



# Liposomal Dexamethasone Reduces A/H1N1 Influenza-Associated Morbidity in Mice

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Virology,  
a section of the journal  
Frontiers in Microbiology

Received: 30 December 2021

Accepted: 23 February 2022

Published: 12 April 2022

### Citation:

Kwon JW, Quan H, Song J,  
Chung H, Jung D, Hong JJ, Na YR  
and Seok SH (2022) Liposomal  
Dexamethasone Reduces A/H1N1  
Influenza-Associated Morbidity  
in Mice. *Front. Microbiol.* 13:845795.  
doi: 10.3389/fmicb.2022.845795

Re-emerging viral threats have continued to challenge the medical and public health systems. It has become clear that a significant number of severe viral infection cases are due to an overreaction of the immune system, which leads to hyperinflammation. In this study, we aimed to demonstrate the therapeutic efficacy of the dexamethasone nanomedicine in controlling the symptoms of influenza virus infection. We found that the A/Wisconsin/WSN/333/2009 (H1N1) infection induced severe pneumonia in mice with a death rate of 80%, accompanied by significant epithelial cell damage, infiltration of immune cells, and accumulation of pro-inflammatory cytokines in the airway space. Moreover, the intranasal delivery of liposomal dexamethasone during disease progression reduced the death rate by 20%. It also significantly reduced the protein level of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and the C-X-C motif chemokine ligand 2 (CXCL2) as well as the number of infiltrated immune cells in the bronchoalveolar lavage fluids as compared to the control and free dexamethasone. The liposomal dexamethasone was mainly distributed into the monocyte/macrophages as a major cell population for inducing the cytokine storm in the lungs. Taken together, the intranasal delivery of liposomal dexamethasone may serve as a novel promising therapeutic strategy for the treatment of influenza A-induced pneumonia.

**Keywords:** H1N1 influenza, dexamethasone, liposome, macrophage, cytokine

## INTRODUCTION

Lower respiratory tract infections account for approximately 7% of infection-related deaths per year worldwide, and viruses are a common cause of community-acquired pneumonia (Li et al., 2004; Russell and Webster, 2005; Shieh et al., 2010; Worobey et al., 2014). The 2009 H1N1 influenza virus caused more than 18,000 confirmed deaths due to lung damage in the United States (Itoh et al., 2009; Walsh et al., 2011). These infections are accompanied by aggressive pro-inflammatory responses along with insufficient control of the anti-inflammatory responses, a combination of events that is referred to as the “cytokine storm (Peiris et al., 2010; To et al., 2010).”

Following the primary exposure of respiratory epithelial cells to the influenza virus, progeny viruses that replicate within these cells can infect other cells, including alveolar macrophages (Bender and Small Jr., 1992; La Gruta et al., 2007; Liu et al., 2016). The inflammatory response begins when the pathogen-associated molecular pattern (PAMP) of the virus is recognized by pattern recognition receptors (PRRs) of innate immune cells (Julkunen et al., 2000; Julkunen et al., 2001; Oslund and Baumgarth, 2011). Specific pro-inflammatory cytokines are expressed and lead to the recruitment of neutrophils, monocytes, macrophages, and T cells into the site of infection (La Gruta et al., 2007; Liu et al., 2016). Hyperinflammation and macrophage activation syndrome (MAS) result in the overproduction of proinflammatory cytokines, such as the IL-1 $\beta$ , IL-6, and TNF $\alpha$ , as well as coagulation abnormalities, which contribute to organ failure and other fatalities (Shinya et al., 2012; Merad and Martin, 2020; Tay et al., 2020). Therefore, the reduction of pro-inflammatory cytokine levels is as important as anti-viral therapies. Since monocytes and macrophages are the main cells that secrete pro-inflammatory cytokines, the efficient control of these cells can be used as a therapeutic target to alleviate inflammation.

Corticosteroids are a class of steroid hormones that exhibit anti-inflammatory activity by binding to the cytoplasmic corticosteroid receptor, which regulates the transcription of anti-inflammatory genes (Rocksén et al., 2000; Darwish et al., 2011). As the outcome of severe influenza is determined by both viral virulence and host resistance, the use of immunomodulatory therapy in combination with conventional antiviral therapy is highly warranted. During the 2009 H1N1 influenza pandemic, nearly 40% of patients in France were treated for ARDS using adjuvant systemic corticosteroids (Brun-Buisson et al., 2011); however, the evidence supporting the use of corticosteroids in severe influenza was unclear. Because immunosuppression resulting from systemic administration of dexamethasone can favor persistent viral replication and further limiting host defenses (Giannella et al., 2011). However, Lammers et al. (2020) recently proposed that targeting alveolar macrophages *via* the nano-formulation of dexamethasone within liposomes improve the management of respiratory viral infections. Liposomes, microscopic phospholipid bubbles with a bilayered membrane structure, have been successfully applied in clinics as drug carriers to improve the delivery of drugs to target cells and tissues that play a key role in the acute and progressive phases of many diseases (Torchilin, 2005). Although dexamethasone nanomedicine has been used as a therapeutic agent for the coronavirus disease 2019 (COVID-19) (Lammers et al., 2020), it has not yet been tested for the influenza virus infection in pre-clinical studies.

In this study, we demonstrated the therapeutic efficacy of liposomal dexamethasone (DEX/lipo) in a mouse model of influenza virus infection. We manufactured DEX/lipo that can specifically deliver into monocytes and macrophages and found that DEX/lipo outperformed the free dexamethasone in reducing the morbidity and mortality caused by the A/H1N1 influenza virus infection. Therefore, our findings offer valuable insights into the efficiency of the currently available corticosteroids

for the treatment of influenza virus infection *via* the targeted delivery of these drugs.

## MATERIALS AND METHODS

### Mice, Virus, and Cell Line

Male C57BL/6J mice of 7-weeks-of-age were purchased from ORIENT BIO (Orient Bio, Gyeonggi-do, South Korea). Animal experiments were conducted at the Institute for Experimental Animals, College of Medicine and cared for according to the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Seoul National University at Seoul (accession number SNU-160307-6-1). Mice were housed in cages with a constant-flow air exchange, supporting specific pathogen-free conditions. Influenza A/Wisconsin/WSLH34939/09 was obtained from the Michael laboratory at the Scripps Research Institute. The adaptation method for influenza A virus in mouse lungs is as follows. The C57BL/6J mice were each anesthetized with isoflurane (HANA PHARM CO., Gyeonggi-do, South Korea), intranasally inoculated with 105 TCID<sub>50</sub> of influenza A/Wisconsin/WSLH34939/09 virus in a volume of 20  $\mu$ l. At day 2 post-infection, the lungs from mice were homogenized in the Dulbecco's modified Eagle medium (DMEM) medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS), centrifuged at 9,520  $\times$  g for 10 min, and the supernatant was stored at  $-80^{\circ}\text{C}$ . The first adapted virus was amplified on the MDCK cells and virus titer detected using TCID<sub>50</sub>. Briefly, the assay was performed by adding a serial dilution of the virus sample to cells in a 96-well plate. After incubation, the percentage of infected wells was observed for each dilution, and the results were used to calculate the TCID<sub>50</sub> value. This calculation can generally be performed using the Spearman-Kärber method. MDCK cells were cultured in DMEM supplemented with 10% FBS and 1% PS.

Mice under isoflurane anesthesia were infected intranasally with 105 TCID<sub>50</sub> of the adapted influenza A virus. One hour after infection, the mice were anesthetized by isoflurane inhalation for the intranasal delivery of vehicle (20  $\mu$ l of PBS/lipo) or dexamethasone (30  $\mu$ g/kg dissolved in PBS) or DEX/lipo (30  $\mu$ g/kg encapsulated in liposomes) for 3 days. On day 3 post-infection, the mice were injected intravenously with 100  $\mu$ l of PBS/lipo, dexamethasone, or DEX/lipo. The mice monitored for survival.

### Reagents

Dexamethasone were purchased from Sigma (Sigma, St. Louis, MO, United States).

### Preparation of Liposomes

Dexamethasone was encapsulated into liposomes with a diameter of 1,000 nm at a concentration of 1 mg/ml. Briefly, L-alpha-phosphatidylcholine (Sigma, St. Louis, MO, United States) and cholesterol (Sigma, St. Louis, MO, United States) were dissolved in a 2:1 mixture of chloroform and methanol and dried under a nitrogen stream, followed by a vacuum pump. The lipid film

was hydrolyzed with PBS buffer and sized to 1,000 nm in a mini-extruder equipped with a 1,000 nm pore polycarbonate membrane (Avanti Polar Lipids, Alabama, AL, United States). The diameter of liposomes was checked by dynamic light scattering (DLS). Liposomes were labeled with DiI (Molecular Probes, Eugene, OR, United States) for visual detection.

## Flow Cytometry

The mice lungs were obtained at 24 h post-injection with DiI-stained liposomes and digested with collagenase type I (Sigma, St. Louis, MO, United States) to obtain single cells. Single cells were prepared for flow cytometric analysis. Anti-mouse CD16/32 antibody (clone number 93) was pre-added to block the non-specific binding of immunoglobulin to macrophage Fc receptors. For surface marker analysis, live cells were re-suspended in staining buffer [1% bovine serum albumin (BSA), 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% NaN<sub>3</sub> in PBS] and stained with anti-mouse CD45 (30-F11), F4/80 (BM8), CD11b (M1/70), CD3 (145-2C11), CD64 (X54-5/7.1; BD Biosciences), Ly6C (HK1.4), and Ly6G (1A8-Ly6g) antibodies at 4°C for 20 min. All antibodies were obtained from eBioscience, unless otherwise indicated. Data were acquired using LSR Fortessa (BD Biosciences) and analyzed using the FlowJo software (FlowJo LCC, Ashland, OR, United States).

## Immunofluorescence Analysis

The mice lungs were obtained at 24 h post-injection with DiI-stained liposomes. Frozen tissues were sectioned to a thickness of 5 μm and fixed with 4% paraformaldehyde (Merck, Kenilworth, NJ, United States). Slides were incubated with 1% BSA in PBST for 1 h to block the non-specific antibody binding. Primary antibodies were pre-diluted in blocking buffer to 1:200 for F4/80 (CI-A3-1; Abcam) or EpCAM (G8.8; eBioscience) and applied to tissue sections overnight at 4°C in a humidified chamber. The next day, appropriate secondary antibodies were applied, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Waltham, MA, United States) before mounting. Fluorescence signals were detected using a Leica TCS SP8 confocal microscope (Leica, Buffalo Grove, IL, United States).

## Cytokine and Chemokine Analyses

On day 2 post-infection, the trachea of euthanized mice was exposed, transected, and intubated with a blunt 18-gage needle that delivered 0.8 ml ice-cold PBS. Infusion of the 0.5 ml volume was repeated twice, and the fluid was recovered. The recovered BALF was centrifuged at 3,000 × *g* for 3 min at 4°C and stored at −80°C until further use. ELISAs were performed on the bronchoalveolar lavage fluid (BALF) using TNFα, IL-6 ELISA kits (BD Biosciences, Palo Alto, CA, United States), IL-1β, CXCL1, and CXCL2 Duoset ELISA kits (R&D systems, Minneapolis, MN, United States).

## Cell Counting

BALF was obtained *via* cannulation of the trachea and lavaging the airway lumen with 0.8 ml ice-cold PBS for three times on day 2 post-infection. The recovered fluid was centrifuged, and the

cell pellets were resuspended in PBS to count the total number of cells. Differential cell counts in the BALF were performed using the Diff-Quik staining reagent (Sysmex, Tokyo, Japan), according to the manufacturer's instructions. The numbers of macrophages, neutrophils, and T cells were calculated by multiplying the percentages obtained by the total yield. Slides were imaged using the QWin program (Leica, Leica Lane Buffalo Grove, IL, United States).

## Histopathological Scoring

Lung tissue samples were fixed in 4% paraformaldehyde neutral buffer solution for 24 h, dehydrated in a graded ethanol series, embedded in paraffin, sliced at 5 μm, and stained with hematoxylin and eosin. Lung histopathological score was assessed using the following parameters: a scale of 0–3 (0 = absent and appeared normal, 1 = light, 2 = moderate, and 3 = severe) according to the histologic features: (1) edema, hyperemia, and congestion; (2) neutrophil margination and tissue infiltration; (3) intra-alveolar hemorrhage and debris. Each parameter was scored from 0 to 3 based on severity. The total score was used to assess lung injury, which was calculated as the sum of all scores for each parameter (0–1, normal to minimal injury; 2–3, mild injury; 4–6, moderate injury; and 7–9, severe injury). The maximum score per animal was 12. Lung section scoring was performed at a low power (×40).

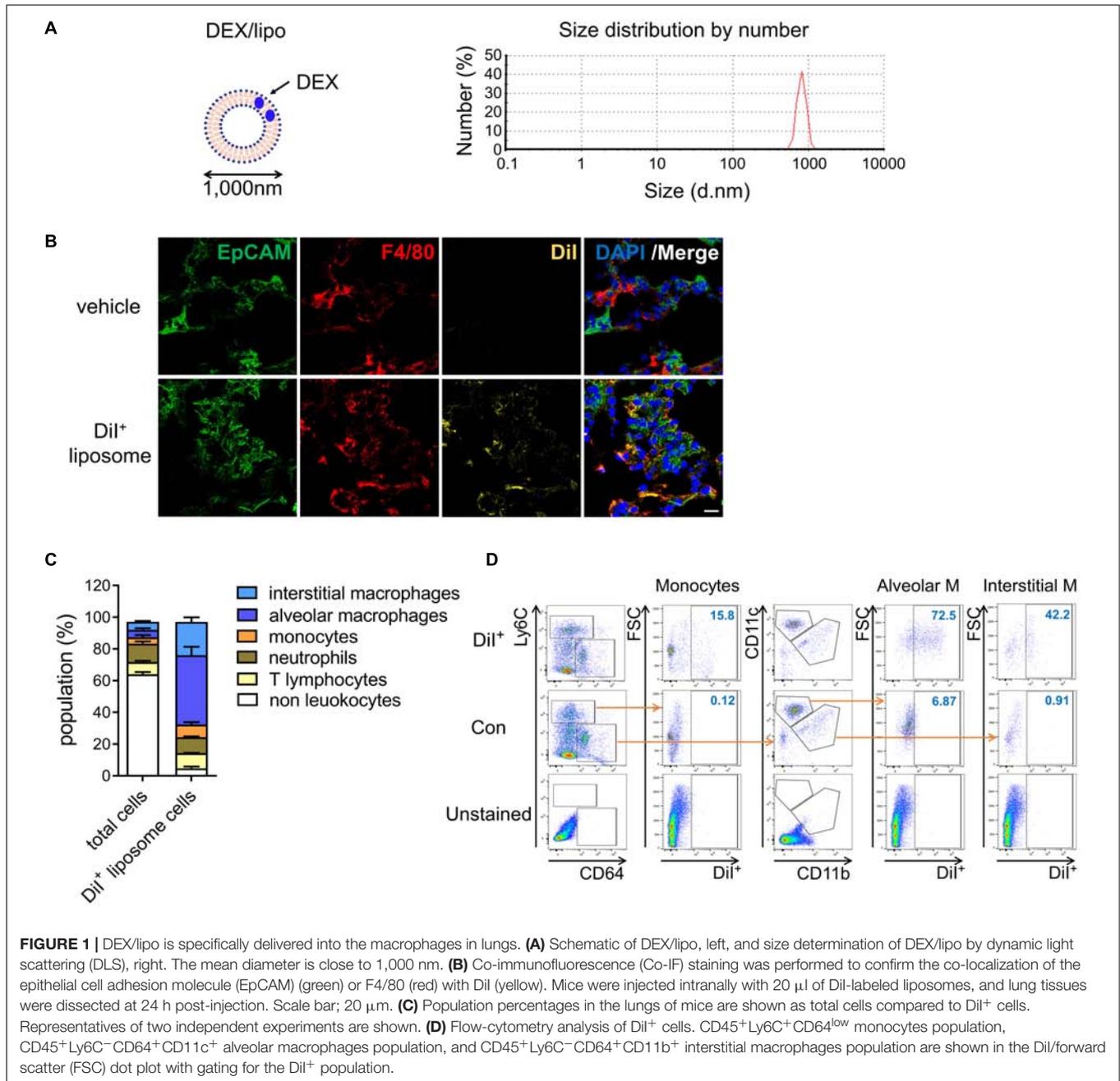
## Quantification and Statistical Analyses

GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, United States) was used for the statistical analysis. Results are presented as the mean ± SEM for the experiments, unless otherwise indicated. Unpaired two-tailed Student's *t*-tests were used to compare the two groups of independent samples. Sample size (*n*) is indicated in the figure legends. Statistical significance was set at *P*-values of less than 0.05.

## RESULTS

### DEX/Lipo Is Specifically Delivered Into the Macrophages in the Lungs

As monocytes and macrophages seem to be implicated in cytokine storm (Giavridis et al., 2018; Norelli et al., 2018), we encapsulated dexamethasone into liposomes with a particle size of 1,000 nm (DEX/lipo) (**Figure 1A**) based on many other studies showing that liposomes with relatively larger diameters were more effectively distributed into phagocytes (Chono et al., 2006, 2007; Kelly et al., 2011). After 24 h of intranasal injection in mice, DiI-labeled DEX/lipo was detected in the cytosol of F4/80<sup>+</sup> macrophages in the lungs (**Figure 1B**). We further confirmed the distribution of injected liposomes as up to 70% of DiI<sup>+</sup> cells were alveolar macrophages, interstitial macrophages and monocytes (**Figure 1C**). In contrast, less than 10% of non-leukocytes and lymphocytes can uptake DiI<sup>+</sup> liposomes. In particular, up to 15.8% of monocytes, 72.5% of alveolar macrophages, and 42.2% of interstitial macrophages in the lungs were DiI<sup>+</sup> (**Figure 1D**; gating schemes are shown in

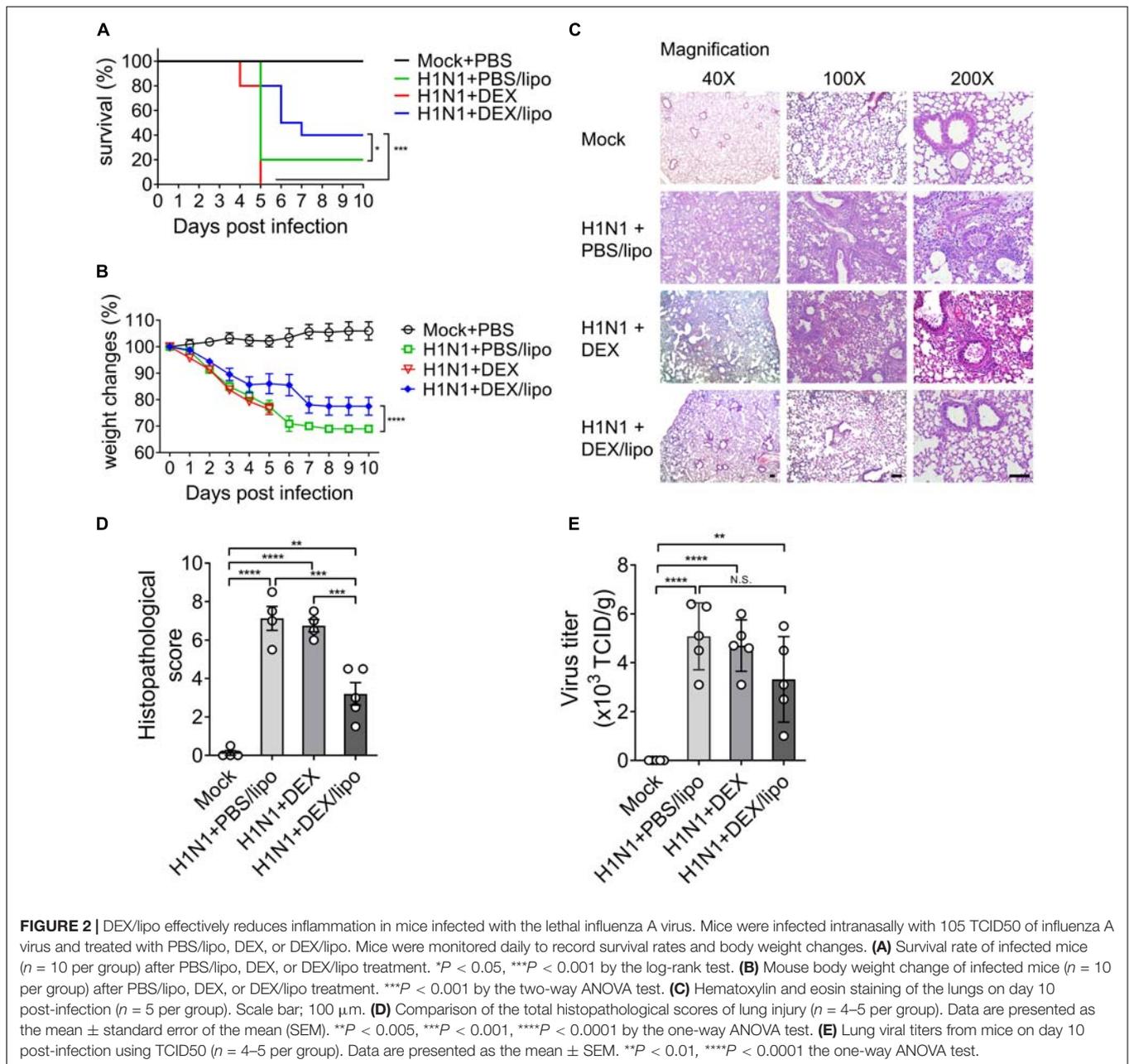


**Supplementary Figure 1).** Collectively, these results confirm that locally delivered DEX/lipo is mostly distributed into monocytes/macrophages in the lungs.

## DEX/Lipo Effectively Reduces Inflammation in Mice Infected With the Lethal Influenza A Virus

We determined that DEX/lipo has a therapeutic effect on a mouse influenza model infected with influenza A/Wisconsin/WSN/33/59. Mice under isoflurane anesthesia were infected intranasally with 105 50% tissue culture infectious

dose (TCID<sub>50</sub>) of influenza virus. One hour after infection, mice were anesthetized by isoflurane inhalation for intranasal delivery of PBS/lipo, dexamethasone (DEX), or DEX/lipo for three days. On day 3 post-infection, mice were treated with 100  $\mu$ l of PBS/lipo, DEX, or DEX/lipo intravenously. Mice were monitored for survival for 10 days. While 20% of PBS/lipo-treated mice survived on day 5 post-infection, mice treated with DEX/lipo showed the highest survival rate (40%). However, all mice treated with DEX died on day 5 (Figure 2A). Also, all mice treated with DEX rapidly lost body weight and died by day 5 post-infection. Control group (H1N1 + PBS/lipo) showed significant illness as determined by loss in body weight up to



27–30% whereas the DEX/lipo treated mice showed body weight loss of 15% on day 6 post-infection. From day 7 post-infection, both control group and H1N1 + DEX/lipo group lost body weight, but H1N1 + DEX/lipo group significantly higher than control (**Figure 2B**). To verify the anti-inflammatory activity of DEX/lipo on the infiltration of inflammatory cells, mice were euthanized at day 10 post-infection to obtain lung tissues for histopathological examination. In mice treated with PBS/lipo, there was a severe inflammatory response characterized by alveolar congestion, thickening of the alveolar wall, and infiltration and aggregation of immune cells in airspaces or vessel walls. However, fewer inflammatory infiltrates were observed in the lungs treated with DEX/lipo than in the lungs

of mice treated with PBS/lipo or DEX (**Figure 2C**). Consistent with these findings, the total histopathological scores also decreased significantly in the H1N1 + DEX/lipo group but not in H1N1 + DEX group (**Figure 2D**). We also measured the virus titer to determine whether this histopathological score improvement in the H1N1 + DEX/lipo group was due to the difference in viral titers between groups. The viruses isolated from lungs plaqued on Madin-Darby canine kidney (MDCK) cells using TCID<sub>50</sub>. However, virus isolated from infected mouse lungs did not decrease in the H1N1 + DEX/lipo group (**Figure 2E**). Collectively, these results support our hypothesis that targeting macrophages using DEX/lipo has a therapeutic effect in reducing inflammation.

## DEX/Lipo Decreases Infiltrated Cells in the Bronchoalveolar Lavage Fluid of Mice With Lethal Influenza Virus Infection

We analyzed the infiltrated cells in bronchoalveolar lavage fluid (BALF) collected from the lungs of mice infected with influenza A virus. Many inflammatory cells, such as monocytes, neutrophils, and lymphocytes are recruited in the lungs of mice infected with influenza A virus. The cell infiltrates in the BALF of mice treated with PBS/lipo, DEX, or DEX/lipo were statistically analyzed for cell number and type (Figure 3). DEX/lipo treatment greatly reduced the infiltration of total cells (Figures 3A,B). Among the infiltrating cells, not only macrophages, which are known to cause acute lung injury in influenza (Dukhinova et al., 2020), but also neutrophils and T cells were decreased in the H1N1 + DEX/lipo group. Rather, DEX treatment increased the total number of infiltrated cells, especially macrophages and T cells, as compared to PBS/lipo treatment (Figures 3A,C–E).

## DEX/Lipo Significantly Reduces the Pro-inflammatory Cytokines and Chemokines in Mice With Lethal Influenza Virus Infection

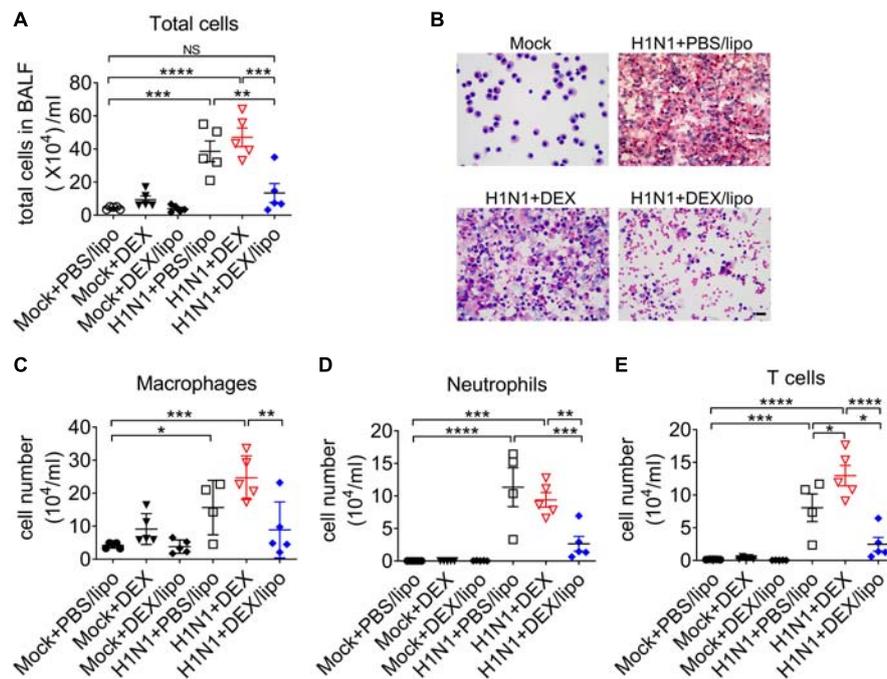
Given that inflammatory cytokines and chemokines are linked to lung damage in severe influenza pneumonia, we determined

the role of DEX/lipo in mice with influenza virus infection. The protein levels of pro-inflammatory cytokines and chemokines, TNF $\alpha$ , IL-1 $\beta$ , IL-6, CXCL1, and CXCL2 increased significantly in the H1N1 + PBS/lipo and H1N1 + DEX group (Figures 4A–E). However, cytokines and chemokines were decreased significantly in the H1N1 + DEX/lipo group, except for CXCL1 (Figures 4A–E). In particular, TNF $\alpha$  and IL-1 $\beta$  was significantly increased in H1N1 + DEX compared to that in the H1N1 + PBS/lipo group. These results correlated with the infiltrated total cell number.

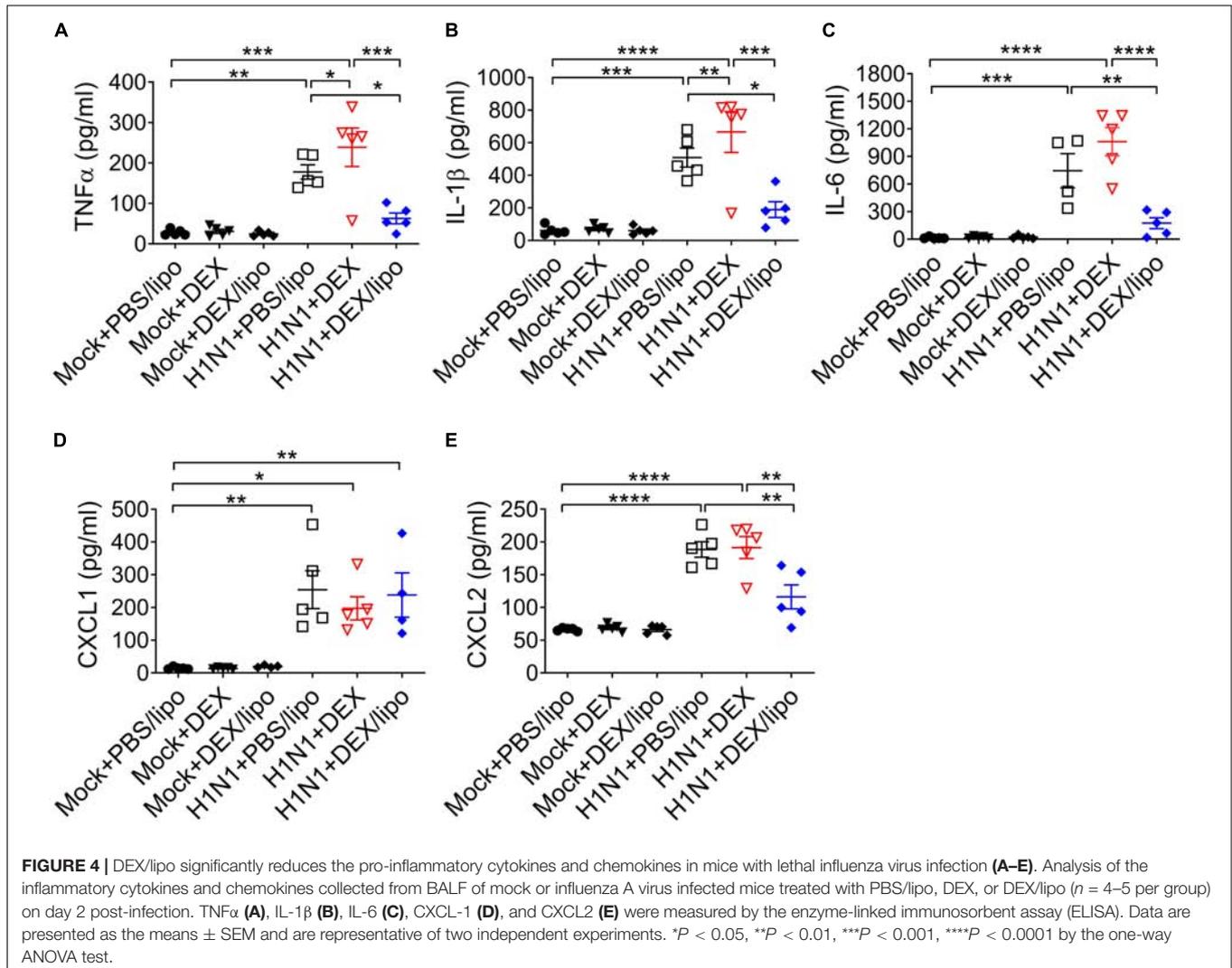
## DISCUSSION

Many patients with severe influenza virus die from overwhelming viral pneumonia and serious complications caused by cytokine storms. In this study, we revealed that DEX/lipo effectively reduces pro-inflammatory responses in hosts with pathogenic A/H1N1 influenza virus infection, demonstrating the possible availability of DEX/lipo in controlling disease symptoms of cytokine storm in respiratory infections.

Pathogenesis in human influenza virus infection is accompanied by diffuse epithelial sloughing in tracheal, bronchial, and bronchiole biopsy, predominant presence of mononuclear cells, and bloody exudate in the airway lumen with interstitial swelling (Walsh et al., 1961; Taubenberger and Morens, 2008). In line with this, a mouse model of human pathogenic pandemic A/Wisconsin/WSLH34939/09 influenza



**FIGURE 3 |** DEX/lipo decreases the total number of cells in the bronchoalveolar lavage fluid of mice with lethal influenza virus infection. **(A)** Analysis of the total number of infiltrated cells in the BALF collected from the mock or influenza A-virus infected mice treated with PBS/lipo, DEX, or DEX/lipo ( $n = 4-5$  per group) on day 2 post-infection **(B)**. Differential cell counts of BALF cells. Representative pictures of Diff-quick staining of cytospin preparation. Scale bar; 20  $\mu\text{m}$ . Changes in the number of macrophages **(C)**, neutrophils **(D)**, T cells **(E)**. BALF cells counted using the QWin program (Leica Microsystems). Data are presented as the mean  $\pm$  SEM and are representative of two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  by the one-way ANOVA test.



virus infection previously showed 80% of death within 12 days post-infection with marked tissue injury, including hemorrhage, pulmonary edema, and mononuclear cell accumulation (Walsh et al., 2011). The mouse adapted influenza virus infection model established in this study showed a faster pathogenesis than the previous study, showing 80% death on day 5 post-infection, but the characteristic histopathological findings, including alveolar congestion and infiltration of macrophages in airway spaces, showed comparable results (Figures 2, 3). Of note, cytokines, such as TNF $\alpha$  and IL-6, all of which are known to be directly correlated with host morbidity and pulmonary injury (Kozak et al., 1997; Hayden et al., 1998; Kaiser et al., 2001), were significantly elevated in bronchoalveolar lavage fluids on day 2 post-infection (Figures 4A,C), implying the importance of immune responses in the disease pathogenesis of our influenza virus infection model.

The most notable results in this study were the differences in the therapeutic responses of DEX/lipo compared to the free dexamethasone. Intranasal delivery of free dexamethasone did not alleviate tissue injury in the infected

mice (Figure 2C). DEX/lipo could be delivered specifically to monocyte/macrophages, whereas DEX could be delivered to other cells, such as lung epithelial cells. Based on the previous study that corticosteroid induce apoptosis of airway epithelial cells (Dorscheid et al., 2001), it could be speculated that DEX treatment induced robust damages such as epithelial cells death resulted more severe inflammatory responses in lungs. In our study, we treated DEX immediately within 1 h after influenza virus infection. If DEX treatment is delayed by 24–48 h after influenza virus infection, it is getting difficult to suppress the initial excessive inflammatory responses even most DEX treatments would be delivered after symptom onset. This was further supported by the results shown in Figures 3, 4, because free dexamethasone did not block the accumulation of macrophages and T cells or decrease of TNF $\alpha$ , IL-1 $\beta$ , IL-6, and CXCL2 in bronchoalveolar fluids. In fact, free dexamethasone accelerated the fatality rate of infected mice from 80 to 100% (Figure 2B), possibly due to increased T cells, TNF $\alpha$ , and IL-1 $\beta$  in airway spaces. In contrast, DEX/lipo treatment rescued infected mice from death compared to free dexamethasone, with a

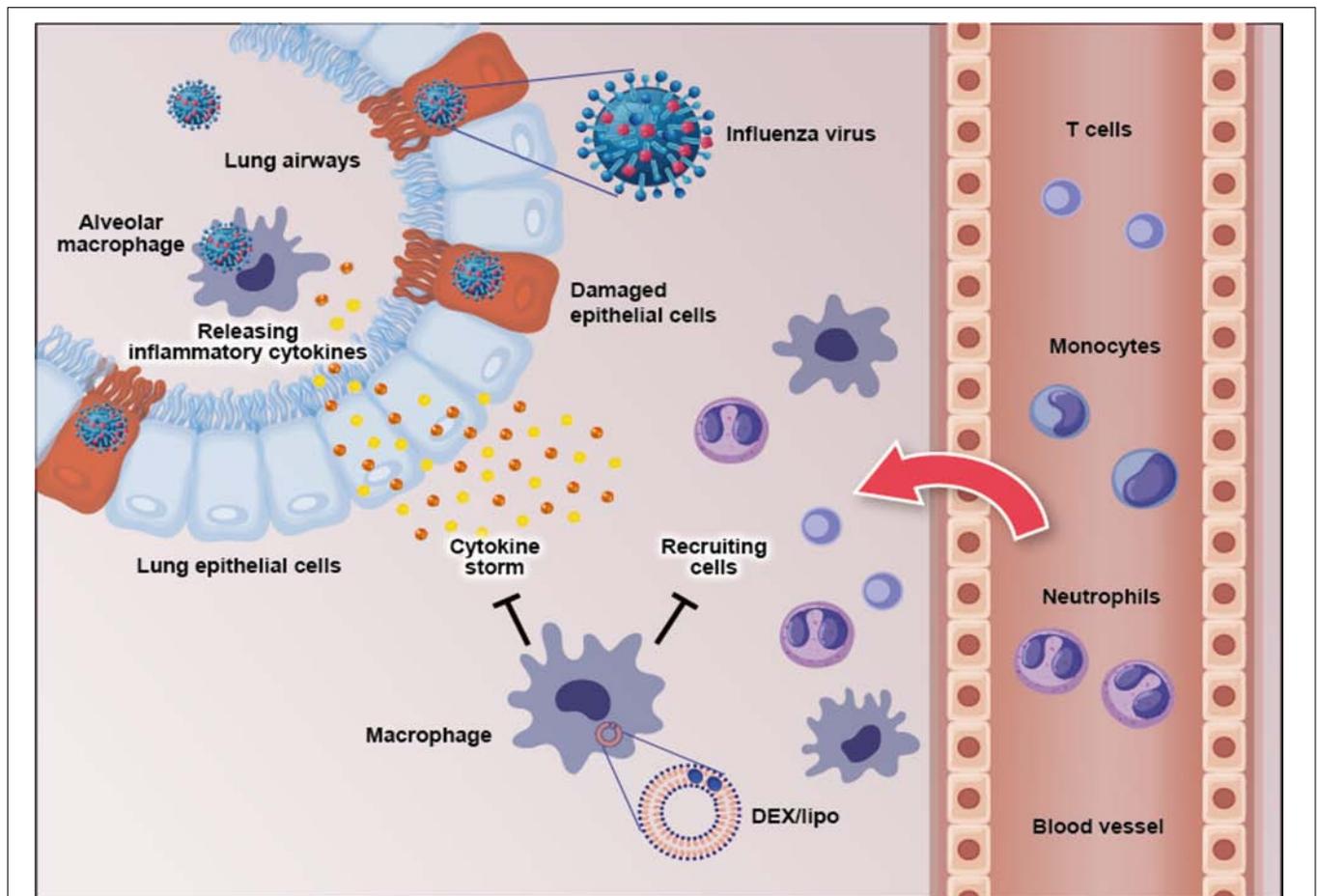
significant decrease in tissue damage, infiltration of macrophages and T cells, and accumulation of  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$ , and  $\text{CXCL2}$  in the lungs. These results are consistent with concerns from previous observations of dexamethasone toxicities in the clinic (Belgaumi et al., 2003; Palladini et al., 2004; Palladini et al., 2005), and further demonstrate the usability of liposomes as delivery cargo in improving the safety and effectiveness of dexamethasone in controlling influenza virus infection.

There have been many cases in which DEX/lipo, mainly targeting macrophages, was delivered to reduce inflammation in various diseases. Polyethylene glycol (PEG)-free formulation of macrophage-targeting DEX/lipo reduces the dose and/or frequency required to treat adjuvant arthritis, with the potential to enhance or prolong therapeutic efficacy and limit side effects in the treatment of rheumatoid arthritis (Anderson et al., 2010). Additionally, administration of tumor-associated macrophage (TAM)-targeting DEX/lipo resulted in a significant inhibition of tumor growth and metastasis in a model of prostate cancer bone metastases (Kroon et al., 2015). In particular, macrophages are a major producer of inflammatory cytokines in influenza

virus infection, and we showed that DEX/lipo were effectively distributed into monocytes/macrophages (Figure 2C). The fact that DEX/lipo did not reduce  $\text{CXCL1}$ , which is mostly produced by epithelial cells, and not by macrophages (Figure 4D), further supports our hypothesis that liposomal encapsulation specifically delivered dexamethasone into macrophages, but not epithelial cells.

Antiviral therapy should be accompanied by an immunomodulatory agent because antiviral drugs do not directly inhibit inflammation in pulmonary injury. In our study, even with the single treatment of DEX/lipo decreased inflammatory cytokines and increased the survival rate.

In conclusion, our data demonstrate that the macrophage-targeting DEX/lipo plays crucial roles in the prevention of pneumonia induced by influenza virus as well as the reduction of pro-inflammatory cytokines/chemokines and infiltration of inflammatory cells (Figure 5). Therefore, targeting macrophages using DEX/lipo may be used as a promising therapeutic approach for the treatment of cytokine storm-induced influenza virus infection with antiviral drugs.



**FIGURE 5 |** Schematic figure of the effects of DEX/lipo on the pathogenesis of the influenza virus infection. Influenza virus infects the lung epithelial cells and subsequently alveolar macrophages. This results in pro-inflammatory cytokine, and chemokine secretion by mainly macrophages. Specific pro-inflammatory cytokines lead to the recruitment of neutrophils, monocytes, macrophages, and T cells into the site of infection. DEX/lipo delivery targeting macrophages reduces cytokines/chemokines secretion and recruitment of leukocytes to the lung of mice with lethal influenza virus infection.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Seoul National University Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JK, YN, and SS conceived, designed, and supervised the experiments. JK and YN performed most experiments, analyzed the data, and wrote the original draft. HQ analyzed the data. JS, HC, DJ, and JH conducted some experiments. All authors reviewed and approved the manuscript.

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## FUNDING

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2020R1A2C2010202 and 2020R1A4A2002903), the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program (KGM4572121), the NRF (NRF-2021R1A2C2010219), and SUNH Research Fund (0320210190).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.845795/full#supplementary-material>

**Supplementary Figure 1** | Gating strategies to determine the cellular compositions of the lungs of mice (Related to **Figure 1**). Single cells were isolated from the lungs of Dll1 injected mice and analyzed by flow cytometry. Sequentially gated cells were **(A)** CD45<sup>-</sup> non-leukocytes, **(B)** CD45<sup>+</sup> CD11b<sup>-</sup> CD3<sup>+</sup> T cells, **(C)** CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>+</sup> neutrophils, **(D)** CD45<sup>+</sup> Ly6G<sup>-</sup> CD64<sup>low</sup> Ly6C<sup>+</sup> monocytes, **(E)** CD45<sup>+</sup> Ly6G<sup>-</sup> CD64<sup>+</sup> CD11c<sup>+</sup> alveolar macrophages, **(F)** CD45<sup>+</sup> Ly6G<sup>-</sup> CD64<sup>+</sup> CD11b<sup>+</sup> interstitial macrophages. Representative dot plots are shown.

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