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BRD4 targeting nanotherapy prevents lipopolysaccharide induced acute respiratory distress syndrome

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a life threatening respiratory disease associated with pulmonary edema, alveolar dysfunction, hypoxia, and inflammatory cell accumulation. The most contagious form of COVID-19 associated with ARDS caused by SARS-CoV-2. SARS-CoV-2 majorly produces the cytokine storm and severe lung inflammation and ultimately leads to respiratory failure. ARDS is a complex disease and there is no proper therapeutics for effective therapy. Still, there is a huge scope to identify novel targets to combat respiratory illness. In the current study, we have identified the epigenetic regulating protein BRD4 and developed siRNA based nanomedicine to treat the ARDS. The liposomes were prepared by thin-film hydration method, where BRD4 siRNA complexed with cationic lipid and exhibited 96.24 ± 18.01 nm size and stable even in the presence of RNase. BRD4 siRNA lipoplexes (BRD4-siRNA-LP) inhibited inflammatory cells in lungs and suppressed the lipopolysaccharide (LPS) induced the neutrophil infiltration and mast cell accumulation. Also, BRD4 siRNA based nanomedicine significantly reduced the LPS induced cytokine storm followed by inflammatory signaling pathways. Interestingly, BRD4-siRNA-LP suppressed the LPS-induced p65 and STAT3 nuclear translocation and ameliorated the lung inflammation. Thus, BRD4-siRNA-LP could be a plausible therapeutic option for treating ARDS and might be useful for combating the COVID-19 associated respiratory illness.

1. Introduction

Acute respiratory distress syndrome (ARDS) is the pathological condition in critically ill patients associated with pulmonary infiltration and emphysema. ARDS is caused by various etiological factors such as inhalation of toxicants, trauma, adverse drug reactions, drug overdosage, sepsis, and pancreatitis that accounts for 35–46% mortality (Bellani et al., 2016; Diamond et al., 2020). Recently, the Coronavirus Disease 2019 (COVID-19 Map, n.d) represents respiratory illness from Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection, and became a potential pandemic with more than 132,662,731 sufferers and around 2,877,835 deaths all over the world (Worldometer, 2021). ARDS is one of the main complications in COVID-19 patients that causes diffuse alveolar damage in the lungs in 42% of patients and 61–81% of that requiring intensive care unit (ICU) support. The COVID-19 induced ARDS is showing wreaking havoc in countries such as the USA, Brazil, India, Russia, UK, Spain, and Italy while ARDS patients have been sustaining in ICU under the ventilation. This virus-associated severe ARDS has a poor prognosis with no proper treatment and various researchers are working on repurposing drugs for symptomatic relief (Möhlenkamp and Thiele, 2020).

LPS (Lipopolysaccharide) is a bacterial endotoxin present on the outer membrane of gram-negative bacteria, an agonist for Toll-like receptor 4 (TLR-4) that mimics the sepsis induced ARDS both *in vitro* and *in vivo* (Pu and Wang, 2014). LPS stimulates macrophages, mast cells, and neutrophils and produces pneumonia-related severe respiratory distress syndrome ultimately causing respiratory failure (Xu and Shi, 2012). Macrophages are large phagocytic cells found in tissues and blood and mainly appear predominantly in inflammatory conditions (Fujiwara and Kobayashi, 2005). Additionally, LPS induces oxidative stress and further induce pulmonary edema and impairs pulmonary endothelial barrier function, which is the characteristic phenomenon of the severe acute respiratory syndrome (Grinnell et al., 2012). LPS stimulation enhances the proinflammatory cytokine levels by activating the p65 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Protein kinase B (Akt), mitogen-activated protein kinase (MAPK) signaling pathways

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which are involved in the perpetuation of the inflammatory responses (Alvira, 2014; Guha and Mackman, 2002; Selvaraj et al., 2015). LPS stimulation also triggers the translocation of transcription factors p65 NF-κB as well as Signal transducer and activator of transcription 3 (STAT3) from cytoplasm to nucleus, thus initiates the transcription of various proinflammatory genes responsible for cytokine storm (Bagaev et al., 2019). LPS induced inflammation may mimic the sepsis condition and most of the aspects of COVID-19 associated ARDS, although, it does not completely resemble the SARS-CoV-2 induced ARDS, but the deleterious effects associated can be resembled.The bromodomain (BD) is a structural motif within the proteins family that recognizes the acetylated lysine residues and employed in epigenetic modification through histone regulation as "readers" that orchestrate gene transcription (Miller et al., 2016). The bromodomain and extra terminal domain (BET) proteins are identified as BRD2, BRD3, BRD4, and testis-specific BRDt, among all, BRD4 plays an important role in inflammation and cancer (Klein, 2018). Generally, BET proteins have two conserved BDs, they involve recognizing acetylated histones and act as scaffolds to recruit transcriptional co-activators to promoters and super-enhancers, thereby drive gene transcription (Cochran et al., 2019). In inflammation, BDs identify the acetylated lysines on non-histone proteins such as RelA known as p65 NF-κB, which plays an important role in this condition (Hajmirza et al., 2018). BRD4 inhibitors were used in alleviating various diseases in the lung inflammation, cancer etc., and most of these are under clinical trials (Duan et al., 2018). However, BRD4 mechanism and its role in ARDS condition are not clear. Hence, in the current study, we evaluated the role of BRD4 in mediating inflammatory responses. Furthermore, BRD4 was attenuated with siRNA to abrogate its function and the effect was investigated.

Lipoplexes are the cationic liposomes, classically used as nonviral synthetic lipid carriers of DNA. These lipoplexes enhance the therapeutic bioavailability of drugs or genes through enhanced permeability and retention (EPR) effect (Deshpande et al., 2013). These lipoplexes are widely used in gene delivery including small interfering RNA (siRNA), micro RNA (miRNA), and short hairpin RNA (shRNA) (Shim et al., 2013). siRNAs are artificially synthesized 20–30 nucleotides long double-stranded RNA molecules, which are routinely used in molecular biology for transient silencing of a gene of interest. They elicit RNA interference (RNAi) response upon binding to their target transcript based on the sequence complementarity and siRNA therapeutics are employed as an alternative to traditional drugs for the management of various diseases (Dana et al., 2017). However, safe and effective siRNA delivery to the affected site is hampered by poor stability, highly hydrophilic nature, and low cellular uptake of siRNA (Wang et al., 2010). siRNA is negative in charge and forms the electrostatic interactions with cationic lipid DOTAP and forms lipoplexes. Further, these siRNA lipoplexes were uptaken by cells through endocytosis mediated pathway (Alshehri et al., 2018; Schroeder et al., 2010). Mainly, in lung diseases, siRNA lipoplexes are administered to lungs through inhalation or intravenous route, as this route of administration of siRNA lipoplexes have the advantage of being less invasive (Qiu et al., 2016). In the current study, for delivering the BRD4 siRNA into the lungs, we have prepared the nanosized cationic lipoplexes for the downregulation of corresponding BRD4 mRNA and further investigated the mechanistic insights of BRD4 inhibition in LPS induced ARDS conditions both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

BRD4 siRNA was procured from Santa Cruz Biotechnology, USA. 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP) (chloride salt) was purchased from Avanti polar lipids, USA. LPS from Escherichia coli (055: B5), PFI-1, Cholesterol (Chol), Trizma Base, SDS, acrylamide, DCFDA, and FITC were procured from Sigma Aldrich, USA. Agarose was procured from Merk, USA. SYBR Green I was purchased from Thermo Fisher Scientific, USA. The primary and secondary antibodies were purchased from Cell Signaling Technologies, USA and Santa Cruz Biotechnology, USA, respectively. All the chemicals which are used in this study are analytically pure and molecular grade.

2.2. Preparation of BRD4 siRNA DOTAP lipoplexes

BRD4 siRNA lipoplexes (BRD4-siRNA-LP) were prepared using the previously reported methods by dissolving DOTAP and Chol in chloroform in a 1:1 M ratio (Pandi et al., 2018). Thin-film was formed by rotary evaporation, which is hydrated with Diethylpyrocarbonate (DEPC) treated water at 37 °C to form multilamellar vesicles (MLV). Further, MLV containing liposomes were subjected to probe sonication to form nanosized liposomes. The stable complex of siRNA lipoplexes was prepared by mixing the siRNA/lipid (N/P) molar ratio by incubating at 37 °C for 30 min. Whereas, blank lipoplexes (Blank-LP) were prepared similar to siRNA lipoplexes without BRD4 siRNA.

2.3. Gel retardation and integrity assay

siRNA easily undergoes ribonucleolytic degradation, while the lipoplex form enhances the stability, agarose gel electrophoresis is used to study the formation of BRD4-siRNA-LP (Patil et al., 2011). In this study, to test the stability of BRD4 siRNA in lipoplexes, they were prepared and loaded on to the gel by mixing in 1X loading dye. Plain BRD4 siRNA was employed for comparison. 3% agarose gel was prepared by adding ethidium bromide and electrophoresis was performed under the conditions of 60 mA for 35 min. The bands were visualized by the Chemidoc system at 365 nm.

2.4. Determination of particle size and entrapment efficiency

Lipoplexes particle size and size distribution were measured by Zetasizer Nano-ZS in DTS0012 plastic cells (Malvern instrument Ltd. UK). The entrapment of siRNA in cationic lipoplexes was determined as described earlier (Jain et al., 2017), where the standard curve of siRNA was plotted by using the nanodrop spectrophotometer and calculated the unknown concentration of siRNA in the supernatant after centrifugation of lipoplexes at 16000 rpm for 10 min from the standard curve followed by measured the bounded and unbounded siRNA concentration from the values. The percentage of entrapment efficiency was calculated using the following formula.

$$\%Entrapmentefficiency = \frac{EncapsulatedsiRNAconcentration}{InitialsiRNAconcentration} \times 100$$

2.5. Cell culture

Mouse macrophages (RAW 264.7 cells) were procured from National Center for Cell Sciences (NCCS), Pune, India. Cells were grown in 10% FBS supplemented 1% Antibiotic and anti-mycotic solution containing DMEM high glucose medium by incubating in a humidified CO_2 incubator at 37°C temperature. Cells were trypsinized and sub-cultured when they attained 80% confluency.

2.6. DCFDA staining

RAW 264.7 cells were pretreated with 10 nM of BRD4-siRNA-LP for 48 h and stimulated with LPS for 30 min. Later cells were stained with 10 μM concentration of DCFDA and incubated for 15 min. Images were captured at × 200 magnification using fluorescent microscopy.

2.7. Animals

6–8 weeks old male C57/BL6 mice (weight 25–30 g) were purchased

from Palamur Biosciences, Mahabubnagar, India. Animals were housed in specific pathogen-free conditions with 12 h day/night cycle. All animals were acclimatized at least one week prior to initiating the experiment. All procedures of the study were approved by the Institutional Animal Ethics Committee (IAEC), NIPER-Hyderabad, India. All the experiments were conducted following the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, the government of India.

2.8. ARDS model development and treatment

In *in vivo*, mice were divided into 7 groups (n = 8) including normal control (NC), LPS control, BRD4-siRNA-LP control, blank lipoplexes (Blank-LP), BRD4-siRNA-LP pretreatment (Pre) and concurrent (Con) treatment and PFI-1 (5 mg/kg) groups. In the pretreatment group, animals were administered with BRD4-siRNA-LP 5 days before LPS stimulation, where the concurrent group animals received BRD4-siRNA-LP along with LPS. Here, mice were treated with 500 nM siRNA. Whereas, NC group animals received only PBS. Here, the lipoplexes, PFI-1, LPS, and PBS were administered by the oropharyngeal route. Animals were sacrificed by isoflurane overexposure after 12 h of LPS post-stimulation. The collected lung tissues were stored in −80°C.

2.9. Physiological parameters

Net body weight changes measured by taking the values before and after LPS stimulation and lung weight index was calculated as described earlier (Pooladanda et al., 2019).

2.10. Bronchoalveolar lavage (BAL) fluid analysis

After sacrifice, lungs were lavaged with ice-cold PBS for 3 times, bronchoalveolar samples were collected, pooled, and centrifuged for 10 min at 300 × g. Cell pellets obtained were suspended in PBS (1 ml) and subjected to differential cell counter ADVIA 2120i hematology system (Siemens, Germany).

2.11. Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-6, IL-17A, IL-22, and TNF-α levels in lung tissue lysates were analyzed by ELISA according to the manufacturer instructions (Thermo Fisher Scientific, USA).

2.12. Immunofluorescence

Specific Antigen-antibody interaction, location, and distribution can be determined by immunofluorescence by using the flourochrome detection system. In this method, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, whereas 5 μm sections of lung tissues were deparaffinized, rehydrated and then antigen was unmasked by treating with proteinase k for 15 min. Non-specific binding portions were eliminated by 3% BSA blocking solution and incubated with primary antibodies (1:100 dilutions) overnight at 4°C and later suitable dye conjugated secondary antibodies were added and incubated. Cells and tissue sections were washed thrice with immune wash buffer and probed with rhodamine or fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA) conjugated anti-mouse or anti-rabbit (1:200 dilutions) for 1 h and nuclei was stained with DAPI stain (Sigma-Aldrich, USA), after mounting slides with Fluoreshield™ histology medium, visualized under the confocal microscope.

2.13. Haemotoxylin and eosin (H&E) staining

Lung tissues were moulded in paraffin wax, and tissue sectioning was performed by microtome (Leica, Germany). Poly-L-Lysine coated slides were used to collect the tissue sections. For histological evaluation, tissue sections were stained with H&E and visualized under the microscope at × 400 magnification Histopathological score was determined as described earlier (Pooladanda et al., 2021).

2.14. Toluidine blue staining

Tissue sections were stained with toluidine blue (TB) and evaluated the mast cell infiltration. Images were captured at × 400 magnifications by bright-field microscope (Olympus CX21i, Japan). Mast cells were counted and represented as described earlier (Pooladanda et al., 2019).

2.15. Western blotting

Protein lysate was extracted from cells as well as from lung tissues as described earlier (Thatikonda et al., 2020). Bicinchonic acid (BCA) calorimetric assay kit (Sigma-Aldrich, USA) was used for the estimation of protein concentration. Protein samples were loaded and subjected to Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred according to their molecular weight onto nylon membrane (Sigma-Aldrich, USA); later proteins were visualized based on their molecular weights by placing membranes in Ponceau S stain. Non-specific binding of antibodies was avoided by a 3% blocking solution and incubated overnight with primary antibody at 4°C, later secondary antibody was added and detected by using enhanced chemiluminescence (ECL) substrate (Bio-Rad, USA). Blots were developed by the Chemidoc imaging system (Vilber Fusion Fx, France). Images were analyzed by ImageJ software, NIH, USA. Protein normalization was done by β-Actin and their respective total proteins.

2.16. Statistical analysis

All the results were analyzed by Graphpad Prism, USA (version 6.01) software, where n represents the number of replicates. One-way analysis of variance (ANOVA) was applied along with the Bonferroni post hoc test for statistical analysis. Here, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of BRD4-siRNA-LP

Lipoplexes were prepared by thin-film hydration method. The particle size, polydispersity index (PDI), and charge were determined by Zetameter. Where, the particle size of Blank-LP and BRD4-siRNA-LP were found to be 95.25 ± 9.49 (Fig. 1**A**) and 96.24 ± 18.01 nm (Fig. 1**B**), respectively, whereas the PDI was found to be 0.24 ± 0.02 and 0.25 ± 0.03, respectively. The Blank-LP and BRD4-siRNA-LP exhibited charge of 32.78 ± 2.71 (Fig. 1**C**) and 30.25 ± 2.01 mV (Fig. 1**D**), respectively. Stable complexes are a prerequisite to deliver the siRNA and to overcome serum instability or a high clearance rate *in vivo* (Pal Singh et al., 2020). We first analyzed the integrity of BRD4 siRNA and gel retardation by agarose gel electrophoresis. Equimolar concentrations of BRD4-siRNA were complexed with different molar ratios of cationic lipoplexes. The assay results reveal that with gradual increase in the lipoplexes concentrations 1:10, 1:50, 1:100 and 1:200 ratio with equimolar concentration of BRD4-siRNA, there was enhanced resistance towards serum nuclease with preserved RNAi activity at 1:200 with significant stable ionic interactions as compared to other concentrations. While the naked siRNA was found to be degraded in the presence of nuclease (Fig. 1**E**). Further, we chose 1:200 ratio stable complex of BRD4-siRNA-LP for performing all the biological and molecular studies. The entrapment efficiency that determines the amount of siRNA present in prepared lipoplexes was evaluated by nanodrop method. The entrapment efficiency of BRD4-siRNA-LP (1:200) was found to be 85.65 ± 0.98%, and also shows enhanced stability of BRD4-siRNA-LP, whereas plain BRD4 siRNA was easily degraded in presence of RNase (Fig. 1**F**).

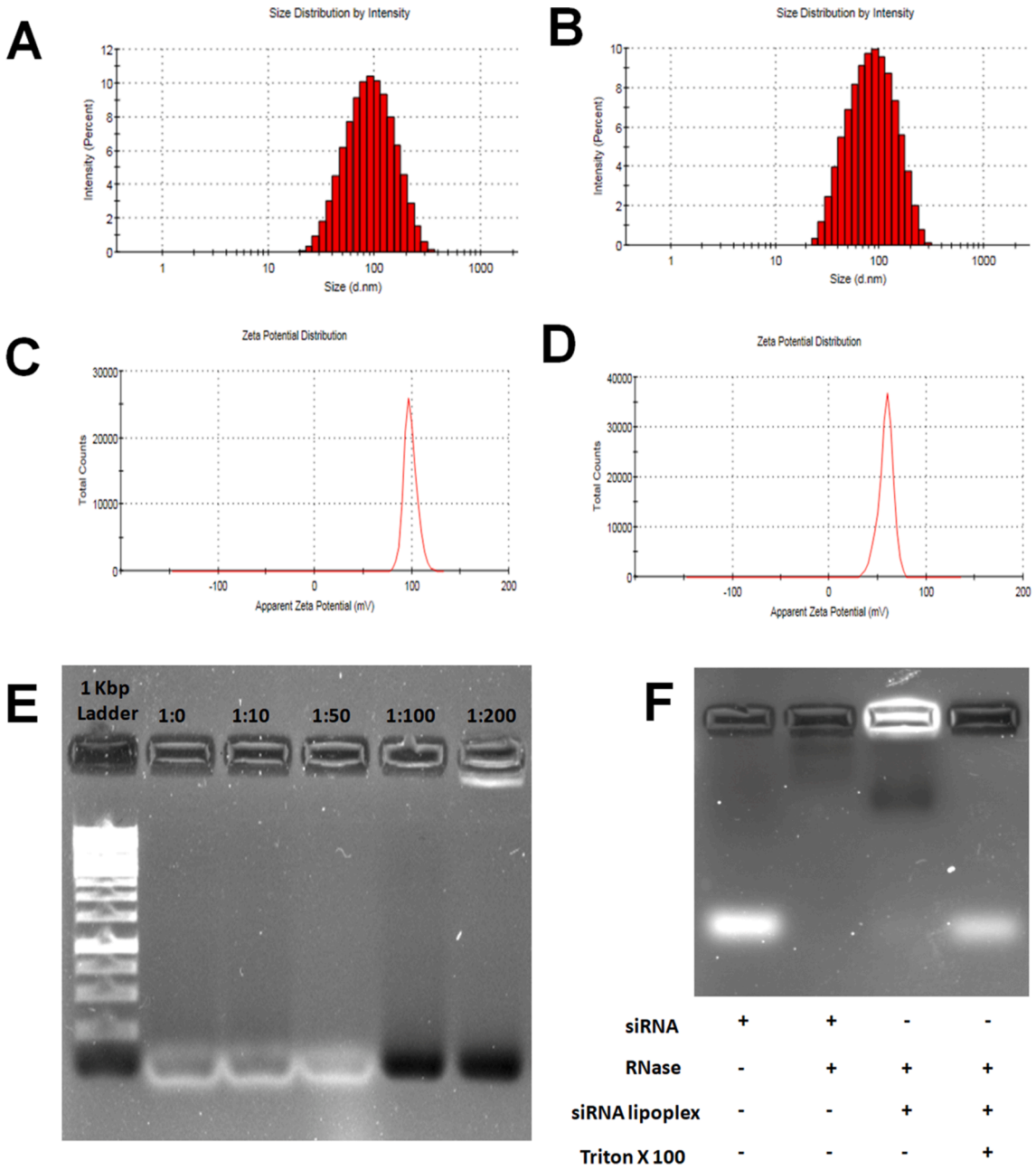

Fig. 1. BRD4-siRNA-LP particles size and charge characterization. The cationic lipoplexes were prepared by thin-film hydration method by taking an equal ratio of DOTAP and Chol. The particle size of (**A**) Blank-LP and (**B**) BRD4-siRNA-LP as well as potential of (**C**) Blank-LP and (**D**) BRD4-siRNA-LP were determined by Zetasizer. (**E**) Gel retardation and integrity assay and (**F**) RNase protection assay were performed by agarose electrophoresis using different molar ratios of BRD4 siRNA and cationic lipoplexes.

Collectively, the prepared BRD4-siRNA-LP were nanosized, exhibited uniform size with positive charge and formed stable complexes.

3.2. BRD4-siRNA-LP modulate physiological changes and control the proinflammatory cells by targeting BRD4 in LPS induced ARDS mouse model

Western blot results revealed that BRD4-siRNA-LP significantly suppressed LPS induced BRD4 expression in lung tissues, similar kind of decreased expression was also observed with BRD4 inhibitor PFI-1 (Fig. 2**A** and **Figure S1A**). The oropharyngeal administration of LPS in mice led to a decrease in net body weight (Fig. 2**B**), and significantly increased the lung weight index (Fig. 2**C**), which further contributes to pulmonary edema. Interestingly, oropharyngeal administration of BRD4-siRNA-LP showed prominent results in modulating LPS induced physiological changes. Both BRD4-siRNA-LP pretreatment and concurrent treatment groups showed satisfactory results as compared to small molecule inhibitor PFI-1. However, in Blank-LP group, there were no significant LPS induced changes were observed. Whereas, BRD4-siRNA-LP alone group exhibited no apparent changes and appeared to be similar to normal control (NC). Targeting BRD4 and its inhibition significantly reduced the endothelial barrier dysfunction followed by suppression of alveolar disruption. The inflammatory responsive cells, including total BAL cells (Fig. 2**D**), white blood cells (WBC) (Fig. 2**E**), neutrophils (Fig. 2**F**), basophils (Fig. 2**G**), and macrophages (Fig. 2**H**) were upregulated in LPS treated animals. Whereas BRD4-siRNA-LP treatment including pre-treatment and concurrent groups exhibited decreased inflammatory cell number as compared to LPS stimulated

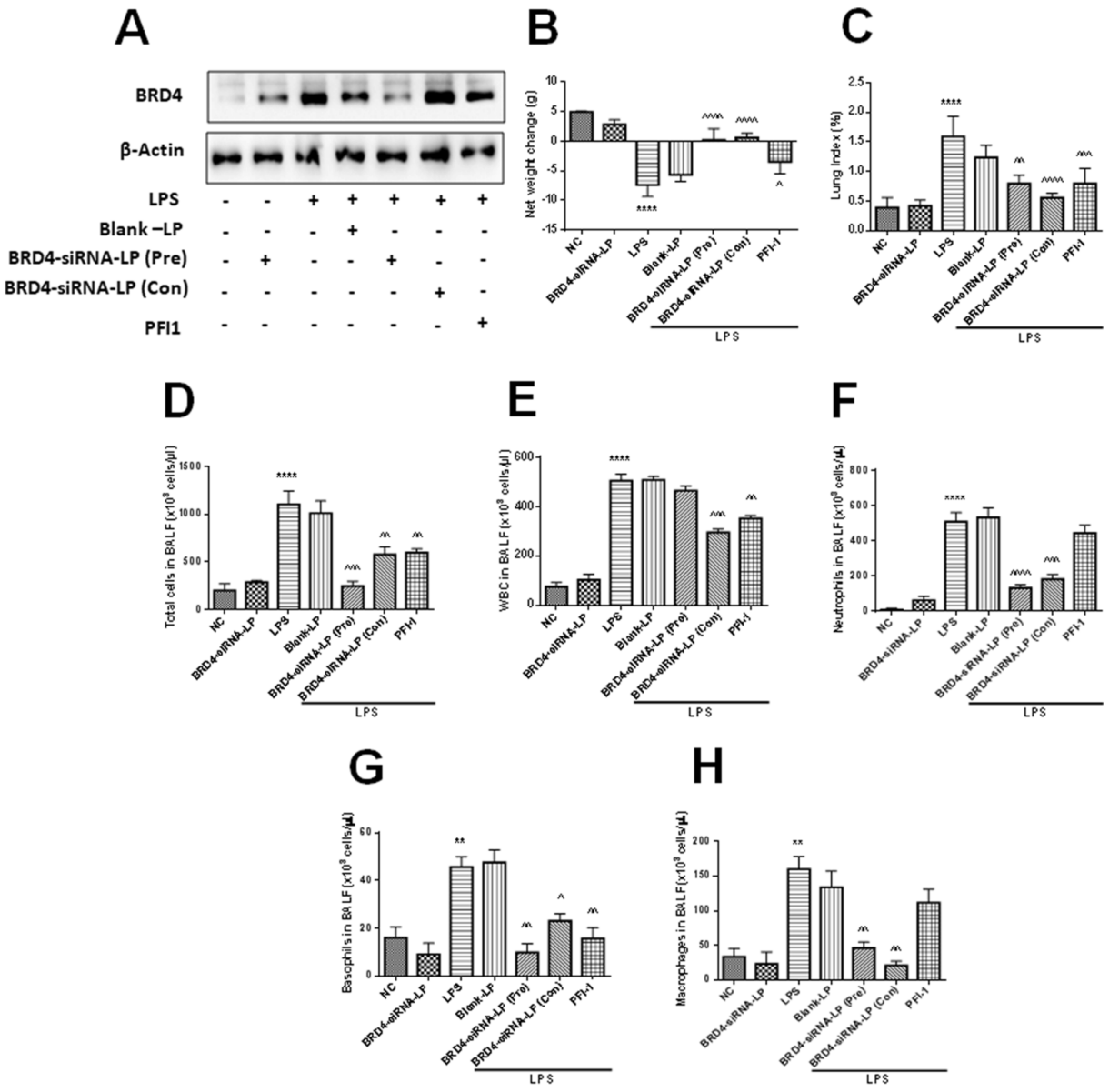

Fig. 2. BRD4-siRNA-LP inhibit LPS induced physiological changes and suppress inflammatory responsive cells in BALF. BRD4-siRNA-LP (500 nM/mouse) were administered through oropharyngeal route at 5 days pre-treatment and concomitant modes, and the effect on LPS induced (**A**) BRD4 expression, physiological changes such as (**B**) net body weight changes and (**C**) lung weight indexes were observed in C57/BL6 mice model. BALF was aspirated with chilled PBS and subjected to differential blood cell counter for estimating the count of inflammatory responsive cells include (**D**) total cells, (**E**) WBC, (**F**) neutrophils, (**G**) basophils, and (**H**) macrophages. Data presented as mean ± SD (n = 8 animals per group). $^{**}P < 0.01$ and $^{****}P < 0.0001$ are significantly different from the NC group; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$, and $^{\wedge\wedge\wedge\wedge}P < 0.0001$ are significantly different from the LPS group. Here, Blank-LP = Blank Lipoplexes; BRD4-siRNA-LP = BRD4 siRNA Lipoplexes; BRD4-siRNA-LP (Pre) = BRD4 siRNA Lipoplexes pretreatment; BRD4-siRNA-LP (Con) = BRD4 siRNA Lipoplexes concurrent treatment.

group animals. Here, we observed that as compared to PFI-1 treated group, BRD4-siRNA-LP treatment showed superior effects, whereas BRD4-siRNA-LP alone group exhibited safer profiles with the results similar to NC group. Thus, targeting BRD4 with lipoplexes reduced pulmonary edema and cell-mediated inflammation.

3.3. BRD4-siRNA-LP inhibit nitrosative stress and pro-inflammatory cytokine levels in ARDS mouse model

Nitrosative and oxidative stress play an important role in inducing alveolar disruption and irreversible damage to critical biomolecules such as lipids, proteins and DNA further responsible for respiratory collapse (Thimmulappa et al., 2019). The TLR4 agonist LPS induces respiratory illness by exacerbating the oxidative stress non-selectively, with accompanying nitrosative stress (Soodaeva et al., 2019). Both pre-treatment and concurrent BRD4-siRNA-LP treated groups showed reduced nitrite levels as compared to LPS control group. The treatment groups which include PFI-1, concurrent BRD4-siRNA-LP group showed a prominent reduction in nitrite levels, whereas the BRD4-siRNA-LP alone group exhibited no significant changes in healthy animals (Fig. 3**A**). Nitrosative and oxidative stress were neutralized by anti-oxidant defensive switches that include superoxide dismutase (SOD) and glutathione (GSH) (Kurutas, 2015). In our study, we have observed that SOD (Fig. 3**B**) levels were significantly increased by BRD4-siRNA-LP treated animals as compared to LPS control group animals, however, there were no changes observed in PFI-1 treatment group. Apparently, we observed that both BRD4-siRNA-LP and PFI-1 moderately enhanced the GSH levels but not to a significant extent (Fig. 3**C**),

LPS induction increased IL-1β (Fig. 3**D**), IL-6 (Fig. 3**E**), IL-17A (Fig. 3**F**), IL-22 (Fig. 3**G**), and TNF-α (Fig. 3**H**) levels, whereas these

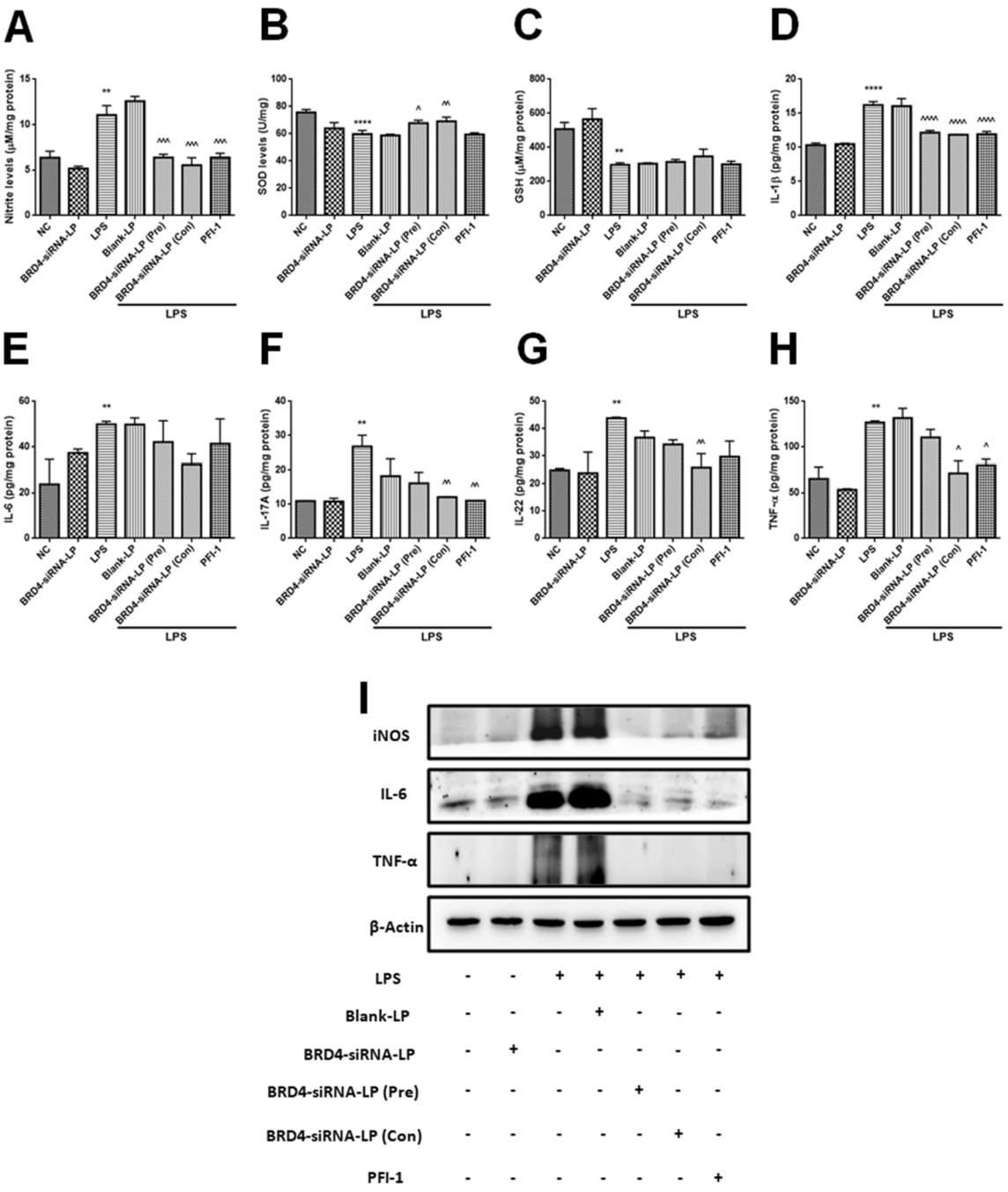

Fig. 3. BRD4-siRNA-LP suppress the nitrosative, oxidative stress, and cytokine storm in LPS induced ARDS mouse model. Mice were pretreated with BRD4-siRNA-LP and PFI-1, whereas the inflammation was induced by LPS oropharyngeal instillation. (**A**) Nitrite, (**B**) SOD, and (**C**) GSH levels were measured in lung tissues. The pro-inflammatory cytokine levels include (**D**) IL-1β, (**E**) IL-6, (**F**) IL-17A, (**G**) IL-22, and (**H**) TNF-α were measured by ELISA in lung tissue lysates. (**I**) Additionally, the inflammatory iNOS, IL-6, and TNF-α protein expression was evaluated by western blotting. Data presented as mean ± SD (n = 8 animals per group). $^{**}P < 0.01$ and $^{****}P < 0.0001$ are significantly different from the NC group; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$, and $^{\wedge\wedge\wedge\wedge}P < 0.0001$ are significantly different from the LPS group.

cytokines were significantly reduced by BRD4-siRNA-LP concurrent group, and showed superior effect compared to pre-treatment with siRNA lipoplexes and PFI-1. Here, PFI-1 slightly reduced IL-6 and IL-22 but non-significant. BRD4-siRNA-LP alone group did not induce the pro-inflammatory cytokines and the levels were in the normal range. Furthermore, we evaluated the expression of iNOS, TNF-α, and IL-6 by immunoblotting. Fig. 3**I** and **Figure S2A-C** infers that targeting BRD4 with lipoplexes as well as PFI-1 significantly suppressed the LPS induced iNOS, IL-6, and TNF-α expression, whereas BRD4-siRNA-LP alone group did not alter the expression of these inflammatory proteins. Collectively, targeted inhibition of BRD4 and inhibiting the expression by BRD4-siRNA-LP hampers the cytokine storm, and we speculate that it has beneficial effects in protecting the lungs from COVID-19 associated respiratory failure.

3.4. BRD4-siRNA-LP modulate the LPS induced histopathological changes in LPS stimulated mice

Excessive inflammatory cytokines promote neutrophil infiltration and mast cell accumulation, which is responsible for respiratory illness

(Moldoveanu et al., 2008). The clinical sign of neutrophilia has been demonstrated the indicator of COVID-19 associated respiratory illness (Cavalcante-Silva et al., 2021). The recent reports infer that COVID-19 associated clinical subjects showed enhanced mast cells (Ribeiro dos Santos Miggiolaro et al., 2020). Histopathological studies such as H&E (Fig. 4**A** and **Figure S1B**) and toluidine blue (Fig. 4**B** and **Figure S1C**) staining and microscopic observations show an increase in the neutrophils and mast cell number in both LPS and Blank-LP with concurrent changes in lung histology that reveal the extent of lung damage. By targeting BRD4, a remarkable improvement in pathological changes with a reduction in neutrophil infiltration and mast cell accumulation was observed. Moreover, we found that when compared to the BRD4-siRNA-LP pre-treatment group, concurrent group exhibited a more significant reduction in contrast to the LPS challenged group. Furthermore, epithelial barrier dysfunction was indistinguishable in BRD4-siRNA-LP alone group with normal pulmonary architecture. However, PFI-1 treatment exhibited similar results, but in comparison, BRD4-siRNA-LP exhibited better effects. Thus, BRD4 inhibition mitigated the LPS induced alveolar disruption and further protected against pulmonary edema and alveolar damage.

3.5. BRD4-siRNA-LP inhibit LPS induced oxidative stress through the inhibition of Akt/MAPK signaling

The next goal of this study was to evaluate the molecular mechanism by BRD4-siRNA-LP in lung inflammation. The oxidative stress plays a crucial role in exacerbating ARDS and further involve in cytokine mediated inflammation (Kellner et al., 2017). To examine this effect; the *in vitro* experimentation was performed in RAW 264.7 cells. Initially, BRD4 knockdown studies were performed by treating the cells with different concentrations of BRD4-siRNA-LP at 1, 2.5, 5, and 10 nM and incubated for 48 h, then stimulated with LPS for 30 min. Then we quantified the BRD4 protein expression and observed a significant knockdown of BRD4 expression at 10 nM (Fig. 5**A** and **Figure S3A**) and

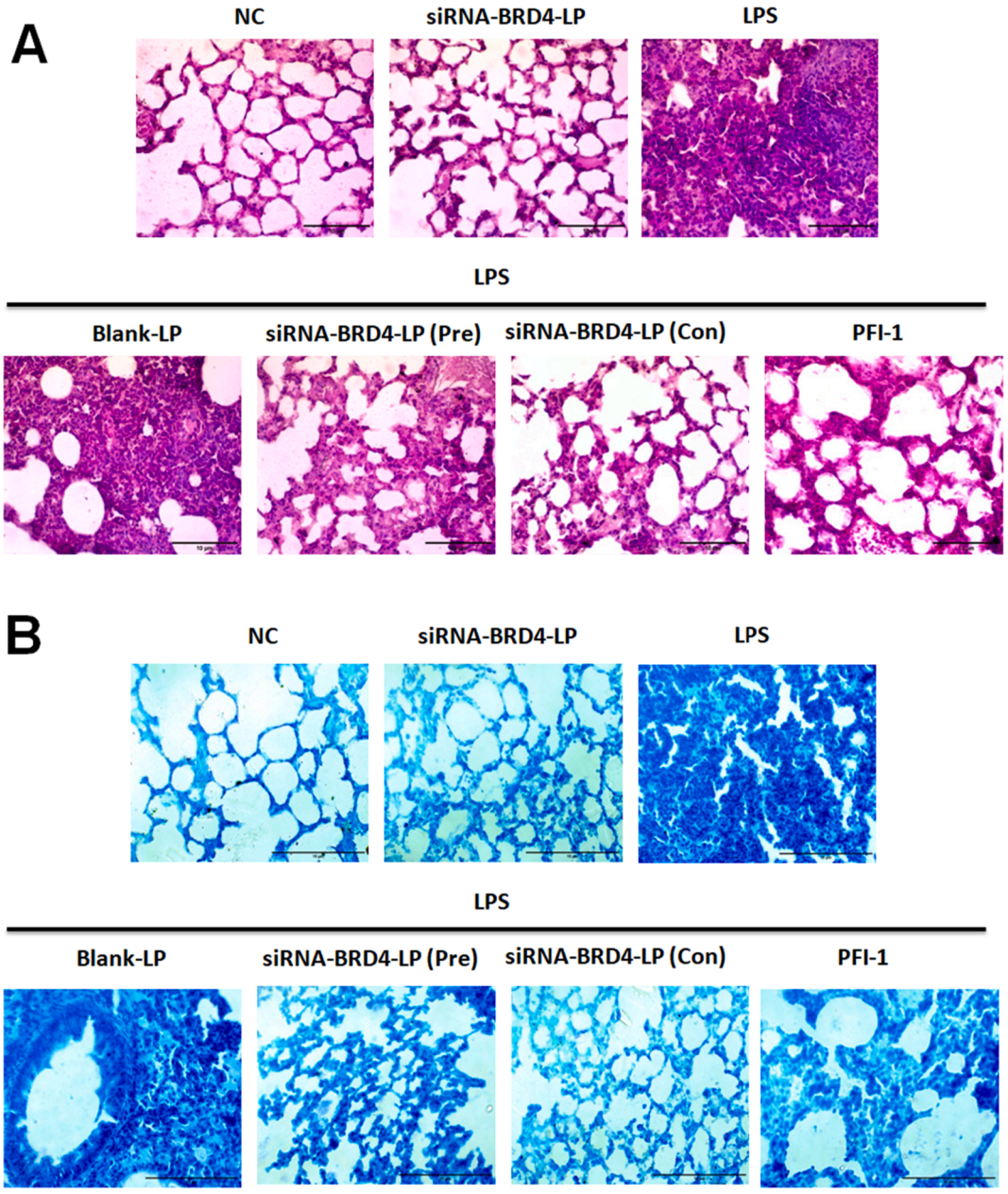

Fig. 4. BRD4-siRNA-LP inhibit LPS induced inflammatory and mast cell accumulation. The 5 μm sized lung tissues were subjected to (**A**) H & E staining and (**B**) toluidine blue staining. The pathological changes were observed under the microscope and images were captured at × 400 magnification.

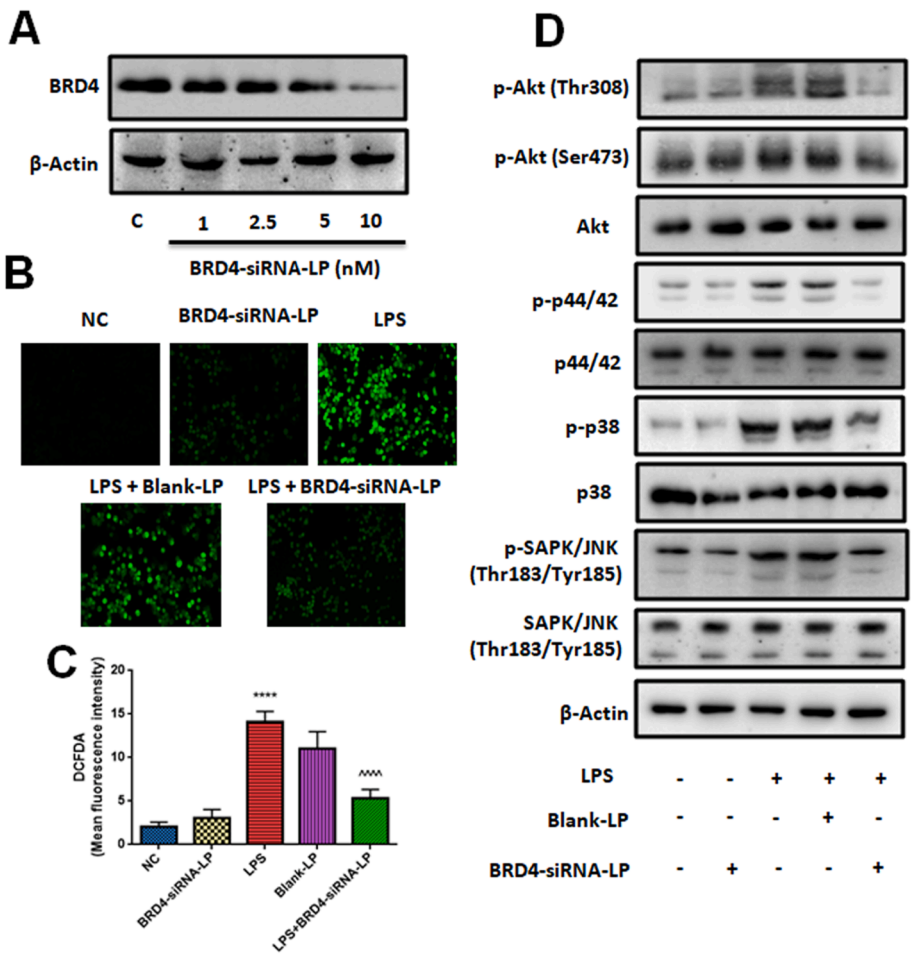

Fig. 5. BRD4-siRNA-LP show antioxidant effect by downregulating Akt/MAPK signaling in mouse macrophages. (A) Initially, RAW 264.7 cells were treated with BRD4-siRNA-LP (1, 2.5, 5, and 10 nM) for 48 h and expression of BRD4 investigated using western blotting. RAW 264.7 cells were pretreated with BRD4-siRNA-LP for 48 h and later cells were stimulated with LPS for 30 min. (**B**) The intracellular ROS levels were determined by DCFDA staining. The images were taken by fluorescent microscope at × 200 magnification and (**C**) DCFDA intensity was measured by Image J, NIH, USA software. (**C**) Similarly, the phosphorylation of Akt (both Thr308 and Ser473 sites), p38, p44/42, and SAPK/JNK were determined by immunoblotting. Data presented as mean ± SD (n = 3 independent experiments). ****$P < 0.0001$ is significantly different from the NC group; ^^^^$P < 0.0001$ is significantly different from the LPS group.

further studies were executed with this concentration. LPS induced oxidative damage in macrophages at 1 μg/ml, while in BRD4-siRNA-LP transfection at 10 nM concentration resulted in significant decreased the ROS levels, and no distinguishable changes occured in the BRD4-siRNA-LP alone treatment (Fig. 5**B & C**). We next explored whether BRD4 has a direct impact on Akt and MAPK signaling using a loss of function of BRD4 by BRD4-siRNA-LP as it plays a crucial role in both inflammation and aggravate respiratory illness and LPS mimics this condition. It was found that LPS induced Akt (at Thr308 and Ser473), p38, p44/42, SAPK/JNK phosphorylation, where activation was also found with Blank-LP, and knock down of BRD4 significantly inhibited protein expression (Fig. 5**D** and **Figure S3B-C**). These results show that BRD4 is a selective target for oxidative stress and inflammation in ARDS.

3.6. BRD4 inhibition suppressed the Akt/mTOR/MAPK signaling in LPS challenged mice

Previous reports suggest that the anti-inflammatory potential of BET bromodomain binding to acetylated histones disrupt the LPS-induced expression of inflammatory cytokines and the formation of the chromatin complexes (Nicodeme et al., 2010). Consistently we observed that LPS enhanced the BRD4 mediated inflammation in ARDS mouse model, and was involved in the upregulation of Akt (Thr308 and Ser473), mTOR and GSK-3β phosphorylation (Fig. 6**A** and **Figure S4A-E**). However, targeting BRD4 with BRD4-siRNA-LP and PFI-1 suppressed these inflammatory genes expression. Additionally, BRD4-siRNA-LP and PFI-1 inhibited the LPS induced phosphorylation of SAPK/JNK, p44/42, and p38 (Fig. 6**B** and **Figure S4F-H**). Here, we observed that BRD4-siRNA-LP showed superior activity over PFI-1 treatment, and BRD4-siRNA-LP alone did not promote the activation of inflammatory cascade in the mouse upon oropharyngeal administration of LPS. Thus, targeting BRD4 inhibits the Akt/mTOR/MAPK signaling and ameliorates the LPS induced ARDS. P

3.7. BRD4-siRNA-LP inhibit LPS/TLR-4 mediated inflammation in macrophages by suppressing p65 and STAT3 nuclear translocation

TLR-4 agonist LPS induces the inflammation by stimulating the p65 NF-κB signaling and causes severe respiratory illness. In our study, we found that LPS induced the phosphorylation of IKK-α/β, IκB-α, p65 NF-κB, and STAT3, which were significantly inhibited by BRD4-siRNA-LP (Fig. 7**A** and **Figure S5A-D**). Additionally, we observed that LPS induced nuclear p65 NF-κB and STAT3 protein expression was significantly ameliorated by BRD4-siRNA-LP (Fig. 7**B** and **Figure S5E & F**). The transcriptional factors, p65 and STAT3, did not exhibit activity when they reside in the cytoplasm. However, these transcriptional factors were phosphorylated in the presence of inflammatory stimuli, and then enter into the nucleus, where they induced the expression of a wide variety of inflammatory genes, which are responsible for the respiratory collapse. Confocal microscopy results infer that LPS induced nuclear translocation of p65 and STAT3 is significantly downregulated by BRD4-siRNA-LP (Fig. 7**C**). Collectively, BRD4-siRNA-LP ameliorated the LPS induced ARDS by suppressing p65 and STAT3 crosstalk.

3.8. Targeting BRD4 inhibits p65 and STAT3 nuclear translocation in LPS induced ARDS mouse model

TLR-4 agonist LPS activated TRAF-6 mediated p65 signaling and

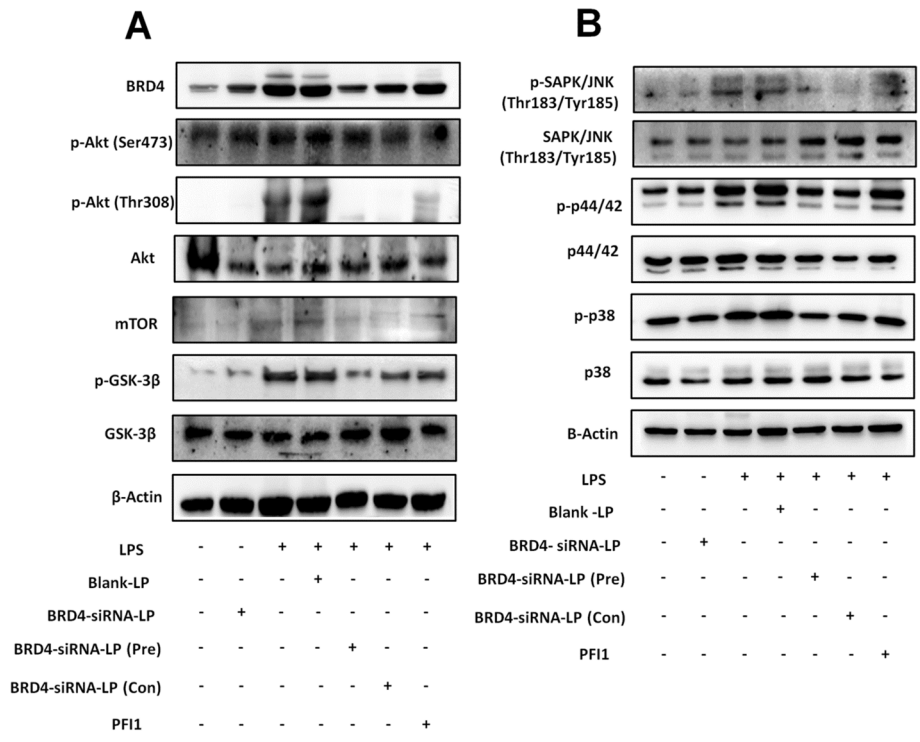

Fig. 6. BRD4-siRNA-LP suppress Akt/mTOR/MAPK signaling in LPS challenged mice. Mice were pretreated with 500 nM concentration of BRD4-siRNA-LP (pretreatment and concurrent) and PFI1 (5 mg/kg) for 2 h and later mice were stimulated with LPS for 12 h. Later protein was isolated from lung tissues and evaluated the (**A**) Akt and GSK-3β phosphorylation, mTOR expression as well as (**B**) MAPK signaling by western blotting.

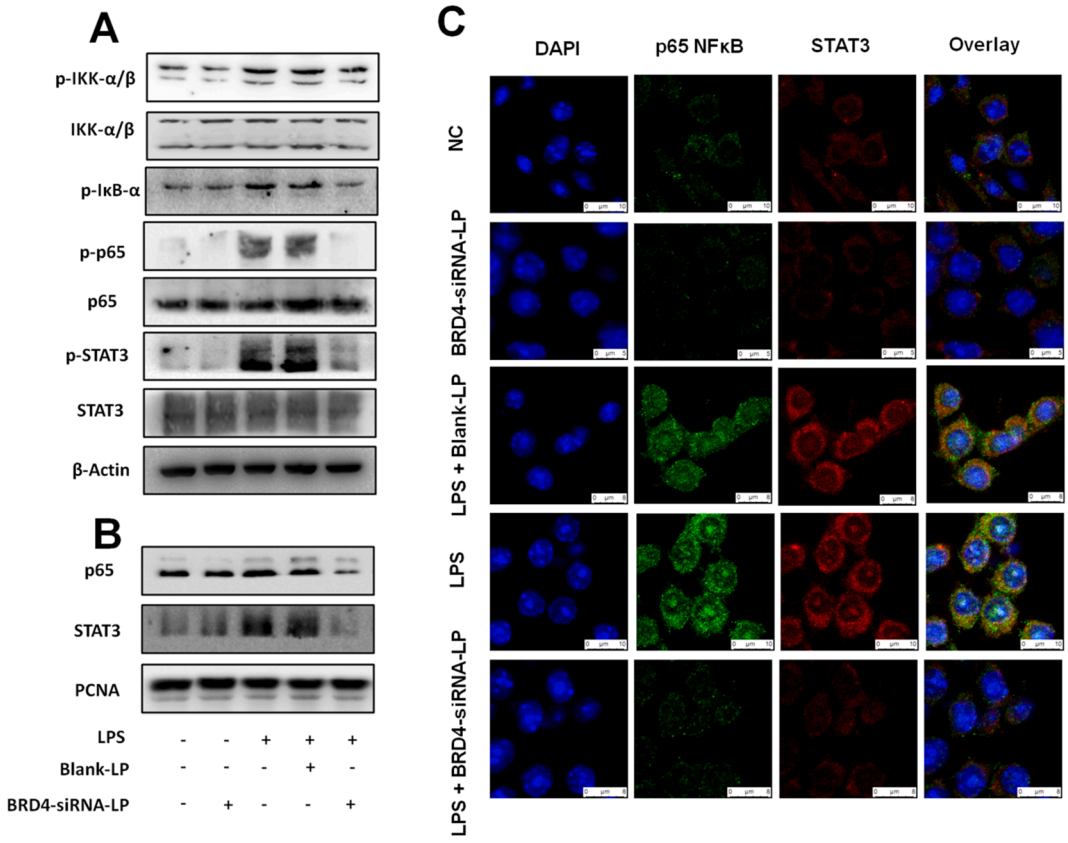

Fig. 7. BRD4-siRNA-LP inhibit p65 and STAT3 nuclear translocation. RAW 264.7 cells were pretreated with 10 nM of BRD4-siRNA-LP for 48 h and later cells were stimulated with 1 μg/ml of LPS for 30 min. Protein lysates were isolated and subjected to SDS-PAGE. (**A**) Phosphorylation of IKK-α/β, IκB-α, p65, and STAT3 and (**B**) nuclear p65 and STAT3 protein expression was evaluated by western blotting. (**C**) Nuclear translocation of p65 and STAT3 was studied by confocal microscopy. The images were captured at × 630 magnification.

actively participated in inflammatory signaling cascade observed in C57/BL6 mice. Targeting BRD4 with novel drug delivery system BRD4-siRNA-LP and small-molecule inhibitor PFI-1 inhibited the phosphorylation of IKK-α/β, IκB-α, p65 and STAT3 along with TRAF6 expression. However, the Blank-LP group did not reverse the LPS mediated changes and BRD4-siRNA-LP alone did not produce any inflammation and remained similar as NC (Fig. 8**A** and **Figure S6A-E**). Similar to *in vitro* results, here, we observed that BRD4-siRNA-LP suppressed LPS induced nuclear p65 NF-κB and STAT3 expression (Fig. 8**B** and **Figure S6F & G**). Additionally, BRD4-siRNA-LP inhibited the nuclear translocation of p65 and STAT3 in LPS challenged mice (Fig. 8**C**). We also noticed that the small molecule inhibitor PFI-1 significantly inhibited the expression of p65 NF-κB and STAT3. Collectively, BRD4 is a novel target, which is upregulated by inflammatory stimulus and induces the nuclear translocation of p65 and STAT3. Thus targeting it by novel lipoplex delivery system will be an useful aid for treating COVID-19 associated ARDS.

4. Discussion

Acute respiratory distress syndrome (ARDS) is a severe form of lung injury in critically ill patients. It is caused by higher levels of free reactive oxygen species and nitrogen species and is responsible for the activation of various inflammatory mediators leading to hypoxemic pulmonary edema, and the need for mechanical ventilation (Kellner et al., 2017; Ricciardolo et al., 2006). ARDS most often occur with severe trauma, pneumonia, aspiration of gastric contents, bacterial sepsis or with viral infections from human coronaviruses such as severe acute respiratory syndrome coronavirus (SARS-CoV), middle east respiratory syndrome coronavirus (MERS-CoV) and a recent outbreak from COVID-19 associated novel coronavirus SARS-CoV-2 infections, which can progress to refractory pulmonary failure and ultimately increases mortality rates (Petrosillo et al., 2020; Naeem, 2013). Despite its high incidence, there are no specific approved drugs to treat this condition. Supportive therapies such as restrictive fluid therapy, extracorporeal membrane oxygenation (ECMO) and prone positioning are some of the management strategies. These interventions improve the ARDS outcome but do not reduce the lung injury burden (Peck and Hibbert, 2019). In ARDS conditions, persistent inflammatory responses are observed as the air spaces are predominantly filled with infiltration of macrophages and neutrophills (Baudouin, 2006). In an attempt to improve the outcome, corticosteroid treatment was analyzed in ARDS patients, but their efficacy and beneficial effects in patients are divergent and controversial (Khilnani and Hadda, 2011). Unfortunately, sometimes ventilation also exacerbates ARDS by perpetuating local and systemic inflammation that further increases the mortality rates (Hough, 2014; Joseph et al., 2018). Recently, in COVID-19 induced ARDS cases, Aviptadil, a synthetic form of human vasoactive intestinal polypeptide (VIP) and Ruxolitinib, a selective Janus kinase (JAK) inhibitor are being investigated for ARDS treatment, which are under clinical trials (NCT04311697) (NCT04359290). However, there is still a huge scope to find new therapeutic targets and new drugs for this devastating ARDS condition (Mahase, 2020).

The structural motif, BRD4 is a member of the BET family that recognizes acetyl-lysine residues in both histone and non-histone proteins that orchestrate gene transcription by functioning as “readers”. BRD4 is implicated in several lung disease conditions such as chronic obstructive pulmonary disease (COPD), pulmonary fibrosis (Tian et al., 2016), lung cancer (Gao et al., 2018) and, asthma (Sanders and Thannickal, 2019). In the present study, we demonstrated for the first time that BRD4 has a predominant role in exacerbating inflammatory conditions, and thereby promotes ARDS. Further, the inhibition of BRD4 by chemical inhibitor PFI-1 and siRNA approaches were done and explained in preclinical

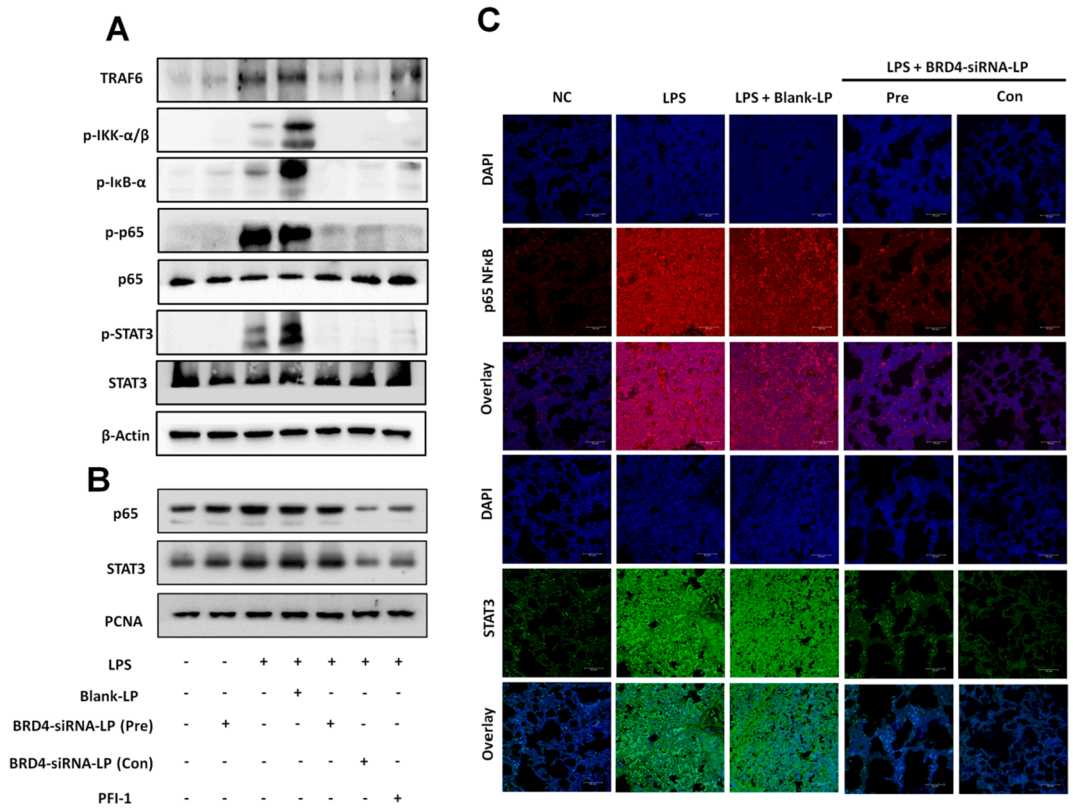

Fig. 8. BRD4-siRNA-LP inhibit LPS induced NF-κB signaling and inhibited nuclear translocation of p65 and STAT3 signaling *in vivo*. C57/BL6 mice were treated with 500 nM of BRD4-siRNA-LP and 5 mg/kg of PFI-1. Further inflammation was induced by LPS instillation. (**A**) Western blotting was performed to evaluate the expression of TRAF6 and phosphorylation of IKK-α/β, IκB-α, p65, and STAT3. (**B**) Nuclear p65 and STAT3 protein expression was studied by immunoblotting. (**C**) The nuclear translocation of p65 and STAT3 was evaluated by confocal microscopy. The images were captured at × 400 magnification.

ARDS model. siRNA acts by RNA interference mechanisms, where it recognises the homologous mRNA sequence in the cell and further induces its degradation and this processes is selective in nature, whereas the chemical small molecular inhibitors suppress not only the target protein expression and also involve in downregulating other proteins and also having various side effects (Chernikov et al., 2019). In the current study, first time, we developed the BRD4-siRNA-LP, where observed the effect of this formulation in ameliorating LPS induced ARDS and its complications by delivering oropharyngeal route of administration. Here, BRD4 siRNA was encapsulated as stable cationic lipoplexes by thin-film hydration method with an equal ratio of cationic lipid DOTAP and neutral charge cholesterol. Here, DOTAP is used to incorporate the positive charge to the lipoplexes, whereas cholesterol is used to maintain stability by ensuring the neutral charge. Both Blank-LP and BRD-siRNA-LP showed nano size and exhibited positive charge with acceptable PDI. The gel retardation assay results suggest that siRNA forms stable ionic interactions at 1:200 siRNA and lipid ratio, and showed 85.65 ± 0.98% entrapment efficiency. These lipoplexes were stable even in the presence of RNase. These siRNA lipoplexes were found to be stable in both *in vitro* and *in vivo*. *In vivo*, lipoplexes were administered via the oropharyngeal route for treating LPS induced respiratory complications. It is affirmed that preclinical demonstration of this novel delivery system will show the way for development of inhalational formulations for ARDS.

Although there are divergent causes for ARDS conditions, a series of acute inflammatory signaling cascades by evoking neutrophil infiltration, mast cell accumulation, oxidative and nitrosative stress, cytokine storm, damage to the epithelial and endothelial cells and respiratory illness are the common manifestations. The LPS induced ARDS model was selected in this study as it is a widely accepted model ,and mimics most of the symptoms of ARDS that are commonly seen in sepsis and COVID-19 conditions (Coperchini et al., 2020; Domscheit et al., 2020). From a pathophysiological perspective, both LPS and COVID19-associated lung inflammation shows ARDS symptoms such as pulmonary edema, endothelial inflammation, impaired pulmonary physiology, altered alveolar homeostasis and infiltration of inflammatory cytokines (Pfortmueller et al., 2020).

Previous reports suggest that the LPS stimulation induces an increase in BRD4 binding with genes, and it has been reported that signal intensity of BRD4 increased with LPS stimulation and BRD4 in macrophages is essential for eliciting TLR Signaling (Dey et al., 2019; Bao et al., 2017) Moreover, Gordon et al. suggest that SARS-CoV-2 transmembrane protein E binds with BRD2 and BRD4, thus, targeting BRD4 may inhibit the viral fusion into host cells as well as respiratory illness (Gordon et al., 2020). These findings raise an intriguing possibility that BRD4 might be involved in ARDS conditions. Consistent with this expectation, after LPS oropharyngeal instillation, the net body weights were found to be decreased, whereas increased lung weight index was observed. Interestingly, BRD-siRNA-LP treated groups, there were no major physiological alterations. Moreover, BRD4-siRNA-LP suppressed the LPS induced total cells, WBC, neutrophils, monocytes, and basophils counts, and protected the epithelial barrier dysfunction. In ARDS patients cytokines such as TNF-α, IL-1β, IL-6, and IL-8 are elevated in BAL fluid and circulating plasma (Han and Mallampalli, 2015). In COVID-19 associated critically ill patients in Intensive care unit (ICU) wards, higher plasma levels of various inflammatory cytokines including MCP −1, MIP −1A, GSCF, TNF-α, IL −2, IL −7, IL −10, and IP −10 were observed (Pooladanda et al., 2020). This cytokine storm perpetuates inflammatory immune cell infiltration, causes accumulation of protein-rich edema fluid into the alveoli with hypoxemia which is caused due to impairment of gas exchange (Pedersen and Ho, 2020). Hence, antioxidant therapy might be useful in ameliorating the ARDS condition (Wang et al., 2020). Here, LPS induced the nitrite levels and reduced the SOD and GSH levels, whereas BRD4-siRNA-LP inhibited nitrosative and oxidative stress by upregulating the antioxidant defensive mechanism. BRD4-siRNA-LP reversed the LPS induced cytokine storm by significantly suppressing pro-inflammatory cytokine levels including IL-1β, IL-6, IL-17A, IL22, and TNF-α. From H & E and Toulidine blue staining, it was observed that BRD4-siRNA-LP suppressed the LPS induced neutrophil and mast cell infiltration and protected the lungs from immune cell-mediated cytokine storm.

Targeting BRD4 suppresses the PI3K-Akt/MAPK/JNK signaling in cancer and inflammatory diseases, and these pathways important in the regulation of inflammatory responses (Liu et al., 2018; Stratikopoulos et al., 2015; Wang et al., 2018). In line with this, previous reports suggest that JNK, p38, and PI3K/Akt were involved in severe acute respiratory distress syndrome-coronavirus (SARS-CoV) infected Vero E6 cells, which suggest the possible involvement of inflammation in COVID-19 associated ARDS through this signaling (Mizutani et al., 2005). BRD4-siRNA-LP potently inhibited the LPS induced mTOR expression and phosphorylation of Akt at Thr308 and Ser473. Additionally, targeting BRD4 suppressed the LPS induced phosphorylation of p44/42, p38, GSK-3β, and SAPK/JNK.

On the other hand, SARS-CoV-2 activates p65 NF-κB signaling similar to SARS-CoV and the Middle East respiratory syndrome-coronavirus (MERS-CoV), which is critical for pulmonary inflammation and associated series of events (Chen et al., 2020). BRD4 coactivates the NF-κB transcriptional activity by acetylating p65 also known as RelA (Huang et al., 2009). Upon IκB-α degradation, p65 moves into the nucleus, and further induces the transcription of various inflammatory genes and evokes the ARDS (Moine et al., 2000). The accumulating evidence suggest that deletion of BRD4 in macrophages showed constant MAP kinase-interacting serine/threonine-protein kinase 2 (Mknk2) and involved in the activation of eIF4E, which further aided in IκBα mRNA translation, which ultimately suppressed the NF-κB mediated inflammatory signaling and demonstrated that conditional knock out of BRD4 in mice were resistant to the sepsis conditions induced by LPS, which depicts the role of BRD4 in regulating the innate immune response (Bao et al., 2017). With respect to this paradigm, in our experimentation, we have observed that LPS induced the phosphorylation of IKK-α/β, IκB-α, and p65, whereas this effect was significantly inhibited by BRD4-siRNA-LP. Accumulating evidence suggest that pro-inflammatory cytokine stimulus activates the STAT3 and NF-κB crosstalk through TRAF6 and p65 dependent mechanism (Yoshida et al., 2004). On the other hand, STAT3 retains the nuclear-bounded p65, thus promotes persistent inflammation (Yu et al., 2009) In addition, targeting BRD4 inhibits the STAT3-dependent MYC expression in cancer, however, it is not yet elucidated in inflammatory condition (Ray et al., 2014). In our study, we noticed that BRD4 inhibition suppressed the STAT3 phosphorylation. We also observed that BRD4-siRNA-LP significantly inhibited p65 and STAT3 and further reduced the activation of inflammatory proteins expression. This might be therefore a promising novel target with the potentially advantageous delivery systems, which may be considered in treating COVID-19 induced ARDS (Fig. 9).

Collectively, our results infer that BRD4 is a novel target for ARDS irrespective of its origin. The LPS induced ARDS model may mimic the COVID-19 associated ARDS. Inhibition of BRD4 by BRD4-siRNA-LP might play an important role in treating clinical COVID-19-ARDS patients potentially through anti-inflammatory and anticytokine storm properties by controlling p65 and STAT3 nuclear translocation. Thus, it could be a better therapeutic strategy for treating COVID-19 induced ARDS in this global pandemic.

CRediT authorship contribution statement

Venkatesh Pooladanda: Conceptualization, Methodology, Writing - original draft, Software, Validation. **Sowjanya Thatikonda:** Visualization, Methodology, Writing - original draft. **Sai Priya Muvvala:** Methodology. **Geetanjali Devabattula:** Methodology. **Chandraiah Godugu:** Supervision.

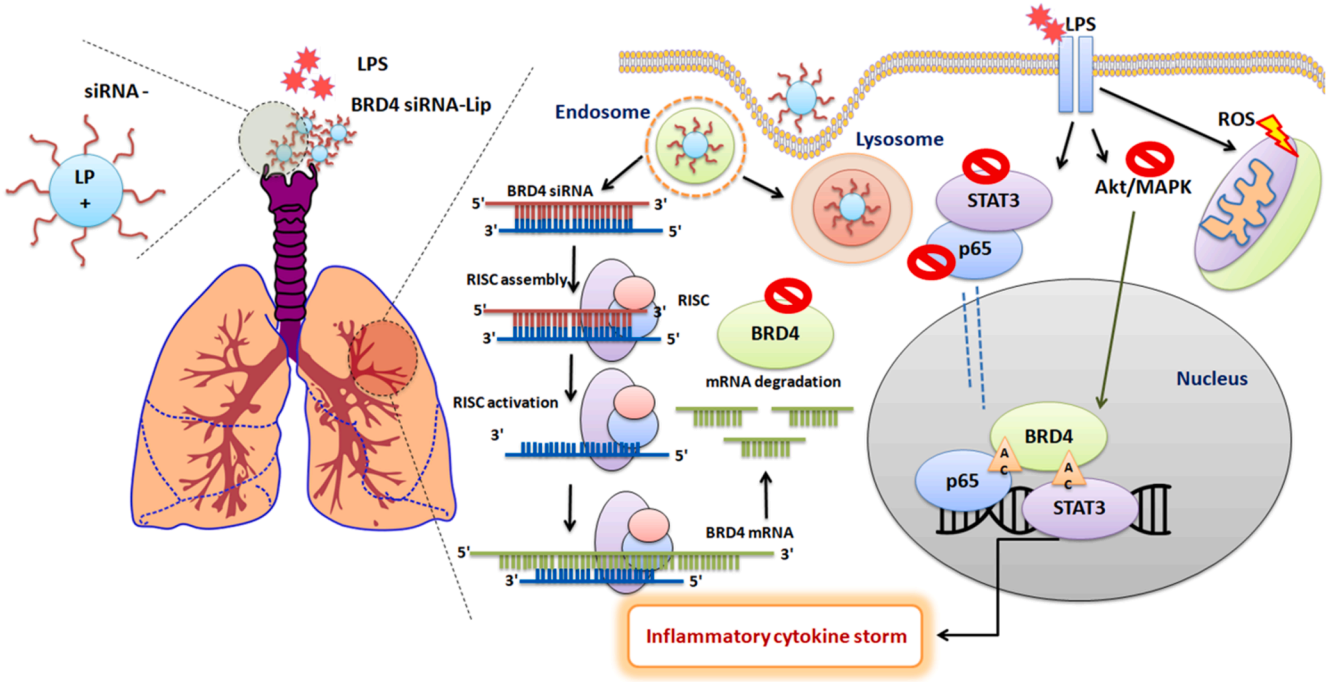

Fig. 9. A schematic diagram represents the therapeutic potential of BRD4-siRNA-LP in LPS-induced ARDS. The novel liposomal delivery system, BRD4-siRNA-LP suppress the oxidative stress, cytokine storm, and pulmonary inflammation by inhibiting the nuclear translocation and p65 and STAT3.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120536.

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