis compared to hemorrhagic fever and healthy controls Correlation with severity miR-147b diagnostic biomarker for patients with bacterial sepsis and septic shock	(247)
miR-150	Adults (223/76) Serum	No significant difference in critically ill patients with and without sepsis Negative correlation with 28-days mortality	(248)
miR-150 miR-4772-5p	Adults (22 SIRS, 23 sepsis, 21 healthy controls) Blood	**Decreased** miR-150 in patients, more decreased in sepsis vs SIRS patients **Increased** miR-4772-5p in sepsis patients	(249)
miR-150	Adults (120/50) Serum	**Decreased** in patients Negative correlation with IL-6 and TNF serum level, renal function and 28-days mortality	(150)
miR-150 Let-7a	Adults (22/20), urosepsis Leukocytes	**Decreased** in patients	(250)
miR-150	Adults (29 survivors and 12 non-survivors of sepsis) Serum	Most strongly **decreased** miRNA in healthy subjects infused with endotoxin	(251)
miR-150	Adults (30, with AKI/15) Serum	**Decreased** in patients	(156)
miR-150	Adults (78 sepsis/62 non-septic trauma patients/10 healthy controls)	**Decreased** in sepsis patients, increased in non-septic trauma patients	(146)
miR-150	Adults (299 survivors and 138 non-survivors of sepsis) Blood	**Decreased** miR-150 associated with 28-day mortality Combination of miR-150 and SOFA score improved prediction of prognosis	(252)
miR-155	Adults (73/83) Plasma	Positive correlation with sepsis-induced ALI and ARDS	(253)
miR-155	Adults (10/10) BALF	**Increased** in patients (all with ARDS)	(254)
miR-181a	Neonates (102/50) Serum	**Decreased** in sepsis neonates	(255)
miR-186-5p	Adults (34 sepsis and 34 respiratory infection/ pneumonia)	**Decreased** in sepsis patients	(172)
miR-206	Adults (93/28) Serum	**Increased** in patients	(256)
miR-218	Adults (53/20) PBMCs and Tregs	**Decreased** in patients **Decreased** according to severity	(257)
miR-223	Adults (187/186) Plasma	**Increased** in patients Positive correlation with APACHE II score, CRP, cytokines Higher in non-survivors than in survivors	(258)

(Continued)

TABLE 2 Continued

miRNA	Subjects (sepsis/controls [n] unless detailed) Sample type	Observations	Reference
miR-223	Adults (143/44) White blood cells	**Increased** in patients Higher in survivors than in non-survivors Negative correlation with lymphocyte apoptosis	(182)
miR-223	Adults (50 sepsis, 30 SIRS, 20 healthy controls) Serum	**Decreased** in sepsis vs SIRS patients and healthy controls	(242)
miR-223	Adults (137/84) Serum	No differential expression	(259)
miR-223	Adults (122/122)	**Increased** in sepsis Positive correlation with APACHE II and SOFA scores, and 28-day mortality	(260)
miR-328	Adults (110/89) Serum	**Increased** in sepsis patients Positive correlation with Scr, WBC, CRP, PCT, APACHE II score, and SOFA score	(261)
miR-410-3p	Neonates (88 sepsis, 86 pneumonia) Serum	**Decreased** in sepsis versus pneumonia patients Correlation with levels of lncRNA NORAD	(262)
miR-451a	Adults (98/65) Serum	**Increased** in patients Positive correlation with sepsis-induced cardiac dysfunction	(263)
miR-452	Adults (47 sepsis with AKI, 50 sepsis without AKI, 10 healthy controls) Serum and urine	**Increased** in serum and urine of sepsis vs controls. Higher in patients with AKI vs no AKI	(264)
miR-494-3p	- Blood	**Decreased** in sepsis patients Downregulates TLR6	(188)
miR-495	Adults (105/100) Serum	**Decreased** in patients **Decreased** in septic shock when compared to non-septic shock patients Negative correlation with sepsis-induced cardiac dysfunction	(265)
miR-1184	Children (30/30) Serum	**Decreased** in sepsis children	(191)
miR-1184	Neonates (72/56) Serum	**Decreased** in sepsis neonates Negative correlation with IL-16	(192)

*Controls were neonates with respiratory infection or pneumonia.
AKI, acute kidney injury; ALI, acute lung injury; APACHE, acute physiology and chronic health evaluation; BALF, bronchoalveolar lavage fluid; CK, creatine kinase; CRP, C reactive protein; lncRNA, long noncoding RNA; NEAT1, nuclear enriched abundant transcript 1; NEC, necrotizing enterocolitis; NGAL, neutrophil gelatinase-associated lipokalin; PCT, procalcitonin; PSP, pancreatic stone protein; Scr, serum creatinine; SELP, P-selectin; SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment; TLR, Toll like receptor; Treg, regulatory T cells; WBC, white blood cell.

migration, microcirculation lesions, tissue ischemia and organ failure (287, 288). Many endogenous and microvesicles-derived miRNAs regulate endothelial cell functions acting on apoptosis, proliferation, migration and inflammation (289–292). For example, miR-155 is increased in pulmonary endothelial cells of sepsis mice, targets the tight junction protein Claudin-1 and induces capillary leakage during infection (159). In a model of ALI, endothelial cell-derived exosomal miRNA-125b-5p downregulates topoisomerase II α resulting in reduced lung injury and inflammatory cell infiltration in the pulmonary mesenchyme (112). Decreased exosomal miR-125b (and miR-30a-5p) is associated with mortality in sepsis patients (197).

Platelets play a role beyond thrombosis and hemostasis, regulating innate immune cells including PMNs, monocytes and macrophages (287, 293, 294). Platelets are an important sources of miRNAs that are released through microvesicles or exosomes and are taken up by endothelial cells and macrophages (113, 295). Reduced miR-26b in platelets is associated with increased P-selectin expression, and with severity and mortality in sepsis patients (92). In fact, miR-26b reduces platelet adhesion and aggregation in mice (296). An increased miR-320a/miR-127 ratio in platelets could help detecting sepsis (236). Platelet microvesicles containing miR-126-3p are taken up by macrophages, strongly affecting the transcriptome and decreasing the expression of cytokines/chemokines/growth factors in the cells (113). Additionaly, miR-126-3p is associated with platelet activation (297). miR15b-5p and miR-378a-3p in platelet-derived exosomes obtained from sepsis patients induce the formation of neutrophil extracellular traps (NETs) involved in organ injury (80). On the contrary, platelet microparticles containing miR-223 reduce intercellular adhesion molecule 1 (ICAM-1) expression and binding to peripheral blood mononuclear cells by endothelial cells, providing a possible protective role against excessive sepsis-induced vascular inflammation (183).

3.1.3 miRNAs and host response to endotoxin (LPS)

Many studies analyzed miRNAs selected based on prior knowledge or miRNA screenings. While very instructive on a case-by-case basis, reductionist explorations tackle a small part of the role of miRNAs. A good illustration comes from reports on endotoxin, which is used as a model system to study host response to Gram-negative bacteria (Figure 3). The sensing of extracellular LPS by innate immune cells involves LPS binding protein (LBP), CD14, MD-2 and TLR4 (298, 299). TLR4, anchored at the cell membrane, recruits the adaptor molecule myeloid differentiation primary response 88 protein (MyD88). MyD88 activates a cascade of phosphorylation initiated at the level of IL-1 receptor (IL-1R)-associated kinase-1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), filling the NF-κB, IRFs and MAPK signaling pathways. These pathways control the transcription of immune response genes. Note that TLR4 shuttling to late endosome induces an alternative signaling through the adaptor molecule TIR domain-containing adaptor inducing IFNβ (TRIF). TRIF initiates IRF3 and late NF-κB activation, involved in the production of type I IFNs and IFN-inducible genes. For reasons of simplicity, this pathway is not described on Figure 3.

A few dozen of miRNAs, among which miR-15a, miR-16, miR-17-5p, miR-21, miR-25, miR-31, miR-98, miR-124-5p, miR-125b, miR-140-5p, miR-141, miR-146a, miR-149-5p, miR-155 miR-181c, miR-203-5p, miR-221, miR-326, miR-378, miR-448 and miR-466I, interfere at different levels with LPS sensing and LPS-induced signaling pathways (Figure 3). Note that miR-15a/16, miR-17-5p, miR-25, miR-125b, miR-141, miR-326 and miR-448 inhibit TLR4 expression, while miR-140-5p increases TLR4 expression. In addition, dozens of miRNAs among which miR-9-5p, miR-19a-5p, miR-21, miR-29, miR-93, miR-98, miR-125, miR-221, miR-222, miR-223 and let-7a-5p target the expression of downstream proinflammatory and anti-inflammatory cytokines. Finally, the inflammatory response itself regulates the expression of proinflammatory and anti-inflammatory miRNAs (273–277, 300–302). These observations provide insight into the complexity of miRNAs interactions during host antimicrobial responses, and highlight the challenge of taking a comprehensive and integrated view of the impact of miRNAs on immune responses.

3.1.4 miRNAs and endotoxin tolerance

Exposure of isolated innate immune cells or whole body to low amounts of LPS induces a transient period of refractory response to subsequent exposure to LPS, generally attested by inhibition of cytokine production. This phenomenon is known as endotoxin tolerance. Expression studies suggest that miR-146a and miR-146b are involved in endotoxin tolerance in THP-1 human monocytic cells (303–305). miR-146a disrupts both transcription and translation of *TNF* gene in tolerant THP-1 cells (305). miR-146b is induced by the anti-inflammatory

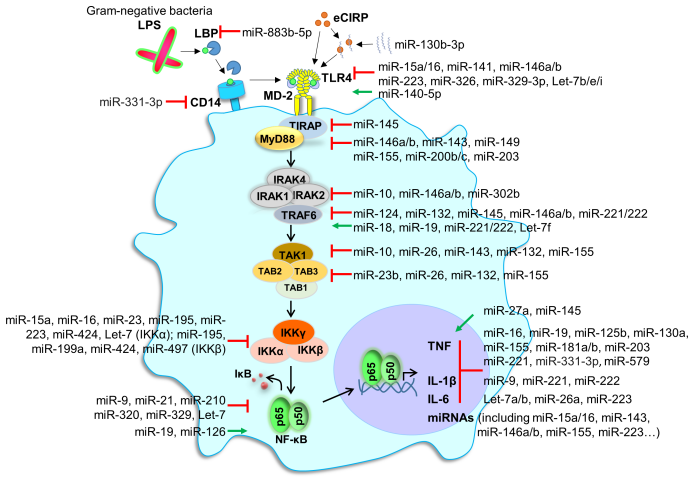

FIGURE 3
miRNAs and endotoxin sensing and signaling. The figure shows the recognition and intracellular signaling events following the sensing by monocytic cells of LPS from Gram-negative bacteria. LPS aggregates are dissociated by the LPS-binding protein (LBP). LPS/LBP complexes are transferred to CD14, a glycosylphosphatidylinositol-anchored molecule expressed on the membrane of monocytic cells. CD14 transfers LPS to TLR4 together with MD-2. This induces the recruitment of TIR domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response gene (MyD88). MyD88 is involved in early nuclear factor-κB (NF-κB) activation and pro-inflammatory gene expression. NF-κB signaling is involved in the expression of many miRNAs. For reasons of simplicity, we did not depict the TIR domain-containing adaptor inducing IFNβ (TRIF)-dependent, MyD88 independent, pathway involved in IRF signaling and late NF-κB activation. Red lines depict inhibition, while green lines depict activation (by miRNAs). eCIRP, extracellular cold-inducible RNA binding protein; IκB, inhibitory kappa B; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase-1; TAB, transforming growth factor-β (TGF-β) activated kinase 1; TRAF, TNF receptor-associated factor; TAK1, TGF-β activated kinase-1.

cytokines IL-10 and transforming growth factor (TGF)-β, but repressed by IFNγ which reverses endotoxin tolerance (304). Tolerance extends beyond LPS and TLR4 signaling. Bacterial lipoproteins recognized through TLR2 increase miR-146a expression and render THP-1 cells hypo-responsive to subsequent stimulation by *Salmonella typhimurium*. This is associated with a strong reduction of IRAK-1, phosphorylated inhibitory kappa B α (IκBα), and TNF production in tolerant THP-1 cells (306). Epigenetic mechanisms are involved in the establishment of tolerance. miR-146a and miR-155 are co-regulated in naïve and tolerant RAW 264.7 mouse macrophages. LPS stimulation induces histone 3 lysine 4 trimethylation ($H_3K_4me_3$, a mark of transcriptionally active genes) and NF-κB p65 binding to miR-146a and miR-155 gene loci. The induction of tolerance is associated with a shift towards repressive $H_3K_9me_3$ mark and the recruitment of CCAAT/enhancer-binding protein (C/EBP) β and p50 inhibitory component of the NF-κB complex to miR-146a and miR-155 genes (307).

3.2 Examples of miRNAs studied as modulators of innate immune responses and biomarkers of sepsis

Table 1 summarizes observations about miRNAs obtained in cells exposed to MAMPs/DAMPs, and in animals and humans with sepsis. Table 2 summarizes observations about miRNAs as potential biomarkers of human sepsis. We will not describe all studies because it would be tedious if not impossible. We will focus on miR-15a, miR-16, miR-122, miR-143, miR-146a/b, miR-150, miR-155 and miR-223 taken as examples of important and versatile miRNAs, and because these miRNAs are discussed in several publications in the sepsis field. This selection is arbitrary, but we will nevertheless see that even a limited sample of miRNAs provides insight into the complexity by which miRNAs on impact sepsis. Observations reported in precedent chapters will not be repeated.

3.2.1 miR-15a/16

miR-15a and miR-16 are members of the miR-15 family comprising miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, and miR-497. *miR-15a/16-1* cluster resides on human chromosome 13. miR-15a and miR-16 share the same seed sequence suggesting that they mediate similar biological functions.

3.2.1.1. Anti-inflammatory activity

miR-15a and miR-16 are commonly viewed as anti-inflammatory miRNAs. Bacterial infection and LPS increase miR-15a/16 in mouse bone-marrow derived macrophages, and in mouse lungs. miR-15a/16 target TLR4 and IRAK-1 in RAW 264.7 mouse macrophages exposed to LPS (78). In agreement, miR-15a/16 deficiency increases the expression of TLR4 through PU.1 (a transcription factor essential for TLR4 expression (308), and the phagocytosis and killing of *E. coli* by macrophages (77). Accordingly, miR-15a/16 knockout mice are resistant to CLP, *E. coli* and LPS-induced lethal sepsis (77). As an example of the connection between ncRNAs, the lncRNA SNHG16 downregulates the expression of miR-15a/16 and counter-regulates the inhibitory effects of miR-15a/16 on the expression of TLR4 in RAW 264.7 macrophages (209).

3.2.1.2 Inflammatory activity

LPS increases miR-16 expression in human monocytic cells and biliary epithelial cells through the MAPK pathway. In a counter-regulatory manner, miR-16 suppresses silencing mediator for retinoid and thyroid hormone receptor, and increases NF-κB transcriptional activity and expression of IL-1α, IL-6 and IL-8 in LPS-stimulated cells (81). Similarly, miR −15a−5p is increased in RAW 264.7 macrophages exposed to LPS, targets TNF-induced protein 3−interacting protein 2, activates the NF-κB pathway and increases cytokine production (79). A miR-15a-5p inhibitor reduces IL-1β, IL-6 and TNF and inflammatory response in mice challenged with LPS (79).

3.2.1.3 Biomarker value

miR-15a and miR-16 are increased in patients with systemic inflammatory response syndrome (SIRS) and sepsis patients when compared to healthy controls (n = 66, 32, 24). miR-15a levels are higher in SIRS than in sepsis patients (206). The screening of 13 miRNAs (miR-15a, miR-16, miR-21, miR-27a, miR-34a, miR-126, miR-150, miR-155 miR-181b, miR-223, miR-125b, miR-146a, miR-486) in 62 adult sepsis patients and 32 healthy controls shows that miR-15a, miR-16, miR-21, miR-125b, miR-126, miR-146a, miR-155, miR-181b, miR-223 are increased in sepsis patients. miR-15a is lower in patients with shock than in patients without shock (309). In a prospective observational study (117 survivors and 97 non-survivors with sepsis), a miRNome analysis shows that miR-15a (together with miR122, miR-193b* and miR483-5p) is increased in sepsis non-survivors, while miR-16 (and miR-223) is decreased (195).

Among seven miRNAs (miR-15a, miR-15b, miR-16, miR-206, miR-223, miR-378 and miR-451) measured in 46 neonatal sepsis patients, only miR-15a and miR-16 are increased, while miR-378 and miR-451 are decreased. Receiver operating characteristic (ROC) curve analyses suggest that miR-15a and miR-16 serum levels are good predictors of neonatal sepsis with area under the curves (AUCs) of 0.85 and 0.87 (78). miR-15a and miR-16 are increased in the serum of neonates with sepsis when compared to neonates with respiratory infection or pneumonia without sepsis (n = 62 and 32) (309). Finally, two recent studies report increased miR-15b and miR-16a in small cohorts of sepsis neonates (25 sepsis and 25 controls) (211, 212).

Overall, miR-15a/16 drive anti-inflammatory or inflammatory action, and are commonly increased in sepsis patients. A link with disease severity seems more uncertain.

3.2.2 miR-122

miR-122 was identified 20 years ago as a liver specific miRNA in mice (310). miR-122 is encoded on chromosome 18 in humans, and has no close paralog. miR-122 has been especially studied in the context of host response to liver-tropic viruses.

3.2.2.1 Anti-inflammatory activity

miR-122 is decreased in the liver of patients with hepatocellular carcinoma (HCC). The upregulation of miR-122 in HepG2 human hepatocellular carcinoma cell lines inhibits TLR4 expression. Moreover, miR-122 decreases the proliferation and the production of TNF and IL-6 by HepG2 and Huh7 hepatocellular carcinoma cell lines (107).

3.2.2.2 Inflammatory activity

miR-122 targets suppressor of cytokine signaling protein (SOCS) 1 and SOCS3, inducing IFNα/β expression and decreasing hepatitis B virus (HBV) replication (103, 104). miR-122 targets the receptor tyrosine kinases (RTKs) insulin like growth factor 1 receptor (IGF1R), fibroblast growth factor receptor and myeloid-epithelial-reproductive tyrosine kinase. Then, miR-122 decreases STAT3 phosphorylation and increases IRF1 signaling and the expression of IFNs in response to hepatitis C virus (HCV) and the synthetic analog of doubled stranded RNA poly(I:C) (105). miR-122 targets heme oxygenase-1 and decreases HBV expression in hepatoma cells (106). Related to sepsis, miR-122-5p is increased in the heart of rats and in H9c2 rat cardiomyocytes challenged with LPS. Inhibition of miR-122-5p reduces myocardial injury through inhibition of inflammation, oxidative stress and apoptosis in endotoxemic rats (108).

3.2.2.3 Biomarker value

At least four studies have reported decreased miR-122 levels in patients with sepsis when compared to healthy controls (201, 207, 225, 227). miR-122 levels are lower in ARDS than in non-ARDS patients and show a negative correlation with 28-days mortality (227). In contrast with these observations, miR-122 is increased in sepsis patients and is an independent risk factor for 30-day mortality (195, 226). Moreover, the levels of miR-122 (but not miR-15a, miR-16, miR-193b*, miR-223 and miR-483-5p) are higher in patients with coagulation abnormalities than in patients with normal coagulation tested at days 1, 3, 7 and 10 of ICU admission (208). Finally, other studies do not point to miR-122 differential expression in sepsis patients and healthy controls (198, 228). Hence, the biomarker value of miR-122 remains questionable.

3.2.3 miR-143

miR-143 is encoded in a bicistronic locus with miR-145, but has no homology with miR-145. miR-143 is considered as an anti-inflammatory miRNA. Few studies looked at the mechanisms of action of miR-143 in the context of innate immune response and sepsis.

3.2.3.1 Anti-inflammatory activity

The quantification of 455 miRNAs in blood leukocytes from heathy volunteers infused 4 hours with endotoxin identified miR-143 as the only upregulated miRNA. High levels of miR-143 are linked to decreased expression of B-cell CLL/lymphoma 2, a regulator of apoptosis an innate immune signaling, and the silencing of inflammation-related target genes (128). Mycobacterial cell wall glycolipid (Ac2PIM) and muramyl dipeptide (MDP) are recognized by TLR2 and NOD2. In mouse macrophages, Ac2PIM induces miR-143. In turn, miR-143 targets the NOD2 signaling adaptors TGF-β activated kinase-1 (TAK1) and receptor-interacting protein kinase 2. miR-143 suppresses PI3K/PKCδ/MAPK/β-catenin-mediated expression of cyclooxygenase-2 (COX-2), SOCS3 and matrix metalloproteinase (MMP)-9 induced by MDP (129). Thus, miR-143 negatively regulates the NOD2 pathway, which may have consequences on the development of vaccines and Gram-positive bacteria sepsis.

miR-143 is the most significantly downregulated miRNA in nasal mucosal tissues from patients with allergic rhinitis (311). miR-143 dampens inflammatory responses in upper airways (130). Bronchial epithelium cells exposed to angiotensin II (AngII) and LPS increase miR-143 which targets angiotensin converting enzyme 2 (ACE2). A miR-143-3p inhibitor increases ACE2 and decreases inflammatory cytokines and apoptosis in cells exposed to AngII and LPS (132). ACE2 protects mice from ALI induced by sepsis (312), so miR-143 may be used to decrease lung inflammation involved in ARDS. In a mouse model of mycoplasma pneumonia, a miR-143-3p mimic reduces IL-2 and TNF, increases IL-10 and reduces alveolar epithelial cell apoptosis. A miR-143 mimic decreases TLR4, MyD88 and phosphorylated NF-κB p50 in lungs. miR-143 might be used to inhibit the TLR4/MyD88/NF-κB signaling pathway and normalize pulmonary inflammation during pneumonia (133).

Mesenchymal stem/stromal cells (MSCs) therapy improves sepsis outcome. Treating human umbilical cord MSCs with poly (I:C) decreases miR-143 and increases the anti-inflammatory power of MSCs on macrophages. miR-143 targets TAK1 involved in TLR3 signaling and COX-2. The infusion of poly (I:C)-activated MSCs improves survival of CLP mice, while the co-delivery of miR-143 reduces the survival benefit provided by MSCs (134). Targeting miR-143 might have therapeutic potential in dampening inflammatory responses in sepsis. No study reported inflammatory activity of miR-143.

3.2.3.2 Biomarker value

Microarray and RT-qPCR analyses have been used to explore miRNAs in T cells and whole blood in 34 healthy controls and 31 sepsis patients. Thirty five miRNAs are differentially regulated in sepsis patients. miR-143 (and miR-15a, miR-16, miR-93, miR-223 and miR-424) is increased in sepsis patients. miR-143 levels correlate with T cell immunoparalysis. The discriminatory power of miR-143 in T cells performs well, with an AUC of 0.95. miR-143 correlates positively with sequential organ failure assessment (SOFA; a clinical score based on the assessment of 6 variables representing an organ system: respiration, coagulation, liver, cardiovascular, central nervous system, renal) score (204). Another study reports higher blood levels of miR-143 in patients with sepsis than in patients with SIRS, and in SIRS patients than in healthy controls (n = 103/95/40). miR-143 levels correlate with disease severity, evaluated by SOFA and Acute Physiology And Chronic Health Evaluation (APACHE) II (a clinical score that estimates ICU mortality based on laboratory values, age and previous health conditions) scores (240).

In a prospective observational study, miR-143 is similarly expressed in sepsis survivors and non-survivors (n = 117/97) (195). In a cohort of 218 critically ill patients, among which 135 sepsis patients, miR-143 levels are similar to those measured in healthy controls (n = 76). In ICU patients, miR-143 levels do not correlate with inflammatory markers, but correlate with indicators of organ failure (239).

Contrary to the above, miR-143 serum levels are higher in sepsis survivors than in sepsis non-survivors. The performance of miR-143 is rather modest (AUC = 0.628), yet it is higher than that of C-reactive protein (CRP), leukocyte count, creatinine and international normalization ratio value (239). In a subsequent report, the same team analyzed the prognostic scoring of combinations of miR-143 (and miR-122, miR-133a, miR-150, miR-155, miR-192, miR-223) in 204 ICU patients of whom 127 with sepsis (228). A "3 miRNAs" score based on higher miR-133a or lower miR-143 and miR-223 levels predicts patient survival in ICU. A "2 miRNAs" score (higher miR-133a and lower miR-150 levels) predicts patient long-term prognosis. The predictive power of the scores is increased by adding age into the calculation.

Overall, miR-143 is consensually anti-inflammatory. It is almost invariably increased in sepsis patients. Its usage as a biomarker remains unsure. miR-143 might be valuable incorporated in combined scores, but this should be confirmed in independent studies.

3.2.4 miR-146a/b

miR-146a and miR-146b are encoded on human chromosomes 5 and 10, respectively. They have nearly identical sequences and might share targets (313).

3.2.4.1 Anti-inflammatory activity

The group of David Baltimore reported in 2006 the negative impact of miR-146a/b on signaling in innate immune cells (314). miR-146a/b is an immediate early-response NF-κB-dependent gene induced by microbial components and proinflammatory mediators. IRAK1 and TRAF6 are targets of miR-146a/b (314). Macrophages from *miR-146a* knockout mice are hyper-responsive to LPS, and miR-146a restrains inflammation, myeloid cell proliferation, and oncogenic transformation *in vivo* (315). miR-146a inhibits NF-κB signaling and expression of cytokines, ICAM-1 and E-selectin, and trafficking induced by MAMPs in monocytes, macrophages, DCs, endothelial cells and keratinocytes (137, 138, 159, 316, 317). miR-146a inhibits the expression of STAT1, IFNγ and TNF, and the cytotoxicity of natural killer cells (318). A miR-146a agomir (a synthetic chemically modified double-strand miRNA) inhibits macrophage inflammatory response and protects mice from LPS-mediated organ damage (141). The delivery of a miR-146a-expressing plasmid decreases inflammatory cytokines and organ injury, and increases survival of mice subjected to CLP (138).

3.2.4.2 Inflammatory activity

Exogenous single stranded miR-146a-5p induces inflammatory responses through activation of TLR7 and proteasome, and downregulation of IRAK-1. miR-146a knockout mice show reduced inflammation and organ injury, improved cardiac function, and increased survival to acute sepsis induced by CLP (144). miR-146a-5p-mediated activation of TLR7 induces TNF, pulmonary inflammation, endothelial barrier disruption and ARDS in sepsis mice (145).

3.2.4.3 Biomarker value

miR-146a is increased in the blood of healthy subjects infused with endotoxin (251). Among 7 miRNAs measured in the serum of healthy controls, SIRS patients, and sepsis patients (n = 20/30/50), miR-146a and miR-223 are lower in sepsis patients (AUC = 0.804 and 0.858) (242). Similarly, reduced miR-146a levels discriminate sepsis from SIRS patients (AUC = 0.813) (243). In a pediatric study (n = 60/55 healthy and sepsis patients), miR-146a is decreased in blood and negatively correlated with the levels of C-reactive protein, procalcitonin (PCT), IL-6 and TNF. miR-146a levels correlate with sepsis severity and mortality, showing lower levels of miR-146a in non-surviving than in surviving patients (244). However, another study does not report differential expression of miR-146a in newborns with or without early-onset sepsis (n = 25/group) (237).

In contrast, miR-146a is increased in two studies analyzing adult patients (241, 245). In the first study (19 healthy controls, 102 sepsis, 44 severe sepsis), the AUCs of miR-146a and miR-155 for predicting 30-day mortality in ALI patients are 0.733 and 0.782 (241). In the second study (180 healthy controls, 180 sepsis

patients), miR-146a and miR-146b expression levels are predictors of sepsis risk (AUC = 0.774 and 0.897) (245). miR-146a and miR-146b positively correlate with APACHE II score, SOFA score, creatinine, CRP, IL-1β, IL-6, IL-17 and TNF. miR-146a and miR-146b are higher in survivors than in 28-day non-survivors. miR-146b has a better predictive value than miR-146a (AUC = 0.703 *vs* 0.599).

Overall, miR-146a is traditionally considered as anti-inflammatory, but 2 recent studies seem to contradict the uniform view. In the same manner, it remains unclear how miR-146a/b are modulated in human sepsis.

3.2.5 miR-150

miR-150 is encoded on human chromosome 19. miR-150 plays a role in hematopoiesis (319). miR-150 affects apoptosis, maturation and differentiation of lymphocytes and NK cells, and autoimmune diseases (320, 321). miR-150 is one of the four miRNAs (with miR-146b, miR-342, and let-7g) down-regulated in healthy subjects infused with LPS (128).

3.2.5.1 Anti-inflammatory activity

miR-150 targets notch receptor 1, STAT1 and NF-κB to inhibit LPS-induced apoptosis and IL-1β, IL-6 and TNF, ICAM-1, VCAM-1 and E-selectin in RAW 264.7 macrophages, THP-1 monocytic cells and endothelial cells (150, 154, 155). miR-150-5p is decreased in the heart of rats challenged with LPS. miR-150 decreases Akt2, cleaved caspase-3, Bax and apoptosis in rat heart and H9C2 cardiomyocytes (152). In a similar way, miR-150 binding to MALAT1 lncRNA inhibits the NF-κB pathway, cytokine production, ER stress and apoptosis in LPS-stimulated human umbilical endothelial cells, H9c2 cardiomyocytes, IL-1β-stimulated chondrocytes, and pulmonary arterial endothelial cells from CLP mice (150, 151, 153). miR-150-5p interacts with X-inactive specific transcript lncRNA to regulate the c-Fos axis, thioredoxin-interacting protein-mediated pyroptosis and sepsis-induced myocardial injury (157). In sepsis mice with acute kidney injury (AKI), miR-150 targets MEKK3, inhibits LPS-induced c-Jun N-terminal kinase (JNK) pathway, apoptosis and inflammation (156). miR-150$^{-/-}$ mice show increased mortality from LPS and CLP. Rescuing miR-150 in lung endothelial cells decreases EGR2-dependent Ang2 expression, restores adherent junction reannealing and endothelial barrier function, and reduces mortality (149). miR-150 inhibits ARG1 and the expansion and immunosuppressive function of MDSCs (146) that expand during severe infections and have been associated with nosocomial infections, morbidity, and mortality in critically ill patients (17, 28, 322). miR-150-3p may have similar expression pattern and activity as miR-150-5p. miR-150-3p is one of the most downregulated exosomal miRNAs (with 146a-5p, 150-3p, 151a-3p) in heat stroke, associated with inflammatory response and coagulation cascade (323).

3.2.5.2 Inflammatory activity

There is no formal demonstration of a proinflammatory activity of miR-150. Though, miR-150 is increased (and not decreased) in the serum of mice ongoing CLP-induced sepsis and in rats challenged with LPS (147, 148).

3.2.5.3 Biomarker value

Many studies have reported decreased miR-150 expression in sepsis conditions. An initial miRNome study identifies 17 differentially expressed miRNAs in sepsis patients and healthy subjects (n = 17/32). miR-150 is decreased in patients. miR-150 positively correlates with diseases severity evaluated by SOFA score, and inversely correlates with cytokine levels (203). The authors propose that miR-150 could be used as a biomarker of early sepsis.

miR-150 is decreased in healthy subjects infused with endotoxin (251), and in patients with urosepsis (250), sepsis with AKI (156), and other sepsis conditions (146, 150, 204, 249). Several studies have reported negative correlations between miR-150-5p and IL-1β and TNF serum levels, renal dysfunction and T cells immunoparalysis (150, 156, 204). Accordingly, sepsis patients with fatal outcomes have reduced miR-150 levels (150, 248, 251, 252). A combination of miR-150 and SOFA score improves prognosis prediction (252). However, while patients with sepsis show lower levels of miR-150 than patients with SIRS and non-sepsis trauma patients (146, 249), no significant difference is observed between critically ill patients with and without sepsis (248). It is proposed that miR-150 may be a useful biomarker or target in the diagnosis, prognosis and treatment of sepsis. This suggestion should be tempered since miR-150 is not differentially expressed in adult sepsis patients tested for 13 miRNAs (309) and, more annoying, in unbiased studies looking at miRNome (195, 196, 198, 202).

Several reasons explain why miRNAs biomarkers are not confirmed in miRNome studies. In any case, it shows that we could increase robustness of the methodology (including cohort constitution) to accurately demonstrate miRNA differential expression in sepsis. On the other side, all studies so far reported anti-inflammatory mode of action of miR-150.

3.2.6 miR-155

miR-155 is encoded on human chromosome 21. Its expression is increased by MAMPs, bacteria, viruses and parasites (267, 324–333). Captivatingly, the induction of miR-155 in macrophages is controlled by the molecular clock controller Bmal1, which in turn is repressed by miR-155. Thus, miR-155 is a regulatory component of the circadian rhythm, and of the circadian control of inflammation (267).

3.2.6.1 Anti-inflammatory activity

miR-155 targets TGF-β activated kinase 1 binding protein 2 (TAB2) and negatively regulates the TLR/IL-1 signaling cascade

in human DCs exposed to microbial stimuli (271). miR-155 inhibits caspase 1 and IL-1β by increasing autophagy through inhibition of TAB2. miR-155 agomir reduces lung pathology in mice with CLP (254). miR-155 inhibits IRF8-mediated antiviral response in Japanese encephalitis virus infected microglial cells (333). In *Francisella tularensis*-infected human macrophages, miR-155 downregulates MyD88 (327). The delivery of miR-155 inhibitor to mice challenged with LPS increases SOCS1, and reduces JAK and STAT3, cytokines, and kidney injury (325). In mice with CLP, a miR-155 mimic decreases JNK and β-arrestin 2 expression, reduces infiltration of macrophages and PMNs in the myocardium, and attenuates late sepsis-induced cardiac dysfunction (162). miR-155-deficient mice infected with H1N1 influenza virus and challenged 5 days later with *Staphylococcus aureus* show a robust induction of IL-17 and IL-23 and reduced bacterial burden in lungs. In a similar way, a miR-155 antagomir (*i.e.* anti-miRNAs, in the form of oligonucleotides silencing endogenous miRNAs) enhances lung bacterial clearance in mice (326). This could be relevant since post influenza bacterial pneumonia is an important cause of morbidity and mortality.

The infection of astrocytes with *Escherichia coli* induces miR-146a and miR-155 expression. In a feedback loop mechanism, miR-146a and miR-155 inhibit TLR- and epithelial growth factor receptor (EGFR)-mediated NF-κB signaling pathway and inflammation. miR-146a and miR-155 antagomirs increase brain inflammation in mice infected with *E. coli*. Thus, miR-155 acts coordinately with miR-146a to safeguard the central nervous system from neuroinflammatory damages (334).

3.2.6.2 Inflammatory activity

Pioneer studies published in late 2000's linked miR-155 with inflammation and innate immunity. miR-155 has been identified as a target induced by inflammatory mediators in macrophages (324). Subsequently, miR-155 is shown to repress SOCS1 and Src homology 2 domain containing inositol polyphosphate 5-phosphatase 1 to increase LPS-induced cytokine production by mouse macrophages (269, 270).

miR-155 transgenic mice produce more TNF in response to LPS and are more sensitive to endotoxemia (335). miR-155 deficient mice have a reduced capacity to clear *Streptococcus pneumoniae* colonization from the nasopharynx, which is associated with impaired recruitment of macrophages and induction of protective T helper (Th) 17 immune responses (328). PMNs from miR-155-deficient septic mice express less NETs. miR-155 deficiency is associated with reduced accumulation of PMNs, NETs, edema and lung damage in mice with CLP (158). miR-155 is increased in endothelial cells from endotoxemic mice, and in the serum and bronchoalveolar lavage fluid (BALF) from septic patients with ARDS. miR-155 promotes vascular permeability and capillary leakage (159). miR-155 deficiency reduces endothelial activation and leukocyte adhesion and infiltration into the myocardium, myocardial edema and dysfunction, vasoplegia, and mortality in mice with endotoxemia or CLP. miR-155 targets CD47 and angiotensin type 1 receptor to promote nitric oxide (NO)-mediated vasorelaxation and vasoplegia (161). Injection of a miR-155 inhibitor reduces inflammation and intestinal barrier dysfunction in mice with CLP (160).

3.2.6.3 Biomarker value

The measure of 13 miRNAs in the plasma of 32 healthy controls and 62 patients with sepsis shows that 11 miRNAs including miR-155 are increased in patients. miR-155 levels are not associated with severity or outcome (309). A miRNome identifies 11 differentially expressed miRNAs in sepsis patients compared to healthy controls (n = 60/30), but only miR-155 is confirmed by PCR. miR-155 is elevated in patients, and positively correlates with SOFA score. miR-155 shows a good prediction value of 28-day survival (AUC = 0.763). Interestingly. miR-155 levels are proportional to the percentage of $CD39^+$ regulatory T cells (201). Another study reports that miR-155 is increased in septic patients and is a valuable predictor of mortality (241). In a study analyzing 10 healthy controls and 10 sepsis patients with ARDS, miR-155 levels are elevated in BALF samples from sepsis patients (254). In a cohort of 156 sepsis patients of whom 41 with ALI and 32 with ARDS, miR-155 levels are higher in patients with ALI or ARDS, positively correlate with IL-1β and TNF, and negatively correlate with $PaO2/FiO_2$ ratio. miR-155 AUC for diagnosing sepsis with ALI/ARDS is 0.87 (253). A study comparing 218 critically ill patients (135 with sepsis) with 76 healthy controls shows that, in critically ill patients ≤ 65 years, high miR-155 levels are associated with increased survival. This is not the case in patients older than 65 years (336). Finally, miR-155 is similarly expressed in peripheral blood from newborns with or without sepsis (237).

To summarize, there are strong arguments in favor of anti-inflammatory and proinflammatory activities of miR-155. miR-155 is usually increased in adults with sepsis, and associated with worse outcome. This is not observed in elderly and newborns, suggesting that miRNA-based biomarkers should be interpreted according to patient's age.

3.2.7 miR-223

miR-233 is encoded on chromosome X in mammals, and is highly conserved among species. miR-223 regulates hematopoiesis and triggers granulopoiesis and macrophage differentiation (337–340). miR-223 targets NLRP3, IGF1R, HSP90, C/EBPα, C/EBPβ, E2F1, forkhead box protein O1, NF-κB p65, nuclear factor I A, PBX/knotted 1 homeobox 1, STAT3 and STAT5, which accounts for a broad range of biological effects (338).

3.2.7.1 Anti-inflammatory activity

miR-223 is predominantly expressed in myeloid cells and drives anti-inflammatory functions. miR-223 is involved in

macrophage polarization and activation, and negatively regulates neutrophil functions. miR-223 inhibits NF-κB p65 phosphorylation and IL-1β, IL-6, TNF and IL-12p40 expression in U-937 human monocytic cells stimulated with LPS and IFNγ (341). NLRP3 is a sensor of the classical inflammasome involved in gasdermin-D processing, pyroptosis and secretion of IL-1β and IL-18 (342). miR-223 suppresses NLRP3 expression and IL-1β production in mouse macrophages and PMNs (343). Stimulation of macrophages with LPS, CpG DNA or poly(I:C) decreases miR-233 expression, which results in increased STAT3, NF-κB and MAPK signaling and production of IL-1β, IL-6 and TNF (344, 345). In the same line, PMN-derived miR-223 inhibits NLRP3 and IL-1β expression, and reduces pathogenesis in mice with DAMPs-induced ALI (346). miRNA-223 is upregulated in blood and lung parenchyma during experimental and human tuberculosis (347), and in monocytes from patients with tuberculosis (341). In a mouse model, miR-223 restricts the expression of CCL3, CXCL2 and IL-6 and the recruitment of PMNs into the lungs. miR-223 knockdown sensitizes mice to *Mycobacterium tuberculosis* lung infection through exacerbated PMN-dependent lethal inflammation (347). miR-223 promotes MMP-1 and MMP-9 activity in macrophages. *M. tuberculosis* infection increases the expression of miR-223, MMP-1 and MMP-9 in lungs. In doing so, it favors bacteria dissemination. On the contrary, miR-223 impedes BMAL1, which influences the expression of the circadian clock genes CLOCK, PER1 and PER2. Thus, *Mycobacterium tuberculosis* interferes with circadian rhythm *via* a miR-223/BMAL1 axis to subvert host defenses (348). Mechanical ventilation and *Staphylococcus aureus*-induced ALI is increased in miR-223 deficient mice. Pulmonary delivery of miR-223 using nanoparticles inhibits ALI. Interestingly, the transfer of miR-223 from PMNs to alveolar epithelial cells may be involved in attenuating lung inflammation (349).

3.2.7.2 Inflammatory activity

miR-223 increases in lungs of mice exposed to cigarette smoke and LPS and human in pulmonary cells and monocytes exposed to inflammatory cytokines. miR-223 targets histone deacetylase 2 (HDAC2), resulting in increased expression of fractalkine. miR-223 negatively correlates with HDAC2 expression in lungs from chronic obstructive pulmonary disease (COPD) patients (181). High miR-223 levels might contribute to stimulate the NF-κB pathway, and decrease corticosteroid response and disease severity in asthma and COPD (350).

3.2.7.3 Biomarker value

Studies evaluating miR-223 as a sepsis biomarker have generated contradictory results. When compared to healthy controls, miR-223 serum levels are either reduced (197, 237, 242), increased (182, 199, 204, 207) or not affected (259, 309) in patients. Observations using severity as a variable appear more consistent since miR-223 levels are lower in sepsis patients than in SIRS patients (242), and in patients with sepsis-induced cardiomyopathy than in healthy controls (238). Yet, miR-223 levels are either lower (182, 195) or higher (260) in sepsis non-survivors than in sepsis survivors (351). Finally, miRNome studies have not pointed to miR-223 as a differentially expressed miRNA in sepsis (195, 196, 198, 202).

Overall, miR-223 is considered anti-inflammatory, albeit it might drive inflammatory effects by targeting HDAC2 in specific conditions. Clinical studies yielded heterogeneous results when assessing the potential of miR-223 as a biomarker of sepsis. However, a meta-analysis of 22 records, including 2210 sepsis, 426 SIRS, and 1076 healthy controls suggested that miR-223 could be used as an indicator for sepsis (351). It should be stressed however that miR-223 values were available in a subset of 6/22 studies.

3.2.8 Other miRNAs

Finally, we will describe few studies analyzing miRNAs in an unsupervised manner or in the context of specific clinical questions. A miRNome analysis in critically ill patients with intra-abdominal sepsis or non-infective SIRS and healthy controls (n = 29/44/16) has detected 116 blood miRNAs increased in SIRS patients. miRNAs are more abundant in non-infectious SIRS than in sepsis patients. The top five differentially expressed miRNAs, miR-23a-5p, miR-26a-5p, miR-30a-5p, miR-30d-5p and miR-192-5p, discriminate severe sepsis from severe SIRS (AUC = 0.74-0.92). miRNA levels inversely correlate with IL-1, IL-6, IL-8, CRP and pancreatic stone protein (PSP), but not SOFA score. Hence, sepsis and non-infective SIRS are characterized by distinct changes in blood miRNAs, which may be used for diagnostic approaches in critically ill patients (196). However, except miR-23a, none of the short listed miRNAs are considered as sepsis biomarkers in previous studies (89, 249).

A recent study evaluated blood changes of miR-15a-5p, miR-155-5p, miR-192-5p, miR-423-5p in 46 sepsis patients treated with gentamicin, vancomycin (*i.e.* nephrotoxic antibiotics) or non-nephrotoxic antibiotics (n = 20/7/19). Small changes of miRNAs are observed in the different groups. miR-15a-5p at day 7 of gentamicin treatment provides good discrimination between AKI and non-AKI. miR-155-5p and miR-192-5p positively correlate with creatinine and neutrophil gelatinase-associated lipokalin in patients receiving vancomycin (210). These data suggest that miRNAs expression might be modulated by antimicrobials, and may serve as diagnostic markers in sepsis patients receiving nephrotoxic antibiotics.

The expression of miR-146-3p, miR-147b, miR-155 and miR-223 (associated with inflammation, see **3.2**) was assessed in the plasma of patients with bacterial sepsis or dengue

hemorrhagic fever and healthy controls (n = 130/69/82). miRNAs are increased in patients with sepsis when compared to patients with hemorrhagic fever or to healthy controls. miR-147b, alone or in combination with PCT, discriminates septic shock (AUC ≥ 0.8). Thus, miR-147b may be a biomarker to support clinical diagnosis of severe sepsis (247).

Necrotizing enterocolitis (NEC) is the most common and severe gastrointestinal pathology in preterm infants. A microarray-based screening has identified 230 upregulated miRNAs and 16 downregulated miRNAs in NEC when compared to sepsis and non-NEC/non-sepsis groups. Targeted analyses in a large cohort shows that miR-1290 can efficiently differentiate NEC from neonatal sepsis and neonatal inflammatory conditions such as bronchopulmonary dysplasia (200). Plasmatic miR-1290 expression may help differentiating NEC from neonatal sepsis.

4 Conclusions

Over the past decade, miRNAs have been the focus of intense research in the field of critical illness and sepsis. Our understanding of the modes of action and impact of miRNAs on host inflammatory and antimicrobial defenses has increased dramatically. However, this has not yet improved clinical management. Possibly, intervention strategies with miRNA mimics or miRNA antagomirs could rebalance the dysregulated host response during sepsis (Figure 1). Unfortunately, no miRNA-based clinical trials have been registered for sepsis so far.

The data summarized in Table 1 and Figure 3 illustrate the complexity and wide range of action of miRNAs in inflammatory and infectious conditions. Some miRNAs have been ascribed both anti-inflammatory and proinflammatory activities. Many reasons may account for diverse observations, including differences between *in vitro*, *ex vivo* and *in vivo* settings, sterile and infectious models, organs and cell types examined, and kinetics. In *in vivo* sepsis models, a mediator may be beneficial or harmful depending on disease condition. For example, inhibition of macrophage migration inhibitory factor (a pleiotropic cytokine and central regulator of innate immune responses (352, 353) increased susceptibility to infection but protected from lethal sepsis (354–357). Similarly, blocking TLR4 at the onset of infection induced mortality from otherwise non-lethal peritonitis, while therapeutic administration of anti-TLR4 antibodies protected mice from lethal Gram-negative bacterial sepsis (299).

Using miRNA as biomarker in sepsis holds more short-term potential than therapeutic opportunities. Many studies reported that miRNAs: 1) discriminate healthy donors from sepsis patients, 2) distinguish sepsis from non-infectious clinically-related diseases, 3) predict severity and/or the mortality, and 4) correlate with clinical parameters or cytokines. However, conflicting observations currently make translation to clinics challenging. So, how to use more efficiently miRNAs as biomarkers?

There is a crucial need for improvement and standardization of clinical studies in order to generate comprehensive views of miRNome during sepsis. We advocate for more stringent methodologies, in terms of both study design, clinical data collection, and miRNA investigation strategies. Importantly, small cohorts tends to exacerbate individual variations, whereas targeted techniques (e.g. RT-qPCR) fail to generate a global landscape of the miRNA fluctuations. Even if constraining, derivation and validation cohorts should be envisaged to corroborate and improve robustness of observations. A key objective would be to run unbiased miRNome analyses in large cohorts of well-defined critically-ill patients with or without sepsis.

Sepsis is a heterogeneous syndrome. Mediators detrimental during the overwhelming phase of sepsis might be beneficial during the later immunosuppressive phase, and miRNAs should not deviate from this principle (Figure 1). In fact, new types of clinical trials using a precision-medicine approach have been launched to adjust treatment (immunosuppressive or immuno-stimulant) given patients' inflammatory status (see https://www.immunosep.eu/ as an example). We believe that studies should take into consideration the causative agent, the site of infection, medications and the inflammatory status to stratify patients. Bearing in mind disease progression, the timing of sampling should be recorded. Ideally, blood samples should be collected at hospital admission (ED, medical/surgical ICUs), and continued over time to have a longitudinal view of the expression miRNAs. For translational perspectives, it would be easier be detected miRNAs in serum or blood than in PBMCs or isolated vesicles.

Finally, miRNA expression levels are prone to be affected by individual parameters (age, sex, genetic, comorbidities…). Therefore, combination scores (including one or several miRNAs, demographic and/or clinical data) should also be considered. Along these lines, whole blood or single cell transcriptomic identified rather simple gene expression signatures to distinguish sterile inflammation from sepsis, sepsis from infection, viral infections from fungal and bacterial infections, peritonitis, and sepsis caused by community-acquired pneumonia (358–363). Ideally, polymorphisms affecting pri-, pre- and mature miRNA sequences or affecting the target gene sequence should be investigated as well (364).

Based on our current knowledge, clinical use of miRNA targeting in sepsis cannot be realistically envisaged. miRNAs might be used as biomarkers. However, further studies will be required to obtain robust results, in order to safely recommend the use of miRNAs as biomarkers of sepsis. This is a certainly an ambitious, but promising goal.

Author contributions

TR conceived the manuscript. TR and NA wrote the manuscript. NA, CG, CT, ITS and TR revised the manuscript. 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.

Publisher's note

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Integrated analysis to reveal potential therapeutic targets and prognostic biomarkers of skin cutaneous melanoma

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Skin cutaneous melanoma (SKCM) is a malignant tumor with high mortality rate in human, and its occurrence and development are jointly regulated by genes and the environment. However, the specific pathogenesis of SKCM is not completely understood. In recent years, an increasing number of studies have reported the important role of competing endogenous RNA (ceRNA) regulatory networks in various tumors; however, the complexity and specific biological effects of the ceRNA regulatory network of SKCM remain unclear. In the present study, we obtained a ceRNA regulatory network of long non-coding RNAs, microRNAs, and mRNAs related to the phosphatase and tensin homolog (*PTEN*) in SKCM and identified the potential diagnostic and prognostic markers related to SKCM. We extracted the above three types of RNA involved in SKCM from The Cancer Genome Atlas database. Through bioinformatics analysis, the *OIP5-AS1*-hsa-miR-186-5p/hsa-miR-616-3p/hsa-miR-135a-5p/hsa-miR-23b-3p/hsa-miR-374b-5p-*PTPRC*/*IL7R*/*CD69* and *MALAT1*-hsa-miR-135a-5p/hsa-miR-23b-3p/hsa-miR-374b-5p-*IL7R*/*CD69* ceRNA networks were found to be related to the prognosis of SKCM. Finally, we determined the *OIP5-AS1-PTPRC*/*IL7R*/*CD69* and *MALAT1-IL7R*/*CD69* axes in ceRNA as a clinical prognostic model using correlation and Cox regression analyses. Additionally, we explored the possible role of these two axes in affecting gene expression and immune microenvironment changes and the occurrence and development of SKCM through methylation and immune infiltration analyses. In summary, the ceRNA-based *OIP5-AS1-PTPRC*/*IL7R*/*CD69* and *MALAT1-IL7R*/*CD69* axes may be a novel and important approach for the diagnosis and prognosis of SKCM.

KEYWORDS

immune infiltration, skin cutaneous melanoma, long non-coding RNA, microRNA, mRNA, DNA methylation

Introduction

Although melanoma accounts for only 5% of all skin cancers, it results in 75% of skin cancer deaths and is highly aggressive and fatal (1). The prognosis of skin cutaneous melanoma (SKCM) is poor without surgical resection (2). SKCM causes 55,500 deaths every year, and its global incidence is increasing every year at a faster rate than that of other cancers. The morbidity and mortality rates of this disease vary greatly worldwide, mainly depending on the availability of early detection and primary care (3). Although there is still no satisfactory clinical treatment for aggressive skin malignant tumors, the approval of tyrosine kinase and immune checkpoint inhibitors has led to revolutionary changes in the treatment of melanoma as these inhibitors have a considerable impact on melanoma prognosis. However, the ensuing problem of drug resistance poses further challenges to current clinical management. Moreover, melanoma cells closely interact with the tumor microenvironment and immune system, and non-coding RNAs have recently been reported to play key roles in the occurrence and development of tumors (4). This information can help determining changes in tumor treatment targets and strategies. Therefore, the discovery of new diagnostic and therapeutic targets and prognostic indicators for SKCM is essential for advancing its clinical diagnosis, treatment, and prognosis.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with a length greater than 200 nucleotides (5) that are widely distributed in human organs and tissues. An increasing number of studies has shown that lncRNAs are involved in the progression of a variety of cancers and generally function as tumor diagnostic and prognostic markers (6). Most reports have demonstrated that lncRNAs exert its biological role *via* the competing endogenous RNAs (ceRNAs) mechanism (7). The ceRNA hypothesis suggests a new mechanism of interaction between the different types of RNAs by which microRNAs (miRNAs) bind to target mRNAs, inhibiting their translation or leading to their degradation; ceRNAs participate in the post-transcriptional regulation of gene expression by competitively binding to miRNAs (7). These single-stranded non-coding RNAs with a length of 21–23 nucleotides (8) have been particularly investigated regarding their role in tumor formation (9). miRNAs can function directly by interacting with mRNAs or other non-coding RNAs to indirectly affect mRNA expression (10). Many studies have focused on the ceRNA network related to lncRNAs in SKCM (11), but the pathogenesis of SKCM is intricate, and therefore, such studies are limited. Many other studies have only focused on lncRNA-miRNA interactions. However, in complex disease environments, one lncRNA can often bind to multiple miRNAs or multiple lncRNAs can bind to one miRNA (12), forming a complex network, which remains unclear. Interactions between RNAs in SKCM can be understood through ceRNA networks, which can reveal the pathogenesis of SKCM comprehensively and provide clinical diagnostic and/or prognostic markers for SKCM.

Phosphatase and tensin homolog (*PTEN*) is the most widely studied tumor suppressor gene regarding ceRNA and the only tumor suppressor gene with dual phosphatase activity (lipids and proteins) specificities identified to date. *PTEN* is also involved in the regulation of multiple intracellular signal transduction pathways and indispensable for preventing the occurrence and development of many cancers, as its deletion or mutation allows malignant cells to grow and metastasize, leading to cancer progression (13). Accordingly, *PTEN* is generally mutated in tumor tissues, including SKCM (14). Its low expression or inactivation can lead to SKCM tumor cell proliferation and invasion, and the low expression level of *PTEN* is directly related to SKCM tumor size, severity, metastasis, and marker levels (15, 16).

Research on SKCM-related disease models and potential prognostic biomarkers has mainly focused on the impact of single genes (17) and on the construction and prediction of ceRNA networks (11). However, the prognosis of SKCM in patients varies greatly because it is related to individual differences and to the complexity of molecular pathological changes in SKCM. Single and broad-use prognostic indicators of SKCM have been proposed in previous studies but there is a lack of in-depth and precise investigation on the distinct gene regulatory networks of SKCM. Although *PTEN* is widely expressed in SKCM and its low expression usually affects the prognosis SKCM patients, the ceRNA network associated with *PTEN* in SKCM has not yet been identified. Therefore, the present study aimed to identify the expression profile of the lncRNA-related ceRNA network in *PTEN*-expressing SKCM.

The lncRNA-related ceRNA network analysis for the *PTEN* gene based on The Cancer Genome Atlas (TCGA) public database allowed classifying SKCM tissues as $PTEN^{high}$ and $PTEN^{low}$ according to their high or low expression of *PTEN*, respectively. These two groups were compared and analyzed, as well as cancer tissues and normal skin tissues, considering three types of RNA: lncRNA, miRNA, and mRNA. Through function

Abbreviations: BP, biological process; CC, cell component; CCLE, Cancer Cell Line Encyclopedia; ceRNAs, competing endogenous RNAs; DE, differentially expressed; DEGs, differentially expressed genes; DElncRNAs, differentially expressed long non-coding RNAs; DEmRNAs, differentially expressed mRNAs; DEmiRNAs, differentially expressed microRNAs; FC, fold-change; GEO, Gene Expression Omnibus; GO, gene ontology; GTEx, Genotype-Tissue expression database; HPA, Human Protein Atlas; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; MF, molecular function; miRNAs, microRNAs; OS, overall survival; PTEN, phosphatase and tensin homolog; RNA-Seq, RNA sequencing; SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas; TIMER, Tumor Immune Estimation Resource; UALCAN, The University of Alabama at Birmingham Cancer data analysis portal.

enrichment analysis of related genes, the possible biological functions and roles of the ceRNA network in SKCM were described. Two main ceRNA axes were constructed, and the relevant biological processes and molecular pathways of these main networks in SKCM were obtained through correlation, survival, and gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. These led to the determination of the essential role of *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69* in SKCM. Furthermore, the methylation and immune infiltration analyses of *PTPRC*, *IL7R*, and *CD69* revealed the potential biological functions of these three RNAs in SKCM.

Materials and methods

Data collection and processing

A scheme of our research design is shown in Figure 1. The fragments per kilobase of transcripts per million reads mapped data from high-throughput RNA sequencing (RNA-Seq) were downloaded from the TCGA (https://portal.gdc.cancer.gov/; version 1.28.0) SKCM project and then converted into transcripts per million data followed by log2 transformation for correlation analysis. For performing differential analysis, the high-throughput RNA-Seq-counts data from the TCGA SKCM project was downloaded and normalized using the DESeq2 package (version 1.26.0) (18); the ggplot2 package (version 3.3.3) (19) of R (https://www.r-project.org) was then used to visualize the differential analysis results. The data included SKCM lncRNA, mRNA, and miRNA sequences. The reference genome included in the TCGA database was from the GENCODE database (GRCh38) (20). To further validate our results, the gene expression datasets of GSE3189 (including 45 SKCM samples and seven normal skin samples; platform GPL96) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (21). The expression levels of differentially expressed (DE) mRNAs (*IL7R*, *CD69*, and *PTPRC*) in different human tissues were verified using the Cancer Cell Line Encyclopedia (CCLE; https://sites.

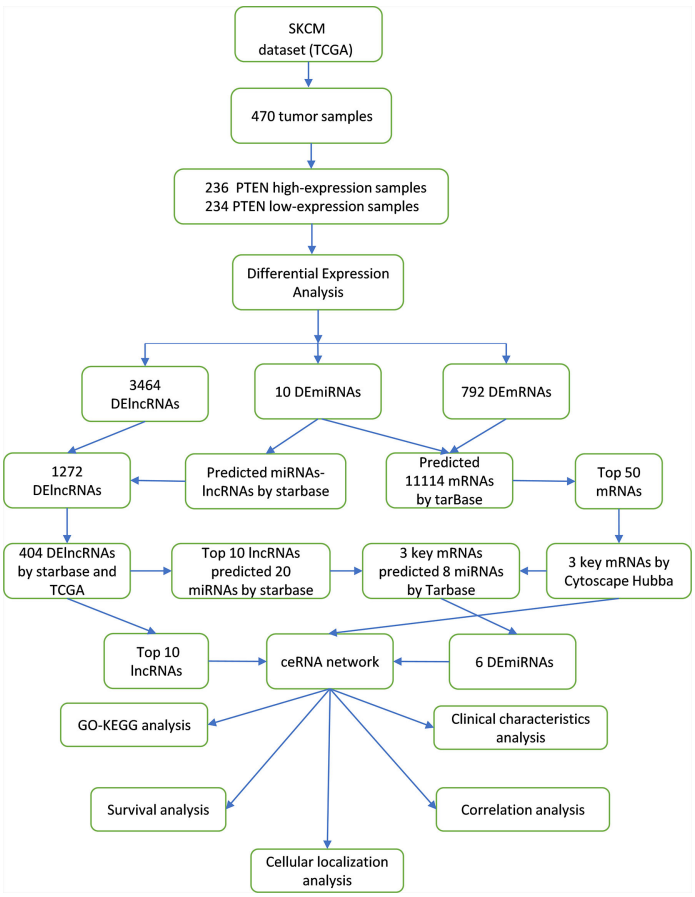

FIGURE 1
Construction and analysis of the ceRNA network in SKCM.

broadinstitute.org/ccle/), and data preprocessing and normalization were performed as described previously (22). The expression levels of *PTEN* and related genes in SKCM and normal skin tissues were verified using the Human Protein Atlas (HPA) database (https://www.proteinatlas.org/; version 21.1), and data preprocessing and normalization were performed as described previously (23). Based on HPA data, HPA031335 was selected as the anti-*PTEN* antibody and purchased from Sigma-Aldrich (St Louis, MO, USA). The mutation status of *PTEN* and related genes was obtained from the cBioPortal database (https://www.cbioportal.org/; version 4.1.13) (24). The data were retrieved on 16 August 2021.

Differential expression screening

When analyzing the expression of *PTEN* in the SKCM dataset, to determine high- and low-expression *PTEN* groups, and when performing the differential expression analysis between SKCM and normal skin tissues, lncRNAs, miRNAs, and mRNAs were considered as DE at $|\log \text{fold-change (FC)}| > 0.5$ and adjusted $p < 0.05$. Volcano plots and heatmap clustering of the DERNAs (including DElncRNAs, DEmiRNAs, and DEmRNAs) were visualized using the ggplot2 package of R. Cytoscape (https://cytoscape.org; version 3.8.2) (25) was used to perform GO/KEGG analyses and visualize differentially expressed genes (DEGs).

Construction of the ceRNA network in SKCM

The expression level of mRNA is regulated by miRNA. There is a competitive relationship between lncRNAs and miRNAs, which can regulate mRNA expression through sponge adsorption. We used the starBase (https://starbase.sysu.edu.cn/index.php) (26) database to predict the lncRNA-miRNA interactions and obtained all lncRNAs that could interact with the different miRNAs. By intersecting the 1,272 predicted lncRNAs with the 3,464 DElncRNAs resulting from TCGA database query, 404 lncRNAs were obtained by using VennDiagram package (version 1.7.3) (27) of R and 20 associated miRNAs were predicted using starBase and the top ten most divergent lncRNAs among the 404 lncRNAs. The DEmiRNAs between in the $PTEN^{high}$ and $PTEN^{low}$ groups were used to predict the corresponding DEmRNAs using the TarBase database (https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=tarbasev8; version 8) (28) and intersected with DEmRNAs analyzed using TCGA data, 266 mRNAs were obtained by using VennDiagram package of R. Then, we selected the top 50 DEmRNAs and analyzed them in the CytoHubba plugin (29) of the Cytoscape software to obtain the top three core mRNAs with the most significant correlations. Eight miRNAs corresponding to these three mRNAs were predicted using the TarBase database (28). Based on the intersection of the 20 miRNAs predicted by the top ten lncRNAs and the miRNAs predicted by the mRNAs, six miRNAs were obtained by using VennDiagram package of R. These lncRNAs, mRNAs, and miRNAs were imported into the CytoHubba plugin of Cytoscape to obtain the final two lncRNAs, five miRNAs, and three mRNAs, which were screened to construct the ceRNA network based on the overlapping miRNAs. These were selected by comparing all predicted miRNAs with the DEmiRNAs using the VennDiagram package (version 1.7.3) of R. The sequence information of lncRNAs was acquired from LNCipedia (https://lncipedia.org/; version 5.2) (30). The distribution of the screened DElncRNAs in the cell was obtained from the lncLocator database (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/; version 2.0) (31).

Enrichment analyses of DEGs

To analyze the mechanisms through which the main DEGs play a role in the occurrence of SKCM, we conducted enrichment analyses of DEGs. First, we analyzed the DEmRNAs in the ceRNA network using the Metascape database (https://metascape.org/gp/index.html#/main/step1; version 3.5) (32) and STRING database (https://cn.string-db.org/; version 11.5) (33). Then, we performed single-gene GO enrichment and KEGG analyses on *OIP5-AS1*, *MALAT1*, *PTPRC*, *IL7R*, and *CD69* in the ceRNA network by using DESeq2 package and clusterProfiler package (version 3.14.3) of R, and the analysis results were visualized using the ggplot2 package of R.

Relationship between the ceRNA network and the survival rate of SKCM patients

The fragments per kilobase of transcripts per million reads mapped data from high-throughput RNA-Seq were downloaded from the TCGA (https://portal.gdc.cancer.gov/; version 1.28.0) SKCM project and then converted into transcripts per million data followed by log2 transformation. Kaplan–Meier analysis of survival data was performed by the survival package (version 3.2-10), and the analysis results were visualized by the survminer package (version 0.4.9). The Logistic regression analysis model was constructed by the basic package of R, where the independent variable was OIP5-AS1, the low expression of OIP5-AS1 was used as the reference, and the dependent variables were TNM stage, Age, Gender, BMI, Pathologic stage, and Breslow depth. IL7R, PTPRC, CD69 and MALAT1

were analyzed in the same way as OIP5-AS1. We analyzed the survival rate of SKCM patients by Cox regression by using survival package (3.2-10 version), considering the DElncRNAs, DEmiRNAs, and DEmRNAs in the ceRNA network, and used these data to determine important biological markers related to the prognosis of SKCM.

CD69, *IL7R*, and *PTPRC* methylation and expression analysis

DNA methylation can cause changes in chromatin structure, DNA conformation, DNA stability, and the way DNA interacts with proteins, thereby regulating gene expression and affecting the characteristics of tumor cells. We used the DiseaseMeth (http://biobigdata.hrbmu.edu.cn/diseasemeth/; version 2.0) (34) and The University of Alabama at Birmingham Cancer data analysis portal (UALCAN, http://ualcan.path.uab.edu) (35) databases to evaluate the methylation levels of genes *CD69*, *IL7R*, and *PTPRC* in tumor and para-carcinoma tissues of SKCM patients. Simultaneously, the correlation between their methylation levels and important genes was analyzed by MEXPRESS (https://mexpress.be; new version) (36). Finally, MethSurv (https://biit.cs.ut.ee/methsurv/) (37) was used to perform a multivariate survival analysis to evaluate the dispersion of different CpG islands. The version numbers of UALCAN database and MethSurv database were not found on the official website.

The correlation between immune infiltration and CD69, *IL7R*, and *PTPRC* in SKCM

The correlation between immune infiltration and *CD69*, *IL7R*, and *PTPRC* genes and selected variables, was analyzed using TIMER (http://timer.cistrome.org/; version 2.0) (38). The relationship between *CD69*, *IL7R*, and *PTPRC*, their mutations and prognosis, with SKCM immune-infiltrating B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells were also analyzed using TIMER (38).

Statistical methods

SPSS (version 22.0) was used for data analysis. The results are presented as the median and 95% confidence interval. The Mann-Whitney test and independent *t*-test were used to evaluate the differences between any two groups of data. One-way analysis of variance and the Kruskal-Wallis and chi-square tests were utilized to evaluate the differences among different groups of data. Statistical differences were considered at $p < 0.05$.

Results

The expression of *PTEN* in SKCM and its influence on SKCM prognosis

We analyzed the expression of *PTEN* in both normal and cancer human tissues at the mRNA and protein levels using HPA database. The results showed that *PTEN* was expressed at a medium level in normal skin tissue (Figure 2A), but at low level in cutaneous melanoma (Figure 2B). Immunohistochemical staining results in the HPA database also confirmed a medium expression level of *PTEN* in normal tissues and low *PTEN* expression in SKCM tissues, consistent with the TCGA database results (Figure 2C, Figure S1, and Table S1). As *PTEN* is downregulated in SKCM, we analyzed the overall survival (OS) rate of SKCM patients with *PTEN* expression. The results suggested that low *PTEN* expression was associated with poor OS of SKCM patients (Figure 2D). Furthermore, we explored the possible mechanism of the low expression of *PTEN* in SKCM patients and analyzed the associated genome and copy number changes using cBioPortal. The OncoPrint plot showed the deletion of *PTEN* in the TCGA SKCM dataset (Figure 2E); moreover, as evidenced in Figure 2F, more than half of the SKCM dataset had *PTEN* deletions. Similarly, the mRNA expression levels in the *PTEN*-deficient SKCM dataset were significantly higher in the diploid and gain groups. Additionally, as the copy number of *PTEN* increased, the expression level of its mRNA also increased significantly (Figure 2G).

Analysis of DElncRNAs, DEmiRNAs, and DEmRNAs

The above results strongly suggested that the *PTEN*-related ceRNA network could be used as a prognostic evaluation indicator for SKCM. Therefore, we analyzed cancer tissues and their adjacent tissues to determine whether they had differential *PTEN* expression. We first divided the TCGA data into $PTEN^{high}$ and $PTEN^{low}$ expression groups using adjusted $p < 0.05$ and $|\log FC| > 0.5$ as screening conditions for DElncRNAs, DEmiRNAs, and DEmRNAs.

There were 1457 DElncRNAs (779 upregulated and 678 downregulated), 44 DEmiRNAs (33 upregulated and 11 downregulated), and 3339 DEmRNAs (1129 upregulated and 2210 downregulated) in the $PTEN^{high}$ expression group (Figures 3A-C). A heatmap was then constructed for the top 15 lncRNAs, miRNAs, and mRNAs with significant differences in expression between the $PTEN^{high}$ and $PTEN^{low}$ groups (Figures 3D-F). Both the volcano map and heatmap were used to visualize the results obtained using the ggplot2 package of R.

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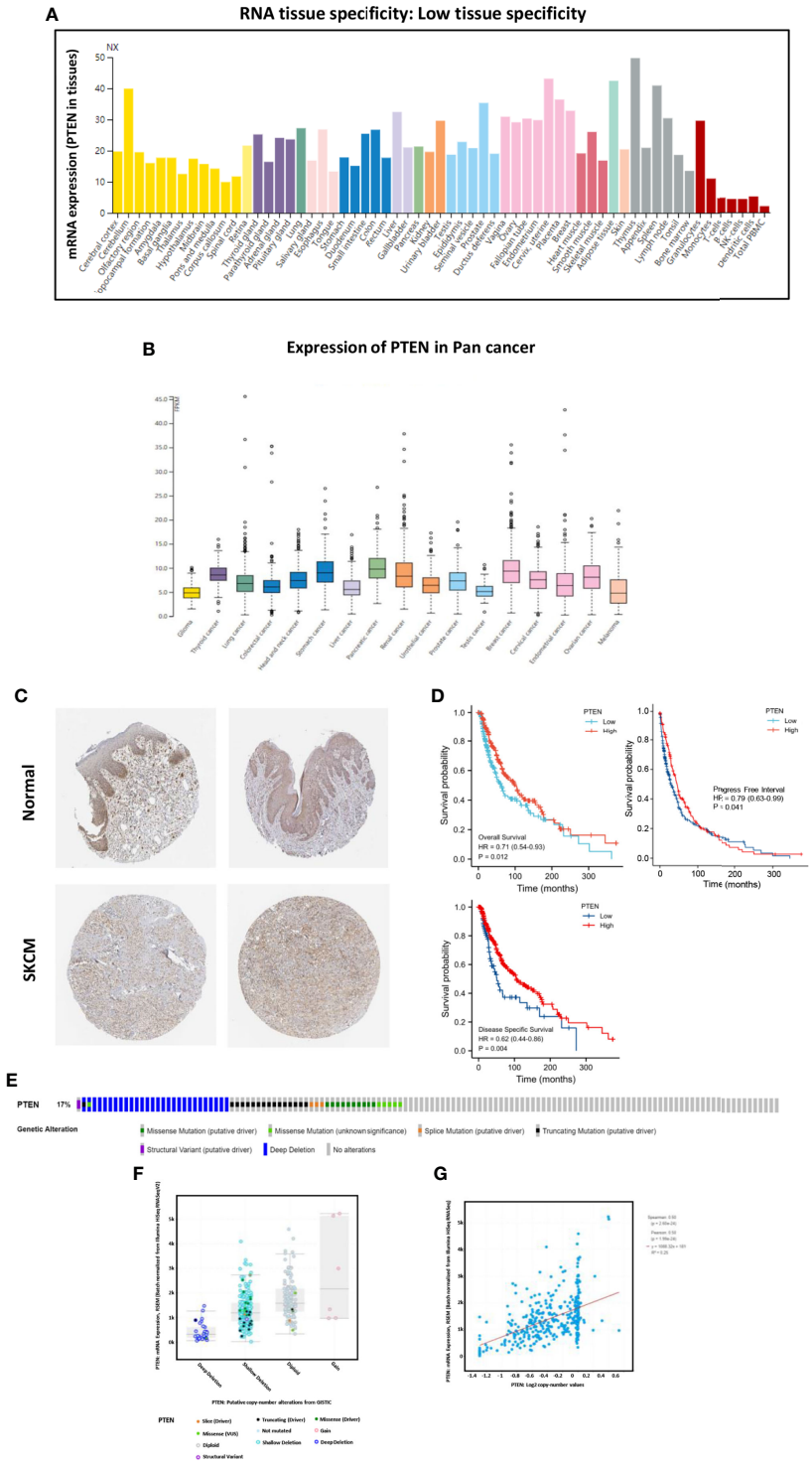

FIGURE 2
The tumor suppressor role of *PTEN* in SKCM. **(A)** Expression specificity of *PTEN* in various human tissues. **(B)** Expression distribution of *PTEN* in para-cancer patients. **(C)** Validation of the expression of *PTEN* at translational level using the Human Protein Atlas database (i.e., as determined by immunohistochemical staining). **(D)** Analysis of the relationship between the expression level of *PTEN* and patient survival rate, including overall survival, progress free interval, and disease specific survival. **(E)** Distribution of *PTEN* genomic alterations in SKCM according to The Cancer Genome Atlas (TGCA) database displayed as a cBioPortal OncoPrint plot. The relationship between *PTEN* copy number and mRNA expression are shown in the dot plot **(F)** and correlation plot **(G)**; they are all positively correlated.

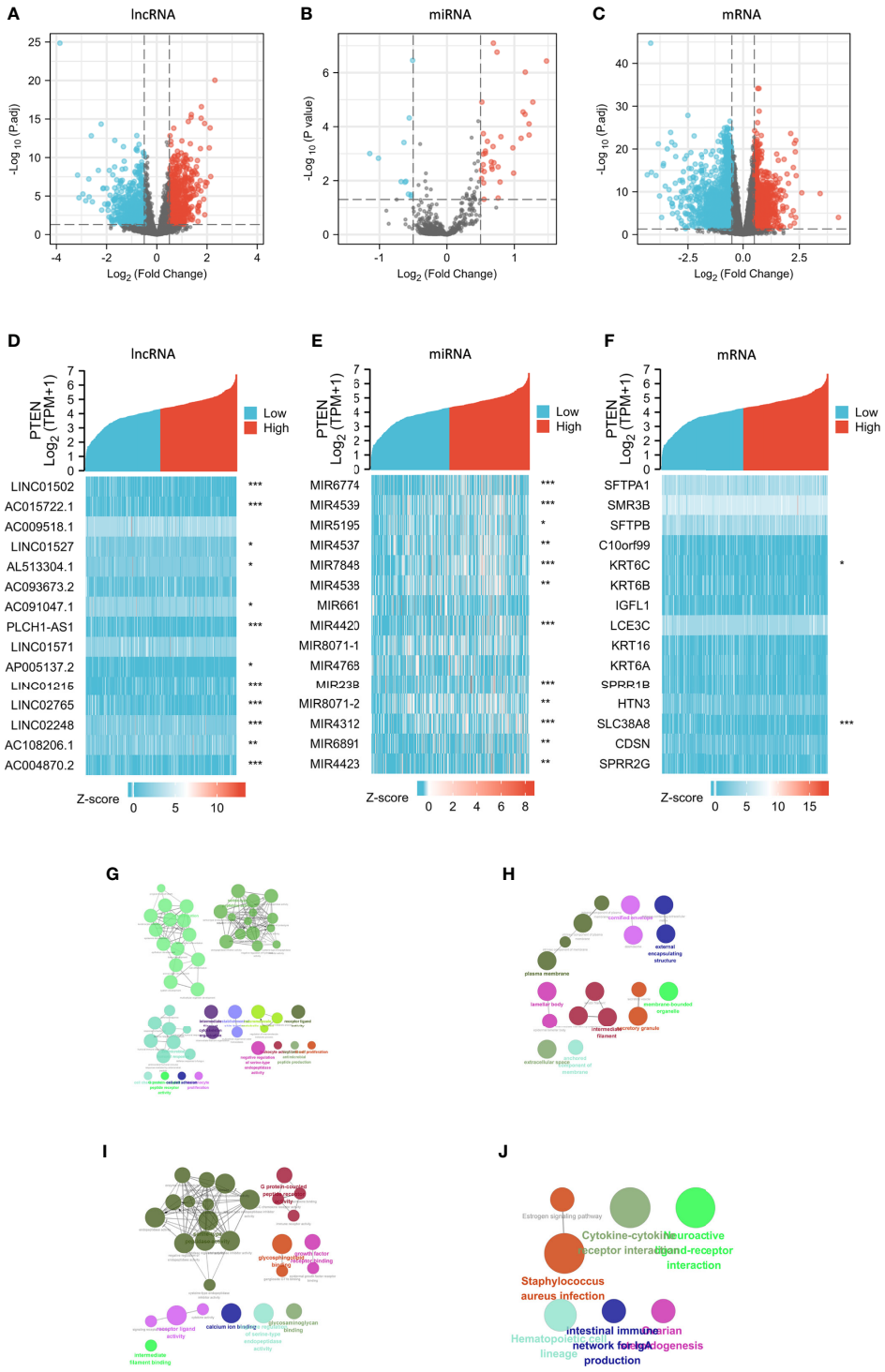

FIGURE 3
Analysis of DElncRNAs, DEmiRNAs, and DEmRNAs in groups characterized by *PTEN*^{high} and *PTEN*^{low} expression in the SKCM dataset. Red represents upregulated genes and blue indicates downregulated genes. Volcano plots describing **(A)** 1457 DElncRNAs (|log2 FC| > 0.5, adjusted p value < 0.05), **(B)** 44 DEmiRNAs (|log2 FC| > 0.5, adjusted p value < 0.05), and **(C)** 3339 DEmRNAs (|log2 FC| > 0.5, adjusted p value < 0.05). **(D–F)** The horizontal axis of the heatmap corresponds to groups and the vertical axis to 15 significant DEGs (lncRNAs, miRNAs, and mRNAs). **(G–J)** GO and KEGG analyses of significantly upregulated DEmRNAs in the *PTEN*^{low} group using Cytoscape. The color indicates a class of biological functions, whereas the size of the circle indicates the degree of significance. *p < 0.05, **p < 0.01, ***p < 0.001.

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We performed GO/KEGG analyses on selected DEGs using ClueGO plug-in of Cytoscape (25) (Figures 3G-J) and analyzed the different expression levels of lncRNAs, miRNAs, and mRNAs in SKCM and their adjacent tissues. In total, 584 DElncRNAs (226 upregulated and 358 downregulated), 23 DEmiRNAs (five upregulated and 18 downregulated), and 3102 DEmRNAs (1510 upregulated and 1592 downregulated) (Figures S2A-C).

Construction of the lncRNA-miRNA-mRNA triple regulatory network

To construct the lncRNA-miRNA-mRNA regulatory network, 1272 lncRNAs were first predicted based on their corresponding DEmiRNAs. Next, 404 lncRNAs were obtained by intersecting the predicted lncRNAs with the 3464 DElncRNAs obtained from the TCGA database analysis

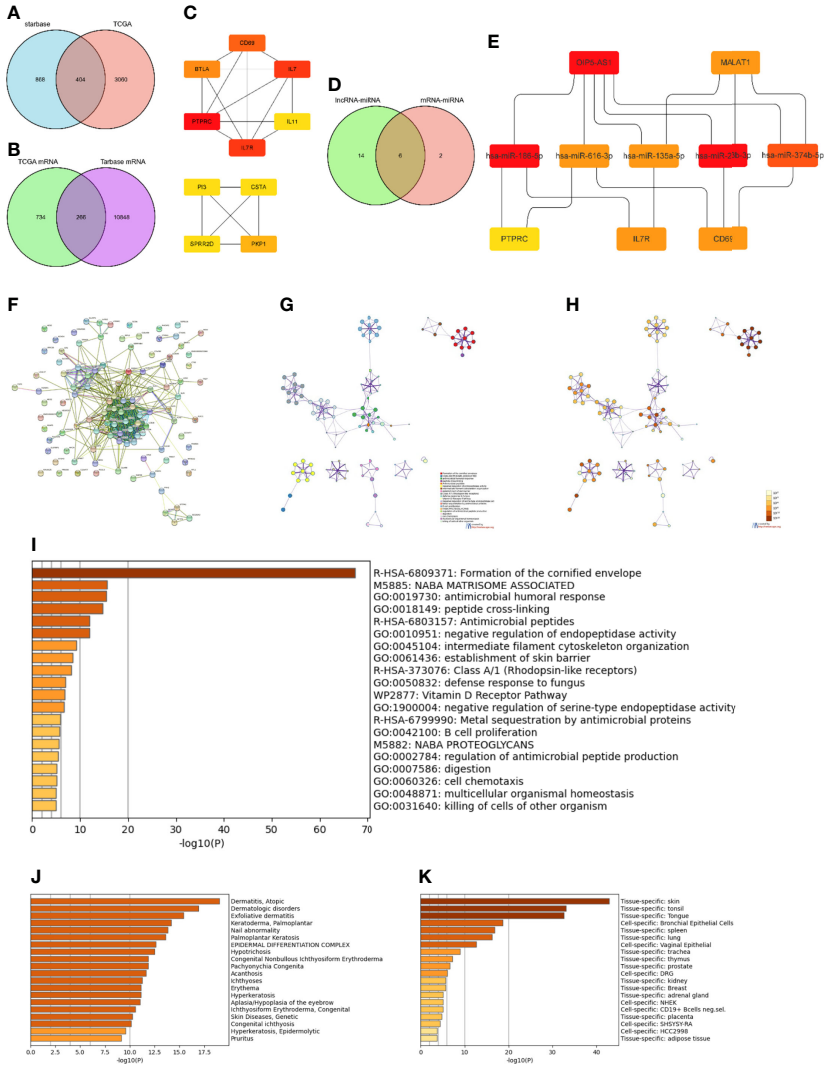

FIGURE 4
Construction and functional enrichment analysis of the lncRNA-miRNA-mRNA triple regulatory network. **(A)** Venndiagram of the 1272 lncRNAs predicted by the starBase database based on corresponding DEmiRNAs and 3464 DElncRNAs selected through the TCGA database analysis. **(B)** Venndiagram of 1000 DEmRNAs selected from TCGA and 11,114 mRNAs predicted from the DEmiRNAs in the TarBase database. **(C)** Interaction network analysis of core DEmRNAs obtained by Cytoscape. **(D)** Eight miRNAs predicted by the three most significant mRNAs intersected with the 20 miRNAs predicted from the top ten DElncRNAs. **(E)** Core ceRNA network predicted by the CytoHubba plug-in of Cytoscape. **(F)** Interaction regulatory network of 100 important DEGs related to the core ceRNA network obtained through STRING. **(G)** Enrichment analysis of the DEGs in **(F)** obtained by Metascape. **(H)** Significance of the enriched KEGG pathways. **(I)** Enriched terms in the DEmRNAs as obtained in Metascape, colored by p-values. **(J)** Enrichment analysis of DEmRNAs in DisGeNET, colored by p-values. **(K)** Enrichment analysis of DEmRNAs in PaGenBase, colored by p-values.

(Figure 4A). The ten lncRNAs with the largest differences (*FTX*, *ENTPD1-AS1*, *AL365361.1*, *OIP5-AS1*, *SNAI3-AS1*, *AL132656.2*, *PSMD6-AS2*, *MALAT1*, *POLR2J4*, and *WDFY3-AS2*) were then further analyzed. Except for *AL132656.2*, all other lncRNAs have been reported to be associated with cancers (39–47). However, no studies have reported that these lncRNAs play a specific role in SKCM. Additionally, we predicted 11,114 mRNAs from the corresponding 1000 DEmiRNAs in the TCGA database and obtained 266 mRNAs by intersecting both datasets (Figure 4B). The top 50 DEmRNAs were then analyzed, and the three core mRNAs, i.e., those with the highest significance, were selected (*PTPRC*, *IL7R*, and *CD69*) (Figure 4C). We identified eight miRNAs corresponding to these three mRNAs (hsa-miR-186-5p, hsa-miR-616-3p, hsa-miR-23a-3p, hsa-miR-135a-5p, hsa-miR-339-5p, hsa-miR-4677-3p, hsa-miR-23b-3p, and hsa-miR-374b-5p). The top ten DElncRNAs were also used to predict 20 DEmiRNAs (Figure 4D), which were intersected with the miRNAs predicted by the mRNAs in the TCGA database to obtain six core miRNAs (Figure 4D). Overall, two lncRNAs (*OIP5-AS1* and *MALAT1*), five miRNAs (hsa-miR-186-5p, hsa-miR-616-3p, hsa-miR-135-5p, hsa-miR-23b-3p, and hsa-miR-374b-5p), and three mRNAs (*PTPRC*, *IL7R*, and *CD69*) were considered to form the ceRNA network (Figure 4E).

Using the STRING database (33), the interactions of 100 DEGs surrounding the final ceRNA network were analyzed (Figure 4F). Functional enrichment analysis of all related DEGs in the interaction network showed that these DEGs were mainly enriched in the organization of intermediate filaments in the cytoskeleton, establishment of the skin barrier, and B cell proliferation (Figures 4G, H and Figures 4I-K). Thus, these results indicate that the ceRNA network obtained is involved in immune response.

Further analysis of DEGs in the ceRNA network and selection of an SKCM-specific prognostic model

In addition to analyzing the expression levels of ceRNA-related genes in normal, primary SKCM, and metastasis groups (Figures 5A-H), we also analyzed the relationship between the different stages of SKCM and ceRNA-related genes (Figures 5I-P) to establish a ceRNA network with important prognostic value for SKCM. *OIP5-AS1*, hsa-mir-186, *IL7R*, *CD69*, and *PTPRC* were significantly upregulated, while hsa-mir-23b was significantly downregulated in the metastasis group compared with the primary SKCM group (Figures 5A-H). The expression levels of *CD69*, *IL7R*, and *PTPRC* decreased with the progression of tumor stages (Figures 5I-P). The expression levels of these genes were confirmed in the SKCM dataset of the TCGA and Genotype-Tissue Expression (GTEx) databases (https://gtexportal.org/home/; version 8) (48) (Figure S2B).

Using Kaplan–Meier analysis and log-rank test, the expression levels of these DEGs were correlated with the OS rate of SKCM patients (Figures 6A-K). Except for hsa-miR-23b-3p, hsa-miR-135a-5p, and hsa-miR-616, the other RNAs involved in the ceRNA network significantly affected the OS rate of SKCM patients. Low expression of *CD69*, hsa-miR-186-5p, *IL7R*, *MALAT1*, *OIP5-AS1*, and *PTPRC* and high expression of hsa-miR-374b-5p and hsa-miR-616-3p were associated with poor OS of SKCM patients.

The functions of lncRNAs are determined by their location in the cell. We analyzed the cellular localization of *MALAT1* and *OIP5-AS1*. *MALAT1* was mainly expressed in the cytoplasm and cytosol, whereas *OIP5-AS1* was mainly expressed in the cytoplasm (Figure 7A), as predicted using lncLocator. These results indicated that these two lncRNAs can affect the binding of miRNAs through sponge adsorption, thereby affecting the expression of *CD69*, *IL7R*, and *PTPRC*. Therefore, we constructed a ceRNA network for *MALAT1* and *OIP5-AS1* (Figures 7B–D). Through correlation analysis, we found that *OIP5-AS1* was positively correlated with *CD69*, *IL7R*, and *PTPRC* and *MALAT1* was positively correlated with *CD69* and *IL7R* (Figures 7E–H). These three ceRNA axes could serve as potential prognostic prediction models. Additionally, we also analyzed the relationship between the expression levels in the ceRNA network and the different expression levels of *PTEN*, which demonstrated that the expression of *CD69*, *IL7R*, *MALAT1*, hsa-miR-186-5p, *OIP5-AS1*, and *PTPRC* was positively correlated with *PTEN* expression (Figure S3). Furthermore, we analyzed the expression levels of DElncRNAs (*OIP5-AS1* and *MALAT1*) and DEmRNAs (PTPRC, IL7R, and CD69) in SKCM and normal skin samples in the TCGA and GTEx databases, respectively. The results showed that *OIP5-AS1* and *MALAT1* were both downregulated in SKCM samples, and *PTPRC* and *IL7R* also showed low expression. In addition, using the GEO dataset, we verified that the expression levels of *PTPRC*, *IL7R*, and *CD69* DEmRNAs were downregulated in SKCM (Figures S4A, B).

Clinical significance of the three ceRNAs axes in SKCM

To confirm whether the three ceRNA axes are affected by clinical factors in patients with SKCM, we analyzed the correlation between the clinical factors and related RNAs in the three ceRNA axes. *OIP5-AS1* expression was correlated with the T stage ($p = 0.026$), N stage ($p = 0.011$), pathologic stage ($p = 0.029$), and Breslow depth ($p = 0.006$) of SKCM (Table S2). However, there was no significant correlation between the expression levels of *MALAT1* and these clinical factors (Table S3). *CD69* expression was significantly correlated with the T stage ($p < 0.001$) and Breslow depth ($p < 0.001$) (Table S4). T stage ($p < 0.001$), age ($p = 0.019$), and Breslow depth ($p < 0.001$) had a significant correlation with *IL7R* expression (Table S5). T stage ($p < 0.001$),

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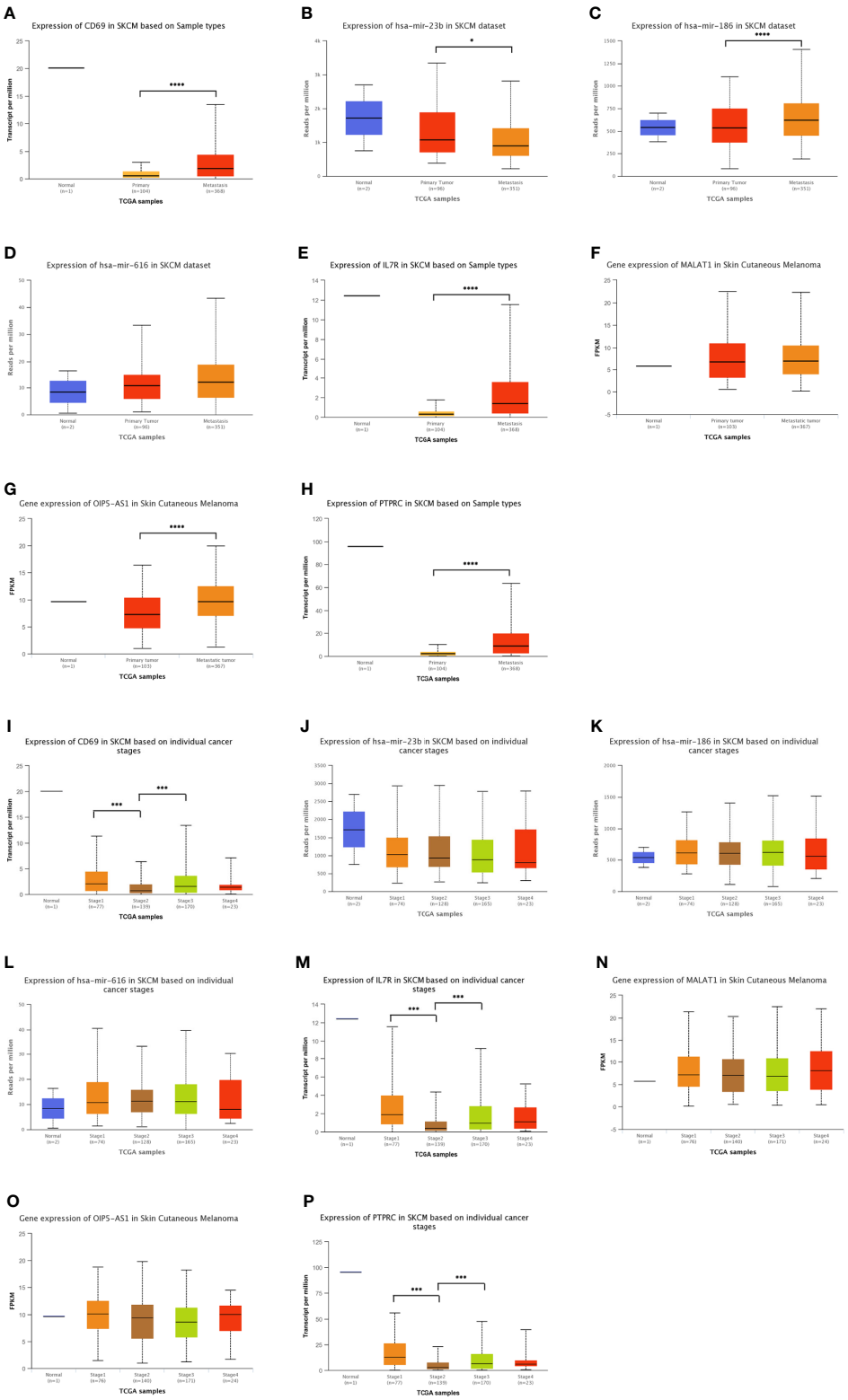

FIGURE 5
Distribution of the expression patterns of ten hub-genes in the triple regulatory network obtained from the UALCAN database. **(A–H)** The expression patterns of ten hub-genes in normal skin, primary SKCM, and metastatic SKCM datasets. **(I–P)** Expression patterns of the same hub-genes in tissue samples at different stages of SKCM. *p < 0.05, ***p < 0.001, ****p < 0.0001.

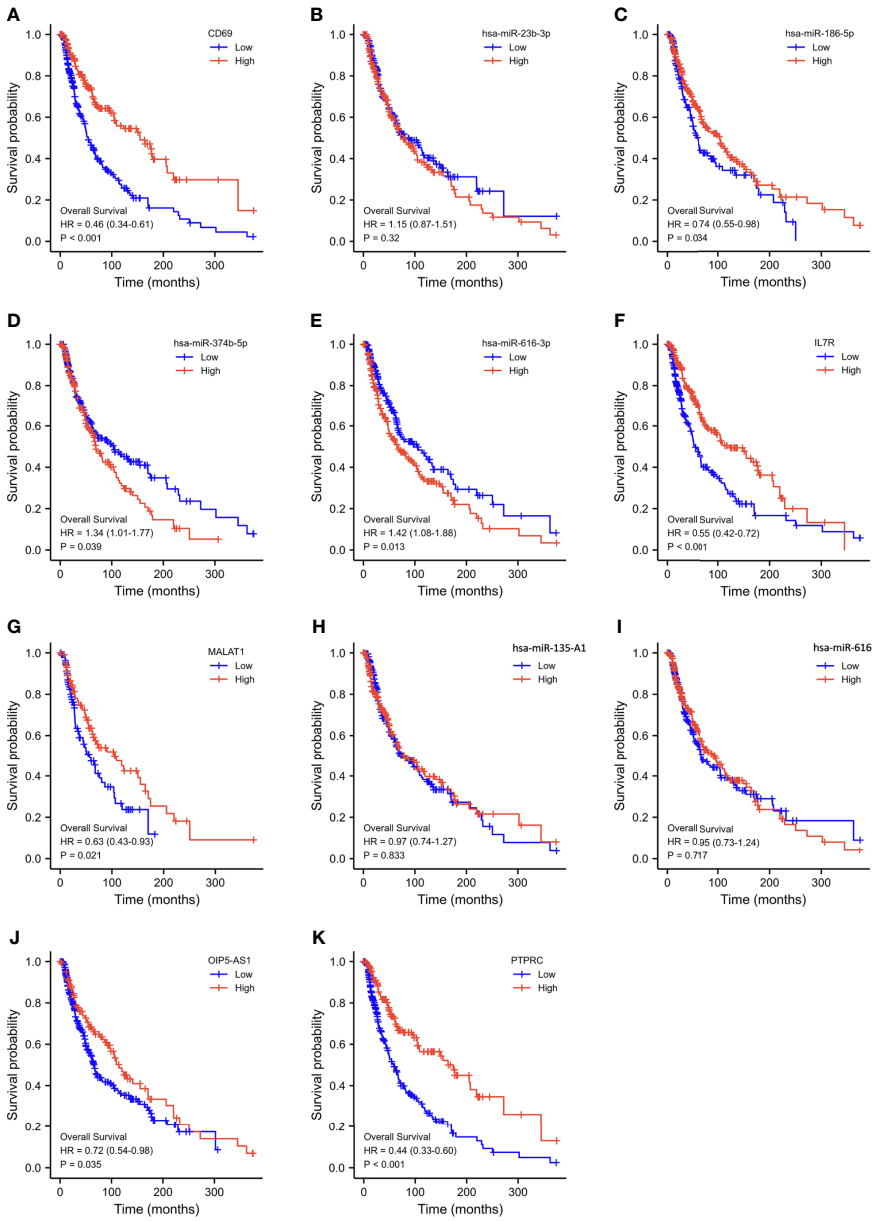

FIGURE 6
Overall survival analysis based on the expression levels of RNAs in the triple regulatory network. **(A–K)** Expression of ten hub-genes in the SKCM dataset with *CD69*^{high} and *CD69*^{low}, hsa-miR-23b-3p^{high} and hsa-miR-23b-3p^{low}, hsa-miR-186-5p^{high} and hsa-miR-186-5p^{low}, hsa-miR-374b-5p^{high} and hsa-miR-374b-5p^{low}, hsa-miR-616-3p^{high} and hsa-miR-616-3p^{low}, *IL7R*^{high} and *IL7R*^{low}, *MALAT1*^{high} and *MALAT1*^{low}, hsa-miR-*135A1*^{high} and hsa-miR-*135A1*^{low}, hsa-miR-*616*^{high} and hsa-miR-*616*^{low}, *OIP5-AS1*^{high} and *OIP5-AS1*^{low}, and *PTPRC*^{high} and *PTPRC*^{low} expression groups, which were compared using a Kaplan–Meier survival curve for TCGA SKCM patient cohort. The horizontal axis indicates the overall survival time in months and the vertical axis represents the survival rate.

age (p = 0.011), and Breslow depth (p < 0.001) were also significantly correlated with *PTPRC* expression (Table S6).

Univariate and multivariate Cox analyses were used to determine the relationships between clinically relevant features and prognosis of SKCM. Results from *MALAT1*, *OIP5-AS1*, *IL7R*, *CD69*, and *PTPRC* single-factor Cox model analysis and *MALAT1*, *IL7R*, *CD69*, and *PTPRC* multi-factor Cox model analysis showed that the TNM staging of cancer patients in the TCGA cohort, age, pathologic stage, and Breslow depth were closely related to the OS rate (p < 0.05) (Table S7-12). The expression level of *MALAT1* (hazard ratio = 0.840, p = 0.005), *PTPRC* (hazard ratio = 0.817, p < 0.001), *IL7R* (hazard ratio = 0.841, p < 0.001), and *CD69* (hazard ratio = 0.747, p < 0.001) was significantly negatively correlated with the OS rate. However, the

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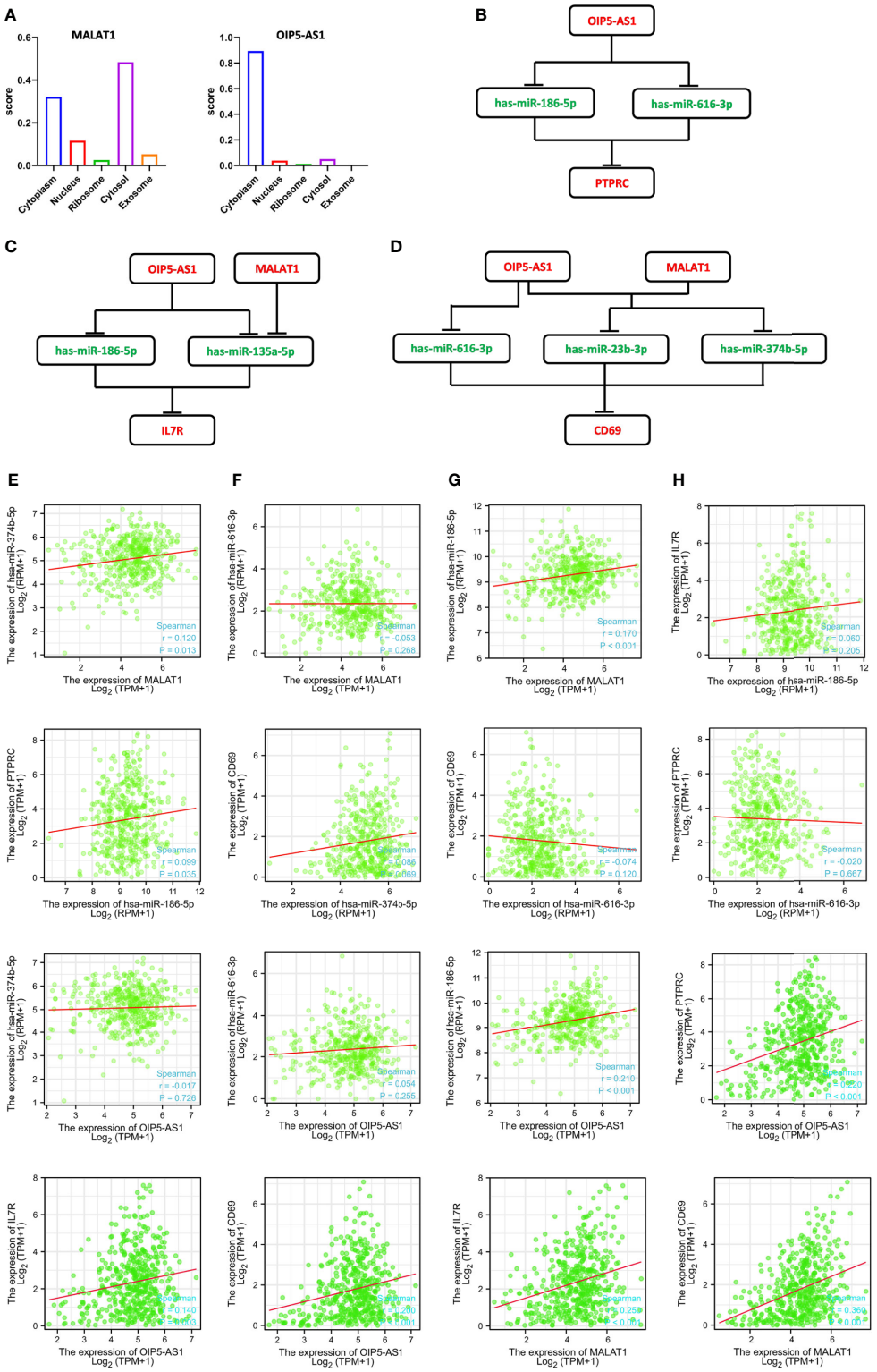

FIGURE 7
Construction and correlation analysis of the ceRNA network. **(A)** Cellular localization for two hub-lncRNAs (*MALAT1* and *OIP5-AS1*) predicted using lncLocator. **(B–D)** Schematic representation of the ceRNA networks. Green indicates downregulated and red indicates upregulated RNAs in SKCM. **(E–H)** Interaction correlation analysis between the ten predicted RNAs in SKCM, consistent with the predicted ceRNA networks.

expression level of *OIP5-AS1* (hazard ratio = 0.850, p = 0.059) was not significantly correlated with the OS rate. The results were obtained using the multivariate Cox model of these genes. The expression levels of *IL7R*, *CD69*, and *PTPRC* and the TNM stages of the tumor were significantly related to the OS rate of SKCM patients. Therefore, we hypothesize that *IL7R*, *CD69*, and *PTPRC* can become important indicators for SKCM prognosis.

Verification of abnormally high expression of *IL7R*, *CD69*, and *PTPRC*

We conducted a detailed analysis of *IL7R*, *CD69*, and *PTPRC* to explore the mechanism underlying the ceRNA network in SKCM. Through analysis of the data in the CCLE database, we found that *IL7R*, *CD69*, and *PTPRC* were highly expressed in SKCM cell lines (Figure S5), supporting our TCGA analysis (Figure 5).

Abnormal gene expression may be caused by gene mutations. The OncoPrint plot showed the amplification of *IL7R*, *CD69*, and *PTPRC* in SKCM in the TCGA dataset (Figure S6A). In the SKCM dataset there was no significant correlation between the expression and copy number of these genes (Figure S6B).

IL7R, *CD69*, and *PTPRC* methylation and expression levels

Exploring the correlation between the expression and methylation levels of *IL7R*, *CD69*, and *PTPRC* may help revealing the possible mechanism for the abnormal upregulation of these genes in SKCM patients. Therefore, we analyzed the methylation levels of these three genes in normal and SKCM tissues and their correlation with age (Figures 8A-I). There was no significant difference in the methylation levels of these genes in normal and SKCM tissues (neither primary nor metastatic tumor tissues) (Figures 8A-F). Moreover, there was no difference in the methylation levels of these three genes with age (Figures 8G-I). However, there was a significant negative correlation between the methylation levels of the three genes and the OS rate of SKCM patients—higher methylation levels were associated with lower OS rate (Figures 8J-L). The methylation levels also had a significant impact on OS outcomes (Figures 8M-O). We found four methylation sites in *CD69* (cg05590294, cg25769852, cg05179921, and cg07354583); cg05590294 was positively correlated with the expression level of *CD69*, and the remaining three sites were negatively correlated with its expression level. Three methylation sites (cg01804183, cg01027405, and cg04312209) were observed in *IL7R*; cg04312209 was negatively correlated with the expression level of *IL7R* while cg01804183 and cg01027405 were positively correlated. We observed 11 methylation sites in *PTPRC*; cg12390585, cg15626828, and cg22073152 were negatively correlated with the expression level of *PTPRC*, while the remaining sites were positively correlated (Figures 8P-U).

Expression levels of *IL7R*, *CD69*, and *PTPRC* in SKCM and immune infiltration

To evaluate the relationship between the expression levels of *IL7R*, *CD69*, and *PTPRC* and the level of SKCM immune infiltration, we performed a correlation analysis using the TIMER database. The results of the somatic copy number alterations module analysis showed that the level of immune cell infiltration in SKCM was related to the number of *CD69* gene copies and positively correlated with the copy numbers of *IL7R* and *PTPRC* genes in B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells (Figures 9A-F). The results also indicated that low levels of immune cell infiltration of B cells, CD8+ T cells, neutrophils, and dendritic cells were associated with poor prognosis in SKCM patients with an OS period of two years or less ($p < 0.05$) (Figure 9G). Our results indicated that the predicted ceRNA network is closely related to tumor immune infiltration, and the positive correlation between the two implies that the ceRNA network may affect the prognosis of SKCM by regulating the level of tumor-infiltrating immune cells. However, this causal relationship requires further exploration and experimental verification.

Enrichment analysis of the related functions of *OIP-AS1*, *MALAT1*, *IL7R*, *CD69*, and *PTPRC* in SKCM

We used GO and KEGG enrichment analyses to explore the putative functions of *OIP-AS1*, *MALAT1*, *IL7R*, *CD69*, and *PTPRC* genes in SKCM (Figure S7). GO enrichment analysis included biological process (BP), cell component (CC), and molecular function (MF) terms. For *OIP*-AS1, enriched BP terms included "epidermis development", "skin development", and "epidermal cell differentiation". CC terms included "lamellar body", "anchored component of membrane", and "basolateral plasma membrane" while MF terms included "structural constituent of epidermis", "peptidase regulator activity", and "receptor ligand activity". KEGG enriched pathways mainly included "alpha-linolenic acid metabolism", "arachidonic acid metabolism", and "carbohydrate digestion and absorption". The GO enrichment analysis of *MALAT1* revealed "epidermis development", "skin development", and "epidermal cell differentiation" as the main BP terms, "chylomicron", "GABA-A receptor complex", and "GABA receptor complex" as the main CC terms, and "inhibitory extracellular ligand-gated ion channel activity", "GABA-A receptor activity", and "chloride channel activity" as the main MF terms. KEGG enrichment

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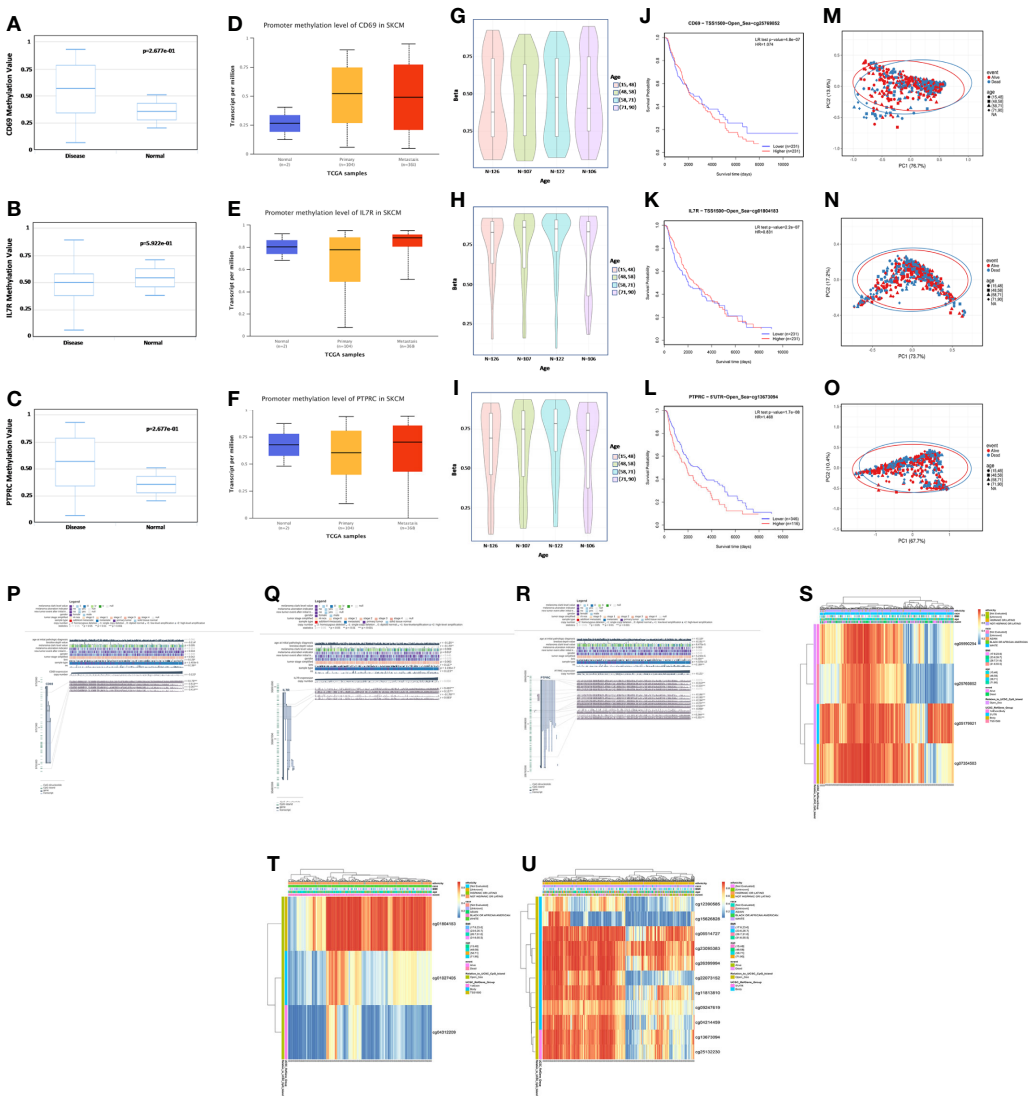

FIGURE 8
Methylation analysis of *CD69*, *IL7R*, and *PTPRC*. **(A–C)** Methylation levels of *CD69*, *IL7R*, and *PTPRC* in normal and disease datasets assessed using DiseaseMeth (version 2.0). **(D–F)** Promoter methylation level of *CD69*, *IL7R*, and *PTPRC* in normal skin, primary SKCM, and metastasis datasets evaluated using UALCAN. **(G–I)** Methylation level of *CD69*, *IL7R*, and *PTPRC* in normal tissues of people with different ages. **(J–L)** Correlation between *CD69*, *IL7R*, and *PTPRC* methylation levels and the survival curve of SKCM patients. **(M–O)** In the three genes, differences between the proportion of PC1 and PC2 is high, showing that the level of methylation has a significant association with survival outcomes of SKCM patients. **(P–R)** Methylation sites of CD69, IL7R, and PTPRC and their expression levels as visualized using MEXPRESS. The expression of *CD69*, *IL7R*, and *PTPRC* is illustrated by the blue line in the center of the plot. Pearson's correlation coefficients and p-values for methylation sites and query gene expression are shown on the right side. **(S–U)** Heatmap of the expression of different methylation sites in *CD69*, *IL7R*, and *PTPRC* in different samples; the ethnicity, race, body mass index, age, event, and other variables are indicated, and the whole analysis was carried using the MethSurv database.

mainly occurred on pathways such as "primary immunodeficiency", "nicotine addiction", and "cholesterol metabolism". The BP enriched terms of *IL7R* were "regulation of lymphocyte activation", "leukocyte migration", and "immune response-activating cell surface receptor signaling pathway". CC enriched terms were mainly "external side of plasma membrane", "plasma membrane receptor complex", and "immunoglobulin complex" and MF terms were mainly enriched in "regulation of lymphocyte activation", "immune response-activating cell surface receptor signaling pathway", and "positive regulation of cell activation". KEGG enriched pathways were mainly "external side of plasma membrane", "plasma membrane receptor complex", and "immunoglobulin complex". The top BP terms enriched for *PTPRC* were

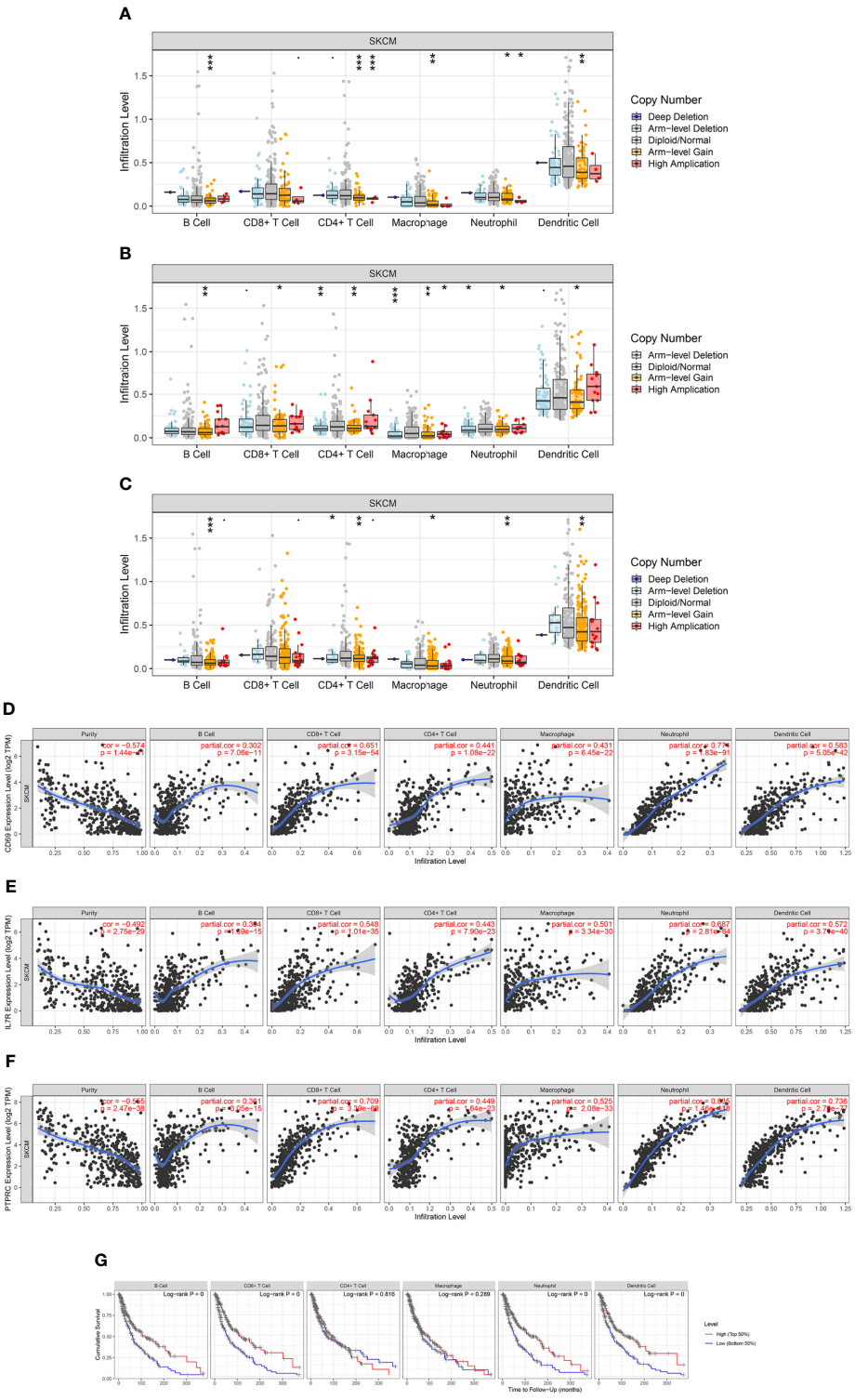

FIGURE 9
Correlation analysis of *CD69*, *IL7R*, and *PTPRC* expression and immune infiltration in SKCM. **(A–C)** Relationship between *CD69*, *IL7R*, and *PTPRC* gene copy number and immune cell infiltration levels in SKCM cohorts, including B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. **(D–F)** Interaction correlation of *CD69*, *IL7R*, and *PTPRC* expression with immune infiltration level in SKCM, including B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. **(G)** Kaplan–Meier plots showing the impact of immune infiltration level of various cells, including B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells, on the overall survival rate of SKCM patients. *p < 0.05, **p < 0.01, ***p < 0.001.

"regulation of lymphocyte activation", "immune response-activating cell surface receptor signaling pathway", and "positive regulation of cell activation". The top CC terms enriched were "external side of plasma membrane", "plasma membrane receptor complex", and "immunoglobulin complex" and MF enriched terms were "antigen binding", "receptor ligand activity", and "cytokine receptor". The main KEGG enriched pathways were "cytokine-cytokine receptor interaction", "hematopoietic cell lineage", and "cell adhesion molecules". The enriched BP terms of *CD69* were mainly "regulation of lymphocyte activation", "immune response-activating cell surface receptor signaling pathway", and "lymphocyte-mediated immunity". The main enriched CC terms were "external side of plasma membrane", "immunoglobulin complex", and "plasma membrane receptor complex" and the main MF terms enriched were "antigen binding", "cytokine receptor binding", and "carbohydrate binding". KEGG enriched pathways were mainly "cytokine-cytokine receptor interaction", "chemokine signaling pathway", and "cell adhesion molecules".

Discussion

Melanoma is one of the most fatal skin cancers. It has a low incidence but a high degree of malignancy, early metastasis, and high mortality. Most melanomas originate in the skin, are more common in men than in women, and frequently affect the feet and lower limbs, followed by the trunk, head and neck, and upper limbs. SKCM may also originate in the eyes and nasal cavity and has a high metastasis rate (3). Current clinical treatments for SKCM are mainly surgical resection, targeted therapy, and immunotherapy. However, problems such as metastasis and drug resistance are prone to occur, which affect the therapeutic efficacy and prognosis of patients (49). In-depth exploration of the pathogenesis of SKCM and development of new therapeutic and prognostic targets are therefore essential. With the continuous improvement in genomics technology, new types and functions of non-coding RNAs are constantly being identified. Among them, lncRNAs, a large category of non-coding RNAs, are abundantly expressed in various tissues of the human body. LncRNAs mainly function through a ceRNA mechanism. LncRNAs distributed in the cytoplasm can bind with miRNAs to affect the expression of target genes and biological functions (12). In tumors, lncRNAs can affect tumor cell growth, migration, and invasion through the ceRNA mechanism (50, 51). As a "star" tumor suppressor gene, *PTEN* is generally reported to be downregulated in tumor tissues, leading to the malignant proliferation of tumor cells (52, 53). Research on the ceRNA network related to *PTEN* in SKCM may not only reveal more complex tumor pathological processes but also lead to the development of novel clinical diagnoses, treatments, and prognostic indicators.

Using Cytoscape, we obtained a ceRNA network composed of 20 lncRNAs, six miRNAs, and eight mRNAs. Enrichment analyses revealed that these regulatory networks are mainly related to "establishment of skin barrier", "multicellular organismal homeostasis", "G protein-coupled peptide receptor activity", and "growth factor receptor binding". Furthermore, we analyzed the key regulatory network using hub analysis to obtain a core ceRNA regulatory network composed of two lncRNAs, five miRNAs, and three mRNAs. Subsequently, we analyzed the cellular localization of *MALAT1* and *OIP5-AS1*. As the ceRNA mechanism primarily occurs in the cytoplasm, the two lncRNAs were mainly distributed in the cytoplasm or cytosol. Thus, we obtained a ceRNA network composed of *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69*, which affects SKCM prognosis.

Several key molecules in the ceRNA network, such as *OIP5-AS1* and *MALAT1*, have been reported to be related to tumor pathology. High expression of *OIP5-AS1* promotes the growth and migration of melanoma cells (54). *MALAT1* promotes the growth and migration of melanoma cells by interacting with miRNAs (55). *PTPRC* is used as a prognostic indicator of cervical cancer (56), but its specific role in SKCM is still unclear. *CD69* is significantly related to the tumor immune microenvironment and participates in the tumor immune infiltration process in melanoma (57). *OIP5-AS1* protects microglia from hypoxia/ischemia-induced neuronal damage by sponging miR-186-5p (58). It also affects the pathological progression of atherosclerosis by acting as a miR-135a-5p sponge (59). Similarly, silenced *MALAT1* reduced myocardial ischemia-reperfusion injury in rat cardiomyocytes by regulating the miR-135a-5p/HIF1AN axis (60). Although there is no report on the ceRNA axis being directly related to *OIP5-AS1* or *MALAT1*, both hsa-miR-23b-3p and hsa-miR-374b-5p are involved in tumor progression (61, 62).

Previous research has reported that the abnormal expression of certain genes in tumors is unrelated to changes in gene copy number. Rather, it is regulated by epigenetics. The expression of the three significant DEmRNAs found here was not significantly associated with gene copy number, and their expressions were regulated by related lncRNAs and miRNAs. Epigenetic modification is a recently proposed mechanism of gene regulation. In addition to the regulation of non-coding RNAs, most studies on epigenetics have focused on DNA methylation (63, 64). Changes in methylation levels are common in tumors and affect tumor outcomes (65). In the present study, we explored the effect of DNA methylation on the expression of the genes related to the non-coding RNAs but found no significant methylation of *CD69*, *IL7R*, and *PTPRC* in tumor tissues compared with that of normal tissues. This may be related to the volume of the included datasets. Nevertheless, methylation levels were significantly negatively correlated with the OS prognosis of SKCM. Therefore, we analyzed the methylation sites as this information may be used as basis to

customize targeted therapy for other tumors and for further tumor-related methylation research in the future.

SKCM is one of the most immunogenic tumors and therefore highly likely to respond to immunotherapy. We analyzed the correlation between related target genes and immune infiltration and found that the expression levels of *CD69*, *IL7R*, and *PTPRC* were all related to the level of immune cell infiltration. Further analysis revealed that these three genes were positively correlated with the infiltration levels of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. These immune-infiltrating cells were significantly related to the OS rate and prognosis of tumor patients. This indicates that the *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69* axes in SKCM may affect the tumor immune microenvironment, leading to SKCM progression.

We further performed GO and KEGG enrichment analyses on the core lncRNAs and mRNAs and found that *OIP-AS1* KEGG was mainly enriched in the IL-17 signaling pathway and *MALAT1* in the primary immunodeficiency pathway. GO (BP, CC, and MF) analysis demonstrated that *OIP-AS1* was mainly enriched in epidermal cell differentiation, cell-cell junction, and peptidase regulator activity. *MALTA1* was enriched in humoral immune response, collagen-containing extracellular matrix, and anion transmembrane transporter activity. KEGG analysis showed that *IL7R*, *PTPRC*, and *CD69* were mainly enriched in chemokine signaling and cell adhesion pathways. GO analysis showed that the three genes were mainly enriched in immune-related terms such as lymphocyte-mediated immunity, immunoglobulin complex, and immunoglobulin receptor binding. These results suggest that these genes are related to the maintenance of the normal structure and function of the skin and immune response.

The *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69* axes obtained here provide possible clinical prognostic indicators for SKCM. However, we could not perform any further clinical experimental verification. Moreover, the specific biological and molecular functions of the *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69* axes in SKCM require further investigation.

In conclusion, we analyzed the *PTEN*-related ceRNA network in SKCM through a variety of bioinformatics analysis methods and obtained the specific expression patterns of the axes *OIP5-AS1-PTPRC/IL7R/CD69* and *MALAT1-IL7R/CD69* in SKCM. These results provide potential indicators for the clinical prognosis of SKCM and lays theoretical foundation for exploring the pathogenesis of SKCM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

XZ, study design, conceptualization, conducting the study, data collection and analysis, methodology, and writing the original draft of the manuscript. RR, conducting the study, data collection and analysis, and writing the original draft of the manuscript. SX, conducting the study and writing, reviewing, and editing the manuscript. WS, conducting the study, writing, reviewing and editing the manuscript, and data analysis. DJ, visualization and writing, reviewing, and editing the manuscript. XX, study design, conceptualization, and writing, reviewing, and editing the manuscript. All authors have contributed to the manuscript 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.914108/full#supplementary-material

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