miR-708 Negatively Regulates TNFα/IL-1β Signaling by Suppressing NF-κB and Arachidonic Acid Pathways

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Two pathways commonly dysregulated in autoimmune diseases and cancer are tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) signaling. Researchers have also shown that both signaling cascades positively regulate arachidonic acid (AA) signaling. More specifically, TNFα/IL-1β promotes expression of the prostaglandin E2-(PGE2-) producing enzymes, cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1). Exacerbated TNFα, IL-1β, and AA signaling have been associated with many diseases. While some TNFα therapies have significantly improved patients’ lives, there is still an urgent need to develop novel therapeutics that more comprehensively treat inflammatory-related diseases. Recently, researchers have begun to use RNA interference (RNAi) to treat various diseases in the clinic. One type of RNAi is microRNA (miRNA), a class of small noncoding RNA found within cells. One miRNA in particular, miR-708, has been shown to target COX-2 and mPGES-1. Previous studies have also suggested that miR-708 may be a negative regulator of TNFα/IL-1β signaling. Therefore, we studied the relationship between miR-708, TNFα/IL-1β, and AA signaling in diseased lung cells. We found that miR-708 negatively regulates TNFα/IL-1β signaling in nondiseased lung cells, which is lost in diseased lung cells. Transient transfection of miR-708 suppressed TNFα/IL-1β-induced changes in COX-2, mPGES-1. Previous studies have also suggested that miR-708 may be a negative regulator of TNFα/IL-1β signaling. Therefore, we studied the relationship between miR-708, TNFα/IL-1β, and AA signaling in diseased lung cells. We found that miR-708 negatively regulates TNFα/IL-1β signaling in nondiseased lung cells, which is lost in diseased lung cells. Transient transfection of miR-708 suppressed TNFα/IL-1β-induced changes in COX-2, mPGES-1, and PGE2 levels. Moreover, miR-708 also suppressed TNFα/IL-1β-induced IL-6 independent of AA signaling. Mechanistically, we determined that miR-708 suppressed IL-6 signaling by reducing expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activator inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ). Collectively, our data suggest miR-708 regulates TNFα/IL-1β signaling by inhibiting multiple points of the signaling cascade.

1. Introduction

Tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) are proinflammatory cytokines crucial for immune responses [1, 2]. It has been shown that both are necessary for effective host defense from bacterial, viral, and parasitic infections [2, 3]. TNFα is produced primarily by macrophages and activated T cells under inflammatory conditions [3]. Through its two cognate receptors, TNFα receptor 1/2 (TNFR1/2), TNFα regulates proinflammatory responses, cell death signaling, tissue repair, and angiogenesis [3]. Mechanistically, TNFα activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), Fas-associated protein with death domain (FADD), and TNF receptor-associated factor 1/2 (TRAF1/2) signaling axes [3, 4]. IL-1β expression is similarly activated, and its proinflammatory signaling can contribute to IL-R1-mediated NF-kB activation [5]. Given TNFα and IL-1β’s pivotal role in immune signaling and survival, their dysregulation has been implicated in various diseases.

Loss of TNFα signaling control has been shown to contribute to numerous diseases, including rheumatoid arthritis (RA), Crohn’s disease, atherosclerosis, cancer, and other autoimmune diseases [3]. Similarly, dysregulated IL-1β expression contributes to autoimmune diseases and cancer [5]. There are several approved TNFα inhibitors and one IL-1R antagonist used to treat autoimmune diseases,
highlighting the importance of this pathway in chronic inflammatory diseases. While these inhibitors have improved patient outcomes, 20–40% of RA patients are nonresponsive to TNFα inhibitors and resistance amongst responders is increasing [6]. More recently, researchers have begun to study the therapeutic potential of TNFα and IL-1β inhibition in cancer. Studies have shown that TNFα/IL-1β inhibition can suppress protumorigenic immune signaling through reduced angiogenesis, metastasis, and immune evasion [7, 8]. While previous work has highlighted the importance of these cytokines in disease, more efficacious therapies are still needed.

It was recently shown that cyclooxygenase-2 (COX-2), the rate-limiting enzyme in the arachidonic acid signaling pathway, was constitutively overexpressed in ulcerative colitis patients who did not respond to TNFα inhibitors [9]. Moreover, COX-2 is also overexpressed in RA and atherosclerosis [10]. Overexpression of COX-2 leads to exacerbated prostaglandin E2 (PGE2) production. While PGE2 is important for hematopoietic stem cell regeneration, inflammation, and gut integrity, increased levels can promote proliferation, invasion, survival, angiogenesis, and immune evasion in cancer [11, 12]. It has also been well documented that TNFα and IL-1β signaling promotes COX-2 expression [13–15]. Lastly, there is also significant crosstalk between TNFα, IL-1β, COX-2, and IL-6, another proinflammatory cytokine implicated in disease [16]. Given these data, it would be worthwhile to examine whether novel COX-2-inhibiting therapies can more efficaciously treat TNFα/IL-1β-related diseases.

One novel method to inhibit gene expression is through microRNA (miRNA). miRNAs are small (21–25 nts), non-coding RNAs that regulate gene expression posttranscriptionally [17]. miRNAs generally suppress gene expression by targeting the transcript’s 3′ UTR with incomplete complimentary, resulting in transcript degradation or translational stalling [17]. One miRNA in particular, miR-708-5p (miR-708), has been shown to target COX-2 in diseased lung cells [18]. miR-708 was also shown to target the NF-κB activator inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), which is intricately involved in canonical TNFα signaling [19]. Additionally, forced overexpression of miR-708 in human airway smooth muscle cells decreased expression of asthma-related genes [20]. miR-708 also decreased alcohol-induced liver inflammation by targeting zinc finger E-box-binding homeobox 1 (ZEB-1) upregulation of TNFα and IL-6 [21]. Lastly, injections of miR-708 mimic into a rodent RA model ameliorated the RA index by inhibiting WNT signaling [22]. Given the various immunoregulatory functions of TNFα, IL-1β, IL-6, and COX-2, paired with the anti-inflammatory functions of miR-708, we decided to investigate whether miR-708 negatively regulates TNFα and IL-1β signaling in disease.

In this article, we show that miR-708 expression is temporally induced by TNFα/IL-1β in nondiseased lung cells. Conversely, miR-708 expression is nonresponsive to TNFα/IL-1β in diseased lung cells. Exogenous miR-708 in nonresponsive lung cells reduced TNFα/IL-1β-induced changes in COX-2, mPGES-1, PGE2, and IL-6 levels. Lastly, we determined that miR-708 is inhibiting TNFα/IL-1β signaling dually by suppression of AA and NF-κB signaling in diseased lung cells. Collectively, these data suggest miR-708 is a potent negative regulator of TNFα/IL-1β, which is lost in diseased lung cells.

2. Methods

2.1. Cytokine Treatment for Lung Cells. First, 4 × 10^5 (A549/Beas2b) cells were plated in 60 mm dishes. The next morning, 3 mL of serum-free DMEM (4 mM L-glutamine and 1% Penicillin/Streptomycin) containing recombinant human 50 ng/mL TNFα (PeproTech, Rocky Hill, NJ) and 10 ng/mL IL-1β (PeproTech) was added to cells for 0–48 hours, followed by RNA/protein isolation or media removal for ELISA. If treated with miR-708, BAY-11-7082, or CEL, cells were pretreated O/N before TNFα/IL-1β treatment.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). A549 PGE2 levels in cell culture media were analyzed using the PGE2 Express ELISA Kit (500141, Cayman Chemical, Ann Arbor, MI) per manufacturer’s instructions. Media was removed, and cells were incubated for 20 min with serum-free media containing 10 μM arachidonic acid (Cayman Chemical) in serum-free DMEM. Collected media was centrifuged at 5000 g × 10 min, 4°C. Media was transferred to new tubes and then centrifuged at 2000 g × 10 min, 4°C, before being transferred to new tubes. Before analysis, samples were diluted 10x with 1x ELISA buffer. Absorbance was read using the SpectraMax M2 plate reader (Molecular Devices, San Jose, CA). PGE2 levels were measured in technical duplicates, normalized to total protein levels, and are an average of ≥3 biological replicates. When probing for IL-6, we used a similar kit from Cayman Chemical (501030) in accordance with the manufacturer’s protocol. In contrast to the PGE2 ELISA, complete media was collected from cells at each time point indicated. Centrifugation was performed as stated above. Media was not diluted prior to performing the IL-6 ELISA. IL-6 levels were measured in technical duplicates, normalized to total protein levels, and are an average of ≥3 biological replicates.

2.3. Mammalian Cell Culture. A549 cells (ATCC, Manassas, VA) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, MilliporeSigma) supplemented with 10% FBS, 4 mM L-glutamine, and 1% Penicillin/Streptomycin. All cells were incubated at 37°C in a 5% CO2 incubator and subcultured using 0.05% Trypsin, 0.53 mM EDTA (Corning, Corning, NY).

2.4. miRNA, BAY-11-7082, and CEL Treatments. A549 cells were seeded in 6-well plates at 3 × 10^5 cells per well. Synthetic versions of hsa-miR-708-5p and nontargeting miRNAs were purchased from Horizon Discovery, Waterbeach, United Kingdom; hsa-miR-708-5p mature miRNA sequence: 5′-AAGGAGCUUACAAUCUAGCUGGG-3′, accession #: MIMAT004926. Horizon Discovery’s mirIDIAN microRNA Mimic Negative Control #1 (sequence is not provided) was used as a nontargeting miRNA. This miRNA has a scrambled sequence with no predicted targets in the human transcriptome. Twenty-four hours after seeding, cells were
transiently transfected with synthetic miRNAs at 25 nM (unless stated otherwise) using INTERFERin (Polyplus, Berkeley, CA) according to the manufacturer’s protocol. Using the same seeding protocol, cells were treated with 10 μM BAY-11-7082 (Abcam) or 10 μM celecoxib (MilliporeSigma) in complete medium [23]. Fresh BAY-11-7082 was added after 24 hours. Cells were treated for a total of 48 hours prior to RNA/protein isolation or media removal for ELISA.

2.5. NF-κB Promoter Assay. We purchased the NanoLuc Reporter Vector with NF-κB Response Element (http://pNL3.2.NF-κB-RE, Promega, Fitchburg, WI). Briefly, this reporter construct has an NF-κB RE upstream of the NLucP luciferase gene. First, we plated 1 × 10^5 A549 cells into each well of a 12-well plate. 24 hours later, we treated cells with mock, 25 nM miR-708, or 10 μM BAY-11-7082 (explained in miRNA, BAY-11-7082, and CEL Treatments). 24 hours later, cell media was replaced with serum-free DMEM. The following morning, cells were treated with TNFa/IL-1β (+/- BAY-11-7082) for 5 hours. Cells were then analyzed using the Nano-Glo Luciferase Assay System (Promega) per manufacturer’s protocol. Data were background subtracted from each treatment (TNFa/IL-1β alone, +miR-708, +BAY-11-7082) at each time point (0, 5h). Samples were then normalized to total protein and TNFa/IL-1β 0h treatment (noninduced control). Luciferase data represent the average of ≥3 biological replicates.

2.6. Quantitative Real-Time PCR (qRT-PCR). Complementary DNA (cDNA) was synthesized by reverse transcription of RNA using the miScript II RT Kit (Qiagen, Venlo, Netherlands). miRNA-specific cDNA was created using HiSpec buffer, while mRNA-specific cDNA was created using HiFlex buffer. qRT-PCR was performed using a Bio-Rad CFX96 Real-Time C1000 Touch Thermal Cycler. mRNA cycling conditions were as follows: (1) 95°C for 15 min, (2) 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s (collection step). mRNA cycling conditions were similar, except for adjusted annealing temperatures on a primer-by-primer basis. miR-708-5p, U6 snRNA, and GAPDH primers were similar, except for adjusted annealing temperatures on a primer-by-primer basis. miR-708 represses NF-κB, TNF, and arachidonic acid (AA) signaling. Given these data, we investigated whether miR-708 was a posttranscriptional regulator of these cytokines.

First, COX-2 and mPGES-1 protein expression is induced by TNFa/IL-1β in Nondiseased Lung Cells. Proinflammatory cytokines have extensive functions in regulating chronic inflammatory-related diseases. Two important proinflammatory cytokines dysregulated in disease are TNFa and IL-1β. Several articles have determined that miR-708 represses NF-κB, TNF, and arachidonic acid (AA) signaling. Given these data, we investigated whether miR-708 was a posttranscriptional regulator of these cytokines.

2.8. Statistical Analysis. We used Prism 7 software to perform one-way ANOVA and Student’s t-test to determine significant differences. Where indicated, the nonparametric tests were used to determine statistical significance. p values less than 0.05 were considered significant.

2.9. Western Blot Analysis. Media was removed from treated cells and lysed in RIPA buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1% protease inhibitor). The cells/supernatant were scraped off wells, collected, and then centrifuged at 14000 × g for 15 min at 4°C. Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA). 25 μg of protein was loaded onto 10% SDS-PAGE gels, separated by electrophoresis, and transferred onto PVDF membrane (VWR) for 2 hours at 4°C. Blots were blocked with 5% nonfat milk+PBSt (5% nonfat dry milk, 1x PBS, 0.1% Tween-20 (MilliporeSigma)) for 1 hour at room temperature (RT). Primary antibody incubations against human COX-2 (160112, Cayman Chemical, Ann Arbor, MI), IKKβ (ab264239, Abcam), mPGES-1 (ab180589, Abcam), and GAPDH (HRP-60004, Proteintech, Rosemont, IL) were performed overnight at 4°C per manufacturer’s recommended dilutions. Blots were washed with PBSt 3x for 5 minutes each and then exposed to secondary HRP conjugated secondary antibodies (goat anti-mouse H+L (31430, Thermo Fisher, Waltham, MA); goat anti-rabbit H+L (31460, Thermo Fisher)) for 1 hour at RT. Blots were developed using Clarity Western ECL Substrate (Bio-Rad) on the ChemiDoc MP Imaging System (Bio-Rad). Western blot images are representative of ≥3 biological replicates.

3. Results

3.1. miR-708 Expression Is Induced by TNFa/IL-1β in Nondiseased Lung Cells. Proinflammatory cytokines have extensive functions in regulating chronic inflammatory-related diseases. Two important proinflammatory cytokines dysregulated in disease are TNFa and IL-1β. Several articles have determined that miR-708 represses NF-κB, TNF, and arachidonic acid (AA) signaling. Given these data, we investigated whether miR-708 was a posttranscriptional regulator of these cytokines.
expression is controlled through the \textit{ODZ4} promoter [25]. In Beas2b cells, \textit{Odz4} mRNA expression mirrors \textit{miR-708} expression, suggesting TNF\textalpha/IL-1\beta regulates \textit{miR-708} transcriptionally through the \textit{ODZ4} promoter (Figure 1(b), \(p < 0.05\) and \(***p < 0.0001, n \geq 3\)).

**3.2. \textit{miR-708} Expression Is Nonresponsive to TNF\textalpha/IL-1\beta in Diseased Lung Cells.** We repeated our experiments in A549 (diseased) lung cells, which are a lung adenocarcinoma cell line. While cancerous, researchers have used this cell line to study other inflammatory-related diseases. A549 cells treated with TNF\textalpha/IL-1\beta dramatically increased COX-2 protein levels through 48 hours, with no resolution observed (Figure 2(a)). Conversely, we saw nonsignificant TNF\textalpha/IL-1\beta-induced increases in \textit{miR-708} expression in A549 cells (Figure 2(b) \(p = \text{n.s.}, n = 3\)). When paired with Beas2b \textit{miR-708} expression changes, we see that A549 \textit{miR-708} levels are significantly lower at baseline (Figure 2(b), \(p < 0.0001, n = 3\)). We have previously shown that reduced \textit{miR-708} expression in A549 cells is due to \textit{ODZ4} promoter methylation [18]. Moreover, A549 \textit{miR-708} levels never increased above 15% of baseline Beas2b expression after TNF\textalpha/IL-1\beta treatment, and \textit{miR-708} levels are significantly lower at every time point in A549 cells (Figure 2(b), \(p < 0.001, n = 3\)). These data suggest that \textit{ODZ4} promoter methylation prevents TNF\textalpha/IL-1\beta-induced \textit{miR-708} expression, disconnecting the
negative feedback loop in diseased lung cells and leading to unresolved COX-2 signaling. As a control, we performed viability (WST-1) analysis on Beas2b and A549 cells untreated or TNFa/IL-1β treated and found that TNFa/IL-1β treatment did not affect cellular viability in either cell line (Supplemental Figure 1). Given these data, we next investigated if transient transfection of exogenous miR-708 could suppress TNFa/IL-1β-induced changes in A549 cells.

3.3. miR-708 Reduces TNFa/IL-1β-Induced Changes in Diseased Lung Cells by Suppressing NF-κB Signaling. We transiently transfected A549 cells with mock (-miR-708) or synthetic miR-708 and then exposed cells to TNFa/IL-1β for 0–48 hours. As seen in Figure 3(a), A549 cells without miR-708 treatment had robust induction of COX-2 protein expression in a time-dependent manner. A549 cells transfected with synthetic miR-708 had a significant reduction in both COX-2 and mPGES-1 protein levels across all TNFa/IL-1β time points (Figure 3(a)). We also measured PGE2 release by ELISA and observed significantly decreased PGE2 levels at every time point in miR-708-treated A549 cells compared to mock samples (Figure 3(b), p < 0.05, n ≥ 3). PGE2 has been shown to form a positive feedback loop to enhance COX-2 activity. Collectively, these data reveal that miR-708 is suppressing TNFα levels in diseased lung cells.

We inhibited COX-2 production of PGE2 with celecoxib (CEL) and repeated our experiments. While CEL efficiently suppressed PGE2 production (Figure 3(d), p < 0.05, n ≥ 3), it only partially reduced TNFa/IL-1β regulation of COX-2 protein expression (Figure 3(c)). Next, we measured IL-6 production, as researchers have shown that it is directly regulated by COX-2/mPGES-1-derived PGE2. While miR-708 prevented TNFa/IL-1β-induced IL-6 expression, CEL had no effect on IL-6 levels after TNFa/IL-1β treatments (Figure 4, p < 0.05, p = n.s., n ≥ 3). These data reveal that miR-708 is suppressing IL-6 production independent of AA signaling. As previously discussed, TNFa/IL-1β has been shown to induce IL-6 both through AA signaling and independent of COX-2, mPGES-1, or PGE2. Given these data, we next examined the mechanism by which miR-708 is suppressing IL-6 levels in diseased lung cells.

Studies have shown that IL-6 can be regulated through the NF-κB pathway [26]. Moreover, miR-708 was also shown to target the NF-κB activator IKKβ, leading to decreased NF-κB-regulated gene expression [19]. Therefore, we examined whether miR-708-mediated IKKβ suppression could be responsible for reduced IL-6 levels in miR-708-treated A549 cells. First, we measured IKKβ protein expression in TNFa/IL-1β-treated A549 cells with mock (-miR-708) or miR-708 (+miR-708). We found that miR-708 treatment decreased A549 IKKβ protein levels up to 24 hours after TNFa/IL-1β treatment (Figures 5(a) and 5(c), p < 0.05, n = 3). Next, we compared A549 cells treated with TNFa/IL-1β+mock, miR-708, or the IKKβ inhibitor BAY-11-7082 and repeated our western blot. BAY-11-7082-treated cells decreased TNFa/IL-1β-mediated COX-2 expression in a similar manner to miR-708 (Figure 5(b)). While it is important to show miR-708 reduces IKKβ protein expression, we must also show miR-708 decreases NF-κB activity.

To achieve this, we utilized the pNL3.2.NF-κB-RE reporter luciferase assay. Briefly, this reporter construct has an NF-κB response element (RE) upstream of the NLucP luciferase gene. Once NF-κB is shuttled to the nucleus, it binds to the NF-κB RE, activating NLucP expression. Changes in NLucP expression can then be quantified via luminescence assays. We transfected A549 cells with mock, 25 nM miR-708, or 10 μM BAY-11-7082, followed by the pNL3.2.NF-κB-RE construct the following day. The next morning, we treated these cells with TNFa/IL-1β for 0 or 5 h and then measured luminescence. We found that TNFa/IL-1β+mock A549 cells had a 16× increase in luminescence after 5 hours (Figure 6(a)). While BAY-11-7082 significantly reduced TNFa/IL-1β-mediated NF-κB activation and luminescence by 28%, miR-708 had a greater suppressive effect than BAY-11-7082 (Figure 6(a), p < 0.0001, n = 3). These data suggest miR-708 is indeed suppressing TNFa/IL-1β-mediated NF-κB activation and downstream signaling. Given these data, we repeated the PGE2 and IL-6 ELISAs in A549 cells. We found that BAY-11-7082 significantly reduced A549 PGE2 production after TNFa/IL-1β treatment similarly to miR-708 (Figure 6(b), p < 0.0001, n = 3). Lastly, BAY-11-7082 also suppressed IL-6 production in A549 cells post-TNFα/IL-1β treatment in a time-dependent manner (Figure 6(c), p < 0.01, n = 3). There was not a significant difference in IL-6 expression between miR-708 and BAY-11-7082 treatments at every time point, suggesting that miR-708’s effects can be attributed to NF-κB signaling inhibition. Collectively, these data reveal that miR-708 is suppressing IL-6 production independent of AA signaling in lung cancer cells. IL-6 is not a predicted miR-708 target, suggesting miR-708’s regulation of IL-6 is indirect. In Figure 7, we propose a model for how miR-708 is suppressing TNFa/IL-1β signaling in A549 cells based on our data and published studies. We surmise that miR-708 suppresses TNFa/IL-1β through multiple mechanisms, by inhibiting both NF-κB and AA signaling in diseased lung cells.

4. Discussion

TNFa and IL-1β are proinflammatory cytokines crucial for immune responses, especially important for host defense from bacterial, viral, and parasitic infections [1, 3]. Uncontrolled TNFa/IL-1β signaling has been shown to contribute to numerous diseases, including RA, Crohn’s disease, atherosclerosis, cancer, and autoimmune diseases [3]. While there are several approved TNFa inhibitors used to treat autoimmune diseases, a significant subset of patients is nonresponsive to TNFa inhibitors or becomes resistant to treatment [6]. Interestingly, it was found that COX-2 is overexpressed in RA and ulcerative colitis patients and is constitutively overexpressed in TNFa inhibitor nonresponders [9, 10]. IL-6 is also overexpressed in many tumors and has been shown to regulate various aspects of oncogenesis [27, 28]. Furthermore, researchers have shown that miR-708 dampens TNFa signaling, possibly by inhibiting NF-κB signaling [29, 30]. Therefore, we examined the ability of miR-708 to mitigate downstream TNFa/IL-1β-related signaling in disease.
Figure 3: miR-708 suppresses TNFα/IL-1β-induced arachidonic acid signaling partially through a positive feedback loop in diseased lung cells. (a) Representative western blot examining COX-2 and mPGES-1 protein expression in A549 cells transiently transfected with mock (left) or 25 nM miR-708 (right) in combination with TNFα/IL-1β for 0–48 hours. (b) ELISA measuring exogenous PGE₂ levels in A549 cells transiently transfected with mock (blue) or 25 nM miR-708 (red) and treated with TNFα/IL-1β for 0–48 hours. (c) Representative western blot examining COX-2 and mPGES-1 protein expression in A549 cells cotreated with vehicle (left) or 10 μM CEL (right) and TNFα/IL-1β for 0–48 hours. (d) ELISA measuring exogenous PGE₂ levels in A549 cells cotreated with vehicle (blue) or 10 μM CEL (red) and TNFα/IL-1β for 0–48 hours. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; n ≥ 3.
In this study, we first confirmed a previous work that showed TNFα upregulated COX-2 and mPGES-1 protein expression within 6 hours and returned to baseline by 48 hours in normal lung cells (Figure 1(a)). Interestingly, miR-708 was temporally upregulated similar to other TNFα-dependent genes in nondiseased lung cells (Figure 1(b)). In diseased lung cells, TNFα/IL-1β highly upregulated COX-2 expression, but AA signaling was never restored to baseline (Figure 2(a)). Conversely, TNFα/IL-1β had no effect on miR-708 expression in lung cancer cells (Figure 2(b)). This suggests that a novel miR-708 negative feedback loop may be lost in diseased lung cells. Therefore, we tested the ability of exogenous miR-708 to suppress TNFα/IL-1β signaling in diseased lung cells.

We found that miR-708 significantly reduced TNFα/IL-1β-induced COX-2, mPGES-1, and PGE2 levels in A549 cells (Figures 3(a) and 3(b)). Reduced AA signaling can partially be contributed to inhibition of an AA signaling positive feedback loop (Figures 3(c) and 3(d)). Next, we tested whether miR-708 could inhibit downstream COX-2-dependent genes, specifically IL-6. We found that miR-708-treated diseased lung cells had significantly lower IL-6 secretion post-TNFα/IL-1β exposure compared to TNFα/IL-1β treatment alone (Figure 4). However, our data suggest that miR-708 suppression of IL-6 expression is independent of AA signaling in diseased lung cells (Figure 4). Given these data, we examined whether miR-708 was modulating the IL-6 regulatory NF-κB.

We found that miR-708 decreased IKKβ protein expression, a potent NF-κB activator (Figure 5). Using a NF-κB promoter luciferase construct, we determined that miR-708 significantly reduced TNFα/IL-1β-induced NF-κB transcriptional activity (Figure 6(a)). Lastly, we inhibited NF-κB signaling using BAY-11-7082 and found that NF-κB inhibition reduced PGE2 and IL-6 production similarly to miR-708 treatment (Figures 6(b) and 6(c)). This finding supports the hypothesis that miR-708’s regulation of IL-6 is through the NF-κB pathway, not AA signaling. Given these data, we created a model showing miR-708’s dampening effects on TNFα/IL-1β in lung cells (Figure 7). We found that miR-708 acts dually to suppress TNFα/IL-1β signaling by inhibiting both AA and NF-κB signaling. Moreover, loss of miR-708 in diseased lung cancer may contribute to uncoupled TNFα/IL-1β signaling. Lastly, these data suggest miR-708 may act as a novel therapeutic to treat TNFα/IL-1β-related diseases. These data provide the foundation for further exploring the role of miR-708 in TNFα/IL-1β-related diseases.

While we have laid the foundation for expanding research on the precise functions of miR-708 in TNFα/IL-1β signaling, several questions remain. First, how is TNFα inducing miR-708 expression in normal lung cells? Previous researchers have shown that encourages the expression/activity of the miR-708 regulators CHOP, CTBP2, C/EBP-β, and CTCF [31–34]. Given these data, there are multiple avenues by which TNFα/IL-1β may stimulate miR-708 expression. Given that TNFα suppresses Grα, MYC, and E2F1 signaling, known miR-708 regulators, it is unlikely that these transcription factors are involved in our study. On the other hand, CHOP and C/EBP-β stimulate miR-708 expression and are TNFα-responsive transcription factors. Therefore, CHOP and C/EBP-β are possibly transcriptional regulators of TNFα/IL-1β-induced miR-708 expression. While this provides a plausible signaling axis, we have not identified why miR-708 is not upregulated after TNFα/IL-1β treatment in diseased lung cells.

There are several possible explanations for how TNFα/IL-1β-mediated activation of miR-708 is lost in diseased lung cells. First, transcriptional activators may not be expressed in these cells. Second, the ODZ4 promoter may be inaccessible to transcription factors due to promoter methylation and chromatin remodeling. Third, miR-708-negative regulators may be preventing transcriptional activation. Previous studies have shown that CHOP signaling is inhibited or defective in cancer [35]. Additionally, C/EBP-β is necessary for effective lung cell inflammatory responses and is lost in cancer [36, 37]. Therefore, loss of CHOP and C/EBP-β activity may prevent activation of miR-708 expression in diseased lung cells. Secondly, it has been previously shown that the ODZ4 promoter, which miR-708 is under the control of, is hypermethylated in diseased lung cells [18]. This may be through EZH2, which is positively influenced by TNFα/IL-1β signaling and has been shown to hypermethylate the ODZ4 promoter in cancer, resulting in repressed miR-708 expression [38, 39]. Lastly, two negative regulators of miR-708 that are positively regulated by TNFα/IL-1β, CTBP2 and CTCF, are overexpressed in lung immune-related diseases [40, 41]. Collectively, these studies suggest that the loss of miR-708 responsiveness to TNFα/IL-1β stimulation may be a multifaceted mechanism. Loss of transcriptional activators, overexpression of negative regulators, and ODZ4 promoter hypermethylation may collectively inhibit miR-708 expression in diseased lung cells. The consequences of this miR-708 suppressive network
may be profound. TNFα-induced chronic inflammatory states frequently result in epithelial cell transformation. Loss of TNFα-negative regulators such as miR-708 further propagates TNFα signaling, increasing the risk of transformation and autoimmune disease. Therefore, miR-708 may be a crucial TNFα/IL-1β signaling-suppressing miRNA involved in early stage inflammatory-related oncogenesis and autoimmunity. Given these proposed circumstances, it would be justifiable to study the therapeutic potential of miR-708 in inflammatory-related cancers and autoimmune diseases.

Given the data we have uncovered, as well as TNFα/IL-1β’s therapeutic relevance in autoimmune diseases and cancer, it would be beneficial to examine miR-708’s ability to reduce autoimmune-related inflammation and oncogenesis. TNFα, IL-1β, and IL-6 are all prominent genes implicated in immune evasion. The ability of miR-708 to suppress TNFα, IL-1β, IL-6, and AA signaling axes may provide a more comprehensive therapeutic intervention compared to current targeted therapies. Additionally, significant subsets of patients receiving TNFα inhibitors become resistant to treatment. In these populations, researchers showed that COX-2 was overexpressed [9]. Moreover, COX-2 inhibition overcame TNFα resistance in these ulcerative colitis patients [9]. While COX-2 inhibitors have shown promise in treating autoimmune diseases and cancer, they have unacceptable long-term side effect profiles. Therefore, using miR-708 to treat these populations may provide a fresh therapeutic alternative. While the efficacy and safety of miR-708 is unknown, future studies will uncover the therapeutic potential of miR-708 in treating TNFα/IL-1β-related diseases.

**Figure 5:** miR-708 and NF-κB inhibition decreases IKKβ and COX-2 protein expression, respectively, in TNFα/IL-1β-treated diseased lung cells. (a) Representative western blot examining COX-2 and IKKβ (IKK-b) protein expression in A549 cells transiently transfected with mock (left) or 25 nM miR-708 (right) in combination with TNFα/IL-1β for 0–48 hours. (b) Representative western blot examining COX-2 and IKKβ (IKK-b) protein expression in A549 cells transiently transfected with mock (left), 25 nM miR-708 (middle), or 10 μM BAY-11-7082 in combination with TNFα/IL-1β for 0–24 hours. (c) Quantification of IKKβ protein expression from representative western blot (b) and others (data not shown). IKKβ expression was normalized to GAPDH protein expression and TNFα/IL-1β 0 h (left black bar). *p < 0.05 and **p < 0.01, n ≥ 3.
**Figure 6:** miR-708 suppresses PGE₂ and IL-6 secretion by inhibiting NF-κB signaling in diseased lung cells. (a) Relative NF-κB promoter reporter NanoLuc luciferase activity in A549 cells. Relative luciferase activities were measured in response to mock (blue), 25 nM miR-708 (red), or 10 μM BAY-11-7082 (green) treatment for 0 or 5 h. Data were normalized to total protein and mock 0 h samples. Differences were compared between samples within 0 and 5 h time points. (b) ELISA measuring exogenous PGE₂ levels in A549 cells cotreated with vehicle (blue), 25 nM miR-708 (red), or 10 μM BAY-11-7082 (green) and TNFα/IL-1β for 0–48 hours. (c) ELISA measuring exogenous IL-6 levels in A549 cells cotreated with vehicle (blue), 25 nM miR-708 (red), or 10 μM BAY-11-7082 (green) and TNFα/IL-1β for 0–48 hours. **p < 0.01 and ****p < 0.0001, n ≥ 3.

**Figure 7:** Proposed model of miR-708-mediated negative regulation of TNFα/IL-1β signaling in diseased lung cells. Model representing the repressive effects of miR-708 on TNFα/IL-1β-mediated regulation of arachidonic acid and IL-6 signaling in A549 cells. Black arrows represent activated signaling pathways, while black dotted lines represent miR-708-inhibited signaling cascades. Green lettering and arrows indicate AA signaling, and red lines represent miR-708 target suppression.
5. Conclusions

Our data suggests miR-708 is a potent negative regulator of TNFα/IL-1β signaling in lung epithelial cells. Loss of endogenous miR-708 expression may contribute to unchecked TNFα/IL-1β signaling, leading to inflammatory-related diseases. Reestablishing miR-708 levels may counteract TNFα/IL-1β-induced inflammation, ultimately resolving disease phenotype.

Data Availability

To request data, please contact Dr. Carol S. Lutz at http://lutzcs@njms.rutgers.edu.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Figure 1 TNFα/IL-1β treatment does not alter viability in lung cells. Nondiseased (Beas2b, (A)) and diseased (A549, (B)) lung cells were untreated (blue) or treated with TNFα/IL-1β (red) for 48 hours. Metabolic rates were measured using the WST-1 assay. Samples were analyzed at 0, 2, 4, 6, 24, and 48 hours and normalized to total protein; n ≥ 3. (Supplementary Materials)

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