

Pharmacoproteomics reveal novel protective activity of bromodomain containing 4 inhibitors on vascular homeostasis in TLR3-mediated airway remodeling

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ABSTRACT

Small molecule inhibitors of the epigenetic regulator bromodomain-containing protein 4 (BRD4) are potential therapeutics for viral and allergen-induced airway remodeling. A limitation of their preclinical advancement is the lack of detailed understanding of mechanisms of action and biomarkers of effect. We report a systems-level pharmacoproteomics in a standardized murine model of toll-like receptor TLR3-NFκB/RelA innate inflammation in the absence or presence of a highly selective BRD4 inhibitor (ZL0454) or nonselective bromodomain and extraterminal domain inhibitor (JQ1). Proteomics of bronchoalveolar lavage fluid (BALF) secretome and exosomal proteins from this murine model revealed increased, selective, capillary leak associated with pericyte-myofibroblast transition, a phenomenon blocked by BRD4 inhibitors. BALF proteomics also suggested that ZL0454 better reduced the vascular leakage and extracellular matrix deposition than JQ1. A significant subset of inflammation-mediated remodeling factors was also identified in a mouse model of idiopathic pulmonary fibrosis produced by bleomycin. BALF exosome analysis indicated that BRD4 inhibitors reduced the induction of exosomes enriched in coagulation factors whose presence correlated with interstitial fibrin deposition. Finally, BALF samples from humans with severe asthma demonstrated similar upregulations of ORM2, APCS, SPARCL1, FGA, and FN1, suggesting their potential as biomarkers for early detection of airway remodeling and/or monitoring of therapy response.

Significance: Repetitive and chronic viral upper respiratory tract infections trigger toll-like receptor (TLR)3-NFκB/RelA mediated airway remodeling which is linked to a progressive decline in pulmonary function in patients with asthma and chronic obstructive pulmonary disease. Small molecule inhibitors of the epigenetic regulator bromodomain-containing protein 4 (BRD4) are potential therapeutics for viral and allergen-induced airway remodeling. A limitation of their preclinical advancement is the lack of detailed understanding of mechanisms of action and biomarkers of effect. Our study revealed that the activation of (TLR)3-NFκB/RelA pathway in the lung induced an elevation in coagulation, complement, and platelet factors, indicating the increased vascular leak during airway remodeling. The mechanism of vascular leakage was chronic inflammation-induced pericyte-myofibroblast transition, which was blocked by BRD4 inhibitors. Finally, proteomics analysis of the bronchoalveolar lavage fluid samples from humans with severe asthma demonstrated similar findings that we observed in the animal model.

Abbreviations: COPD, chronic obstructive pulmonary disease; BALF, bronchoalveolar lavage; BET, bromodomain and extra terminal domain; BRD4, bromodomain containing 4; ECM, Extracellular matrix; GO, Gene ontology; GOBP, Gene ontology biological process; IPF, idiopathic pulmonary fibrosis; MS, Mass spectrometry; PCA, Principle component analysis; Q, Quadrupole; SID, Stable isotope dilution; SIS, Stable isotope labeled internal standard; SRM, Selected reaction monitoring; TGF, Transforming growth factor; TLR, toll-like receptor

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1. Introduction

Epidemiological studies indicate that recurrent viral airway infections are the most common cause of exacerbations in airway remodeling diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung fibrosis [1–4]. These infections accelerate the decline in lung function through poorly-defined mechanisms [5]. The respiratory epithelium plays a significant role in the defense against mucosal viruses by producing inflammatory and antiviral responses [6] such as the releasing epithelial- and matrix-associated growth factors (transforming growth factor [TGF]- β , epidermal growth factor) and secreting cytokines (periostin, IL-17, IL-11) [7]. Although these processes initially promote re-epithelialization after mucosal injury, their prolonged activity is linked to organ fibrosis and progressive decline in lung function [8]. Recently, we developed an animal model that mimics many aspects of recurrent viral respiratory infections, generated by repetitive intranasal administration of poly(I:C), an immunostimulatory molecular pattern associated with many viruses [9,10]. Our mechanistic studies show that repetitive poly(I:C) administration activates the TLR3-NF- κ B/RelA pathway, producing mesenchymal transition and enhancing subepithelial matrix deposition and fibrosis in both the proximal and small-airway epithelia [9–11]. We previously observed that bromodomain-containing protein 4 (BRD4) is functionally required for activation of the NF- κ B/RelA pathway [10,12]. Inhibition of BRD4 activities with small molecule inhibitors blocks airway remodeling programs in response to aerosolized delivery of TLR3 agonists, TGF β and allergens [9–13].

We invented a highly selective BRD4 inhibitor, ZL0454, that shows 30-fold greater selectivity for binding the BRD4 bromodomains over the closely-related BRD2 isoform, and greater cellular potency than the prototypical pan-BET inhibitors JQ1 and RVX208 [14,15]. Despite intriguing preclinical studies, the advancement of BRD4 inhibitors into clinical treatment of airway remodeling will be challenging in the absence of efficacy biomarkers. To address that problem, we conducted a pharmacoproteomics study focusing on alterations of the protein composition of BALF during chronic TLR3-mediated airway remodeling in the presence or absence of ZL0454. Our unbiased systems-level proteomics of secretome and exosomal BALF proteins revealed the induction of extracellular matrix and capillary leak proteins associated with the pericyte-myofibroblast transition. Also, our study provides insights into the protective mechanisms of BRD4 inhibitors against vascular leakage and pericyte transdifferentiation in inflammation-induced airway remodeling. Our discriminant protein panels in TLR3-induced remodeling shared with cytotoxic pulmonary injury were validated in humans with severe asthma, providing tools for biochemical monitoring of airway remodeling.

2. Material and methods

2.1. Materials

Polyinosinic–polycytidylic acid poly (I:C) was obtained from Sigma-Aldrich (St Louis, MO, USA) as the sodium salt. All reagents and solvents in LC-MS/MS analyses were ACS grade. Ammonium bicarbonate (ABC), 2,2,2-trifluoroethanol (TFE), and acetic acid were purchased from Sigma-Aldrich. Iodoacetamide (IDA), dithiothreitol (DTT), acetonitrile (ACN), formic acid, and methanol were purchased from Thermo Scientific (Waltham, MA, USA). Urea ultra was from MP Biomedicals (Santa Ana, CA, USA). Sequencing-grade modified trypsin and LysC were from Promega (Madison, WI, USA). CD140b (platelet-derived growth factor receptor beta [PDGFRB]) monoclonal antibody (APB5) was from Invitrogen (San Diego, CA, USA). Anti-alpha smooth muscle actin antibody (ab5694) was from Abcam (Cambridge, MA, USA). Goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa fluor 568 (A-11036) and goat anti-Rat IgG (H + L) cross-adsorbed secondary antibody, Alexa fluor 488 (A-11006) were from

Invitrogen.

2.2. Animal study

Male 18-week-old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed under pathogen-free conditions in the animal research facility of the University of Texas Medical Branch (Galveston, TX). Mice were given food and water ad libitum. The experimental protocol was approved by the university Institutional Animal Care and Use Committee (IACUC# 1312058A). We used a repetitive poly(I:C) induced pulmonary fibrosis mouse model that we established previously [12]. The study had four experiment groups: control (phosphate-buffered saline, PBS); repetitive poly(I:C); repetitive poly(I:C) + JQ1; and repetitive poly(I:C) + ZL0454. JQ1 and ZL0454 are BRD4 inhibitors [16] [9,12,13]. Each group had six mice. The control group was given 50 μ L of PBS via intranasal every other day for 15 doses. The repetitive poly(I:C) group was given 50 μ L of 10 μ g/ μ L of poly(I:C) via intranasal every other day for 15 doses. The repetitive poly(I:C) + JQ1 group was first injected with JQ1 (25 mg/kg of body weight) via the intraperitoneal route and the animals were given 50 μ L of 10 μ g/ μ L of poly(I:C) via intranasal on the next day; the administration of JQ1 and poly(I:C) was repeated 15 times. The repetitive poly(I:C) + ZL0454 group was first injected with ZL0454 (25 mg/kg of body weight) via the intraperitoneal route, and animals were given 50 μ L of 10 μ g/ μ L of poly(I:C) intranasally on the next day; administration of JQ1 and poly(I:C) was repeated 15 times. Twelve days after the last treatment, mice were euthanized. Bronchoalveolar lavage was performed as detailed previously [12,17]. The lung was fixed with 10% (vol/vol) neutral buffered formalin for 3 days, processed into paraffin blocks, and cut into 5- μ m sections for hematoxylin and eosin (H&E) and trichrome staining. Microscopy was performed on a Nikon Eclipse Ti System [10,11]. Periodic acid–Schiff (PAS) staining was performed in parallel to demonstrate fibrin deposition in airway epithelium. Quantification of fibrin deposition was assessed by two investigators who were blind to the treatment groups on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe fibrin deposition, respectively. Data were expressed as means of scores recorded by two blinded investigators [11,18].

2.3. Isolation of secretome and exosome from BALF

The isolation of secretome and exosome from BALF was performed as described previously [17,19]. Briefly, cells present in the BALF were removed by low-speed centrifugation at 400 $\times$ g for 10 min. The cleared supernatant was then sequentially centrifuged at 2000 $\times$ g for 15 min (Thermo Scientific IEC CL31R multispeed centrifuge, rotor T41*11210435) and 10,000 $\times$ g for 30 min to remove any remaining cell debris/microvesicles (Beckman Optima TLX ultracentrifuge, rotor TLA-100.3, Indianapolis, IN, USA). The supernatant was centrifuged at 100,000 $\times$ g for 2 h. The supernatant was collected as the BALF secretome, and the pellet was exosome fraction. The exosome was washed with PBS, then pelleted ultracentrifugation at 100,000 $\times$ g for 60 min. The BALF secretome fraction was further concentrated using Amicon ultra-4 centrifugal filters-3K (Millipore, Billerica, MA, USA).

2.4. Dynamic light scattering (DLS)

A 10 μ L aliquot from the resuspended exosome sample was diluted in 990 μ L of PBS, mixed well and loaded into the cuvette. Three determinations per sample were taken at room temperature using a Malvern High Performance Particle Sizer (HPPS, incorporating non-Invasive Back Scatter Technologies, Malvern Instruments, Westborough, MA, USA) for each independent experiment. The exosome size was calculated using the Stokes–Einstein equation to determine the particle's hydrodynamic radius (Rh) or diameter. In brief, the Brownian motion of a particle is measured by the fluctuations of

scattered light intensity at a fixed angle (173°), laser wavelength 633 nm, as an indication of the velocity distribution of the particle movement in solution. Exosomes suspended in a sample volume of 1 mL of PBS was measured and a total of three readings per sample were performed. Data acquisition and analysis were performed using Dispersion Technology Software (DTS, V4.1.26.0, Bedford Hills, NY, USA) configured for HPPs analysis. DTS analysis allows one to interpret the data acquired considering several parameters, such as intensity, volume and number distribution, as well as statistical analysis. The average particle diameter results from a peak of a Gaussian model fitting to the particle distribution, and the polydispersity index (Pdl) reflects the width of the primary size distribution present in the solution. To ensure proper operation of the instrument the equipment was calibrated periodically using polymer latex spheres (Malvern).

2.5. Transmission electron microscopy (TEM)

A 10 μ L aliquot from the exosome suspension was diluted in deionized water, applied to 200 mesh Formvar/carbon coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min at room temperature (24°C) and negatively stained with 2% uranyl acetate (UA). The grids were examined in a Philips CM-100 transmission electron microscope at 60 kV FEI (Thermo-Fischer, Waltham, MA, USA). Two independent experiments were carried out and several fields were pictured for each experimental condition. Exosome images were acquired with a Gatan Orius 2001 charge-coupled device (CCD, Pleasanton, CA, USA) camera.

2.6. Western immunoblot

Exosome and cell pellets were lysed in sodium dodecyl sulfate (SDS)-urea lysis buffer [5% SDS, 9 M urea, 125 mM Tris HCl pH 6.8 supplemented with protease inhibitor cocktail (Sigma P8340)]. Protein concentration was determined by bicinchoninic acid (BCA, Pierce, Thermo Scientific) and 10 μ g were dissolved into SDS loading buffer (with 5% β ME) and fractionated on 4–15% Mini-protean TGX gels (BioRad, Hercules CA, USA) in 1 $\times$ Tris Glycine SDS (TGS) 1 $\times$ running buffer at room temperature. Proteins were electro-transferred to PVDF (Immobilon-P, Millipore) in 1 $\times$ TGS buffer-methanol (20%) buffer. The blots were blocked with 5% milk Tween-20 (0.1%) PBS buffer (T-PBS, pH 7.4) for 1 h and incubated overnight at 4°C in primary antibodies. Antibodies were: anti-Alix/PDC61 (Ab76608) and anti-GRP4 (Cell Signaling #2104). Secondary antibodies were HRP-conjugated anti-rabbit IgG and anti-mouse IgG from Cell Signaling and Southern Biotech (Birmingham, AL, USA), respectively.

2.7. Digestion of proteins in BALF exosomes and secretome

The proteins in 80 μ L of the supernatant were reduced with 10 mM dithiothreitol (DTT) for 30 min, followed by alkylation with 30 mM iodoacetamide for 60 min at room temperature in the dark. The proteins were digested with 1.0 μ g LysC-trypsin (Promega) for 12 h at 37°C , then diluted and further digested with 1.0 μ g trypsin (Promega) for 16 h at 37°C . The digestion was terminated with 0.5% trifluoroacetic acid.

The proteins present in the exosomes were dissolved in 45 μ L of 8 M guanidine, proteins were reduced with 10 mM DTT for 30 min, followed by alkylation with 30 mM iodoacetamide for 60 min in the dark. The sample was diluted 1:1 with 50 mM ammonium bicarbonate. Proteins were digested with 1.0 μ g LysC-trypsin (Promega) for 12 h at 37°C , then diluted 4:1 with 50 mM NH_4HCO_3 . The proteins were further digested with 1.0 μ g trypsin (Promega) for 16 h at 37°C and the digestion stopped with 0.5% trifluoroacetic acid. The peptides were desalted on a reversed-phase SepPak C18 cartridge (Waters) and eluted with 80% acetonitrile. The eluate was dried in a SpeedVac and the peptides acidified with 2% acetonitrile-0.1% trifluoroacetic acid.

2.8. LC-MS/MS analysis

A nanoflow ultra-high performance chromatography instrument (Easy nLC, Thermo Fisher Scientific) was coupled online to a Q Exactive mass spectrometer (Thermo Scientific) with a nanoelectrospray ion source (Thermo Scientific). Peptides were loaded onto a C18 reversed-phase column (25 cm long, 75 μ m inner diameter) and separated with a linear gradient of 5–35% buffer B (100% acetonitrile in 0.1% formic acid) at a flow rate of 300 nL/min over 180 min. Mass spectrometry (MS) data were acquired using a data-dependent Top15 method dynamically choosing the most abundant precursor ions from the survey scan (400–1400 m/z) using HCD fragmentation. Survey scans were acquired at a resolution of 70,000 at m/z 400. Unassigned precursor ion charge states, as well as singly charged species were excluded from fragmentation. The isolation window was set to 3 Da and fragmented with normalized collision energies of 27. The maximum ion injection times for the survey scan and tandem MS (MS/MS) scans were 20 ms and 60 ms, respectively, and the ion target values were set to $1\text{E}6$ and $1\text{E}5$, respectively. Selected sequenced ions were dynamically excluded for 30 s. Data were acquired using Xcalibur software.

2.9. Data processing and Bioinformatic analysis

Mass spectra were analyzed using MaxQuant software version 1.5.2.8 using the Andromeda search engine [20,21]. The initial maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. Enzyme specificity was set to trypsin, defined as C-terminal to arginine and lysine excluding proline, and a maximum of two missed cleavages was allowed. Carbamidomethylcysteine was set as a fixed modification, N-terminal acetylation and methionine oxidation as variable modifications. The spectra were searched with the Andromeda search engine against the mouse SWISS-PROT sequence database (containing 17,000 mouse protein entries) combined with 248 common contaminants and concatenated with the reversed versions of all sequences. Protein identification required at least one unique or razor peptide per protein group. Quantification in MaxQuant was performed using the built-in XIC-based label-free quantification (LFQ) algorithm [20]. The required false positive rate for identification was set to 1% at the peptide level and 1% at the protein level and the minimum required peptide length was set to 6 amino acids. Contaminants, reverse identification, and proteins only identified by modified peptides were excluded from further data analysis. The 'match between runs' feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min), and this was also used in quantification experiments. The Maxquant results were further analyzed using Perseus platform [22]. The LFQ MS intensities were log2-transformed. After filtering (at least two valid LFQ values in at least one group), the remaining missing LFQ values were imputed from a normal distribution of log2 LFQ intensity of proteins in each sample by shrinking the distribution of 0.3 of standard deviation and shifting it down by 1.8 of standard deviation. The imputation was performed only once. Six pair-wise comparisons – poly(I:C) vs. control, poly(I:C) + JQ1 vs. poly(I:C), poly(I:C) + ZL0454 vs. poly(I:C), poly(I:C) + JQ1 vs. control, poly(I:C) + ZL0454 vs control, and poly(I:C) + ZL0454 vs poly(I:C) + JQ1 were performed. Two criteria – Student's *t*-test with Permutation-based FDR 0.01 and two-fold change in the abundance – were used to determine the significant hits in each pair-wise comparison. The unsupervised hierarchical clustering and heat map were based on protein expression. The rows of the heat map indicate the proteins, and the columns indicate the samples. The log2 LFQ intensity of each protein were z-score normalized for each row. Hierarchical clustering of the z-normalized log2 LFQ intensity was performed using Euclidean distances between means. The number of clusters was set as 300. Genome ontology enrichment analysis of molecular functions and biological process in differentially expressed proteins was performed using

Panther (<http://pantherdb.org/>). This classification uses an evolutionary framework to infer protein functions in a species-independent manner [23]. The resulting *p*-values were adjusted with Bonferroni correction for multiple testing. The significant hits were those with the adjusted *p*-value better than 0.05.

2.10. Immunostaining of pericytes

Confocal immunofluorescence assays of lung sections were performed on formalin-fixed, paraffin-embedded sections after rehydration using serial concentrations of ethanol. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). The slides were then incubated overnight at 4 °C with primary antibodies. Pericytes were identified by labeling with rat monoclonal antibody against the pericyte marker platelet-derived growth factor beta receptor (PDGFBR) [24]. Myofibroblast transitioned pericytes were identified using a-smooth muscle cell actin (α -SMA). After incubation with primary antibody, sections were washed with 0.1% Triton X-100 in PBS, incubated for 4 h at room temperature with a goat anti-rabbit IgG (h + l) highly cross-adsorbed secondary antibody conjugated to Alexa Fluor 488 (Invitrogen), and washed again. For the second labeling of α -SMA, this procedure was repeated with a rabbit polyclonal antibody against α -SMA and the secondary goat anti-rabbit IgG (h + l) highly cross-adsorbed secondary antibody conjugated to Alexa Fluor 568 (Invitrogen). Lung sections were imaged by tiling under low magnification, and we performed systematic random sampling to obtain five views for each tissue section, counting the number of cells with double positive PDGFBR and α -SMA. Images were acquired with a Zeiss 880 laser scanning confocal microscope and a Zeiss 20× Plan-Apochromat 0.8NA objective lens. DAPI, Alexa Fluor 488, and Alexa Fluor 568 fluorophores were sequentially excited with 405 nm (diode), 488 nm (argon), and 561 nm (DPSS) laser lines and their corresponding fluorescence emissions were collected in the 410–479 nm, 494–550 nm, and 569–647 nm spectral bands using a 3-channel Zeiss QUASAR detector, respectively. Pixel depth was 8 bit. The pinhole diameter was set to 50 μ m for all detectors. Detectors' gain and digital offset, and lasers' intensity settings were adjusted using the brightest sample in the series to avoid pixel saturation. For cell staining and morphology overview, tiled lung images were produced by scanning of overlapping (10% overlap) z-stacks of images. Images were stitched in Zeiss ZEN 2.3 SP1 software (black, ver. 14). Tiled lung images were then used to randomly select and scan lung areas containing both bronchioles and blood vessels. Z-stacks of those selected areas were thresholded to the same levels to remove the background, maximally projected to reduce dimensionality, then used for cell identification and counting using ImageJ. The number of differentiated pericytes (PDGFBR + / α -SMA +) were assessed by investigator who was blind to the treatment groups. Five views for each tissue section were randomly selected and the number of cells with double positive PDGFBR and α -SMA in each view were counted and averaged.

Table 1
Demographic information and diagnosis of human subjects.

	Sex	Race	Age	Asthma status	Pre-bronchodilator spirometry (FEV1/FVC)	Treatment
1	Male	Caucasian	30	No	N/A	–
2	Male	Asian	31	No	N/A	–
3	Male	Asian	36	No	N/A	–
4	Female	Hispanic	56	No	N/A	–
5	Male	White	34	No	N/A	–
6	Female	Caucasian	50	Severe	0.50%	Advair 250/50 μ g
7	Female	African American	46	Severe	0.99%	Dulera
8	Male	African American	44	Severe	0.79%	Advair 250/50 μ g
9	Male	African American	70	Severe	0.65%	Not available
10	Female	Caucasian	57	Severe	0.70%	Symbicort 160/4.5 μ g

2.11. Stable isotope dilution-selected reaction monitoring-MS

The SID-SRM-MS assays of selected proteins were developed as described previously [25]. For each targeted protein, two or three peptides were initially selected, then their sensitivity and selectivity were experimentally evaluated as described previously. The peptide with the best sensitivity and selectivity was selected as the surrogate for that protein. For each peptide, 3–5 SRM transitions were monitored. The signature peptides and SRM parameters are listed in Supplemental Table S1. The peptides were chemically synthesized incorporating isotopically labeled [$^{13}\text{C}_6^{15}\text{N}_4$] arginine or [$^{13}\text{C}_6^{15}\text{N}_2$] lysine to a 99% isotopic enrichment (Thermo Scientific, San Jose, CA, USA). The amount of stable isotope labeled standard (SIS) peptides was determined by amino acid analysis. The proteins were trypsin digested on the beads as described above. The tryptic digests were then reconstituted in 30 μ L of 5% formic acid-0.01% TFA. An aliquot of 10 μ L of 50 fmol/ μ L diluted SIS peptides was added to each tryptic digest. These samples were desalted with a ZipTip C18 cartridge. The peptides were eluted with 80% ACN and dried. The peptides were reconstituted in 30 μ L of 5% formic acid-0.01% TFA and were directly analyzed by liquid chromatography (LC)-SRM-MS. LC-SRM-MS analysis was performed with a TSQ Vantage triple quadrupole mass spectrometer equipped with a nanospray source (Thermo Scientific, San Jose, CA, USA). About 8–10 targeted proteins were analyzed in a single LC-SRM run. The online chromatography was performed using an Eksigent NanoLC-2D HPLC system (AB SCIEX, Dublin, CA, USA). An aliquot of 10 μ L of each of the tryptic digests was injected on a C18 reverse-phase nano-HPLC column (PicoFrit™, 75 μ m $\times$ 10 cm; tip ID 15 μ m) at a flow rate of 500 nL/min with a 20-min 98% A, followed by a 15-min linear gradient from 2 to 30% mobile phase B (0.1% formic acid-90% acetonitrile) in mobile phase A (0.1% formic acid). The TSQ Vantage was operated in high-resolution SRM mode with Q1 and Q3 set to 0.2 and 0.7-Da Full-Width Half Maximum (FWHM). All acquisition methods used the following parameters: 2100 V ion spray voltage, a 275 °C ion transferring tube temperature, a collision-activated dissociation pressure at 1.5 mTorr, and the S-lens voltage used the values in S-lens table generated during MS calibration.

All SRM data were manually inspected to ensure peak detection and accurate integration. The chromatographic retention time and the relative product ion intensities of the analyte peptides were compared to those of the stable isotope-labeled standard peptides. The variation in retention time between the analyte peptides and their SIS counterparts should be within 0.05 min, and the difference in the relative product ion intensities of the analyte peptides and SIS peptides were below 20%. The peak areas in the extract ion chromatography of the native and SIS version of each signature peptide were integrated using Xcalibur® 2.1. The default values for noise percentage and base-line subtraction window were used. The ratio between the peak area of native and SIS version of each peptide was calculated.

2.12. SRM analysis of human BALF

The BALF samples were collected from five healthy individuals and five patients with severe asthma. Human subjects were part of clinical [trials.gov](https://www.clinicaltrials.gov/ct2/show/study?term=NCT02264691) trial [NCT02264691](https://www.clinicaltrials.gov/ct2/show/study?term=NCT02264691). The demographic information and diagnosis of human subjects is summarized in the [Table 1](#). The isolation of proteins from human BALF samples was performed as described above. Briefly, about 500 μ L of BALF was centrifuged at 400 $\times$ g for 10 min to remove the cells from the BALF. The cleared supernatant was then sequentially centrifuged at 2000 $\times$ g for 15 min and 10,000 $\times$ g for 30 min to remove any remaining cell debris. The BALF secretome fraction was further concentrated using Amicon ultra-4 centrifugal filters-3K (Millipore, Billerica, MA, USA). The proteins were digested and analyzed by SID-SRM-MS as described above.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [\[26\]](#) partner repository with the dataset identifier PXD012060.

3. Results

3.1. Quantitative proteomics analysis of BALF secretome of TLR3-induced remodeling

We used an established repetitive poly(I:C) challenge-induced, TLR3-mediated airway remodeling mouse model [\[10\]](#) in the presence or absence of BRD4 inhibitors (ZL0454 and JQ1). The four experimental groups of animals are: control, poly(I:C), poly(I:C) + ZL0454, and poly(I:C) + JQ1. The treatment with repetitive poly(I:C) treatment

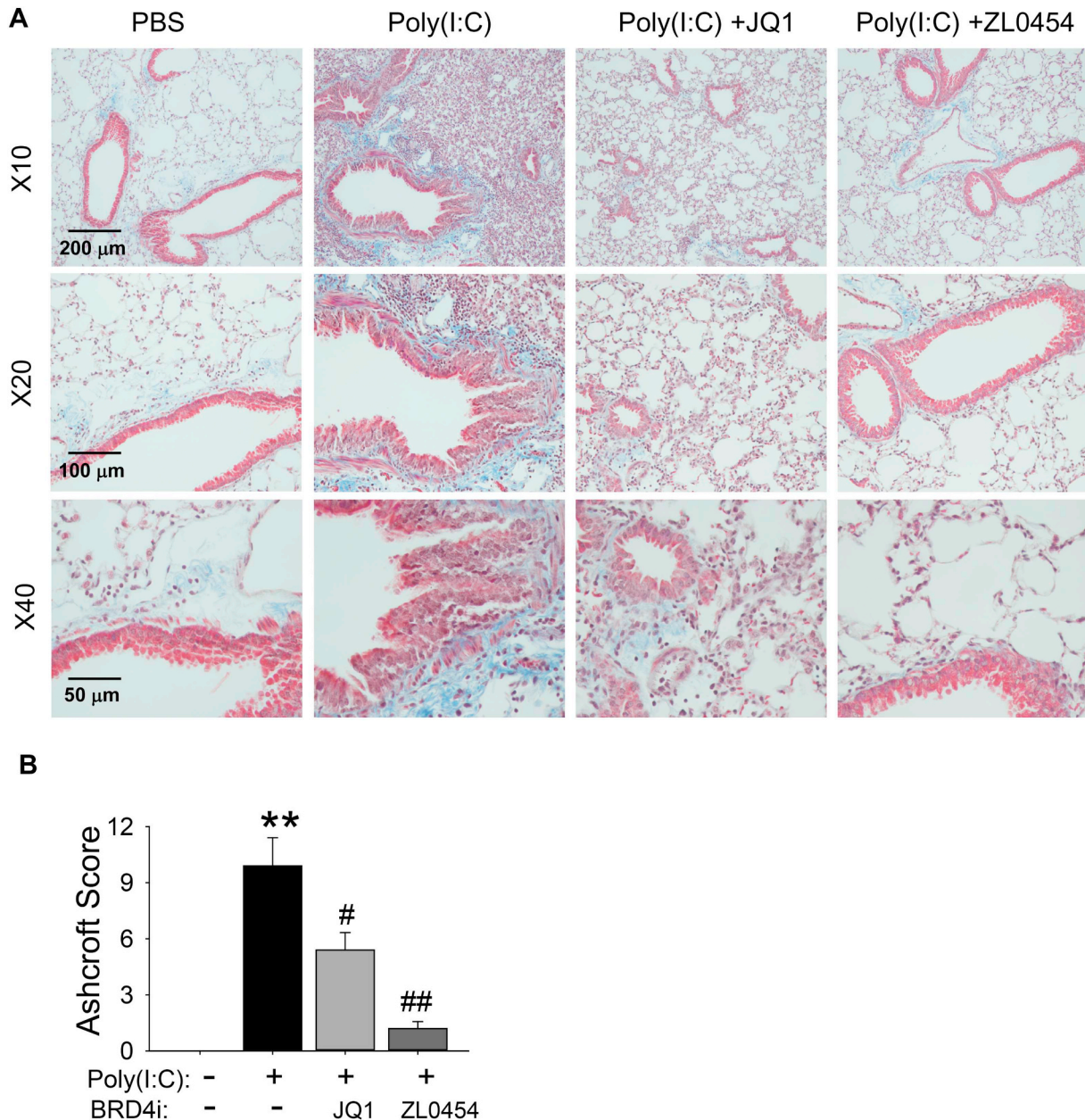


Fig. 1. Histopathology analysis of lung sections from animals treated with repetitive poly(I:C) in the presence or absence of BRD4 inhibitors, ZL0454 and JQ1. C57BL/6J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) ($n = 6$ per group, three times experiments). (A) Morphological changes of the lung after Masson trichrome staining. Top row, representative images at x10; middle row, images at x20; bottom row, images at x40. (B) Modified Ashcroft scoring for treatment groups. **Student's t -test p -value < .001 compared to PBS group. #Student's t -test p -value < .05 compared to poly(I:C) group. ##Student's t -test p -value < .001 compared to poly(I:C) group.

significantly increased leukocyte infiltration throughout the interstitium and induced subepithelial fibrosis with enhanced collagen distribution throughout the parenchyma (Fig. 1A, collagen in blue). The epithelium was flattened with loss of its brush-border appearance, and there were enhanced numbers of glandular cells. At higher power, we observed thickening of the alveolar septae. By contrast, the lungs of animals treated with BRD4 inhibitors had significantly less epithelial changes and interstitial collagen deposition. An over 9-fold increase in

the modified Ashcroft score observed in poly(I:C)-treated mice was significantly reduced by BRD4 inhibitors (Fig. 1B). We noted that ZL0454 had enhanced potency over the JQ1 prototype. The comparisons of the animals treated with JQ1 with those treated with ZL0454 show that the lung of animals treated with ZL0454 had more normal epithelial histology, interstitial cellularity, and reduced fibrosis. We examined the total number of cells in the BALF of four comparison group. We did not find significant difference in the total cell counts in

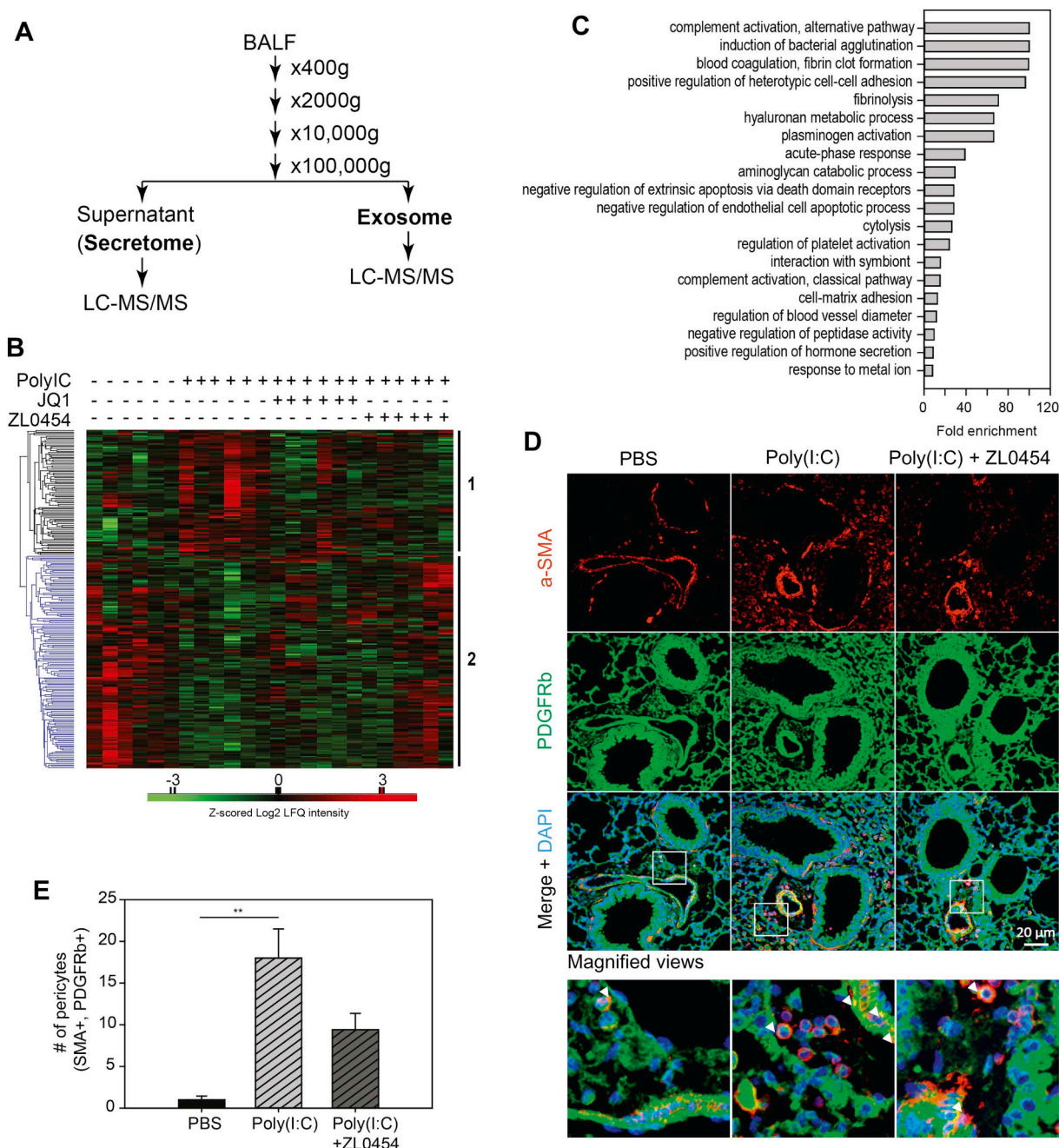


Fig. 2. BALF proteins associated with active remodeling and reversal by BRD4 inhibitors. (A) Workflow of quantitative proteomics analysis of BALF secretome and exosome. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) ($n = 6$ per group). (B). Hierarchical clustering of significantly changed proteins in the BALF secretome. Green, low level of expression, red, high expression. Cluster 1 has proteins upregulated by poly(I:C), but blocked by BRD4 inhibitors administration; cluster 2 has proteins downregulated by poly(I:C). (C). Genome ontology (GO) enrichment of biological process of protein in the upper cluster (black dendrogram) of Fig. 2B. The ranked GOBP are displayed along with fold enrichment of the pathway (grey bars) ($p < .05$ with Bonferroni correction for multiple testing). (D) Immunostaining of pericytes in the lung. Red, α -smooth muscle actin (α -SMA); green, platelet-derived growth factor receptor (PDGFRb); blue, DAPI for nuclear. Yellow, the differentiated pericytes (α -SMA + , PDGFRb +). (E) Immunostaining of the differentiated pericytes (α -SMA + , PDGFRb +) in the lung. Error bars are standard errors. **Student's t -test p -value $< .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BALF among the experimental groups (Supplemental Fig. S1). In contrast to the induction of neutrophilia in the acute poly(I:C) exposure [13], leukocyte infiltration had been cleared 12 days after the chronic poly(I:C) treatment, enabling us to focus on characterizing the remodeling process.

Next, we investigated how TLR3-mediated airway remodeling regulates the protein composition of the airway fluids. For this purpose, we conducted a label-free proteomics analysis of the proteins in BALF collected from the four groups of animals: control, poly(I:C), poly(I:C) + ZL0454, and poly(I:C) + JQ1. The BALF collected was separated into secretome (supernatant) and exosome fractions (Fig. 2A). The proteomics analysis of BALF secretome identified and quantified 939 proteins across all samples (Supplemental Table S2). Comparing the intensity of each protein in biological and technical replicates shows excellent agreement (Pearson correlation $r = 0.62\text{--}0.90$), confirming the robustness and reproducibility of the quantification.

Proteins identified in BALF could also originate from tissue leakage or cell lysis. We examined the composition of the secretome based on the annotation of Gene Ontology cellular components. Of 939 BALF secretome proteins, 297 proteins were previously categorized as “secreted” or “extracellular region”, 56 were transmembrane proteins, and 116 were plasma membrane proteins. In total, 382 proteins were released into BALF by either secretion or shedding of cell surface macromolecules. We also identified 127 nuclear proteins and 106 cytoplasmic proteins. We examined the MS intensity distribution of these categories in the BALF secretome (Supplemental Fig. S2). The secreted proteins and proteins shed from cell surface were highly regulated in BALF in comparison to cytoplasmic and nuclear proteins. For example, the MS intensity of uteroglobin (Scgb1a1), a Clara cell secretory protein, is about four orders of magnitude higher than the MS intensity of histone H4 in the BALF, and three orders of magnitude higher than the MS intensity of lysosomal alpha-mannosidase. Therefore, any proteins originating from tissue leakage or cell lysis artifacts impose minimum effect on our study.

The proteins in BALF secretome come from several types of airway cells. We used the markers of cell types described by Schiller et al. [27] to identify the cellular sources of secretome proteins. As shown in

Supplemental Fig. S3, markers of Clara cells, granulocytes, alveolar type 2 epithelial cells (AEC2), alveolar type 1 epithelial cells (AEC1), macrophages, and monocytes are more abundant than those of other cell types, suggesting that the proteins in the BALF secretome likely originated from innate immune cells and airway epithelial cells.

To identify the BALF proteins regulated by either TLR3-mediated airway remodeling or BRD4 inhibitors, we performed pair-wise comparison between the experimental groups: poly(I:C) vs. control, poly(I:C) + JQ1 vs. poly(I:C), poly(I:C) + ZL0454 vs. poly(I:C), poly(I:C) + JQ1 vs. control, poly(I:C) + ZL0454 vs. control, and poly(I:C) + ZL0454 vs poly(I:C) + JQ1, identifying 168, 7, 109, 62, 14, and 15 significant differences, respectively. As a result, we identified a total of 229 proteins whose abundance in BALF was significantly changed by the poly(I:C)-induced fibrotic response, or BRD4 inhibitors, or both. Unsupervised hierarchical clustering of these 229 proteins identified two clusters (Fig. 2B). The lower cluster includes the proteins whose abundances in BALF were reduced by the TLR3-induced remodeling. The genome ontology biological process (GOBP) enrichment analysis of that cluster found proteins with functions in various catabolic and metabolic processes, lipoprotein particle remodeling, and proteasome regulatory particle assembly (Supplemental Table S3). The top cluster represents proteins induced by repetitive poly(I:C) stimulation but blocked by BRD4 inhibitor administration. The GOBP enrichment analysis of the top cluster found proteins involved in acute inflammatory responses such as serum amyloid P-component (APCS), alpha-1-acid glycoprotein 2 (ORM2) and CD44 antigen (CD44), and also extracellular matrix (ECM) proteins such as fibronectin (FN1), SPARC-like protein 1 (SPARCL1), and collagen alpha-1(VI) chain (COL6A1) (Fig. 2C).

3.2. Chronic inflammation activates pericyte-myofibroblast transdifferentiation

Interesting to us, we found that the BALF levels of alveolar homeostasis proteins were significantly increased by repetitive poly(I:C) stimulation, an induction blocked by BRD4 inhibitors (Fig. 2B and C). For example, TLR3 stimulation increased the abundance of the

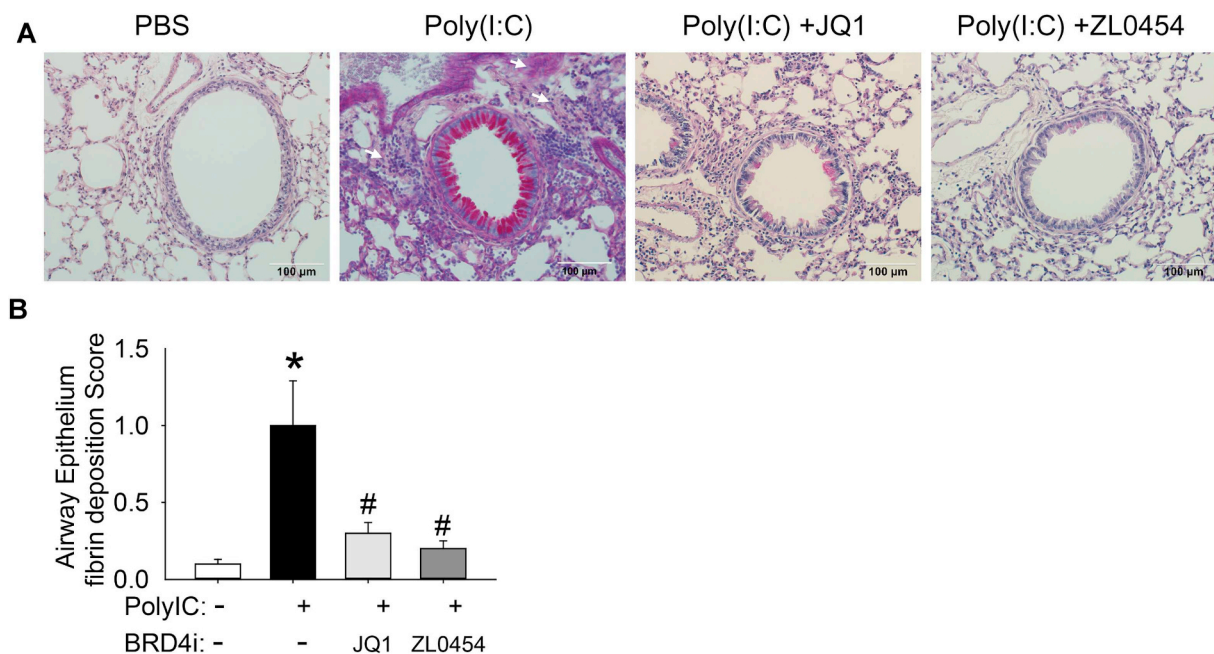


Fig. 3. Chronic poly(I:C) exposure induces fibrin deposition on the airway. C57BL/6J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) ($n = 6$ per group, three times experiments). (A) Histological examination of fibrin deposition on the airway with PAS staining. White arrows indicate the deposition of fibrin. (B) Airway epithelium fibrin deposition score. *Student's t -test p -value $< .05$ compared to PBS group. #Student's t -test p -value $< .05$ compared to poly(I:C) group.

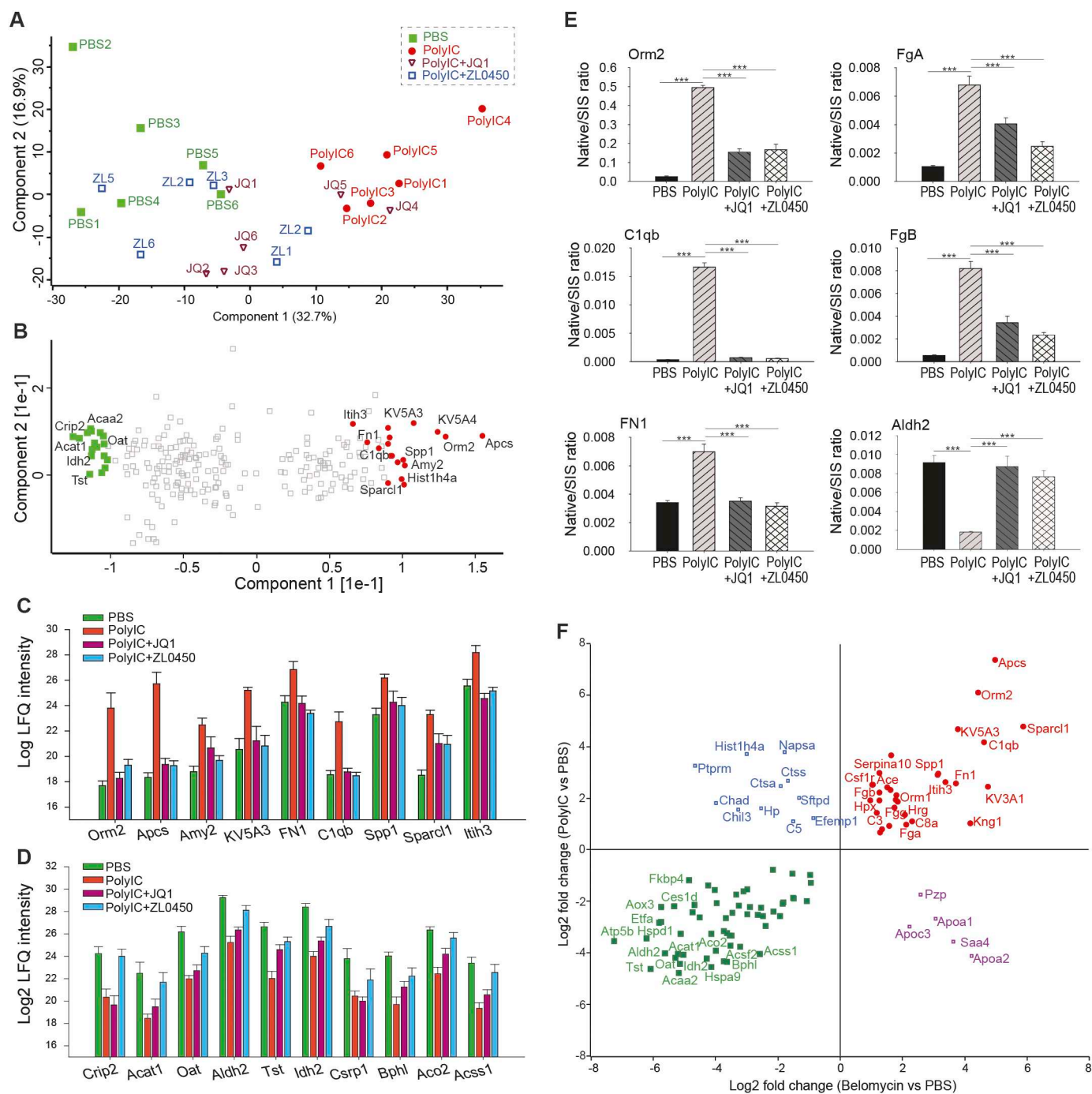


Fig. 4. The BALF secretome markers for pulmonary fibrosis and the efficacy of BRD4 inhibitors. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) ($n = 6$ per group). (A) Principal components analysis (PCA) of the groups. Red are individual animals from poly(I:C) induced fibrosis. PBS controls are in green. Animals with poly(I:C) + JQ1 are in purple. Animals with poly(I:C) + ZL0454 are in blue. (B) Loading plot of the variables (proteins) that lead to the group clustering shown in Fig. 4A. Red proteins are increased by poly(I:C), green proteins are decreased. (C) The abundance of proteins by treatment group. Shown are some proteins upregulated by poly(I:C)-induced airway remodeling and have the highest separation power in the PCA analysis. Bars are the mean of six animals in each group; the error bars are standard errors. (D) The abundance of proteins by treatment group. Shown are proteins downregulated by poly(I:C)-induced fibrosis and have the highest separation power in the PCA analysis. Bars are the mean of six animals in each group; the error bars are standard errors. (E) SID-SRM-MS validation of some selected significant proteins. Error bars are standard errors. **Student's *t*-test *p*-value < .05; ***Student's *t*-test *p*-value < .001. (F) Comparison of proteins which abundance in the BALF was significantly altered by poly(I:C) induced remodeling or bleomycin-induced fibrosis (data from Ref [27]). Note that a subset of the upregulated proteins in poly(I:C) are also upregulated in bleomycin, and similarly proteins downregulated in poly(I:C) are also downregulated by bleomycin treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

components of complement pathways (CFH, CFP, C8b, C8a, C5, C2, C1QB), blood coagulation products (FGG, FGA, FGB), and platelet activation (APOE, FGG, HRG) in the BALF. The presence of these plasma proteins in BALF suggest damage to the endothelial barrier and disruption of vascular homeostasis during chronic inflammation-remodeling. This observation led us to investigate the distribution of pericytes in the lung. Normal pericytes maintain microvascular homeostasis. Upon growth factor stimulation, pericytes transdifferentiate to myofibroblasts and then migrate into the tissue, creating an unstable vascular endothelium prone to leakage and ineffective angiogenesis

[28–30]. We quantified the number of pericyte-derived myofibroblasts using co-immunostaining for PDGFRB, a selective pericyte marker, and α -SMA, a marker for myofibroblasts in lung sections. As shown in Fig. 2D,E, the number of PDGFRB⁺/ α -SMA⁺ cells was significantly higher in animals treated with poly(I:C) than in controls, suggesting that TLR3 activation induced pericyte to myofibroblast transdifferentiation. By contrast, the number of PDGFRB⁺/ α -SMA⁺ cells was decreased in animals treated with poly(I:C) and ZL0454.

We examined the fibrin deposition on the airways of four comparison groups with Periodic acid–Schiff (PAS) staining (Fig. 3). PAS is a

Table 2

The BALF proteins markers for pulmonary fibrosis and efficacy of BRD4 inhibitors in treatment of pulmonary fibrosis.

Proteins Name	Gene Name	Uniprot Accession #	PolyIC vs. PBS		PolyIC vs. PolyIC+JQ1		PolyIC vs. polyIC+ZL0450	
			FC log2	p-value -log10	FC log2	p-value -log10	FC log2	p-value -log10
Serum amyloid P-component	APCS	P12246	7.37	6.69	6.35	5.45	6.44	5.76
Alpha-1-acid glycoprotein 2	ORM2	P07361	6.11	4.12	5.55	3.48	4.51	2.67
Pancreatic alpha-amylase	AMY2	P00688	3.69	4.47	1.81	1.02	2.80	3.41
Histone H4	HIST1H4A	P62806	3.72	3.82	0.21	0.07	4.18	5.21
Napsin-A	NAPSA	O09043	3.78	2.53	2.18	1.01	2.03	1.29
Osteopontin	SPP1	P10923	2.90	4.03	1.91	1.29	2.17	2.21
Microfibril-associated glycoprotein 4	MFAP4	Q9D1H9	3.03	1.92	4.13	2.73	2.58	1.23
Protein Z-dependent protease inhibitor	SERPINA10	Q8R121	2.99	2.05	2.48	1.38	1.66	0.97
SPARC-like protein 1	SPARCL1	P70663	4.78	7.93	2.28	1.83	2.34	2.14
Resistin-like alpha	RETNLA	Q9EP95	3.54	4.17	1.39	1.21	2.35	2.33
Dipeptidase 2	DPEP2	Q8C255	3.52	4.03	2.13	2.18	2.04	1.83
Complement C1q subcomponent subunit B	C1QB	P14106	4.17	4.14	3.94	3.95	4.24	4.36
Apolipoprotein E	APOE	P08226	1.74	1.23	4.13	2.85	5.18	4.95
Fibronectin	FN1	P11276	2.58	2.31	2.68	2.27	3.46	4.27
Angiotensin-converting enzyme	ACE	P09470	2.52	2.20	1.82	1.16	2.38	2.09
Cathepsin S	CTSS	O70370	2.67	2.48	1.08	1.04	1.41	2.64
Receptor-type tyrosine-protein phosphatase mu	PTPRM	P28828	3.27	3.60	0.84	0.95	1.31	1.44
Collagen alpha-1(VI) chain	COL6A1	Q04857	3.31	2.65	3.07	2.00	2.31	1.68
Lysosomal protective protein	CTSA	P16675	2.47	2.32	1.90	2.12	1.67	1.93
Inter-alpha-trypsin inhibitor heavy chain H3	ITI1H3	Q61704	2.63	2.60	3.63	4.55	3.03	4.06
Cysteine-rich protein 2	CRIP2	Q9DCT8	-3.91	3.36	0.69	0.27	-3.65	2.88
Acetyl-CoA acetyltransferase	ACAT1	Q8QZT1	-4.04	3.06	-1.03	0.69	-3.23	2.64
Cysteine and glycine-rich protein 1	CSRP1	P97315	-3.35	2.51	0.44	0.34	-1.45	0.71
Thiosulfate sulfurtransferase	TST	P52196	-4.64	5.49	-2.58	2.50	-3.30	3.59
3-ketoacyl-CoA thiolase	ACAA2	Q8BWT1	-4.78	4.96	-1.02	0.86	-1.84	1.34
Acyl-CoA synthetase family member 2	ACSF2	Q8VCW8	-4.32	5.12	-0.13	0.07	-1.75	1.02
Isocitrate dehydrogenase [NADP]	IDH2	P54071	-4.41	7.44	-1.38	1.68	-2.68	2.72
Stress-70 protein	HSPA9	P38647	-4.56	4.79	-2.10	2.30	-3.17	3.23
Pyruvate carboxylase	PC	Q05920	-4.21	6.83	-0.24	0.16	-2.78	2.64
Acetyl-coenzyme A synthetase 2-like	ACSS1	Q99NB1	-4.05	4.83	-1.23	1.07	-3.22	2.86
Methylmalonate-semialdehyde dehydrogenase	ALDH6A1	Q9EQ20	-4.21	4.96	0.13	0.07	-3.04	3.03
Serine/threonine-protein phosphatase 2B alpha	PPP3CA	P63328	-3.03	2.69	-1.17	0.73	-2.99	2.14
Electron transfer flavoprotein subunit beta	ETFB	Q9DCW4	-4.07	4.40	-0.82	0.40	-2.33	1.63
Valacyclovir hydrolase	BPHL	Q8R164	-4.34	4.96	-1.56	1.10	-2.53	1.68
Ornithine aminotransferase	OAT	P29758	-4.19	6.42	-0.73	0.64	-2.30	2.72
Aconitate hydratase	ACO2	Q99KI0	-3.91	5.13	-1.75	1.42	-3.19	3.33
Aldehyde dehydrogenase	ALDH2	P47738	-4.01	6.09	-1.11	1.06	-2.87	3.29
Glutathione S-transferase theta-1	GSTT1	Q64471	-2.85	2.32	-0.29	0.12	-2.20	0.95
Talin-1	TLN1	P26039	-2.44	2.19	-0.81	0.81	-2.20	1.44
Galactokinase	GALK1	Q9R0N0	-2.58	2.03	-0.82	0.47	-2.00	1.32

FC, fold change. The abundance levels that were significantly changes were highlighted in red (elevated) and green (decreased).

histological marker of epithelial mucin and fibrin deposition. The histological image of the lung shows poly(I:C) induced a significant increase of PAS staining in the epithelial border and the interstitium (Fig. 3A). The interstitial PAS is due local fibrin deposition; we observe that BRD4 inhibitors reduced the fibrin deposition. We further measured the interstitial fibrin deposition score for the four comparison groups. As shown in Fig. 3B, poly(I:C) produced a marked increase in the fibrin deposition score, and this effect was also blocked by the treatment of BRD4 inhibitors.

3.3. BALF secretome profiles are indicative of the remodeling and the response of therapy

A principal component analysis (PCA) of the 229 significant proteins in Fig. 2B confirmed that these proteins contained sufficient information to quantitatively separate the poly(I:C) group from the control group (Fig. 4A). Secondly, BRD4 inhibitor-treated groups, especially the ZL0454 group, were not only separate from the poly(I:C) group but largely overlapped with controls. By contrast, the distribution of individual animals treated with JQ1 was mixed, with two animals still overlapping the poly(I:C) group. These data suggest that selective BRD4 inhibition with ZL0454 more effectively restores the normal BALF secretome than the less potent, nonselective JQ1.

Next, we identified the major proteins responsible for the separation (the “loadings” of the multidimensional PCA) of the inflammation-

remodeling group from the control, JQ1, and ZL0454 treatment groups. Results are highlighted in red and green in Fig. 4B and tabulated in Table 2. The proteins on the far right of Fig. 4B include APCs, ORM2, pancreatic alpha-amylase (AMY2), osteopontin (SPP1), SPARCL1, and FN1. The logarithm-transformed abundance levels of these proteins in BALF are plotted in Fig. 4C. We noted significantly elevated abundance of these proteins in the poly(I:C) treated BALF in comparison to controls, and their abundance was restored to normal with the treatment of BRD4 inhibitors. On the far left of the loading plot (Fig. 4B) is a group of proteins whose abundances were reduced in the BALF of animals treated with poly(I:C) in comparison to controls. The logarithm-transformed abundances of these proteins in BALF are plotted in Fig. 4D. We used quantitative mass spectrometry (SID-SRM-MS) to validate the expression of some proteins related to inflammation response (ORM2), ECM secretion (FN1), homeostasis (C1QB, FGA, FGB) as well as a protein (ALDH2) with reduced abundance in BALF of animals treated with poly(I:C). These assays confirmed the increased inflammation response, elevated secretion of ECM proteins such as FN1, increased plasma proteins (ORM2, C1QB, fibrinogens), as well as the reduced secretion of ALDH2 in the poly(I:C) model. Moreover, the BRD4 inhibitors treatment reversed the secretory profiles of these proteins close to normal (Fig. 4E). These data show that the BALF abundances of these proteins were restored to normal with the highly selective BRD4 inhibitor treatment, but not with JQ1, consistent with the inability of JQ1 to restore protein secretory profiles to normal in PCA.

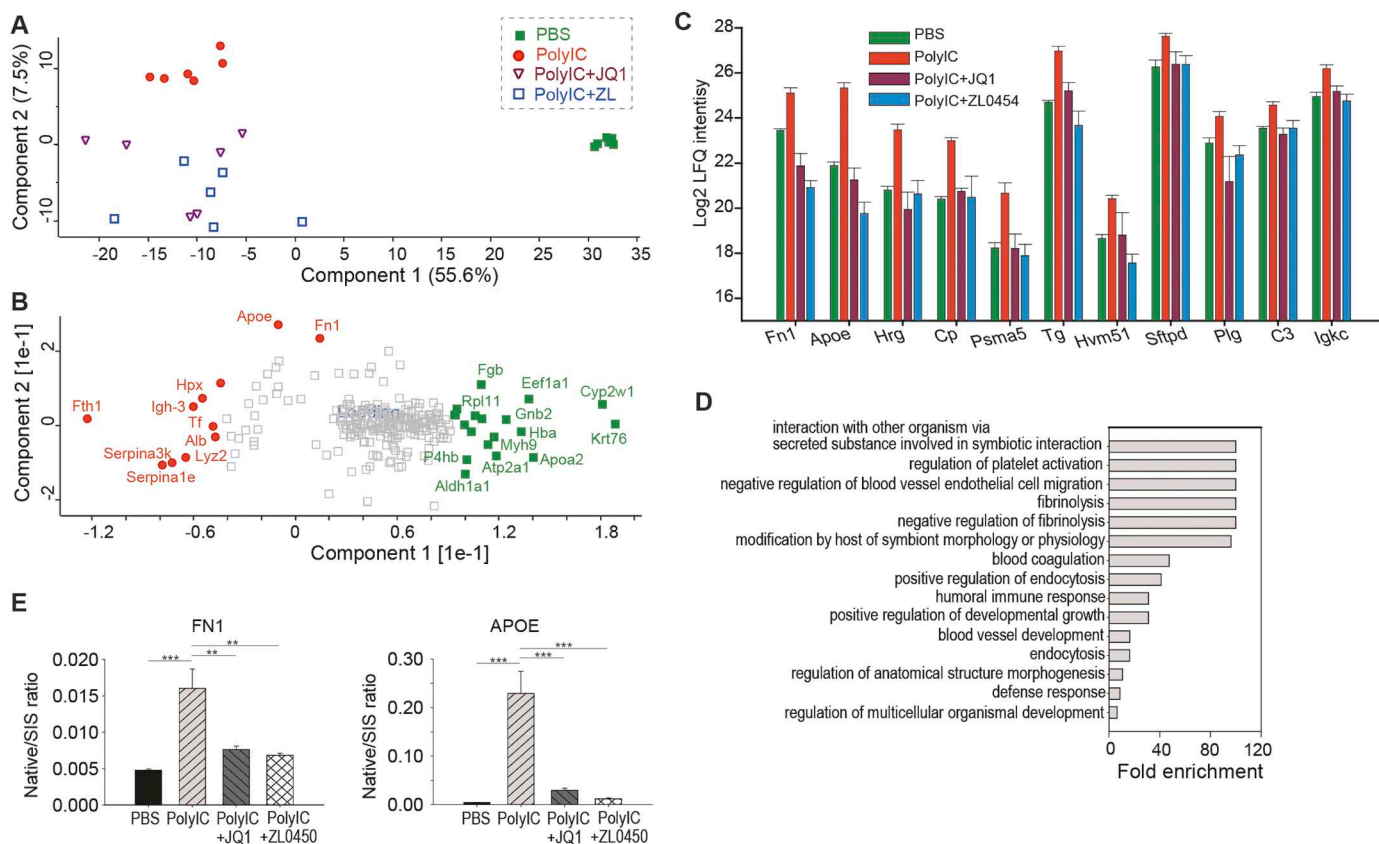


Fig. 5. Exosomal proteins associated with airway remodeling and reversal by BRD4 inhibitors. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) ($n = 6$ per group). (A) Principal components analysis (PCA) of the groups. Red are individual animals from poly(I:C) induced fibrosis. PBS controls are in green. Animals with poly(I:C) + JQ1 are in purple. Animals with poly(I:C) + ZL0454 are in blue. (B) Loading plot of the variables (proteins) that lead to the group clustering shown in Fig. 4A. Red proteins are increased by poly(I:C), green ones are decreased. (C) The abundance of proteins by treatment group. Shown are proteins upregulated by poly(I:C)-induced fibrosis and reversed by BRD4 inhibitors. Bars are the mean of six animals in each group; the error bars are standard errors. (D) The ranked GOBPs are displayed along with fold enrichment of the pathway (grey bars) ($p < .05$ with Bonferroni correction for multiple testing). (E) SID-SRM-MS validation of selected exosome proteins which abundance in the BALF exosome was significantly upregulated by repetitive poly(I:C) stimulation and reversed by BRD4 inhibitors. Error bars are standard errors. **Student's t -test p -value $< .05$; ***Student's t -test p -value $< .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The bleomycin model is a well-accepted murine model of pulmonary fibrosis. It mimics the pathology and gene expression profile of human idiopathic pulmonary fibrosis (IPF) [31]. We compared the proteins identified in the BALF secretome from the poly(I:C) induced remodeling with those identified in a previous proteomics study of BALF collected 14 days after the bleomycin treatment [27]. The reason that we chose this time point is because the maximal fibrogenesis occurred 14 days after bleomycin-induced injury [27]. We found 104 proteins present in both datasets. The majority (88 out of 104) had similar relative changes in the BALF in both models (Fig. 4F), confirmed by a Pearson correlation coefficient of 0.75. For example, we observed significant elevations of APCS, ORM2, SPARCL1, C1QB, FN1, and SPP1 in the BALF of animals treated with poly(I:C), proteins also elevated in the BALF of bleomycin mice. Similarly, we found 57 proteins such as ALDH2, OAT, TST, and ACAA2 with decreased abundances in the BALF of both animal models. Although the poly(I:C) and bleomycin treatment induces different mechanisms of injury, the significant overlap of the

BALF markers suggests that inflammation, vascular injury, and ECM secretion are a common airway-remodeling response in both animal models. Moreover, these BALF proteins have the potential to be universal markers for injury-induced pulmonary remodeling.

3.4. Quantitative proteomics analysis of BALF exosomes

Extracellular nanovesicles (exosomes) contain biological molecules whose abundance is modified by TLR3-induced inflammation [17]. Because of their potential for biomarker development, we investigated whether the protein content in the BALF exosomes was regulated by TLR3-induced inflammation and/or affected by BRD4 inhibitors. For this purpose, we conducted a label-free proteomics analysis of BALF exosomes collected from the four treatment groups. In our previous study, we showed the method we used for isolating exosomes from BALF to yield high-quality exosomes with minimal contamination [17]. We examined the quality the exosomes isolated with this method. First,

Table 3

The BALF exosome proteins markers for pulmonary fibrosis and efficacy of BRD4 inhibitors in treatment of pulmonary fibrosis.

Proteins Name	Gene Name	Uniprot Accession #	PolyIC vs. PBS		PolyIC vs. PolyIC+JQ1		PolyIC vs. polyIC+ZL0450	
			FC log2	p-value -log10	FC log2	p-value -log10	FC log2	p-value -log10
Ferritin heavy chain	FTH1	P09528	5.89	3.98	0.47	0.44	0.90	1.19
Ig gamma-2B chain C region	IGH-3	P01867	3.50	2.46	0.59	0.21	1.50	0.68
Apolipoprotein E	APOE	P08226	3.45	6.66	4.09	4.47	5.58	5.84
Hemopexin	HPX	Q91X72	3.21	7.40	1.22	2.65	1.28	2.14
Serine protease inhibitor A3K	SERPINA3K	P07759	2.22	3.31	-1.37	1.64	-1.75	1.51
Serotransferrin	TF	Q92111	2.09	6.91	-0.03	0.09	0.03	0.05
Alpha-1-antitrypsin 1-5	SERPINA1E	Q00898	2.06	1.31	-1.73	2.38	-1.48	1.67
Lysozyme C-2	LYZ2	P08905	1.95	4.75	-1.24	1.54	-1.10	1.59
Serum albumin	ALB	P07724	1.70	6.17	-0.33	0.76	-0.76	2.21
Fibronectin	FN1	P11276	1.66	4.42	3.24	3.54	4.20	6.16
Fibrinogen beta chain	FGB	Q8K0E8	-3.17	3.37	2.49	2.38	2.08	1.57
Hemoglobin subunit beta-1	HBB-B1	P02088	-3.49	2.68	1.57	0.99	0.37	0.16
78 kDa glucose-regulated protein	HSPA5	P20029	-3.65	5.11	1.00	0.77	0.05	0.02
Membrane-associated progesterone receptor component 1	PGRMC1	O55022	-3.69	5.16	0.00	0.00	0.00	0.00
40S ribosomal protein S11	RPS11	P62281	-3.74	6.01	0.00	0.00	0.00	0.00
60S ribosomal protein L11	RPL11	Q9CXW4	-4.47	6.59	0.00	0.00	0.00	0.00
Retinal dehydrogenase 1	ALDH1A1	P24549	-4.52	2.75	0.43	0.10	-2.24	0.70
Glutamine synthetase	GLUL	P15105	-4.68	7.96	-0.24	0.14	0.00	0.00
40S ribosomal protein S4, X isoform	RPS4X	P62702	-4.69	8.16	0.00	0.00	0.00	0.00
Myosin-14	MYH14	Q6URW6	-4.99	7.11	0.48	0.42	-0.96	0.83
Protein disulfide-isomerase	P4HB	P09103	-5.04	6.48	-0.80	0.43	-1.72	0.78
Myosin-9	MYH9	Q8VDD5	-5.11	7.04	0.23	0.17	-0.45	0.21
Elongation factor 1-alpha 1	EEF1A1	P10126	-5.41	6.81	0.00	0.00	0.00	0.00
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2	P62880	-5.43	7.19	0.00	0.00	0.00	0.00
Sarcoplasmic/ER calcium ATPase 1	ATP2A1	Q8R429	-5.75	7.48	-1.84	0.62	-0.33	0.12
Hemoglobin subunit alpha	HBA	P01942	-6.04	5.93	-1.03	0.46	0.00	0.00
Apolipoprotein A-II	APOA2	P09813	-7.10	6.21	-1.16	0.35	-3.89	1.23
R3H and coiled-coil domain-containing protein 1	CYP2W1	E9Q816	-7.63	6.80	0.00	0.00	0.00	0.00
Keratin, type II cytoskeletal 2 oral	KRT76	Q3UV17	-8.34	7.77	0.00	0.00	0.00	0.00

FC, fold change. The abundance levels that were significantly changes were highlighted in red (elevated) and green (decreased).

we used transmission electron microscopy (TEM) to examine the ultrastructure of exosomes. As shown in Supplemental Fig. S4A, the BALF exosomes appeared in TEM as isolated, membrane-encapsulate nanovesicles. Next, we measured the average size of exosomes from the four experimental groups ($n = 6$ for each experimental group). The average size of BALF exosomes is about 100 nm, which is in the range of nanovesicles. We do not find the significant variation in the exosome size from one experimental group to another (Supplemental Fig. S4B). To check the purity of the BALF exosomes, we measured the abundance of exosomal markers including tumor susceptibility gene 101 protein (TSG101), annexin A2 (ANXA2), fructose-bisphosphate aldolase A (ALDOA), and guanine nucleotide-binding protein G(i) subunit alpha-2 (GNAI2) with SID-SRM-MS (Supplemental Fig. S4C) and programmed cell death 6-interacting protein (PDCD6IP/Alix) with western blot. These proteins are top exosome markers according to ExoCarta, a compendium of exosome cargo [32]. As shown in Supplemental Fig. S4D, these exosomal markers were significantly enriched in BALF exosomes in comparison to the lysate of lung tissue, while the GRP94, a marker of endoplasmic reticulum, was only detected in the tissue lysate, which indicates the purity of the exosome preparation.

We identified and quantified 340 proteins (Supplemental Table S4). Comparing the intensity of each protein in biological and technical replicates shows excellent agreement ($r = 0.69$ – 0.97), confirming the robustness and reproducibility of the quantification. We performed pair-wise comparison between the experimental groups – poly(I:C) vs. control, poly(I:C) + JQ1 vs. poly(I:C), poly(I:C) + ZL0454 vs. poly(I:C), poly(I:C) + JQ1 vs. control, poly(I:C) + ZL0454 vs. control, and poly(I:C) + ZL0454 vs. poly(I:C) + JQ1 – and identified 220, 4, 8, 207, 185, and 0 significant hits, respectively. A total of 245 exosome proteins whose levels were significantly changed by either the poly(I:C)-induced remodeling, or BRD4 inhibitor treatment, or both. A PCA of the 245 significant exosomal proteins in Fig. 5A confirmed the successful quantitative separation between the poly(I:C) groups and the control group. We examined the loading plot of the proteins driving the separation in PCA analysis (Fig. 5B), and the proteins with the highest power to separate the inflammation-remodeling group from the control, JQ1 and ZL0454 treatment are listed in Table 3. The logarithm-transformed abundance levels of the proteins significantly elevated in BALF exosome by poly(I:C) and restored to normal by BRD4 inhibitors are plotted in Fig. 5C. The GOBP enrichment analysis identified those protein functions in alveolar hemostasis (C3, HRG, TG, CP, APOE, PLG) and fibrotic response (FN1) (Fig. 5D). We used SID-SRM-MS to validate

the expression of some BALF exosome proteins (FN1 and APOE). These assay confirmed the increased secretion of FN1 and APOE in the BALF exosome in the poly(I:C) model. BRD4 inhibitors treatment reversed the abundance of these proteins to normal (Fig. 5E). One interpretation for these data is that BRD4 inhibitors are affecting secretion of exosomes derived from vascular injury.

3.5. Validation of significant proteins using human BALF from patients with severe asthma

Next, we applied our highly quantitative SRM assays to significantly regulated coagulation and ECM proteins identified in the mouse study to investigate whether the abundance of these proteins is altered in airway fluids of humans with severe asthma. BALF samples were prepared from patients with severe asthma and normal volunteers undergoing research bronchoscopy after informed consent. These assays showed that the the BALF of severe asthmatics had significantly increased 3-fold abundance of ORM2, APCS, SPARCL1, FGA, and FN1 (Fig. 6). These data validate the presence of vascular leak and ECM proteins in severe asthma, suggesting the activation of a common pathway of inflammation-remodeling in this disease.

4. Discussion

Recurrent viral upper respiratory tract infections are linked to a progressive decline in pulmonary function in patients with asthma and obstructive lung diseases [33,34]. Previously, we standardized an animal model of repetitive TLR3 activation which produces many clinically-observed aspects of airway remodeling [10,11]. We found that cellular adaptive epigenetic reprogramming and structural remodeling produced by innate inflammation is mediated by the BRD4 histone acetyltransferase [9–12]. We here used systems-level pharmacoproteomics to understand the effects of TLR3-NF κ B/RelA-mediated airway remodeling in the presence or absence of BRD4 inhibitors. Our major findings are vascular injury, pericyte transdifferentiation into myofibroblasts, and increased vascular leakage consistently present during airway remodeling. These protein signatures of vascular leakage in airway secretions are all reversible by BRD4 inhibition. We observed that these selective plasma leak proteins include components of the classical complement pathways, blood coagulation products, and platelet activation. This selective vascular leakage is not due to microhemorrhage because the composition of plasma proteins is not identical

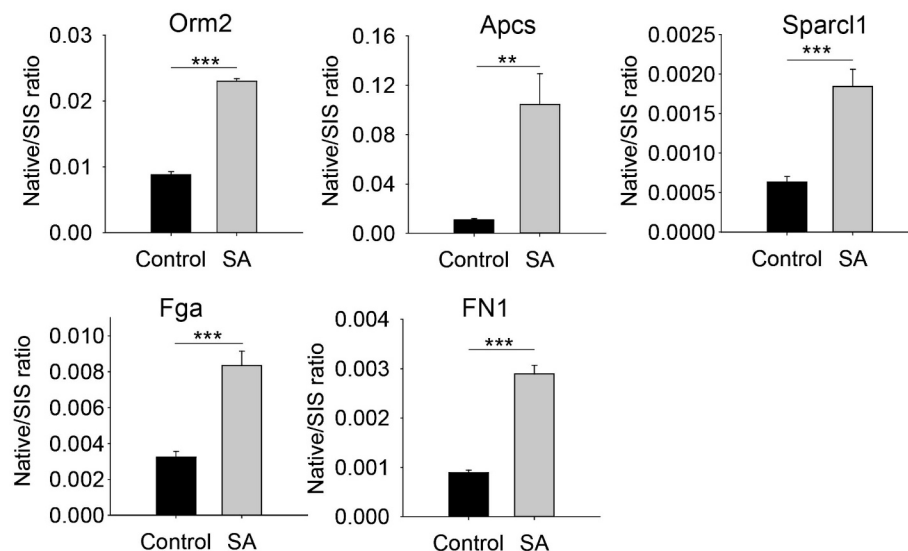


Fig. 6. SID-SRM-MS validation of selected significant proteins using the BALF from patients with severe asthma. Error bars are standard errors. **Student's t -test p -value < .05; ***Student's t -test p -value < .001.

to that of plasma. Moreover, the presence of fibrin deposition suggested by the PAS staining in the lung interstitium, suggests that the coagulation pathway is activated in TLR3-induced remodeling. The persistent and excessive deposition of extravascular fibrin and collagen observed in our model is also typical in fibrotic lung diseases such as sarcoidosis, hypersensitivity pneumonitis and IPF [35,36], potentially indicating overlapping pathomechanisms.

Our previous mechanistic studies pointed to BRD4 as a central mediator of the epithelial-mesenchymal transition by activating fibrotic gene programs as a result of NF κ B/RelA mediated chromatin remodeling [9,11–13,37]. Previously, we developed and validated a BRD4 inhibitor, ZL0454, which shows no evidence of systemic toxicity at doses that interfere with BRD4 action in vivo [10,14,15]. At the molecular level, ZL0454 blocks BRD4 activity by displacing BRD4 from high-affinity chromatin binding sites and disrupting the multiple protein-protein interactions necessary for BRD4 function [9,13]. We previously showed that ZL0454 reversed TLR3-associated airway hyperresponsiveness and increased lung compliance in vivo [10]. Here, our pharmacoproteomics findings indicate that ZL0454 is more potent in reducing the vascular leakage and ECM deposition than the non-selective BET inhibitor, JQ1.

In the present study, we found that dual-labeled PDGFR⁺/ α -SMA⁺ cells accumulate in the subepithelial region in the lung (Fig. 2D), and BRD4 inhibitors reverse this process in the poly(I:C) model. Also, our finding that BRD4 inhibition effectively reduced inflammation-induced vascular leakage and its protein markers in lung secretions suggests that BRD4 mediates selective alveolar capillary leakage. Several mechanisms could explain this finding. One is that TLR3 activation induces the release of a plethora of fibrogenic cytokines, enzymes, metabolites, and growth factors that induce the transdifferentiation of homeostatic pericytes as a paracrine mechanism. A second explanation is that BRD4 also directly acts in myofibroblast transdifferentiation gene expression in the pericyte. BRD4 mediates myofibroblast transdifferentiation in hypertrophic scar dermal fibroblasts [38]. Which pathway of pericyte transdifferentiation is operative in inflammation-mediated remodeling will require further investigation.

Airway exosomes are involved in regulating bronchial hyperresponsiveness, inflammation, antiviral responses, and macrophage chemotaxis [39–42]. In previous proteomics analysis of the exosome released from the airway epithelial cells infected with RSV, we found that the protein content of exosomes differed by cell type (small airway epithelial cells vs. bronchial epithelial cells) and RSV infection reprogrammed their protein cargo. Data provided herein show that BALF exosome content is affected by inflammation-remodeling. The increased presence of exosome components originating from blood such as C3, HRG, TG, CP, APOE, and PLG suggest that blood exosomes may leak into the alveolar space as a result of vascular leakage and pericyte transdifferentiation.

BALF has long been used for clinical differential diagnosis of lung diseases because of its vicinity to airway cells and simpler protein composition than plasma [43,44]. We here identified many BALF secretory and exosome proteins (Tables 2 and 3) that could differentiate remodeling from control and also indicate treatment efficacy. Of these, APCS, SPP1, FN1 and SPARCL1 show particular mechanistic importance. APCS is a member of the pentraxin family with a profound effect on fibrosis development and innate immune system regulation [45–48]. SPP1 is a pro-fibrotic cytokine released from the alveolar epithelium and a well-established marker of IPF [49]. The elevation of SPP1, FN1, and SPARCL1 are consistent with our previous findings that inflammation-remodeling activates TGF β signaling and excessive production of ECM proteins [12]. Interestingly, we found a significant overlap of over 101 proteins in the BALF markers in repetitive TLR3-stimulation model and those induced by bleomycin-mediated epithelial injury. The similarity of secretome responses implies some shared cellular and molecular mechanisms. This finding paves the way to identify common mechanisms of airway remodeling that could be used to target

many respiratory disorders therapeutically.

5. Conclusion

In summary, our pharmacoproteomics study has significantly advanced our understanding of the mechanisms of TLR3-NF κ B/RelA-mediated remodeling, and the effects of epigenetic inhibitors in preventing this phenomenon. Our validation of the upregulation of ORM2, APCS, SPARCL1, FGA, and FN1 in BALF of patients with severe asthma, suggests that these may be candidate biomarkers of airway remodeling or components of dimensionless panels. Because imaging is not sensitive enough to detect airway remodeling, developing sensitive biochemical markers of remodeling will have substantial clinical impact on the monitoring of airway remodeling disease and monitoring the efficacy of anti-remodeling treatment.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2019.103415>.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Author contributions

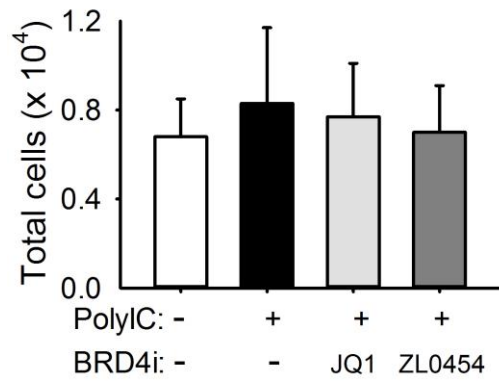
YXZ and ARB conceived, designed and initiated the study. BT, HS, JZ, YQZ, YXZ, RM, and ARB conducted the experiments. YXZ, JZ, YQZ, BT, MI, HS, RM, and ARB analyzed the data and interpreted results. YXZ and ARB wrote the manuscript. All authors read the manuscript and discussed the interpretation of results.

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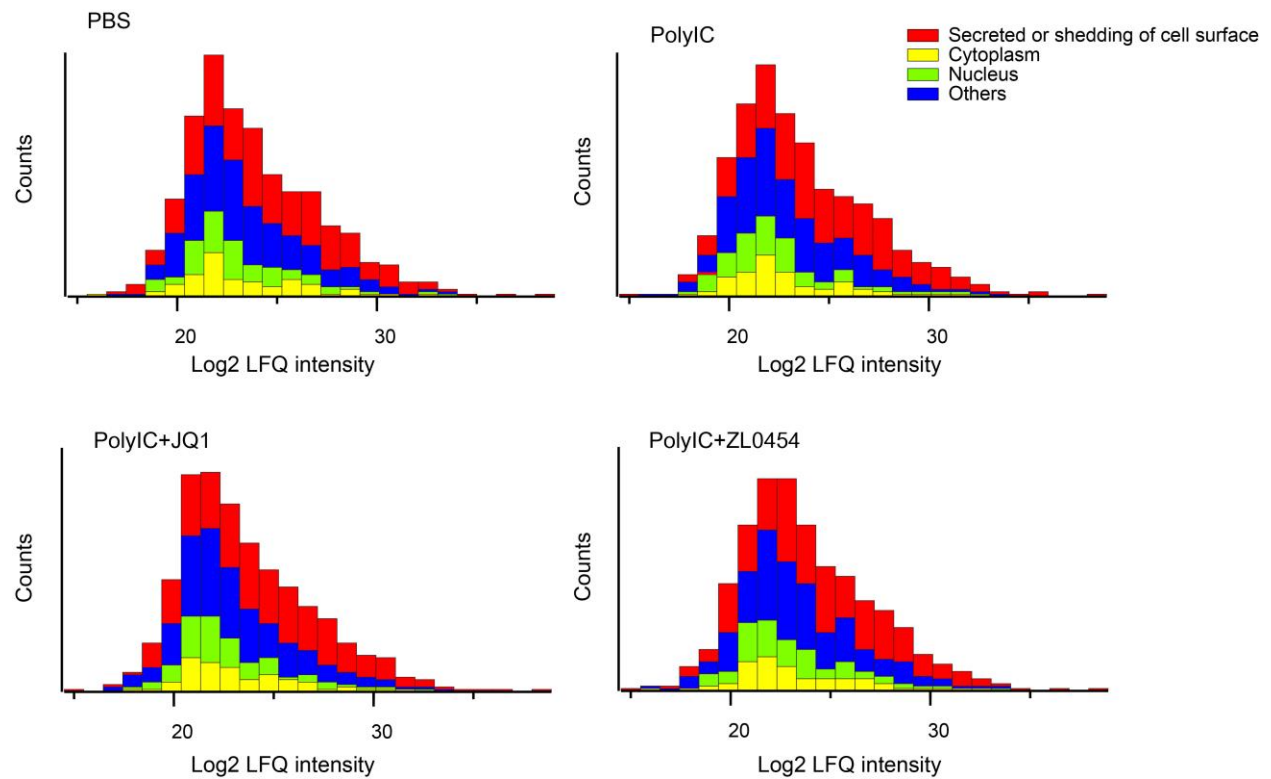
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Supplemental Figure S1



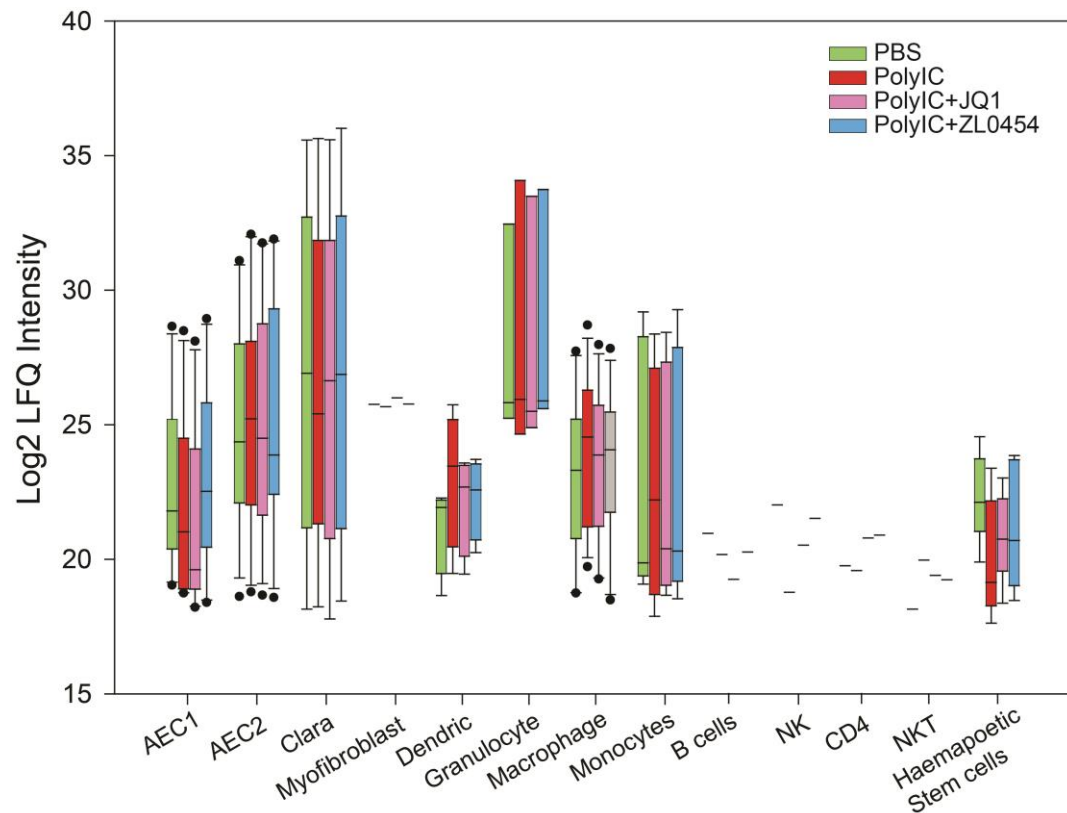
Supplemental Figure S1 Total cell counts in the BALF. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) (n=6 per group, three times experiments).

Supplemental Figure S2



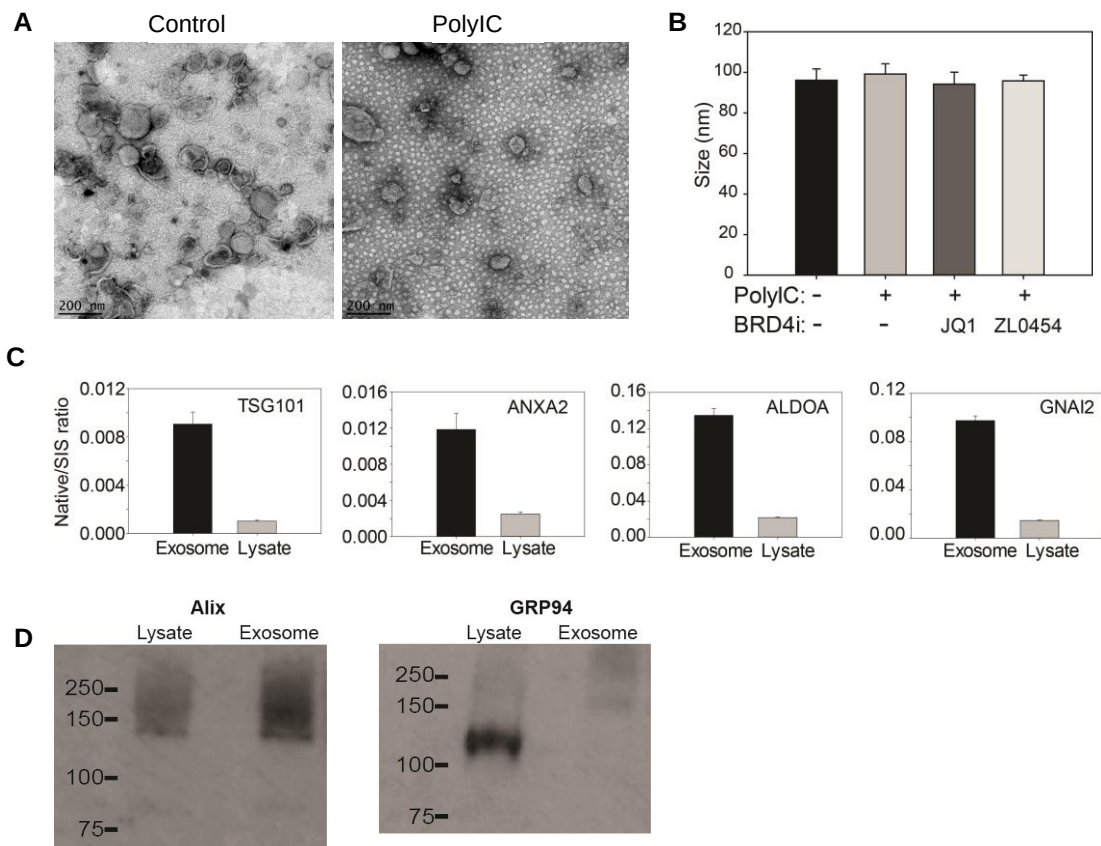
Supplemental Figure S2. The histogram depicts MS intensity distributions of the indicated categories in the BALF secretome. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) (n=6 per group). X-axis, the median MS intensity of proteins (n=6 per group).

Supplemental Figure S3



Supplemental Figure S3. Box plots of MS intensity distributions of the markers of airway cells in the BALF secretome. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) (n=6 per group). AEC, alveolar type 2 epithelial cells; NK, natural killer cells; NKT, natural killer T cells.

Supplemental Figure S4



Supplemental Figure S4. Characteristics of BALF exosomes. C57BL6/J mice were pretreated with and without BRD4 inhibitors (JQ1 or ZL0454) and underwent repetitive intranasal administration of poly(I:C) (n=6 per group, three times experiments). (A) Transmission electron microscopy images of BALF exosomes. (B) Size of BALF exosomes. Shown is the average size for the BALF exosome preparations (n=6 for each experimental group). Error bar, the standard deviation. (C) SID-SRM-MS analysis of exosome markers. The abundance of exosome markers in the BALF exosome was compared with the abundance in the tissue lysate. Error bar, the standard deviation. (D) Western blot of exosomal marker Alix and marker of endoplasmic reticulum, GRP94.