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iRGD conjugated nimbolide liposomes protect against endotoxin induced acute respiratory distress syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is a deadly respiratory illness associated with refractory hypoxemia and pulmonary edema. The recent pandemic outbreak of COVID-19 is associated with severe pneumonia and inflammatory cytokine storm in the lungs. The anti-inflammatory phytochemistry nimbolide (NIM) may not be feasible for clinical translation due to poor pharmacokinetic properties and lack of suitable delivery systems. To overcome these barriers, we have developed nimbolide liposomes conjugated with iRGD peptide (iRGD-NIMLip) for targeting lung inflammation. It was observed that iRGD-NIMLip treatment significantly inhibited oxidative stress and cytokine storm compared to nimbolide free-drug (f-NIM), nimbolide liposomes (NIMLip), and exhibited superior activity compared to dexamethasone (DEX). iRGD-NIMLip abrogated the LPS induced p65 NF- κ B, Akt, MAPK, Integrin β 3 and β 5, STAT3, and DNMT1 expression. Collectively, our results demonstrate that iRGD-NIMLip could be a promising novel drug delivery system to target severe pathological consequences observed in ARDS and COVID-19 associated cytokine storm.

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Key words: Nimbolide; iRGD-NIMLip; Oxidative stress; Cytokine storm; Inflammation; ARDS

Acute respiratory distress syndrome (ARDS) is a predominant cause of death among various lung inflammatory diseases. It is a frequent complication in critically ill patients, which can be commonly caused by sepsis, pneumonia, aspiration, pancreatitis, trauma, viral diseases such as influenza, herpes simplex virus (HSV), as well as cytomegalovirus (CMV), and coronaviruses including HCoV-NL63, SARS-HCoV, MERS-CoV, and SARS-CoV-2.¹ The main reason for morbidity and mortality observed with the current global pandemic disease COVID-19 is severe pneumonia and ARDS.² The bacterial endotoxin lipopolysaccharide (LPS) activates the toll-like receptor-4 (TLR-4) on the macrophages, dendritic cells, and other inflammatory cells. This leads to the induction of various inflammatory cytokines, which are further responsible for severe inflammation in the lungs.³ For simulating the human ARDS complications, LPS is used frequently

as an inducing agent in both *in vitro* and *in vivo* models.^{4,5} Song and colleagues illustrated the antiviral activity of baicalin in SARS-CoV-2 infected mice, where inflammatory cell infiltration in lungs was observed in LPS induced ARDS mice.⁶ Similarly, Su et al supported the COVID-19 associated ARDS with LPS induced cytokine storm mice model and demonstrated the therapeutic efficiency of entecavir and imipenem.⁷

Signal transducer and activator of transcription 3 (STAT3) is activated by IL-6 and LPS and further, it interacts with p65 NF- κ B at multiple levels and involved in inflammation.^{8,9} The existing evidence suggests that STAT3 activates DNA methyltransferase 1 gene (DNMT1) transcription in neoplastic T lymphocytes and is involved in cancer; however, STAT3 mediated DNMT1 expression was not elucidated in ARDS.¹⁰ Additionally, it was observed that STAT3 may cause the

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Conflict of interest

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epigenetic gene silencing by inducing the DNMT1 expression.¹¹

Integrins are the transmembrane receptors, which exist as heterodimers and have α and β subunits. Integrins $\beta 3$ and $\beta 5$ are widely expressed in tumor vasculature and involved in inflammation and metastasis.^{12,13} Integrin $\beta 3$ is upregulated in response to LPS. It promotes PI3K/Akt induced p65 NF- κ B transcriptional activity and induces the inflammatory genes expression and cause cytokine storm.¹⁴ iRGD peptide consists of CRGDKGPDC sequence, which has been elucidated as a tumor penetrating peptide and is involved in enhancement of the therapeutic index of various antineoplastic drugs by selectively targeting the $\alpha v\beta 3/\beta 5$ integrins.¹⁵ This peptide is proteolytically processed into CRGDK/R, exhibited as an active CendR motif at the C-terminus and interacts with neuropilins (NRPs), which will increase the vascular and tissue permeability.¹⁶ Existing reports suggest that RGD peptide ameliorated the LPS-induced ERK signaling and also mitigated the inflammatory WISP1-integrin $\beta 6$ signaling pathway in lung inflammatory conditions.^{17,18}

Nimbolide (NIM) is an active chemical constituent of the neem (*Azadirachta indica*) tree. It possesses multiple pharmacological activities, including antibacterial, antimalarial, anticancer, antifungal and anti-inflammatory effects.¹⁹ In our recent work, we have demonstrated that NIM, when administered intraperitoneally (i.p.) exhibited anti-inflammatory and antioxidant effects in LPS-induced ARDS model by inhibiting TNF- α mediated p65 NF- κ B and HDAC-3 nuclear translocation.²⁰ However, we found that NIM has poor solubility and is orally not bioavailable, which hampers its pharmacological activity and clinical development.²¹ Therefore, to overcome this limitation, we have designed novel liposomes to encapsulate NIM and improve its pharmacological activity. In the present study, we have evaluated the lung-protective effects such as the anti-inflammatory and antioxidant activity of NIM liposomes, which are conjugated with iRGD and evaluated the pharmacological activity in LPS induced experimental ARDS *in vitro* and *in vivo* models.

Materials and methods

Materials

NIM was purchased from Aptus therapeutics, Hyderabad, India. iRGD is a 9-amino acid cyclic peptide (Sequence: CRGDKGPDC), which was custom synthesized by Biotech Desk Pvt. Ltd, Hyderabad, India. LPS from *Escherichia coli* O111: B4, cholesterol, and phosphatidylcholine were purchased from Sigma-Aldrich, USA. 3-Maleimidobenzoic acid N-succinimidyl ester (MBS) was purchased from TCI chemicals, Japan. The antibodies used in this study were described in Supplementary Information. All the chemicals used in this study were pure and analytical grade.

Preparation and characterization of liposomes

iRGD conjugated nimbolide liposomes (iRGD-NIMLip) were prepared by a well-known thin-film hydration method.²² NIM, cholesterol, MBS, and phosphatidylcholine (1:1:1:2.5

molar/weight ratio) were dissolved in chloroform in a round bottom flask and the organic solvent was evaporated by rota evaporator. The thin film was hydrated by distilled water. Then liposomes were subjected to probe sonication and further incubated with iRGD peptide (drug and peptide 1:2 ratio) for 4 h at room temperature. The iRGD blank liposomes (iRGDLip) were prepared as described above without NIM, whereas nimbolide liposomes (NIMLip) were prepared without iRGD conjugation. The size distribution was determined by Zeta Sizer (Malvern Panalyticals, UK) with standard protocol. The morphology of liposomes was evaluated by transmission electron microscopy (TEM). The percentage of NIM entrapment was determined by liquid chromatography–mass spectrometry (LC–MS). The extent of iRGD peptide conjugation was determined by LC–MS analysis, circular dichroism (CD) spectra, and the Bradford assay methods (Detail methods were described in Supplementary Information).

Cell culture

Human bronchial epithelial cells (BEAS-2B) were a kind gift sample from Dr. Anurag Agrawal, IGIB, New Delhi, India, and these cells were cultured in a 1:1 ratio of DMEM low glucose and F12K Ham's media. Mouse macrophages (RAW 264.7) were procured from National Centre for Cell Science (NCCS), Pune, India and cells were cultured in DMEM medium. The cells were grown in a humidified CO₂ incubator supplemented with 10% FBS (Gibco, USA).

Determination of cell viability and oxidative stress

Effect of NIM formulations on cell viability was evaluated by MTT (Sigma Aldrich, USA) and Annexin V Alexa Flour 488/Propidium Iodide (PI) staining assays (Thermo Fisher Scientific, USA). The reactive oxygen species (ROS) levels were determined by 2',7'-dichlorofluorescein diacetate (DCFDA) (Sigma Aldrich, USA) and MitoSOX red (Thermo Fisher Scientific, USA) stainings. These procedures were clearly described in Supplementary Information.

LPS induced ARDS animal model

Male C57BL/6 mice (5-6 weeks old) were procured from Palamur Biosciences, Mahabubnagar, Telangana, India. All the animal experiments were conducted by following the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (GoI). To evaluate the efficacy of NIM as a liposomal drug delivery system as compared to the free drug form in attenuating the LPS-induced ARDS, animals were divided into seven groups with $n=8$ animals per group. The animals were randomized into different groups such as normal control (NC), LPS control (LPS), iRGD blank liposomes (iRGDLip), nimbolide (f-NIM), nimbolide liposomes (NIMLip), iRGD conjugated nimbolide liposomes (iRGD-NIMLip), and dexamethasone (DEX). Mice were treated with NIM and its formulations, where animals were administered with 0.3 mg/kg equivalent dose of NIM in either free form or liposomal forms by oropharyngeal route. In case of iRGD-NIMLip treatment group, equivalent amounts of liposomal components needed to entrap

required amount of NIM were used to treat the animals. Similarly, mice were oropharyngeally administered with standard drug DEX (0.3 mg/kg). Initially, animals were pretreated with iRGDLip, f-NIM, NIMLip, iRGD-NIMLip, and DEX for 2 h and stimulated with LPS (50 µg/mice) for 12 h. Later, animals were sacrificed by isoflurane and lungs were collected and stored at -80 °C. LPS, f-NIM, and its liposomal formulations along with DEX were administered through the oropharyngeal route.²³ Briefly, animals were anesthetized using isoflurane. After attaining slight plane anesthesia, mice were placed at 60° inclined angle on intubation platform. The blunt forceps were used to pull the tongue, following which 50 µg of LPS and different treatments in 50 µl volume of vehicle were administered into the back of the oral cavity using 100 µl micropipette.

Physiological, bronchoalveolar lavage fluid (BALF) analysis, biochemical parameter, cell uptake, tissue distribution studies, enzyme-linked immunosorbent assay (ELISA), western blot, immunofluorescence (IF) analysis, and histopathology

Detailed methods were explained in Supplementary Information.

Statistical analysis

Here, results are expressed as mean ± SD, and *n* refers to the number of sample replicates. The results were analyzed using Prism software (version 6.01; GraphPad, USA) with one-way, two way ANOVA along with unpaired Student *t* test and the post hoc testing was performed with Tukey's and Sidak's test. Here, *P*<0.05 was considered statistically significant.

Results

Preparation and characterization of iRGD conjugated NIM liposomes

Prepared liposomal formulation size distribution (Figure 1, A), charges (Figure 1, B), and polydispersity index (PDI) were evaluated by zeta meter (Figure 1, C). The prepared iRGD-NIMLip exhibited an average particle size of 171.5 ± 25.02 nm, 0.27 ± 0.02 PDI, and -21.42 ± 0.78 mV zetapotential (Table 1 and Figure S1). The iRGD-NIMLip exhibited a spherical morphology, which was confirmed by TEM analysis (Figure 1, D). As shown in Figure 1, E, the extent of iRGD peptide conjugation was confirmed by LC-MS analysis, where we found that 24.23% ± 0.53% of the peptide was conjugated. Additionally, we determined the extent of conjugation by Bradford assay, where we found that 32.14% ± 0.87% of the peptide was present in a liposomal formulation. Additionally, CD spectroscopic analysis infers that iRGD peptide is significantly conjugated to NIM liposomes and it further supports the LC-MS data (Figure 1, F). Further, we evaluated the entrapment efficiency of NIM in liposomes by LC-MS analysis, where we found 85.24% ± 0.97% of NIM was entrapped in the liposomal formulation (Figure 1, G).

iRGD-NIMLip exhibit anti-inflammatory activity by targeting inflammatory sites

Initially, we have evaluated the viability of both RAW 264.7 and BEAS-2B cells in presence of iRGDLip, f-NIM, NIMLip, and iRGD-NIMLip by MTT assay. iRGD-NIMLip, iRGDLip, f-NIM, and NIMLip have shown minimal toxicity at 0.5 µg/ml concentration in both cell types (Figure S2, A-B). Additionally, we performed Annexin V Alexa Flour 488/PI dual staining to evaluate the effect of NIM liposomal formulations on apoptosis in RAW 264.7 and BEAS-2B cells, where liposomes at 0.5 µg/ml concentration showed minimal apoptotic cell death and did not exhibit any cytotoxicity (Figure S3, A-B). Therefore, further *in vitro* experiments were performed using 0.5 µg/ml concentration.

iRGD peptide non-specifically binds with integrins, which are highly expressed in inflammatory conditions. To mimic this inflammatory condition, we stimulated the mouse macrophages with LPS and evaluated the liposomal formulation uptake. Initially, cells were stimulated with 1 µg/ml LPS for 24 h and further they were incubated with FITCLip and iRGD-FITCLip for 3 h. We observed a significant uptake of iRGD-FITCLip by macrophages which were stimulated with LPS as compared to un-stimulated cells confirmed by both confocal (Figure S4, A) and flow cytometric analysis (Figure S4, B & C). To evaluate the effect of iRGD to increase the retention of liposomes in presence of inflammation, two groups of mice were taken, and 50 µg of LPS was administered through the oropharyngeal route. After 2 h of LPS stimulation, mice received FITC solution, FITCLip, and iRGD conjugated FITC liposomes (iRGD-FITCLip) through the oropharyngeal route. After 3 h of liposomes administration, animals were sacrificed and the lung tissues were sectioned by cryotome and images were captured by confocal microscopy. Data in Figure S4, D suggest that iRGD-FITCLip were more significantly accumulated in LPS instilled lung tissues as compared to FITC solution and FITCLip. In another set of experiment, f-NIM and iRGD-NIMLip were oropharyngeally administered to LPS challenged mice and the drug concentration was estimated using LC-MS analysis, where iRGD-NIMLip showed more deposition of NIM in lung tissues as compared to f-NIM observed at 12 h post-treatment (Figure S4, E).

iRGD-NIMLip restores the LPS-induced physiological and pathological changes

The corticosteroid DEX exhibits anti-inflammatory properties and reduces the mortality of ARDS patients by upregulating the anti-inflammatory cytokines.²⁴ Here, we have used DEX as a standard drug to compare the therapeutic activity of iRGD-NIMLip in LPS-induced ARDS mouse model. In LPS challenged mice group, a significant reduction in the body weights was observed, while in those treated with iRGD-NIMLip (0.3 mg/kg of net NIM concentration) liposomal formulation a significant improvement in the body weights was observed. Moreover, these body weights are comparable with the NC group, while with DEX treatment, a significant enhancement was not observed (Figure S5, A). LPS induction enhanced the lung weight index, which is an indication of pulmonary edema and neutrophil infiltration. Pretreatment of f-

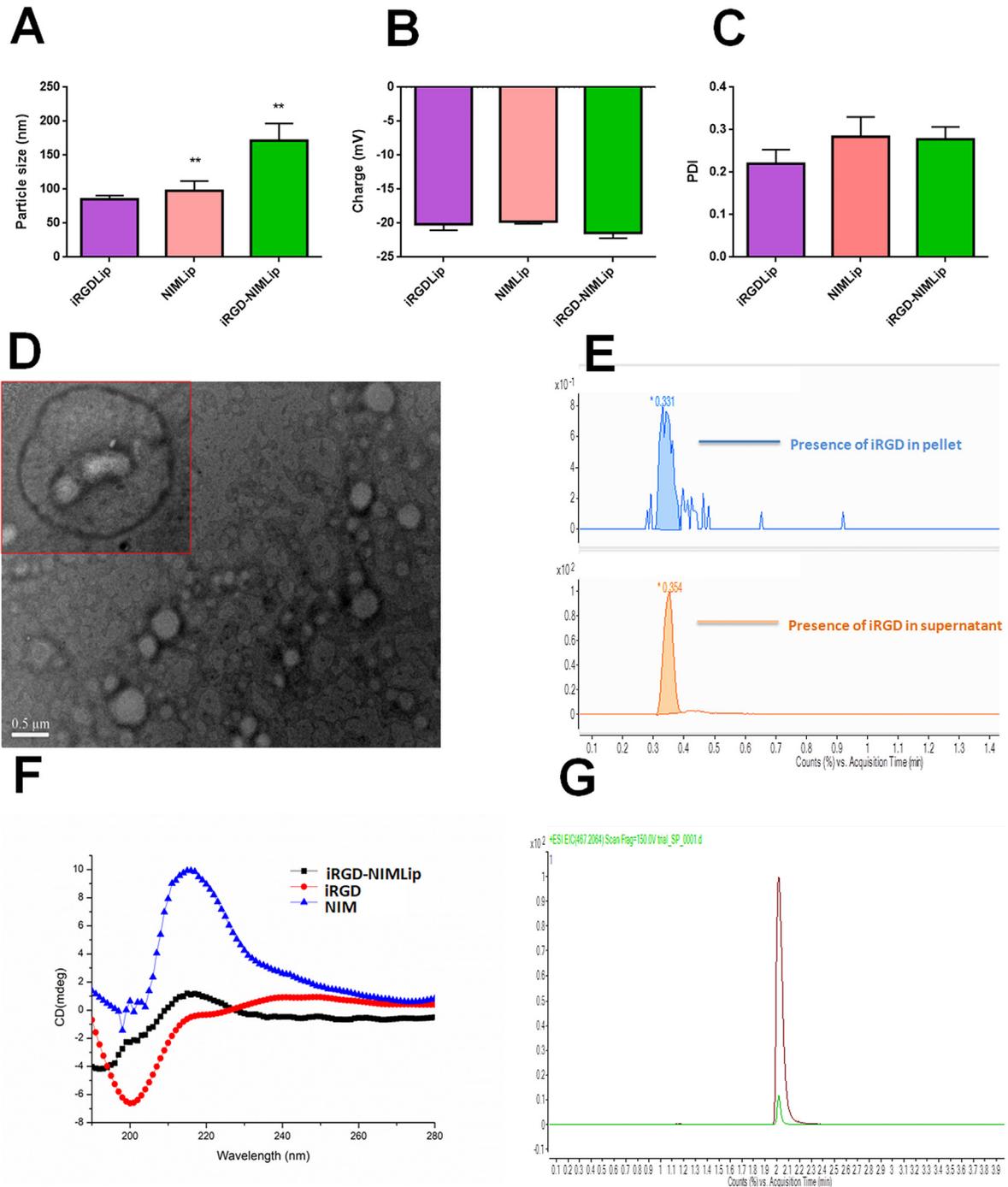


Figure 1. Characterization of nimbolide (NIM) liposomal drug delivery systems. (A) The particle size, (B) charge, and (C) PDI of iRGD conjugated liposomes (iRGDLip), nimbolide loaded liposomes (NIMLip), and iRGD conjugated nimbolide liposomes (iRGD-NIMLip) were evaluated by Zeta sizer. (D) The particle size and shape of iRGD-NIMLip were evaluated by TEM and these liposomes exhibited spherical structure. The extent of peptide conjugation to the liposomes was determined by (E) LC–MS and (F) CD spectra. (G) The percentage of NIM entrapment in liposomes was estimated by LC–MS analysis. Data were presented as mean \pm SD ($n=3$ independent experiments). ** $P<0.01$ is significantly different from the control and iRGDLip group (Statistical significance was determined by one-way ANOVA followed by Tukey's post-test).

NIM at 0.3 mg/kg dose was not effective in controlling the LPS induced edema to a significant level. However, the novel formulation of iRGD-NIMLip significantly reduced the LPS induced lung weight index as compared to DEX (at 0.3 mg/kg) (Figure S5, B).

Besides, the pictorial images of lungs show that LPS induction led to a definite increase in the size of the lungs as a result of edema formation with phenotypic changes such as white and pale color patches as compared to the NC animal lungs, where lungs appeared reddish in color. On the other side, iRGD-

Table 1

Liposomal formulation characteristics.*

S. no.	Liposomal formulation	Size (nm)	PDI	Charge (mV)
1	iRGDLip	85.37 ± 5.38	0.22 ± 0.03	-19.22 ± 0.95
2	NIMLip	97.94 ± 14.02	0.28 ± 0.05	-19.77 ± 0.30
3	iRGD-NIMLip	171.5 ± 25.05	0.27 ± 0.02	-21.42 ± 0.78

* Data presented as mean ± SD (n=3). iRGD conjugated liposomes (iRGDLip); Nimbolide loaded liposomes (NIMLip); iRGD conjugated nimbolide liposomes (iRGD-NIMLip); Polydispersity index (PDI).

NIMLip treated animals exhibited superior changes with reduced signs of edema compared to iRGDLip, f-NIM, NIMLip, and DEX group animals (Figure 2, A). The inflammatory cells were counted by differential blood cell counter, where LPS stimulation elevated the levels of BAL total cells, neutrophils, platelets, procalcitonin (PCT), absolute lymphocytes, basophils, monocytes, eosinophils, and white blood cells (WBC). Remarkably, we found that iRGD-NIMLip significantly reduced the LPS induced inflammatory cells. Additionally, this novel NIM formulation has shown more potent activity as compared to DEX treatment (Figure 2, B-J).

iRGD-NIMLip suppress the LPS induced lipid peroxidation and pro-inflammatory cytokines

In our study, we observed that the bacterial LPS induced the MDA and nitrite levels, while concomitantly reducing the GSH/GSSG ratio. It was found that iRGD-NIMLip treatment significantly reduced LPS induced lipid peroxidation and nitrosative stress by upregulating the antioxidant levels in contrast with DEX (Figure S6, A-C). Moreover, a superior activity of iRGD-NIMLip was observed in comparison to f-NIM, NIMLip, and iRGDLip treated groups. The inflammatory cytokine levels in mouse lungs were evaluated by ELISA, where we found that LPS induced the pro-inflammatory cytokine levels such as IL-1 β , IL-6, IL-17A, IL-22, TNF- α , and TGF- β . iRGD-NIMLip significantly reduced these pro-inflammatory cytokines and reversed the LPS induced inflammation as compared to DEX treatment group (Figure S6, D-I).

iRGD-NIMLip reduce the LPS induced pathological consequences

H&E staining results infer that NC group animals exhibited clear pulmonary alveoli, whereas LPS induced the acute inflammation and showed destructive epithelium. Here, it was noticed that iRGD-NIMLip inhibited the inflammatory cell migration and pulmonary edema and showed better activity as compared to the free-drug of NIM and iRGDLip. Moreover, the iRGD-NIMLip treated group exhibited excellent anti-inflammatory activity as compared to the DEX treatment group (Figure 3, A and Figure S7, A). Additionally, iRGD-NIMLip treatment reduced the LPS induced mast cell accumulation as compared to DEX as well as f-NIM and NIMLip treated groups (Figure 3, B and Figure S7, B).

iRGD-NIMLip inhibit TNF- α mediated p65 NF- κ B signaling and exhibit antioxidant activity through Nrf-2 activation

LPS produces inflammation by enhancing the nitrite levels in macrophages, where f-NIM, NIMLip, and iRGD-NIMLip exhibited prominent anti-nitrosative effect by significantly reducing the nitrite levels observed in RAW264.7 cells (Figure 4, A and Figure S8, A). LPS binds to TLR4/integrins and induces oxidative stress by enhancing various inflammatory cytokines. Total cellular and mitochondrial ROS levels were determined by DCFDA and MitoSOX Red staining, where pretreatment with f-NIM, NIMLip, and iRGD-NIMLip inhibited both total cellular and mitochondrial ROS levels. Moreover, iRGD-NIMLip showed a significant antioxidant effect in comparison with other treatment groups (Figure 4, B & C and Figure S8, B & C).

The anti-inflammatory and antioxidant effects of iRGD conjugated NIM liposomal formulations were evaluated in RAW 264.7 cells. Western blot results revealed that LPS enhanced the expression of TRAF6 and TNF- α . Furthermore, LPS phosphorylated the IKK- α/β and was involved in the degradation of I κ B- α , which further induced the phosphorylation of p65 NF- κ B (Figure 4, D and Figure S9, A-D). Moreover, iRGD-NIMLip significantly reduced the NF- κ B signaling pathway by reducing phosphorylation and nuclear translocation compared to free-drug and NIMLip. Additionally, iRGD-NIMLip significantly reduced LPS induced oxidative stress by upregulating Nrf-2, SOD-1, and HO-1 proteins' expression and neutralized the endotoxin induced inflammation compared to f-NIM and NIMLip groups (Figure 4, E and Figure S9, E-G). Further, we observed that iRGD-NIMLip significantly reduced the p65 activated COX-2 and iNOS expression (Figure 4, F and Figure S9, H & I). To further confirm these results, we performed IF analysis, where iRGD-NIMLip significantly reduced the LPS induced COX-2 and iNOS expression. Overall, it was observed that iRGD-NIMLip exhibited superior results over f-NIM, NIMLip, and iRGDLip treated groups (Figure 4, G). Additionally, LPS enhanced the expression of hypoxia-inducible factor 1-alpha (HIF-1 α), which was significantly reversed by iRGD-NIMLip and showed better results compared to f-NIM and NIMLip groups (Figure 4, F and Figure S9, J).

iRGD-NIMLip downregulate LPS induced inflammatory signaling cascade in bronchial epithelial cells

MAPK and GSK-3 β signaling plays a pivotal role in inflammation, where the novel drug delivery system, iRGD-NIMLip reduced LPS induced phosphorylation of SAPK/JNK, p44/42, p38, and GSK-3 β as compared to f-NIM, NIMLip, iRGDLip, and DEX treatment groups (Figure 5, A and Figure S10, A-D). Additionally, PI3K-Akt-mTOR is signaling involved in multiple diseases and participates in activation of various inflammatory genes. iRGD-NIMLip reversed LPS induced PI3K and mTOR expression and simultaneously reduced the phosphorylation of Akt at both S473 and T308 sites as compared to other groups (Figure 5, B and Figure S10, E-H). The expression of transmembrane receptors Integrins β 3 and β 5 was upregulated upon LPS stimulation in BEAS-2B cells, whereas these proteins were significantly reduced by iRGD-NIMLip compared to other

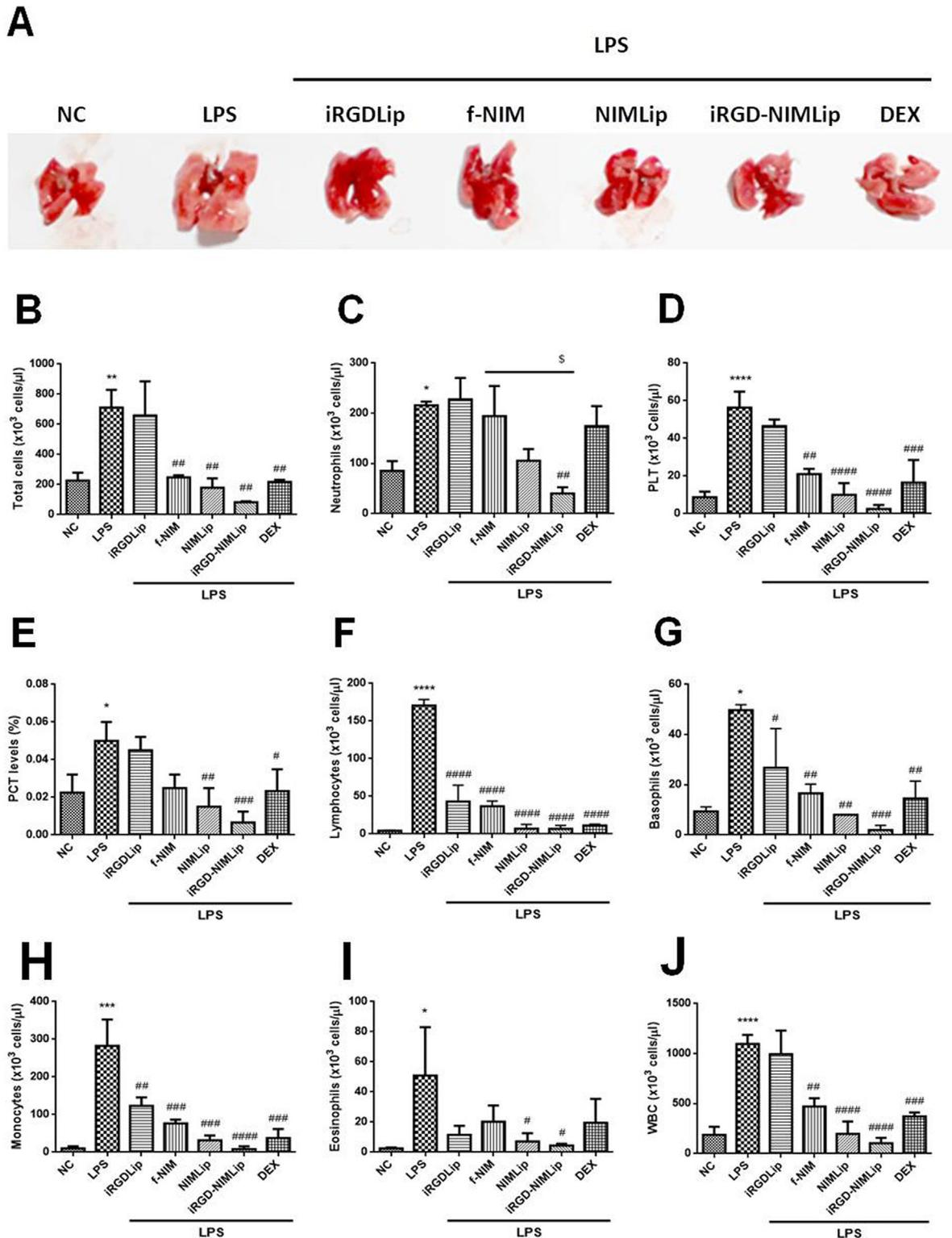


Figure 2. **iRGD-NIMLip modulate the LPS induced inflammatory cells in BALF.** Mice were pretreated with iRGDLip, f-NIM, NIMLip, iRGD-NIMLip, and DEX for 2 h. Then the mice were challenged with LPS for further 12 h. (A) After sacrifice, lungs were isolated followed by photographic images were taken using digital camera. Here, LPS instilled mice lungs were infiltrated in large or disseminated in small areas and showed livid discolorations and hemorrhages along with pulmonary edema. The BALF was collected in chilled PBS and subjected to differential blood cell counter and evaluated the (B) total cells, (C) neutrophils, (D) platelets (PLT), (E) procalcitonin (PCT), (F) lymphocytes, (G) basophils, (H) monocytes, (I) eosinophils, and (J) white blood cells (WBC). Data were presented as mean \pm SD ($n=4$ mice per group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$ are significantly different from the NC group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ and #### $P<0.0001$ are significantly different from the LPS group; ^S $P<0.05$ is significantly different from f-NIM group (Statistical significance was determined by one-way ANOVA followed by Tukey's post-test).

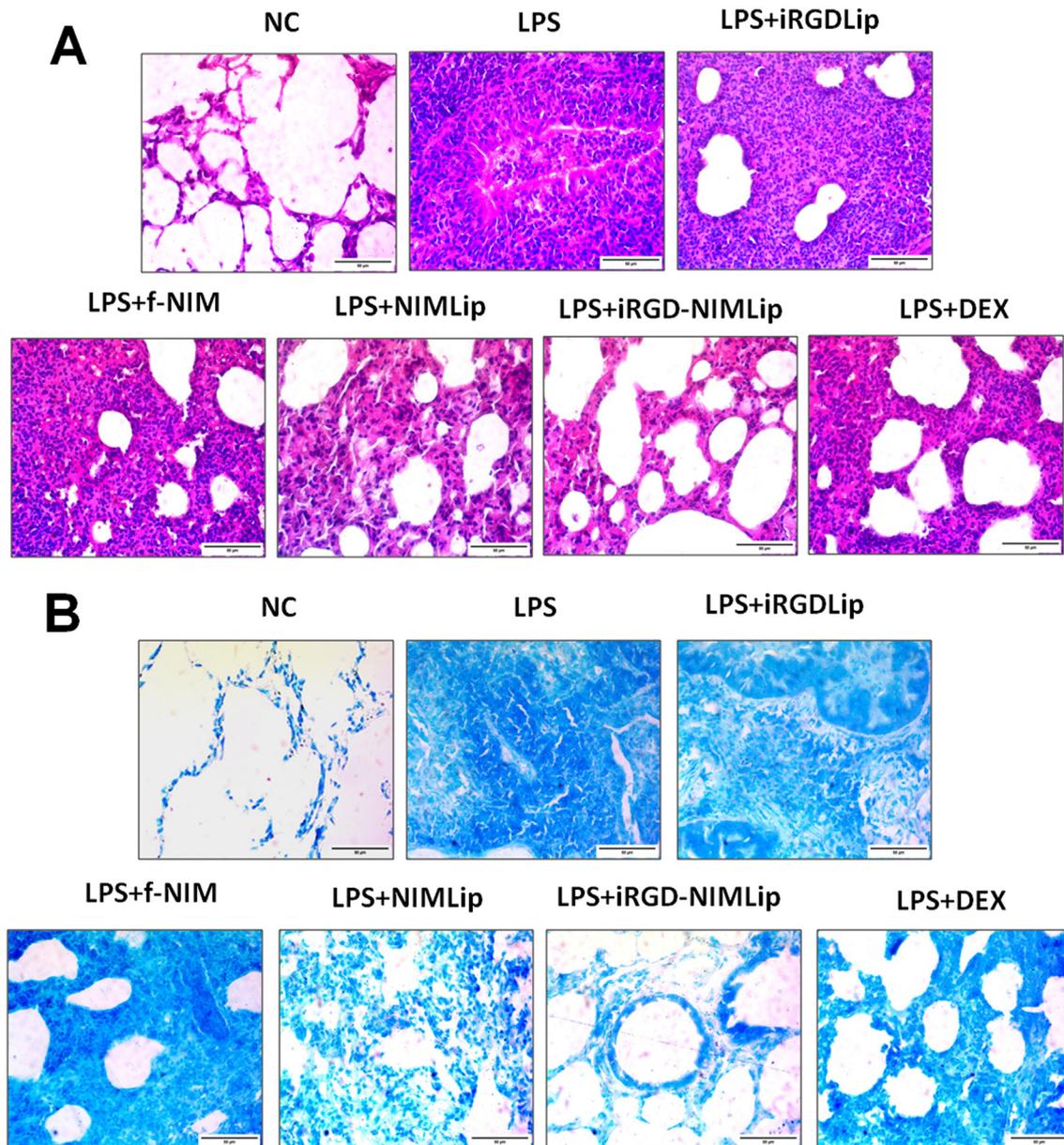


Figure 3. **iRGD-NIMLip re-modulate LPS induced pathological consequences.** The 5 μ m lung sections were stained with H&E and TB. (A) H&E staining images inferred that the NC group showed intact lung tissue, whereas LPS induced thickening of the alveolar wall and accumulation of inflammatory cells. (B) TB staining images showed that LPS enhanced the mast cell accumulation, which was significantly reversed by iRGD-NIMLip. The images were taken by bright field microscope at $\times 400$ magnification.

groups including iRGDLip, f-NIM, and NIMLip (Figure 5, C and Figure S10, I & J). Moreover, iRGD-NIMLip significantly reduced LPS induced phosphorylated STAT3 at Tyr 705 and DNA methyltransferase 1 (DNMT1) expression in bronchial epithelial cells (Figure 5, D and Figure S10, K & L). Confocal data were further supportive of western blot results, where iRGD-NIMLip inhibited STAT3 and DNMT1 expression in BEAS-2B cells (Figure 5, E).

iRGD-NIMLip downregulate the LPS induced inflammatory cascade by suppressing STAT3 and DNMT1 interaction in ARDS mouse model

As mentioned earlier, LPS activated the p65 NF- κ B, Akt, and MAPK signaling, which further resulted in enhanced oxidative-nitrosative stress. Similarly, it was observed that iRGD-NIMLip significantly downregulated LPS-induced IKK- α/β , I κ B- α , and

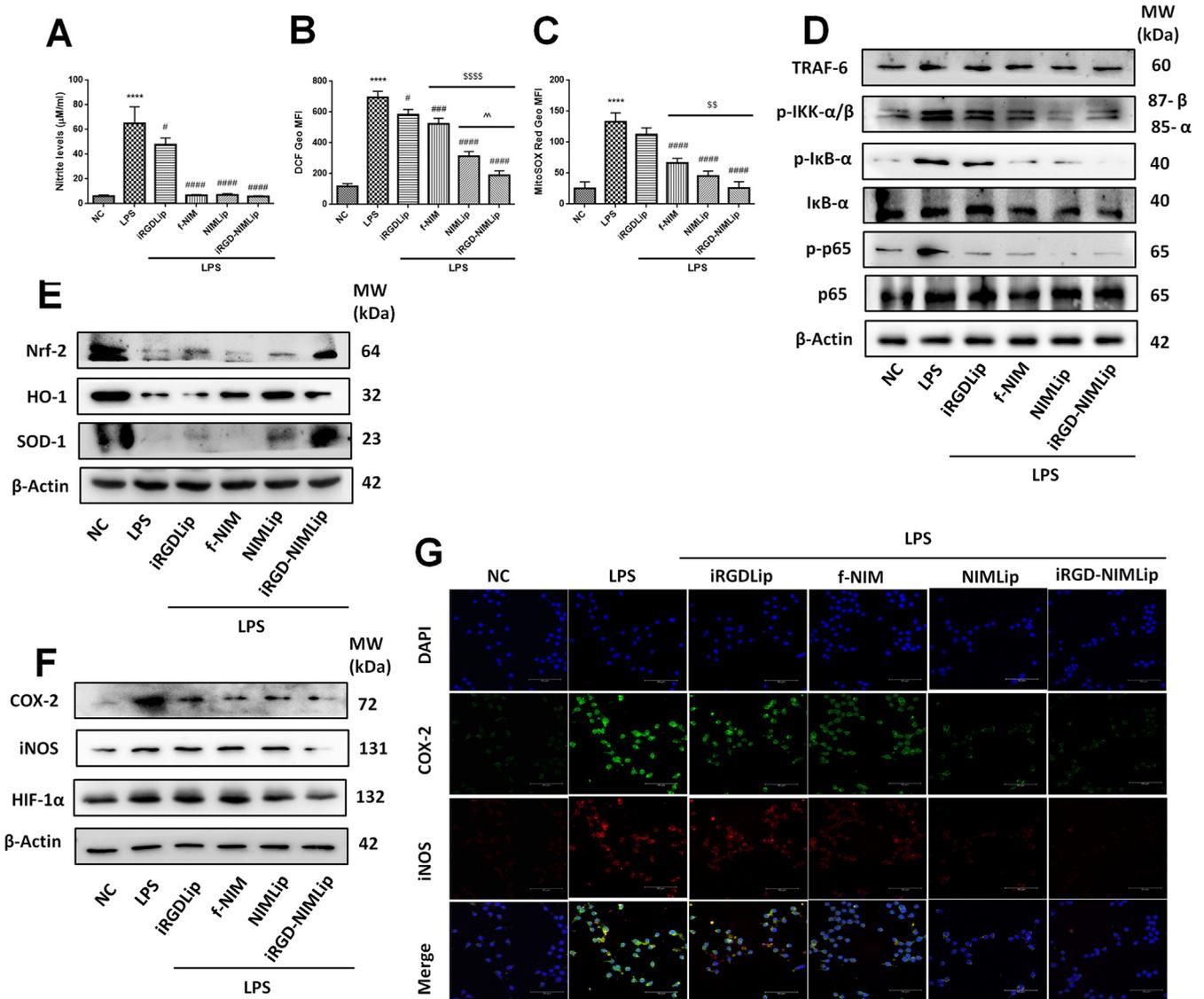


Figure 4. **iRGD-NIMLip** inhibit LPS induced oxidative stress by downregulating the inflammatory signaling cascade in macrophages. (A) Nitrite levels were measured in cell culture supernatants of macrophages by Griess assay. (B) The intracellular and (C) mitochondrial ROS levels were determined by DCFDA and MitoSOX Red staining, respectively and quantitative analysis was performed by flow cytometry. Macrophages were pretreated with NIM and its liposomes (0.5 µg/ml net NIM concentration) for 24 h and stimulated with LPS (1 µg/ml) for 30 min. (D) After 30 min of post LPS stimulation, western blotting was performed to evaluate the expression of TRAF6, p-IKK-α/β, p-IκB-α and p-p65 NF-κB. (E) Nrf-2, HO-1, and SOD-1 expression was evaluated by immunoblotting 24 h after post LPS stimulation. Additionally, (F) COX-2, iNOS, and HIF-1α expression was elucidated by immunoblotting after 24 of post LPS exposure. (G) The confocal analysis was performed and illustrated COX-2 and iNOS expression in macrophages. The images were captured at $\times 630$ magnification. Data presented as mean \pm SD ($n=3$ independent experiments and each experiment was conducted in triplicates). **** $P<0.0001$ is significantly different from the NC group; # $P<0.05$, ### $P<0.001$ and ##### $P<0.0001$ are significantly different from the LPS group; \$\$\$\$ $P<0.0001$ and \$\$ $P<0.01$ are significantly different from the f-NIM group; ## $P<0.01$ is significantly different from the NIMLip group (Statistical significance was determined by one-way ANOVA followed by Tukey's post-test).

p65 NF-κB phosphorylation and concomitantly decreased the TRAF-6 expression, which further downregulated the TNF-α mediated oxidative stress compared to iRGDLip, f-NIM, NIMLip, and DEX treated groups, *in vivo* (Figure 6, A and Figure S11, A-D). Additionally, iRGD-NIMLip suppressed the COX-2 and iNOS along with HIF-1α expression and prevented the LPS induced oxidative damage and hypoxia (Figure 6, B and Figure S11, E-G). Here, iRGD-NIMLip showed superior activity

as compared to f-NIM, NIMLip, and iRGDLip, along with the DEX treatment group. Akt and MAPK signaling plays a crucial role in LPS mediated pulmonary inflammation. We noticed that LPS enhanced the phosphorylation of p-SAPK/JNK, p-p44/42, p-p38, p-GSK-3β, and p-Akt both at S473 and T308 along with PI3K and mTOR, whereas these inflammatory events were significantly more controlled by iRGD-NIMLip as compared to other groups (Figure 6, C & D and Figure S11, H-O). Upon TLR-4

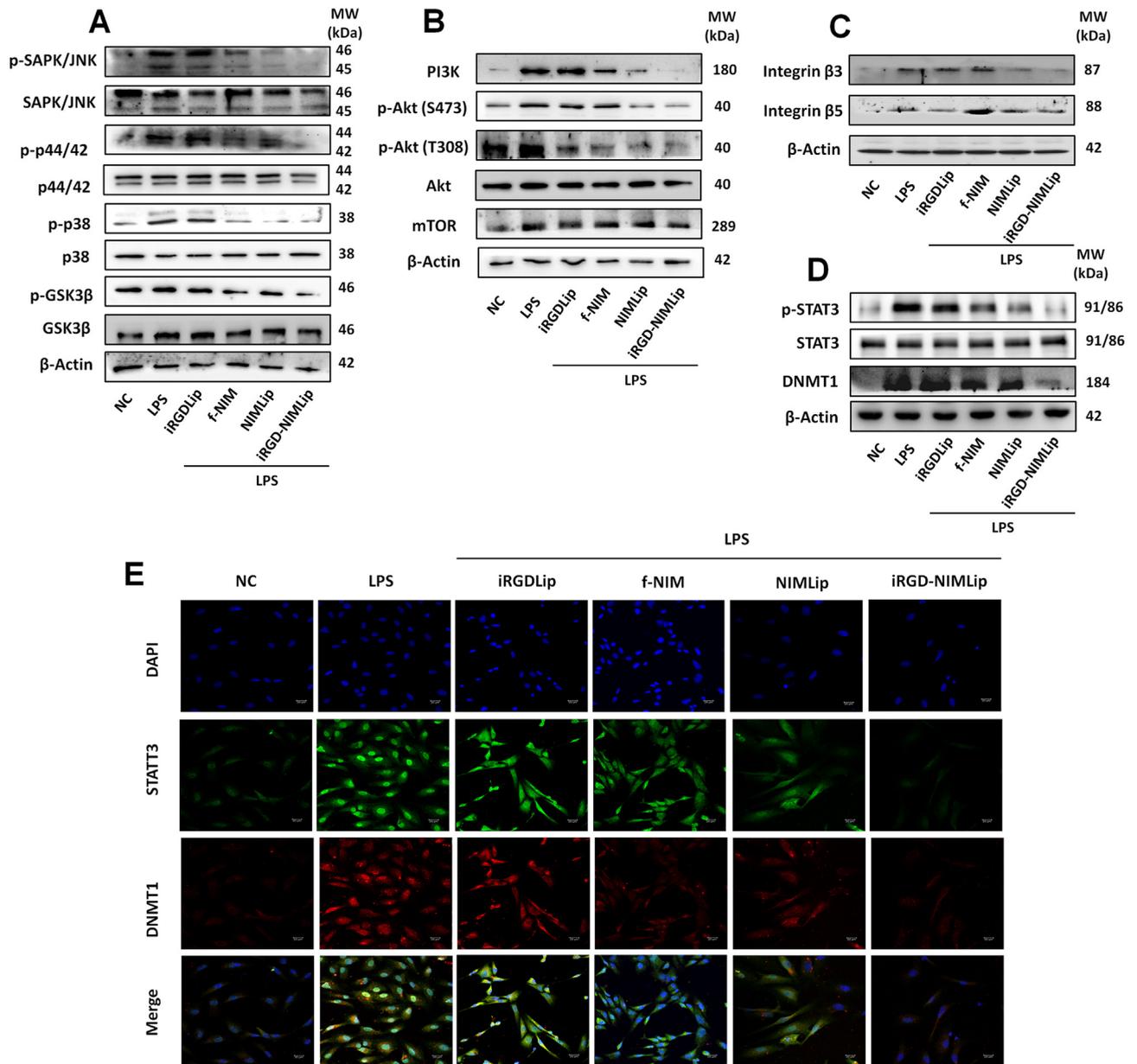


Figure 5. iRGD-NIMLip inhibit LPS induced inflammatory cascade and downregulate STAT3-DNMT1 complex in bronchial epithelial cells. BEAS-2B cells were pretreated with NIM and its liposomes for 24 h and (A) phosphorylation of SAPK/JNK, p44/42, p38, and GSK-3β was evaluated after 30 min of post LPS stimulation. (B) Additionally, PI3K, p-Akt (S473), p-Akt (T308), and mTOR expression was studied by immunoblotting, whereas (C) Integrin β3 and β5 as well as (D) p-STAT3 and DNMT1 expression was evaluated after 1 h of post LPS exposure. (E) After 1 h of post LPS stimulation, STAT3 and DNMT1 expression was evaluated by confocal analysis and images were collected at $\times 400$ magnification.

activation by LPS, Integrins β3 and β5 were expressed aberrantly in inflammatory lungs, whereas iRGD-NIMLip treatment significantly reduced these integrins as compared to other NIM formulations and DEX treated groups (Figure 6, E and Figure S11, P & Q). Additionally, iRGD-NIMLip significantly ameliorated the LPS induced STAT3 and DNMT1 expression in lung tissues (Figure 6, F and Figure S11, R & S). Further, the confocal analysis data infer that iRGD-NIMLip downregulated the STAT3 nuclear translocation and further reduced the DNMT1 expression. Here, we found that iRGD-NIMLip treatment showed more

significant activity as compared to f-NIM, NIMLip, iRGDL and DEX groups (Figure 6, G).

Discussion

ARDS is a serious life-threatening condition, which integrates with acute onset of sepsis, pneumonia and severe trauma.²⁵ In 2019, a newly emerged novel corona virus strain SARS-CoV-2 evidenced a wreaking ARDS case in 67%-85% of

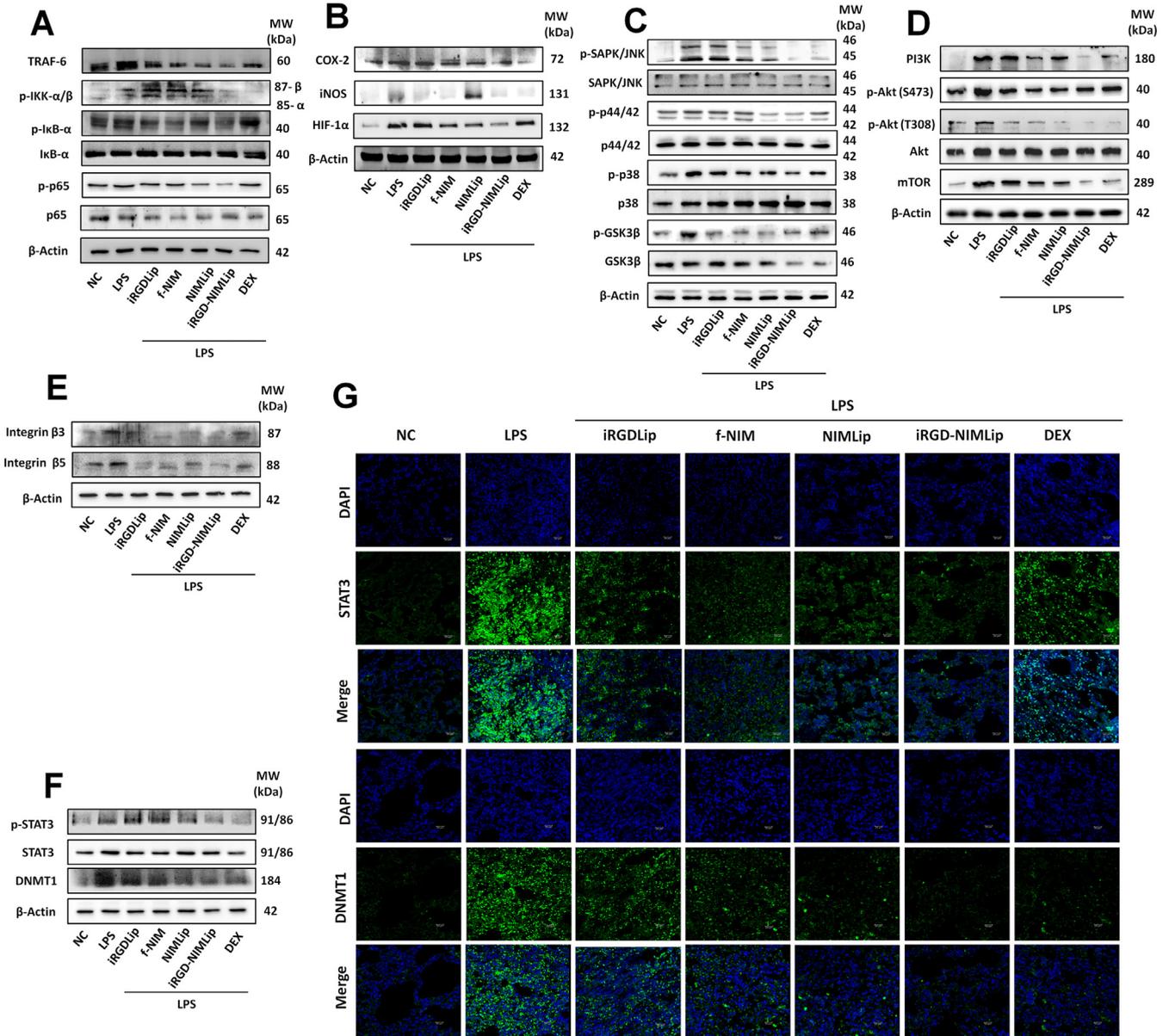


Figure 6. **iRGD-NIMLip suppress LPS induced inflammation and oxidative stress *in vivo*.** Mice were pretreated with iRGDLip, f-NIM, NIMLip, and iRGD-NIMLip (0.3 mg/kg net NIM dose) as well as DEX (0.3 mg/kg) for 2 h. Later, mice were challenged with LPS for 12 h. (A) TRAF-6, p-IKK- α / β , p-I κ B- α , and p-p65 NF- κ B expressions were evaluated by western blotting. (B) COX-2, iNOS, and HIF-1 α expressions were also studied by immunoblotting. (C) Additionally, phosphorylation of SAPK/JNK, p44/42, p38, and GSK-3 β was evaluated by immunoblotting along with (D) PI3K, mTOR, and phosphorylation of Akt at both S473 and T308. (E) Integrins β 3 and β 5 as well as (F) STAT3 and DNMT1 expression was evaluated by western blotting. (G) Additionally, STAT3 and DNMT1 expression in lung tissue sections was studied by IF, where the images were captured at $\times 400$ magnification.

critically ill patients with 61.5% mortality.²⁶ Although the pathogenesis is much evolved, still there is no specific pharmacologic treatment that has been identified to manage ARDS. However, although a low dose of DEX was found to suppress LPS induced pulmonary neutrophil sequestration, bronchoconstriction and alveolocapillary dysfunction, its clinical efficacy in ARDS patients is not known.²⁷ Hence, there is a critical need to explore various safer therapeutic agents for

treating ARDS complications. NIM is widely explored in cancer and inflammatory diseases.²⁸ To expand the therapeutic utility of this phytomedicine, we proposed liposomal formulations conjugated with a peptide to increase the therapeutic effects. Inhalational drug delivery systems show localized drug action and they are non-invasive and may reduce the system adverse effects in comparison to the parenteral routes of administration. One such liposomal formulation is amikacin (Arikace®)

developed to treat cystic fibrosis and administered through the inhalation route (NCT03905642) and these liposomes show the way to develop novel pulmonary delivery systems.

iRGD is a cyclic peptide which preferentially inhibits ligand binding to integrins with an RGD recognition specificity and suppresses their function by selectively targeting the inflammation sites.²⁹ For efficient lung targeting and treating LPS induced ARDS, we designed NIM encapsulated liposomes conjugated with iRGD peptide, which binds with inflammatory sites. The homing is achieved through a mechanism of selective binding of iRGD to integrins, which are overexpressed in inflammatory conditions. In the present study, we found that iRGDLip, NIMLip, and iRGD-NIMLip exhibited nanosize and were negative in charge, whereas iRGD-NIMLip exhibited high entrapment efficiency of NIM. The prepared formulations were spherical in shape with clear liposomal compartments.

To improve the therapeutic response with less deleterious effects, the formulation was opted to deliver through oropharyngeal route to improve the effectiveness at low doses with minimal accumulation in other organs. To further corroborate the target specificity, FITCLip and iRGD-FITCLip were tested in both inflammatory and non-inflammatory conditions. In confocal analysis, we observed that iRGD-FITCLip were uptaken significantly in LPS stimulated RAW 264.7 cells as well as accumulated in LPS challenged mice. We have also observed that iRGD-NIMLip exhibited increased accumulation of NIM as compared to f-NIM treatment.

LPS stimulation in the later phases induces TRAF-6 mediated excessive TNF- α activation through an accompanying switch in mechanisms such as NF- κ B, MAPK, and Akt signaling.³⁰ Consistent with our hypothesis, the prepared iRGD-NIMLip formulation exhibited prominent activity in comparison with NIMLip and f-NIM by abrogating LPS induced ROS. Furthermore, a profound reduction in the nitrite and MDA levels were observed with iRGD-NIMLip along with enhanced GSH/GSSG ratio. Molecularly, iRGD-NIMLip dramatically upregulated the SOD1 and HO-1 expression along with Nrf-2, thus inhibiting the LPS induced oxidative stress.

In the experimental ARDS mouse model, LPS induction causes severe weight loss and hypothermic shock.^{31,32} LPS challenge leads to the influx of protein-rich edematous fluids into the lungs, accompanied by the recruitment of inflammatory cells such as neutrophils, monocytes, and lymphocytes in the microvasculature of airways, resulting in increased lung weight index. In agreement with the previous reports, we found a significant loss in the integrity of lungs with LPS instillation.³³ Remarkably, we observed that iRGD-NIMLip treatment counter-regulated the LPS mediated inflammatory response in comparison with NIMLip and f-NIM.

LPS activates critical inflammatory responsive transcriptional factors such as p65 and STAT3, which orchestrate the inflammatory responses by upregulating the pro-inflammatory cytokines including IL-1 β , IL-6, IL-13, IL-17, TNF- α , and TGF- β .³⁴ On the other hand, LPS significantly induced neutrophil infiltration and mast cell accumulation and disrupted the epithelial and endothelial barrier.³⁵⁻³⁷ Interestingly, iRGD-NIMLip significantly reduced cytokine storm and protected the alveolar epithelium as compared to DEX. Moreover, it was

observed that LPS enhanced phosphorylation of IKK- α/β , I κ B- α , and p65 NF- κ B, which was significantly reduced by iRGD-NIMLip as compared to NIMLip and f-NIM. Interestingly, the other inflammatory mediators such as COX-2 and iNOS were suppressed by this novel formulation and ameliorated the LPS induced inflammation. Accumulating evidences suggest that HIF-1 α enhances the production of pro-inflammatory cytokines especially IL-6 and TNF- α ; moreover iRGD-NIMLip remarkably suppressed the HIF-1 α expression.³⁸

Inflammatory response may trigger the expression of Integrins β 3 and β 5. On the other hand, Integrin β 3 in association with WISP1 strongly contributes to TLR-4 induced TNF- α expression and causes respiratory dysfunction.^{13,39} Interestingly, iRGD-NIMLip significantly reduced the LPS stimulated Integrin β 3 and β 5 expression. On the other side, the phosphorylation of Akt, JNK, p44/42, p38, GSK-3 β were found to be reduced along with PI3K and mTOR expression.

Among various signal transduction pathways, STAT3 aberrantly activates in macrophages, neutrophils, dendritic and endothelial cells and is involved in perpetuating persistent inflammation.^{40,41} Furthermore, altered DNA methylation in airway inflammation by LPS is associated with the macrophage polarization through DNMT1, which preferentially methylates CpG islands on hemimethylated DNA that further promotes the pro-inflammatory cytokines secretion.⁴² Recent reports have emphasized that STAT3 binds to DNMT1, thereby abrogating STAT3 expression and disruption of this complex with DNMT1 will have a beneficial effect in dampening inflammatory responses.^{11,43} In line with these evidences, we found that LPS induced STAT3 and DNMT1 expression in both bronchial epithelial cells and LPS challenged lung tissues. It is noteworthy that iRGD-NIMLip potently boosted the efficacy with remarkable disruption of this reciprocal crosstalk and inhibited a spectrum of cytokines secretions as compared to f-NIM.

Collectively, this study provides mechanistic and preclinical evidence of therapeutic activity of iRGD-NIMLip as down-regulator of STAT3-DNMT1 complex induced cytokine storm. This novel strategy could be a promising therapeutic option to combat the oxidative stress and inflammation associated with severe life-threatening complications of ARDS. Generally, the majority of ARDS and COVID-19 associated pneumonias share common pathophysiology like pulmonary edema and oxidative stress, whereas our novel drug delivery system iRGD-NIMLip may protect COVID-19 associated ARDS by suppressing the cytokine storm and inflammation. Future studies in suitable animal models to simulate the SARS-CoV-2 induced ARDS may provide further evidence of possible clinical translation of our approach to protect patients from COVID-19 associated respiratory complications.

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Credit Author Statement

VP designed and performed the experimental work as well as analyzed the results and wrote the manuscript. ST, OS, and ST performed part of the experimental work and/or analyzed the results. PKV and MVNKT facilitated in manuscript preparation. CG conceptualized the study, wrote and revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2020.102351>.

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