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Macrophage-derived apoptotic bodies promote the proliferation of the recipient cells via shuttling microRNA-221/222

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ABSTRACT

Bacterial pneumonia is a common and serious clinical entity. Alveolar epithelial cells and alveolar macrophages are the first line of defense in the innate immunity against bacterial pathogens. Epithelial cells are known to release chemokines/cytokines that recruit and activate phagocytic cells. However, the signals sent from alveolar macrophages back to the lung epithelial cells remain largely unexplored. We found that LPS, a well-recognized stimulator derived from gram-negative (G^-) bacteria, rapidly and robustly induces the secretion of macrophage-derived extracellular vesicles (EVs). The main type of EVs found in the early stages after LPS stimulation are apoptotic bodies (ABs) and not microvesicles (MVs) or exosomes (Exos). Furthermore, LPS markedly up-regulate the levels of a repertoire of microRNAs (miRNAs) in the macrophage-derived ABs, including miR-221 and miR-222. Functionally, the LPS-induced, macrophage-derived ABs promote the proliferation of malignant and/or normal lung epithelial cells. We next directly transfected miR-221 and/or miR-222 inhibitors into the LPS-induced ABs. Deletion of miR-221/222 in ABs significantly reduces the AB-mediated proliferation of lung epithelial cells. Mechanistically, AB-shuttling miR-221/222 promote cell growth by modulating cyclin-dependent kinase inhibitor 1B (CDKN1B) pathways. Collectively, LPS-induced, macrophage-derived ABs promote the proliferation of their recipient epithelial cells, partially via AB-shuttling miRNAs.
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Introduction

Pneumonia is a very common pulmonary entity in the United States [1]. Lung infections caused by G^- bacteria are of particular concern, given that these organisms are highly efficient in acquiring antibiotic drug resistance [2]. Therefore, innate immunity plays a primary and non-negligible role in lung antimicrobial defenses [3]. The innate immune system includes but is not limited to alveolar macrophages and the epithelial cells lining the alveolar surface.

Macrophages, the first arm of defense in the immune system, play a crucial role in innate immunity and host defenses upon bacterial infection. Macrophages, once activated (classic, or M1 activation), develop an enhanced capacity to engulf bacteria, release inflammatory cytokines/chemokines, NO/reactive oxygen species, and present MHC class II antigen [4–6]. A substantial number of molecules are released from the macrophages during their activation. The effect of macrophage-derived signals on adjacent and distant recipient cells, such as lung cancer cells or normal lung epithelial cells, is incompletely understood. Recent evidence suggests that most cells constantly release EVs, and EVs are believed to augment intercellular communications upon exposure to noxious stimuli [7]. EV generation is a dynamic process, and EVs are classified into 3 main groups, namely ABs, MVs, and Exos, per the International Society for Extracellular Vesicles [8]. In the setting of repetitive bacterial infections, macrophage-derived ABs may play an important role in intercellular communication. In this report, we investigate the effects of macrophage-derived ABs on the proliferation of lung epithelial cells in the setting of G^- bacterial infection.

The 3 classes of EVs mainly differ in size and mechanism of biogenesis. Their contents, size, and membrane composition are heterogeneous and are largely dictated by the cellular sources and environmental stimuli [9]. ABs are the largest EVs, with a diameter in the 1000–5000 nm range, and comparable with

Abbreviations: AB = apoptotic body, BMDM = bone marrow-derived macrophage, CCK-8 = Cell Counting Kit-8, CDK = cyclin-dependent kinase, CDKN1 = cyclin-dependent kinase inhibitor 1, DLS = dynamic light scattering, EV = extracellular vesicle, Exo = exosome, G^-/G^+ = gram negative/positive, miR/miRNA = microRNA, MV = microvesicle, PCNA = proliferating cell nuclear antigen, qPCR = quantitative PCR, WST-8 = 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

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normal platelets in size [10]. ABs are generated through plasma membrane blebbing during apoptosis [11]. The content of ABs is variable and may include mRNAs, long noncoding RNAs, rRNA, miRNAs, or fragments of these intact RNA molecules [12].

Previous reports have suggested that RNA profiles in the 3 EV fractions are distinctive [12]. For example, the RNA components in MVs and Exos are altered later than those in ABs [12]. The findings of miRNAs in EVs have shed light on novel mechanisms of tumor growth and metastasis [13]. Whereas many researchers focus on characterizing the miRNA profiles in Exos or MVs, surprisingly, recent reports suggest that the copy numbers of "highly up-regulated" miRNAs found in tumor cells are, in fact, very low in individual Exos detected in plasma [14–16]. This observation has raised the question of whether the exosomal miRNAs can have physiologic or pathologic effects on their recipient cells. Interestingly, oncogenic DNAs were initially identified in ABs [17]. However, to date, we have very limited understanding of the roles of RNA contained in AB compared with those found in MVs/Exos. Presumably, apoptotic cells, in the process of dying, constantly broadcast the "danger" and "defense" signals to adjacent and distant cells via many different molecules, including but not limited to cytokines, Exos, and MVs, among others.

In this study, we focused on exploring the effects of macrophage-derived AB and AB-containing miRNAs on the fate of lung epithelial cells. We adopted the G^- bacterial pneumonia models and used G^- bacteria-derived LPS to induce macrophage-derived ABs.

MATERIALS AND METHODS

Chemicals and reagents

miRNA-221 mimics (HMI0398), miRNA-222 mimics (HMI0400), and LPS from *Escherichia coli* 026:B6 (L3755) were purchased from Sigma-Aldrich (St. Louis, MO, USA). miRNA-221 and miRNA-222 inhibitors were purchased from Integrated DNA Technologies (Coralville, IA, USA). Mouse anti-p27 (sc-1641) and rabbit anti-PCNA (sc-7907) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Animals

Wild-type C57BL/6 mice (male, 6–8 wk of age) were obtained from Charles River Laboratories (Wilmington, MA, USA). All of the protocols involving animals in this study were approved by the Institutional Animal Care and Use Committee of Boston University. All experimental protocols and methods were approved by Boston University and were carried out in accordance with the approved guidelines.

Cell culture and drug treatment

A549, BEAS2B, and RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM medium with 10% FBS. H1299, MH-S, and THP-1 (American Type Culture Collection) were cultured in RPMI-1640 medium with 10% FBS. The murine alveolar type I epithelial cell-like cell line E10 (obtained from the late Dr. Alvin Malkinson, University of Colorado, Aurora, CO, USA) was cultured in CMRL1066 medium, as previously described [18]. The differentiation of THP-1 monocytes into macrophages was induced by 5 ng/ml PMA, according to a previous report [19]. Cultures were incubated at 37°C in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA). Cells were grown until they reached 70–80% confluence, at which time, they were subjected to the experiment.

LPS was dissolved in PBS, and macrophages were treated with 1 μg/ml, whereas control cells received PBS only as previously described [20].

Isolation and differentiation of BMDMs

Mouse bone marrow was isolated as previously described [21] and was cultured with 30% L929 cell conditioned medium in complete DMEM for 7 d before any further experimental procedure. L929 cells were purchased from American Type Culture Collection.

To prepare L929 cell conditioned medium, L929 cells were cultured in DMEM media with 10% FBS at 37°C in a 5% CO_2 incubator. Cell culture media were collected and filtered using 0.22 μm filters.

Categorization of the EVs

Three types of EVs were prepared by using sequential centrifugation protocols described previously with a minor modification [12, 20, 22]. Cultured cells were incubated with culture medium containing EV-depleted FBS for designated time points. Conditioned medium was collected and centrifuged at 300 *g* for 10 min to remove floating cells. The supernatant was further centrifuged at 2000 *g* for 20 min to pellet ABs. To isolate MVs, the AB-depleted supernatant was passed through a 0.8 μm pore filter, followed by centrifugation at 16,000 *g* for 40 min. Finally, the resulting supernatant was passed through a 0.2 μm pore filter and ultracentrifuged at 100,000 *g* for 1 h to pellet Exos. Each type of vesicle was washed further with cold PBS, then resuspended with PBS, and stored at −80°C. Protein concentration was measured with a Bradford assay.

Uptake of macrophage-derived ABs

ABs from macrophages were stained with Annexin V-FITC (BioVision, Milpitas, CA, USA), with a ratio of 1:200 for 30 min, and incubated with recipient cells for 10 min. The recipient cells were then visualized with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich). Images were obtained with ×400 fluorescent microscopy (Axioskop 40; Zeiss, Thornwood, NY, USA).

DLS analysis

The size of EVs was analyzed using the DLS instrument 90Plus. Nanoparticle Size Analyzer (Brookhaven Instruments, Holtsville, NY, USA). For calculating the absolute EV number, a standard calibration curve (particle number vs. count rate) was generated using DLS ($R^2 = 0.9978$). Count rate of each EV sample was measured by DLS, followed by calculation of the absolute vesicle number.

Immunofluorescence

Immunofluorescence was performed as previously described [23–26]. Images were captured using a fluorescence microscope (Eclipse TS100; Nikon Instruments, Melville, NY, USA) at ×20 magnification and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA preparation, RT, and real-time qPCR

SYBR Green-based real-time qPCR technique was performed for detection of miRNAs, as previously described [23–26]. Total RNAs were purified from isolated ABs using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). Purified RNA amount was measured by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). Reverse Transcription Kit (Thermo Fisher Scientific) was used to generate single-stranded cDNA from an equal amount of purified RNAs.

CCK-8 assay

The CCK-8 detection kit (Sigma-Aldrich) was used to measure cell viability, according to the manufacturer's instructions. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm.

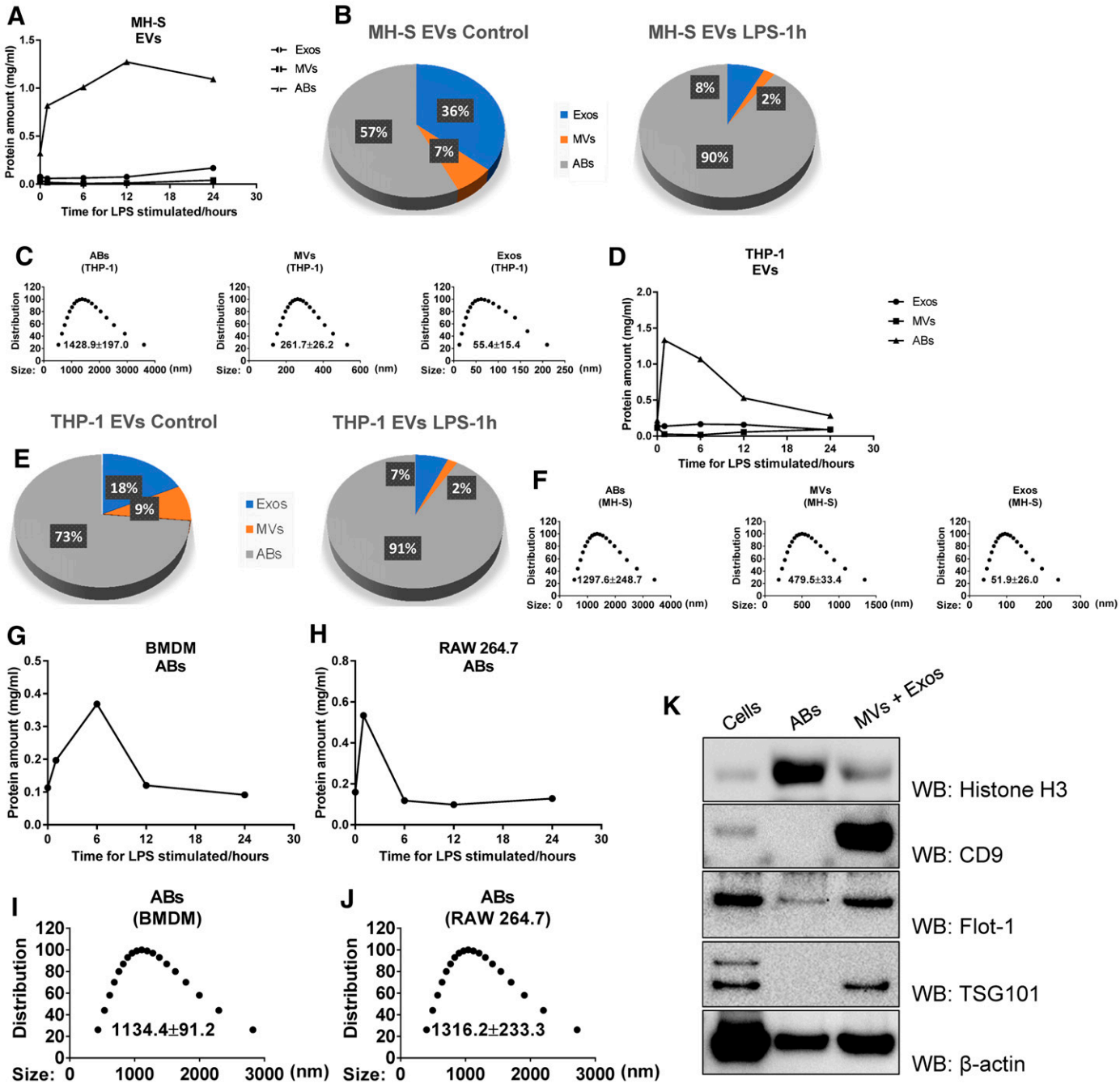

Figure 1. Characterization of the EVs generated from macrophages. (A–C) Three types of EVs were isolated from MH-S alveolar macrophage cells, including ABs, MVs, and Exos. (D–F) Three types of EVs were isolated from THP-1 cells, including ABs, MVs, and Exos. Cells were stimulated with LPS (1 μg/ml) for 0, 1, 6, 12, and 24 h, followed by the isolation of EVs. (A and D) The amount of EV generations after a time course of LPS. (B and E) The percentages of each type of EVs, with or without LPS stimulation, presented in pie graphs. (C and F) The sizes of the isolated EVs measured using DLS. (G–J) ABs were isolated from BMDM and RAW 264.7 after stimulated with LPS (1 μg/ml) for 0, 1, 6, 12, and 24 h. (G and H) The amount of AB generations after a time course of LPS. (I and J) The sizes of the isolated ABs measured using DLS. (K) Western blot (WB) data for characterization of THP-1 macrophage-derived ABs. Data represent means ± SD of 3 independent experiments with identical results. Flot-1, Flotillin 1; TSG101, tumor susceptibility gene 101.

miRNA transfection into ABs

Isolated ABs were incubated with Exo-Fect reagent (System Biosciences, Palo Alto, CA, USA) and miRNA mimics or inhibitor (10 pM) at 37°C for 10 min and placed on ice for 30 min, followed by 3 times washing with PBS using 2000 *g* force centrifugation. The transfected ABs were then used directly for further experiments.

Western blot analysis

Western blotting analysis was performed as described previously [23–26]. Harvested cells, ABs, MVs, and Exos were lysed with radioimmunoprecipitation assay buffer containing 1% Triton X-100, protease inhibitor, and phosphatase inhibitor.

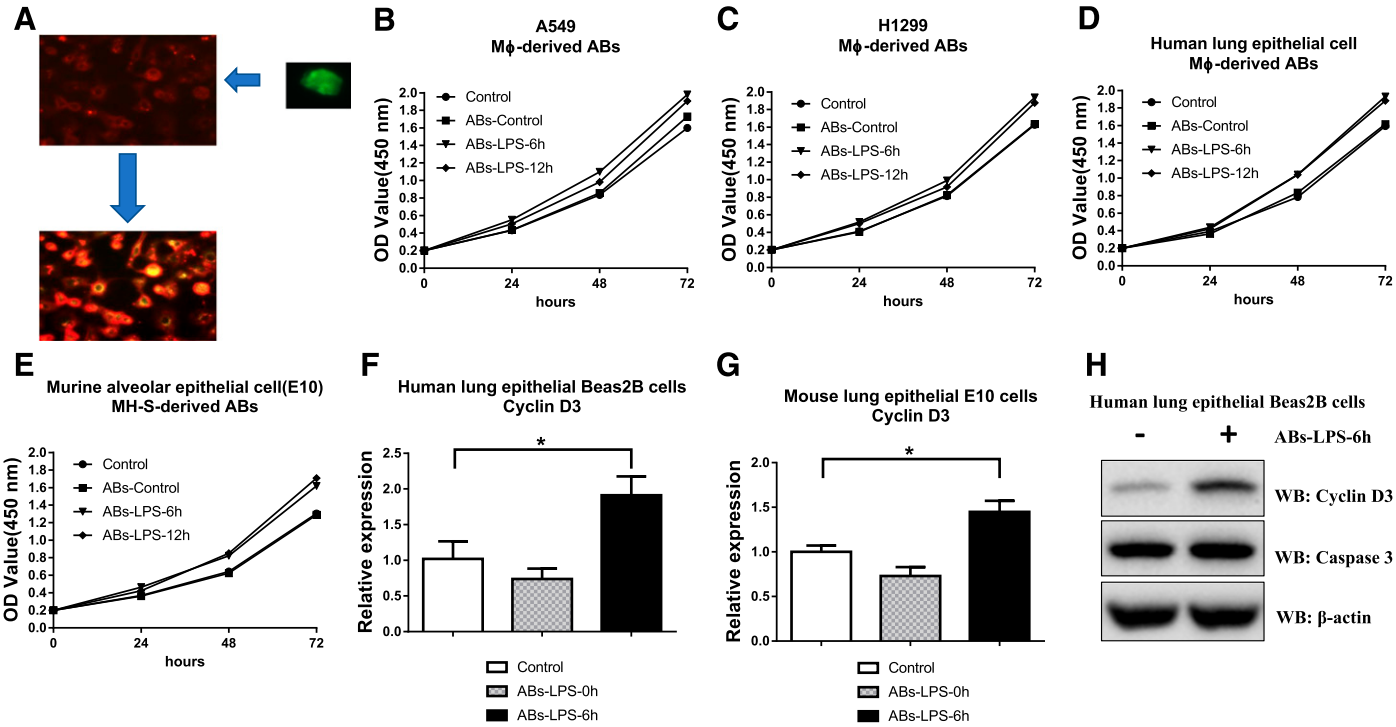

Figure 2. The effects of LPS-induced, macrophage-derived ABs on cell survival and proliferation in lung malignant or normal epithelial cells. (A) Colocalization of the THP-1 macrophage-derived ABs and A549 cells. ABs were labeled with Annexin V-FITC, and the A549 cells membranes were marked with PKH26 Red. ABs (50 μg/2 ml per well) were added into the cell culture of A549 cells; after 10 min, cells were washed with PBS twice. Cells were then examined using the confocal microscopy. (B–E) ABs were isolated from the macrophages after LPS (1 μg/ml) stimulation for 0, 6, and 12 h. The recipient cells were treated with the macrophage (Mϕ)-derived ABs (50 μg/2 ml per well). The effects of macrophage-derived ABs on the proliferation of recipient cells were measured using CCK-8 assay. The values of OD (450 nm) represent cell viability. The data represent means ± SD of 3 independent experiments. (F and G) Cyclin D3 levels were analyzed using real-time PCR. (F) Human lung epithelial Beas2B cells. (G) Mouse alveolar epithelial type I cells (E10). (H) Cyclin D3 levels were analyzed using Western blot analysis in Beas2B cells.

Statistics

All data were presented as means ± SD. Comparisons between 2 groups were performed using a 2-tailed unpaired Student's *t* test. Multiple groups were compared using a one-way ANOVA with Tukey method. $P < 0.05$ was considered statistically significant.

RESULTS

LPS stimulated the generations of macrophage-derived ABs

LPS are derived from the outer membrane of G^- bacteria and can trigger strong immune responses in the host. In the setting of G^- bacterial pneumonia, alveolar macrophages are the first responders to infection [27]. We initially evaluated the generation of alveolar macrophage-derived EVs in response to LPS. As shown in **Fig. 1A**, LPS induced a rapid and robust release of ABs from MH-S cells, a cell line of murine alveolar macrophages. Surprisingly, only minimal up-regulation of MVs or Exos was found. Percentages of each category of EVs in the absence or presence of 1 h treatment of LPS were illustrated using pie graphs (Fig. 1B). Next, we confirmed our findings using human macrophages, THP-1. Although slightly different compared with MH-S cells, the immediate up-regulation of ABs is again observed in these cells (Fig. 1D and E). Furthermore, similar results were also observed in BMDMs and RAW 264.7 macrophages (Fig. 1G and H). To confirm that the up-regulated EVs fall into the category of ABs, we evaluated the sizes of the EVs obtained from MH-S, THP-1, BMDMs, and RAW 264.7 cells using DLS. Shown in Fig. 1C, F, I, and J, we confirmed that the dramatically up-regulated EVs fell into the category of ABs. To characterize further the macrophage-derived ABs, we performed a Western blot analysis, according to a previous paper [28]. As shown in Fig. 1K, Western blot analysis showed that ABs contained ample amounts of histone H3 and lack the MV/Exo maker, CD9. These data can prove that these large particles represent AB-like particles [28].

Macrophage-derived ABs induced proliferation of the recipient epithelial cells

Next, we determined the effects of macrophage-derived ABs on the recipient epithelial cells. In **Fig. 2A**, we treated the A549 alveolar epithelial cells with LPS-induced ABs. These ABs were labeled with Annexin V-FITC. Only after 10 min, uptake of THP-1-derived ABs by A549 cells was observed (Fig. 2A). Next, we treated the A549 cells with macrophage-derived ABs in a time-dependent manner. Surprisingly, we found that ABs induced A549 proliferation (Fig. 2B). To confirm this observation, we treated H1299 cells (lung epithelial cell line; Fig. 2C) and normal lung epithelial Beas2B cells (Fig. 2D) with the macrophage-derived ABs. Similar results were found in both H1299 and Beas2B. Furthermore, we were also able to confirm this

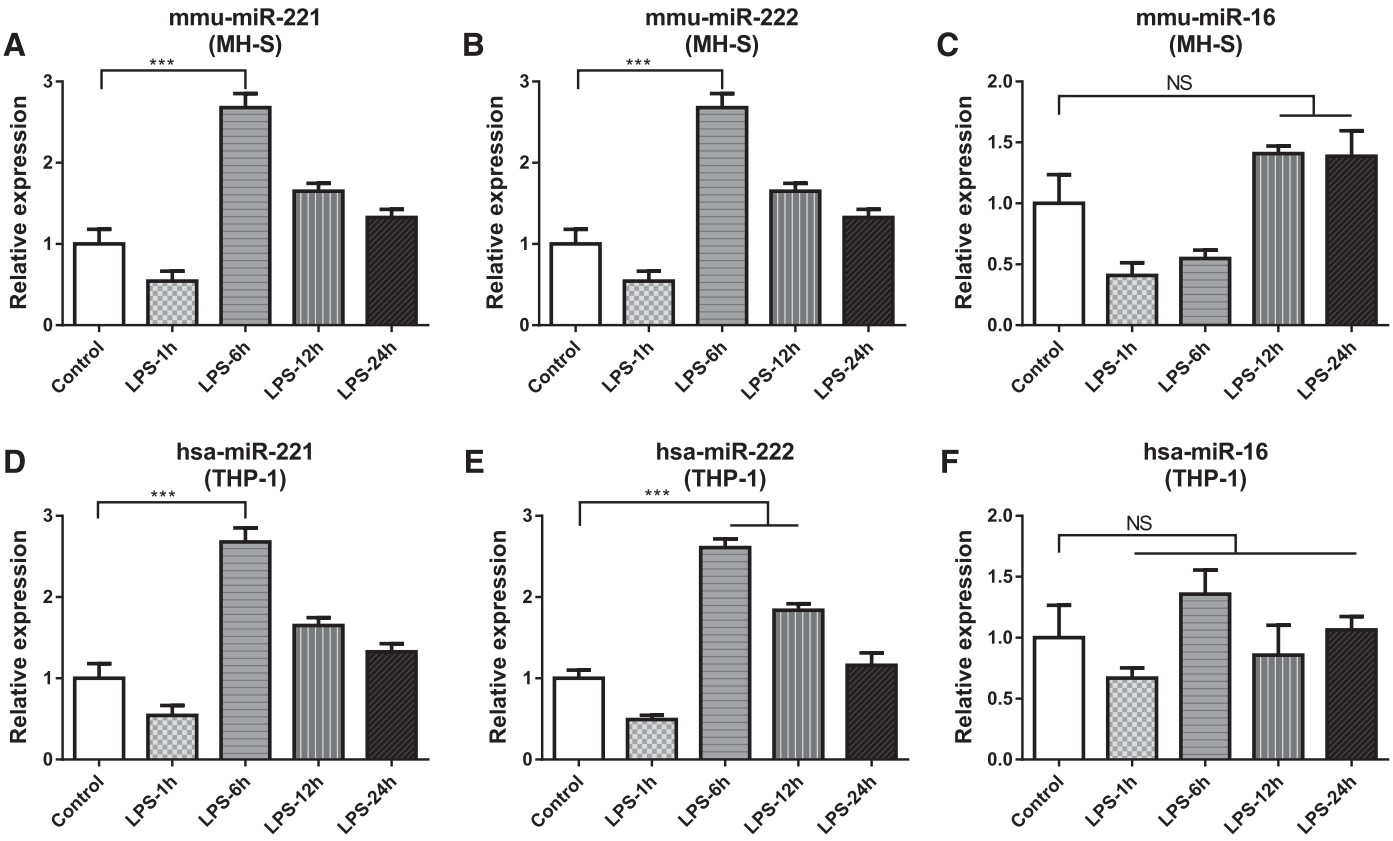

Figure 3. LPS induce miR-221/222 expression in macrophage-derived ABs. RNA was isolated from the macrophage-derived ABs after LPS stimulation for 0, 1, 6, 12, and 24 h and quantified using the real-time qPCR. miRNA expression levels were shown in bar graphs. (A–C) miR-221, miR-222, and miR-16 expressions in MH-S-derived ABs. (D–F) miR-221, miR-222, and miR-16 expressions in THP-1-derived ABs. All of the data represent means ± sd of 3 independent experiments. ***$P < 0.001$. mmu, Mus musculus ; hsa, Homo sapiens.

observation in normal murine alveolar epithelial cells (E10) after being exposed to MH-S-derived ABs (Fig. 2E and Supplemental Fig. 1). Moreover, we confirmed that the cyclin D3 levels in the epithelial cells were altered after exposure to macrophage-derived ABs (Fig. 2F–H) using real-time PCR and Western blot analysis. Additionally, we analyzed the cell death of epithelial cells after exposure to macrophage-derived ABs. Consistently, we found no significant cell death or apoptosis in the recipient cells after the treatment of ABs (Supplemental Fig. 3).

miR-221/222 up-regulated in macrophage-derived ABs

To determine the underlying mechanisms by which AB induces cell proliferation, we investigated the effects of LPS on the modification of AB components. We found that LPS induced miR-221 and -222 up-regulation in MH-S-derived ABs (**Fig. 3A** and **B**). To confirm that this observation is unique to miR-221 or -222, we tested several other miRNAs. For instance, LPS failed to induce a significant change in AB enwrapped miR-16, as shown in Fig. 3C. With the use of THP-1 human macrophages, we confirmed the above observation in MH-S murine macrophages as well (Fig. 3D–F).

Macrophage-derived ABs induced proliferation of the recipient epithelial cells via miR-221/222

To test whether macrophage-derived ABs induced cell proliferation via the up-regulated miR-221/222 in ABs, we first manipulated the level of miR-221/222 in macrophage-derived ABs by introducing miR-221 and/or miR-222 mimics or inhibitors. As shown in **Fig. 4A**, miR-221 or miR-222 mimics enhanced cell proliferation. Additionally, miR-221 and miR-222 mimics exhibited a synergistic effect (Fig. 4A). This result was confirmed using 2 separate methods: WST-8 [29] (Fig. 4A) and PCNA [30] (Fig. 4B). On the other hand, miR-221 or miR-222 inhibitors robustly suppressed AB-induced cell proliferation. miR-221 and miR-222 inhibitors were also seen to exert a synergistic effect consistently (Fig. 4C). This result was again confirmed using 2 separate methods: WST-8 (Fig. 4C) and PCNA (Fig. 4D).

miR-221/222 overexpression promotes cell growth via suppressing CDKN1B in normal lung epithelial cells

The effect of miR-221/222 has been studied in the A549 nonsmall cell lung cancer cell line [31]. We exposed normal murine alveolar epithelial cells (E10) with miR-221 or miR-222 mimics. Overexpression of miR-221/222 promoted E10 cell proliferation, as detected using PCNA methods (**Fig. 5A** and **B**). Interestingly, we observed a marked knockdown of CDKN1B ($p27^{Kip1}$) in murine alveolar epithelial cells after introducing miR-221 or miR-222 mimics (Fig. 5C and D). The inhibition by miR-221/222 is unique to CDKN1B, given that opposite effects were found in CDKN1A and CDKN1C (Fig. 5E and F). Similar results were confirmed in human lung epithelial cells (Fig. 5G–I).

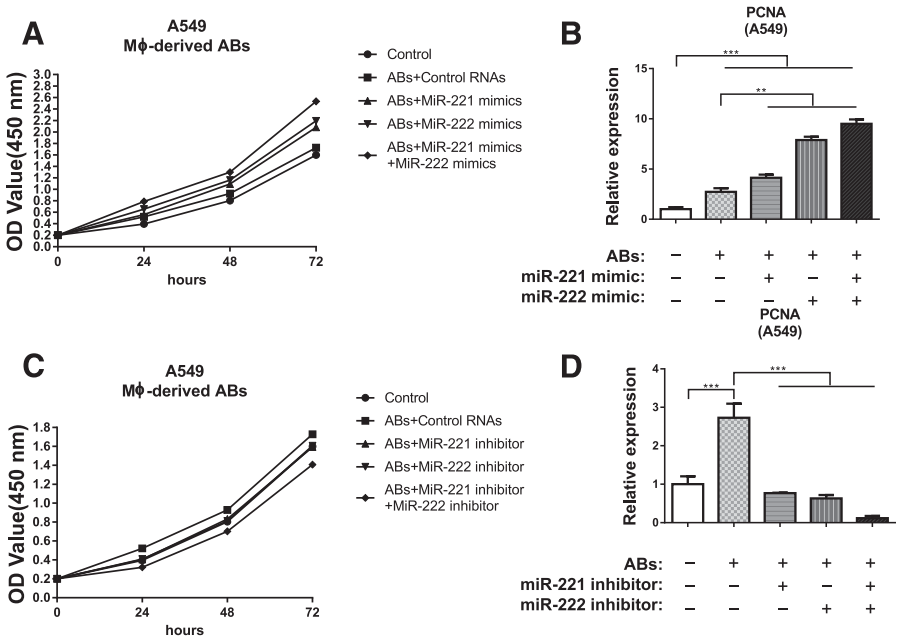

Figure 4. Synergistic effects of AB-containing miR-222 and miR-222 on the cell proliferation. (A and B) Transfection with miR-221 and/or miR-222 mimics induces the proliferative effect of macrophage-derived ABs. (C and D) Transfection with miR-221 and/or miR-222 inhibitors suppresses the proliferative effect of macrophage-derived ABs. ABs were isolated from the THP-1 cells and directly transfected with miR-221 and/or miR-222 mimics or inhibitor, as described in Materials and Methods. The recipient A549 cells were treated with the LPS-induced ABs, ABs with control RNAs, or ABs with miRNA mimics or inhibitor (50 μg/2 ml per well). Cell viability was determined using the CCK-8 assays. (B and D) Relative mRNA level of PCNA was detected in A549 cells treated with ABs transfected with miR-221 and/or miR-222 mimics (B) or inhibitor (D). Data represent means ± SD of 3 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

Macrophage-derived ABs suppress CDKN1B expression via miR-221/222

Next, we determined the effects of macrophage-derived ABs on the expression of miR-221/222. We treated human lung cancer A549 cells with LPS-induced ABs. LPS-induced ABs suppressed the expression of CDKN1B. This effect was observed in the ABs induced as early as 6 h after exposure of LPS (**Fig. 6A** and Supplemental Fig. S2). On the other hand, ABs failed to exert significant effects on the expression of CDKN1A, whereas an up-regulation, but not suppression, was observed in the expression of CDKN1C (Fig. 6B and C). Similar results were also found using normal lung epithelial cells (Fig. 6D–F). We introduced the miR-221/222 mimics or inhibitors into LPS-induced, macrophage-derived ABs, and the A549 cells were treated with these manipulated ABs. Addition of miR-221 or miR-222 mimics synergistically exaggerated the AB-mediated suppression of CDKN1B in A549 cells (Fig. 6G). Consistently, the miR-221 inhibitor or miR-222 inhibitor reversed the effects of ABs on CDKN1B in A549 cells. Moreover, miR-221 and -222 and ABs exerted a synergistic effect on CDKN1B in A549 cells (Fig. 6H).

Macrophage-derived ABs induce the cyclin D3 and Cdk4 expression via the miR-221/222

Given that CDKN1B prevents the activation of cyclin D-CDK4 complexes and subsequently controls the cell-cycle progression at G1, it is often considered a tumor suppressor [32–34]. We treated the A549 cells with macrophage-derived ABs, miR-221 mimic-enhanced ABs and miR-222 mimic-enhanced ABs. Up-regulation of cyclin D3 was found in all of the above-treated cells (**Fig. 7A**). More importantly, a significant synergistic effect was found in the cells treated with miRNA mimic-enhanced ABs (Fig. 7C). Consistently, miRNA inhibitor-enhanced ABs markedly reversed the effects of ABs on cyclin D3 expression (Fig. 7D). Likewise, miRNA mimic-enhanced ABs up-regulated the Cdk4 level, whereas miRNA inhibitor-enhanced ABs suppressed the Cdk4 level in A549 cells (Fig. 7B, E, and F).

DISCUSSION

EVs have been considered as novel targets for the development of diagnostic, prognostic, and therapeutic agents [35]. EVs potentially act as a carrier to transport and exchange molecular information among different types of cells, tissues, and organs [35, 36]. Whereas intensive studies have focused on the role of EVs originated from cancer cells, the generation and function of EVs from immunomodulatory cells remain largely unexplored. Furthermore, it is also unclear whether these EVs mediate the cross-talk between the immunomodulatory cells and benign or malignant epithelial cells. Our studies show that after LPS stimulation, the macrophage-derived EVs, particularly the ABs, regulate lung epithelial cell growth. To our best knowledge, this is the first study exploring the intercellular communications via EVs between the activated macrophages and lung epithelial cells. Our study potentially provides novel insights into lung epithelial cell propagation and tumorigenesis (**Fig. 8**).

Previously, most EV research focused on the roles of MVs or Exos. Our studies demonstrate that after LPS stimulation, ABs, rather than MVs or Exos, are the main type of EVs generated by stimulated macrophages. Therefore, our studies have focused on the role of ABs in the survival and death of epithelial cells. As previously mentioned, ABs fall into the 1000–5000 nm range in diameter and are comparable with the size of platelets. As the largest one among all 3 types of EVs, ABs are much more diverse in their morphology, composition, function, and biogenesis, as compared with MVs and Exos [8]. Despite the fact that classifications of EVs are primarily made on their sizes,

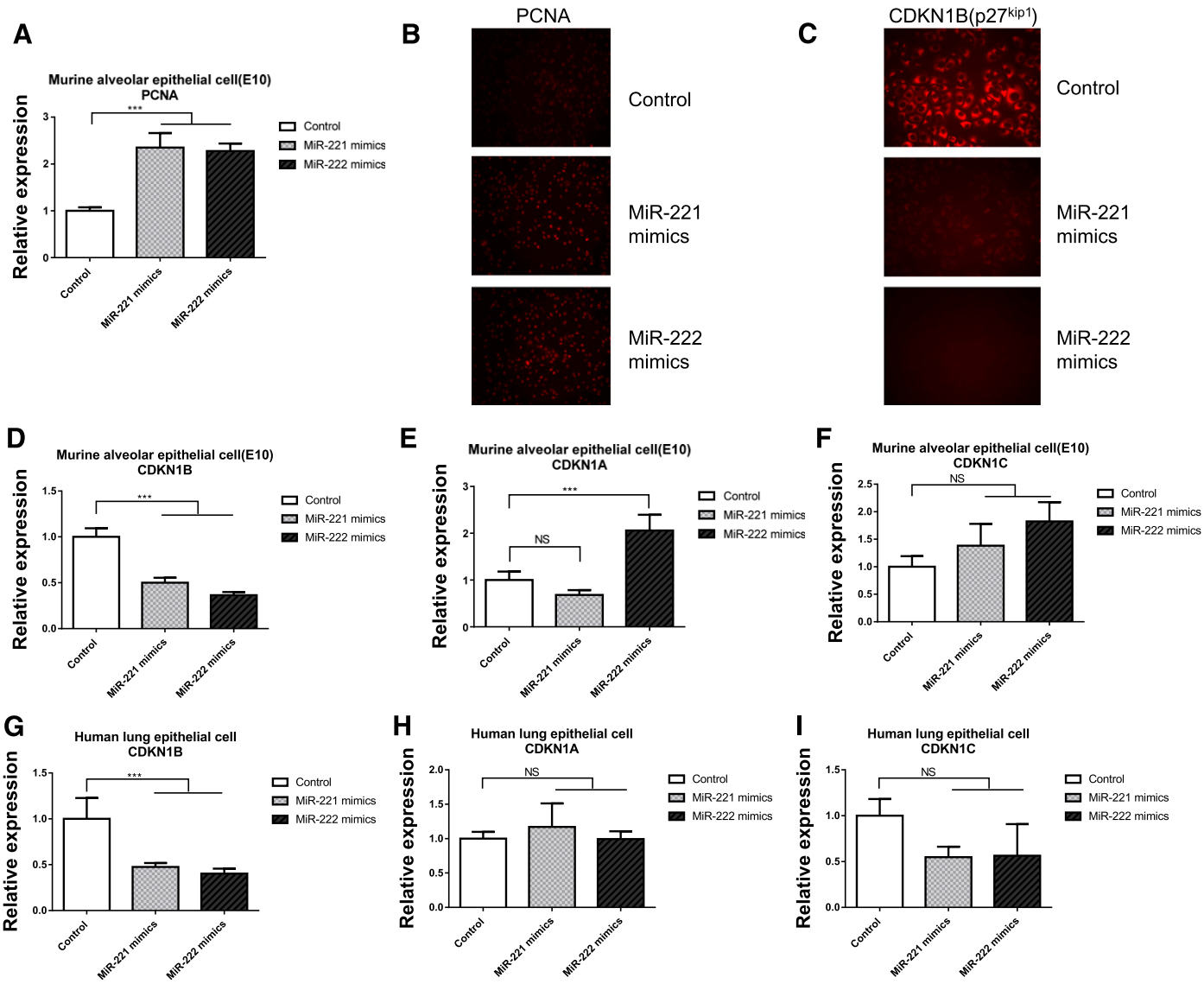

Figure 5. miR-221 and miR-222 overexpression promotes cell growth through CDKN1B inhibition in lung epithelial cells. (A–F) Murine alveolar epithelial E10 cells were transfected with control or miR-221/222 mimics for 48 h. (A) The relative PCNA mRNA level was detected by qPCR. (B) PCNA immunofluorescence was used to detect proliferation in E10 cells transfected with control or miR-221/222 mimics. Representative images were captured using a fluorescent microscope at ×100 magnification. (C) CDKN1B immunofluorescence was used to confirm the CDKN1B mRNA change in E10 cells transfected with control or miR-221/222 mimics. Representative images were captured using a fluorescent microscope at ×200 magnification. (D–F) The relative CDKN1B, CDKN1A, and CDKN1C mRNA levels were detected using qPCR after transfection. (G–J) The relative CDKN1B, CDKN1A, and CDKN1C mRNA levels were detected in human lung epithelial BEAS-2B cells using qPCR after transfection. Data represent means ± SD of 3 independent experiments. $***P < 0.001$.

differential compositions do exist within the various types of EVs. For example, recent reports suggest that RNA profiles in the 3 types of EVs are different [12]. Particularly, ABs and not MVs and/or Exos are believed to contribute the majority of RNA compositions analyzed in the EVs [12]. Moreover, as a result of the significantly larger size of ABs, more diverse contents are found in the ABs, including but not limited to proteins, lipids, RNA, and DNA molecules [12]. Presumably, ABs would confer more robust effects on their downstream cells or recipient cells, given their larger pool of encapsulated molecules. That said, major advances on EVs and cancer biology were achieved by targeting Exos and/or MVs. Malignant cells are shown to "engulf" the nano-sized Exos in a variety of reports [37]. Caveolae-dependent or -independent endocytosis are thought to play essential roles in this process [38]. These findings provide crucial bases for the development of novel drug delivery systems using Exo-shuttling methods, which potentially can be cancer cell specific. On the other hand, ABs, as a result of their larger size, are presumably difficult to be taken up by the recipient cells via endocytosis. Initially, we assumed that ABs would exert effects via contact with surface proteins on the recipient cells. As shown in Fig. 2A, we found that Annexin V-FITC-labeled ABs

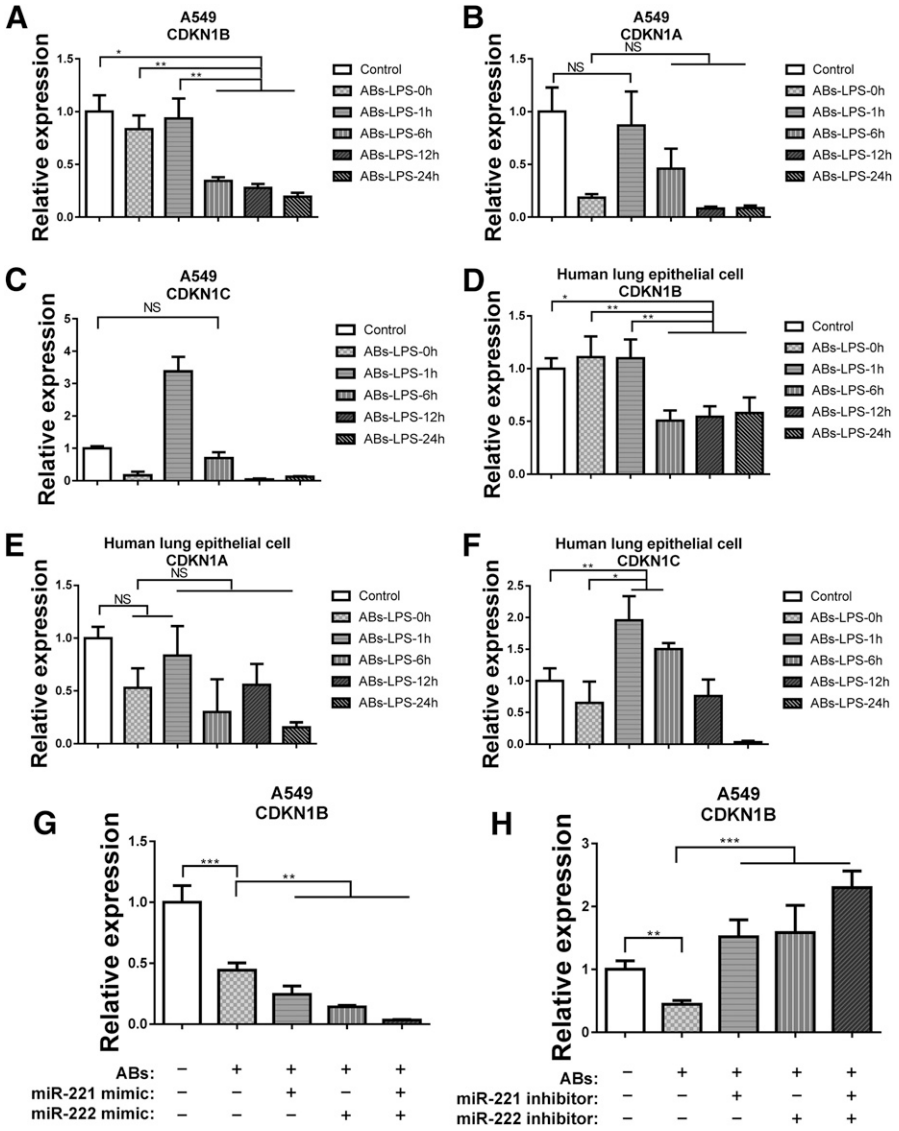

Figure 6. Macrophage-derived ABs suppress the CDKN1B expression through the miR-221/222. (A–C) A549 cells were treated with macrophage-derived ABs, as described in Materials and Methods. The relative CDKN1B, CDKN1A, and CDKN1C mRNA levels were detected using qPCR. (D–F) Human BEAS-2B lung epithelial cells were treated with macrophage-derived ABs. The relative CDKN1B, CDKN1A, and CDKN1C mRNA levels were detected using qPCR. (G) Macrophage-derived ABs transfected with miR-221 and/or miR-222 mimics induced the down-regulation of CDKN1B($p27^{Kip1}$) in A549 cells. (H) Macrophage-derived ABs transfected with miR-221 and/or miR-222 inhibitor reduced the down-regulation of CDKN1B($p27^{Kip1}$) in A549 cells. ABs were isolated from the THP-1 cells and directly transfected with miR-221 and/or miR-222 mimics or inhibitor, as described in Materials and Methods. The recipient A549 cells were treated with the LPS-induced ABs, ABs with control RNAs, or ABs with miRNA mimics or inhibitor (50 μg/2 ml per well). Data represent means ± SD of 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(green) colocalized with the PKH26 Red-labeled A549 cell membranes. However, given that confocal microscopy cannot be used to examine 3-dimensional subjects, it is unclear whether the ABs were engulfed partially or entirely into the epithelial cells or if they were mainly interacting with the cell-surface molecules. That said, this contact probably does cause structural changes to the lipid bilayer cell membrane via either a caveolae-dependent or -independent mechanism. The alteration of the cell-surface membrane, in fact, has been widely observed and reported in EV research, including the modification of cell-surface molecules through glycosylation and palmitoylation [39].

We discovered that miR-221 and miR-222, 2 known miRNAs involved in cancer cell growth [40], were up-regulated in the macrophage-derived ABs after LPS (Fig. 3). More interestingly, these 2 AB-shuttling miRNAs were highly functional in the process of promoting epithelial cell proliferation (Fig. 4). Both miR-221 and -222 have been predicted to target the CDKN1B ($p27^{Kip1}$) gene, and our studies confirm this result (Fig. 5). Therefore, we believe that ABs or AB-shuttling compositions, such as miRNAs, are at least partially engulfed by the recipient epithelial cells. This probably occurs via recipient cell-surface modification triggered by AB/recipient cell contact.

Among all the AB-encapsulated compositions, compared with the larger and more complex proteins—RNA and DNA molecules—miRNAs are potentially the most powerful ones to result in significant effects on the recipient cells. They are ~20 nt noncoding, small RNAs and directly target the designated genes in the nucleus [41], which in our study, was the CDKN1B ($p27^{Kip1}$) gene. miRNAs are small and relatively more stable, as compared with the larger RNA or DNA molecules. They probably interact with certain permeable peptides and/or "flip-flop" proteins on the cell surface and subsequently, get transported intracellularly to the recipient cells. The detailed mechanism behind this process, however, remains to be explored.

Despite previous reports of only endogenous miR-221/222-mediated cell proliferations in A549 cells [40], our studies were

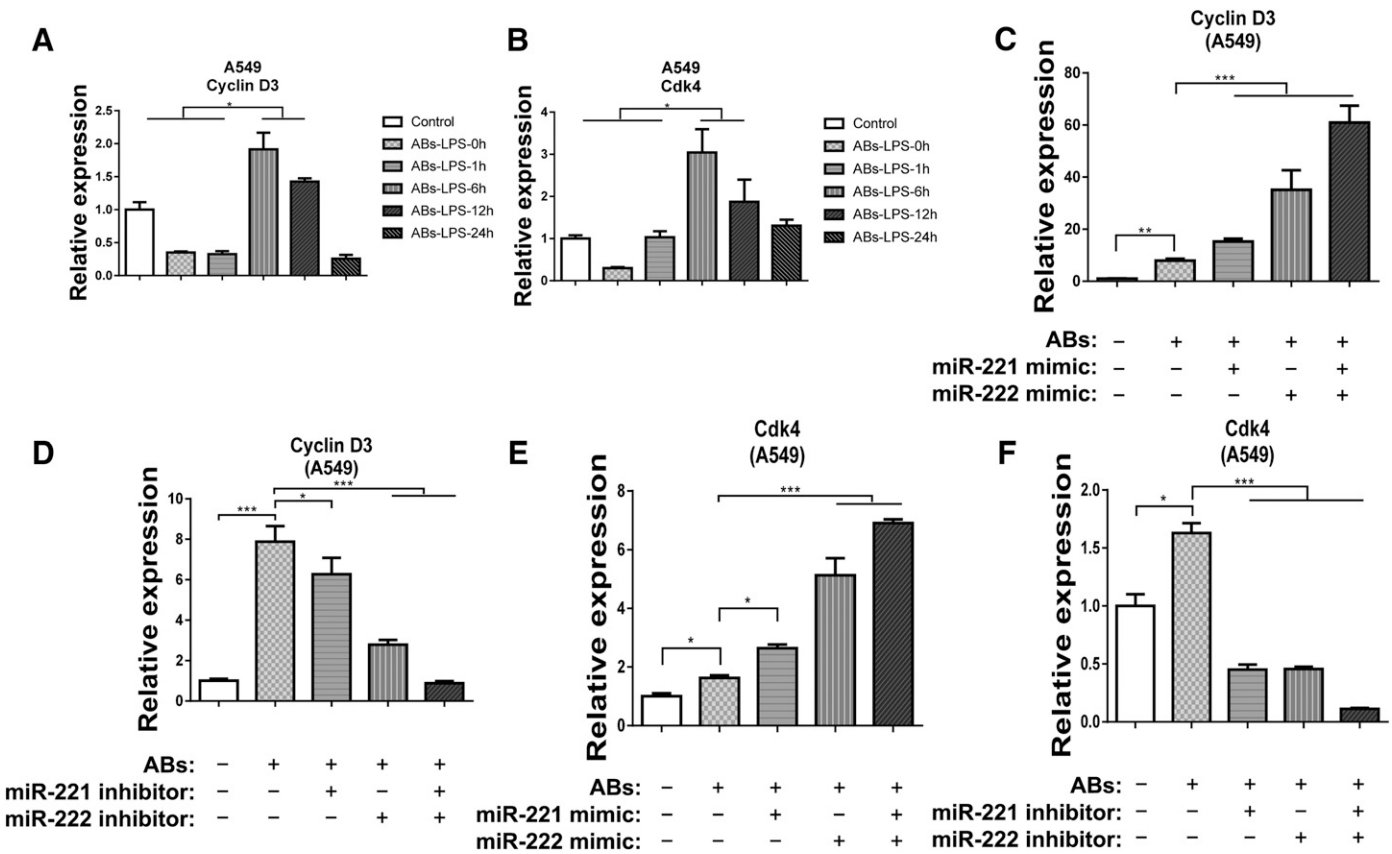

Figure 7. Macrophage-derived ABs induce the cyclin D3 and Cdk4 expression through the miR-221/222. (A and B) A549 cells were treated with macrophage-derived ABs. The relative cyclin D3 and Cdk4 mRNA levels were detected using qPCR. (C and D) Macrophage-derived ABs transfected with miR-221 and/or miR-222 mimics induced the up-regulation of cyclin D3 and Cdk4 in A549 cells. (E and F) Macrophage-derived ABs transfected with miR-221 and/or miR-222 inhibitor reduced the up-regulation of cyclin D3 and Cdk4 in A549 cells. ABs were isolated from the THP-1 cells and directly transfected with miR-221 and/or miR-222 mimics or inhibitor, as described in Materials and Methods. The recipient A549 cells were treated with the LPS-induced ABs, ABs with control RNAs, or ABs with miRNA mimics or inhibitor (50 μg/2 ml per well). Data represent means ± SD of 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

able to extrapolate this observation to an exogenous source of miR-221/222, i.e., the LPS-induced, macrophage-derived, AB-containing miR-221/222. This observation demonstrates for the first time that a cross-talk exists between the infection-activated macrophages and lung epithelial cells in the setting of G^- bacterial infections. Additionally, we report that the AB-shuttling miR-221/222 induces cell proliferation in primary lung epithelial cells too. However, it is premature to conclude that repeated bacterial infections may contribute to lung epithelial cell tumorigenesis. Many questions remain to be answered through further studies . These questions include but are not limited to the concentration and amount of specific miRNAs in each ABs induced by LPS or G^- bacterial infection; the effective "dose" or "amount" of ABs/AB-shuttling miRNAs to trigger cell growth; and the efficacy of AB-shuttling miRNAs to enter the recipient cells. There is yet to be a study comparing the half-lives between exogenous, AB-delivering miRNAs and the endogenous miRNAs; looking at the underlying mechanisms by which AB-shuttling miRNAs enter the recipient cells; or comparing the generation of macrophage-derived ABs in G^+ bacterial infection versus G^- bacterial infection. Currently, our studies adopted the miRNA mimics and/or inhibitors to manipulate directly the amount of miR-221/222 in the ABs. This approach certainly carries a disadvantage, which is that it is less likely to reflect the precise and pathophysiological level of miRNAs in the ABs and/or recipient cells. However, on the other hand, it suggests that manipulation of AB-containing miRNAs can potentially be applied as a novel therapeutic target.

Our studies also illustrate the complexity of developing therapeutic targets using EV or EV compositions. The differential type of EVs involved in the pathogenesis requires a detailed characterization that is probably stimulation dependent and cell-type dependent. Multiple compositions in each type of EVs probably collectively participate in a disease process and so do the synergistic effects of different EV compositions. The other significance of our studies includes the following: LPS-induced, macrophage-derived ABs functioned as a messenger to lung epithelium and helped to maintain its integrity via promotion of epithelial cell proliferation. This may contribute a novel concept in lung epithelial cell turnover in the presence of infectious insults. Conventionally, type II epithelial cells are believed to be responsible for replacing/repairing damaged

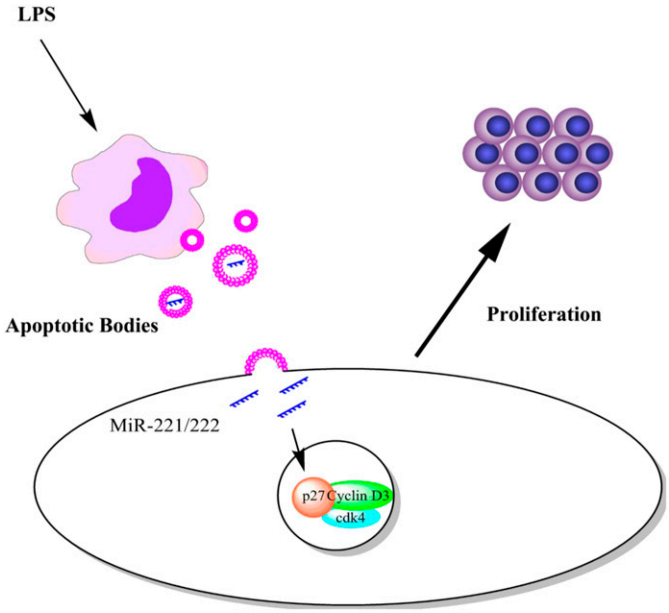

Figure 8. Schematic review of the effects of AB-containing miRNAs on the recipient cancer cells. LPS rapidly and robustly induces the secretion of macrophage-derived ABs and markedly up-regulates the levels of a repertoire of miRNAs in the macrophage-derived ABs, including miR-221 and miR-222. These ABs promote the proliferation of malignant and/or normal lung epithelial cells via up-regulated miR-221/222 in ABs. AB-shuttling miR-221/222 promote cell growth through modulating CDKN1B-cyclin D3/Cdk4 pathways.

type I cells. Our study suggests that AB-stimulated type I epithelial cell proliferation may also contribute to the integrity of epithelium in the presence of infectious insults. However, whether repetitive exposure to the proproliferative ABs triggers unregulated tumor-like proliferation of epithelial cells requires further investigation.

In summary, our report finds that macrophages release a significant amount of ABs after LPS, rather than MVs and Exos. The LPS-induced, macrophage-derived ABs promote the proliferation of recipient epithelial cells via AB-shuttling miR-221/222 and miR-221/222-targeted CDKN1B pathways.

AUTHORSHIP

Y.J. supervised the project. Y.J., Z.Z., A.A.M., and K.H. wrote and revised the manuscript. Z.Z., D.Z., H.L., and J.W. designed the experiments, performed each individual assay, analyzed the data, and wrote the manuscript. All authors reviewed the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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