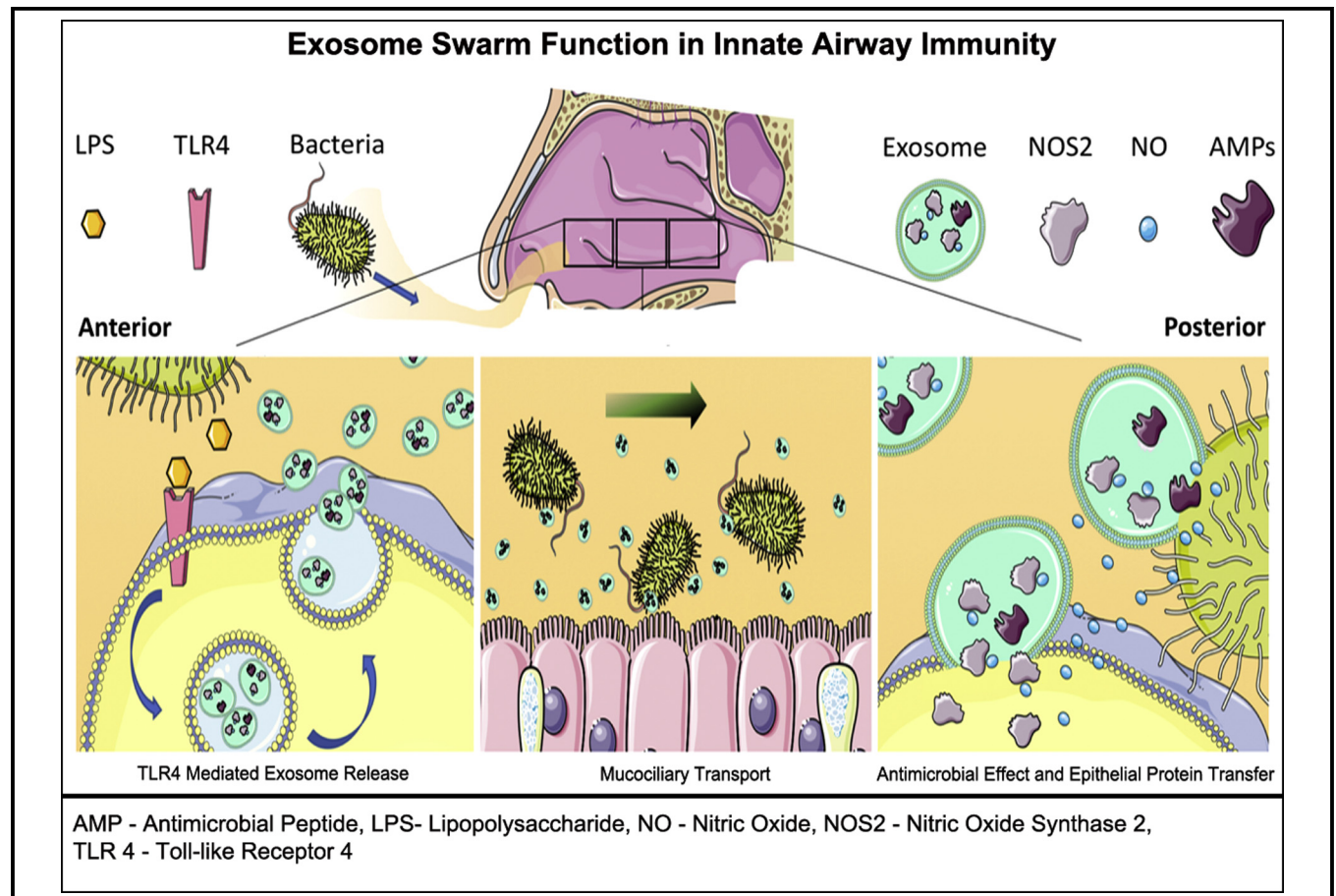


Exosome swarms eliminate airway pathogens and provide passive epithelial immunoprotection through nitric oxide

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GRAPHICAL ABSTRACT



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Canon, Arrinex, and Storz; has provided expert testimony on ENT-related cases; has a patent for P-gp inhibition for chronic rhinosinusitis and receives royalties from this patent; and has stock/stock options in Interscope. The rest of the authors declare that they have no relevant conflicts of interest.

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Background: Nasal mucosa–derived exosomes (NMDEs) harbor immunodefensive proteins and are capable of rapid interepithelial protein transfer.

Objectives: We sought to determine whether mucosal exposure to inhaled pathogens stimulates a defensive swarm of microbiocidal exosomes, which also donate their antimicrobial cargo to adjacent epithelial cells.

Methods: We performed an institutional review board–approved study of healthy NMDE secretion after Toll-like receptor (TLR) 4 stimulation by LPS (12.5 µg/mL) in the presence of TLR4 inhibitors. Interepithelial transfer of exosomal nitric oxide (NO) synthase and nitric oxide was measured by using ELISAs and NO activity assays. Exosomal antimicrobial assays were performed with *Pseudomonas aeruginosa*. Proteomic analyses were performed by using SOMAscan.

Results: *In vivo* and *in vitro* LPS exposure induced a 2-fold increase in NMDE secretion along with a 2-fold increase in exosomal inducible nitric oxide synthase expression and function through TLR4 and inhibitor of nuclear factor κB kinase activation. LPS stimulation increased exosomal microbiocidal activity against *P aeruginosa* by almost 2 orders of magnitude. LPS-stimulated exosomes induced a 4-fold increase in NO production within autologous epithelial cells with protein transfer within 5 minutes of contact. Pathway analysis of the NMDE proteome revealed 44 additional proteins associated with NO signaling and innate immune function.

Conclusions: We provide direct *in vivo* evidence for a novel exosome-mediated innate immunosurveillance and defense mechanism of the human upper airway. These findings have implications for lower airway innate immunity, delivery of airway therapeutics, and host microbiome regulation. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: Exosome, innate immunity, CD81, epithelium, sinonasal mucus, nitric oxide, proteomics

The human nasal cavity is one of the first sites of contact between the external environment and the interior of the body. Consequently, the nasal mucosal barrier elaborates an array of protective mechanisms to defend against constant exposure to inhaled extrinsic pathogens.

Among the most widely studied components of nasal mucosal innate immunity are bulk mucociliary clearance (MCC)¹ and epithelial expression of Toll-like receptors (TLRs). TLRs are germline-encoded pattern recognition receptors (PRRs) stimulated by microbial structural motifs known as pathogen-associated molecular patterns (PAMPs).^{2,3} Activation of anterior nasal mucosal TLRs by PAMPs have been shown to induce release of antimicrobial peptides (AMPs),^{4–6} which exert direct microbiocidal effects on the invading pathogens,⁷ into the mucus microenvironment, whereas MCC mechanically removes the foreign material within 15 to 20 minutes of exposure.¹ A paradoxical side effect of MCC is that posterior epithelial cells are, by necessity, exposed to trapped microbes within the mucus, which might still be viable as they are swept posteriorly into the nasopharynx. During this time, the protective AMPs are subject to degradation through a host of proteolytic enzymes,⁸ which limit their effective half-life and leave the posterior epithelium vulnerable to the pathogen. Therefore it remains an open question as to how the immune system mitigates these maladaptive consequences of MCC.

Abbreviations used

AMP:	Antimicrobial peptide
BEGM:	Bronchial epithelial growth medium
CTCF:	Corrected total cell fluorescence
HSNECC:	Human sinonasal epithelial cell culture
IKK2:	Inhibitor of nuclear factor κB kinase
MCC:	Mucociliary clearance
NMDE:	Nasal mucosa–derived exosome
NO:	Nitric oxide
NOS2:	nitric oxide synthase
PAMP:	Pathogen-associated molecular pattern
PRR:	Pattern recognition receptor
PVA:	Polyvinyl alcohol
TLR:	Toll-like receptor
TSB:	Tryptic soy broth
UCF:	Ultracentrifugation

Exosomes are 30- to 150-nm, tetraspanin-enriched micro-vesicles,⁹ which are secreted into almost all body fluids, including blood, lymph, and nasal mucus.^{10–13} Exosomal biogenesis is controlled by the endosomal sorting complex yielding cargo that includes mRNA, microRNA, cytoplasmic proteins, integral membrane proteins, and others.^{13–16} Prior proteomic analyses of nasal mucosa–derived exosomes (NMDEs) have identified multiple proteins that are specifically involved in the innate immune response, including inducible nitric oxide synthase (NOS2).¹² Our laboratory has previously shown that NMDEs are capable of rapid interepithelial transfer of proteins that function to propagate inflammation in the setting of chronic rhinosinusitis.¹³ Furthermore, previous studies in gastrointestinal models have demonstrated that constitutive exosome secretion from epithelial cells can be upregulated by TLR4 activation.¹⁷ These stimulated exosomes were capable of shuttling AMPs, such as human β-defensin 2 and cathelicidin-27, directly to the surfaces of invading pathogens, where they induced a dose-dependent reduction of viability and infectivity.¹⁷

Taken together, the prior findings that exosomes are hyper-secreted after TLR4 stimulation, convey both NOS2 and an array of AMPs, are microbiocidal, and rapidly transfer protein cargo between epithelial cells point to a novel arm of human upper airway innate immunity. We hypothesized that on inhalation of pathogens expressing TLR4-stimulating PAMPs, the epithelial cells of the anterior nasal mucosa release a swarm of exosomes, which exert a direct antimicrobial response within the mucus microenvironment. We further hypothesized that a population of these exosomes is simultaneously swept backward in the nose through MCC, where they donate their immunoprotective cargo to posterior naive epithelial cells to defend against the advancing pathogen (see the Graphical Abstract).

To test these hypotheses, we leveraged our unique access to human NMDEs and autologous tissue samples to explore the effect of TLR4 stimulation using LPS on exosome secretion, composition, and antimicrobial defense both in primary nasal epithelial cell culture and *in vivo*.

METHODS

Primary human nasal tissue and mucus sampling

Tissue and mucus sampling was approved by the Massachusetts Eye and Ear Infirmary Institutional Review Board. All samples were taken from

patients who had not been exposed to antibiotics or steroids for at least 4 weeks before harvest. Exclusion criteria included ciliary dysfunction, autoimmune disease, cystic fibrosis, immunodeficiency, and smoking. Mucus samples were extracted from the nasal cavity by placing a compressed polyvinyl alcohol (PVA) sponge (Medtronic, Minneapolis, Minn) against the head of the inferior turbinate for 5 minutes.

Whole mucus extraction

Mucus samples were extracted from the PVA sponge by means of centrifugation (1500g at 4°C for 30 minutes) after addition of 150 μ L of 1 $\times$ PBS (Life Technologies, Carlsbad, Calif) with Protease Inhibitor Cocktail (1:100; Sigma, St Louis, Mo). The sponges were centrifuged a second time after addition of 150 μ L of 1 $\times$ PBS with Protease Inhibitor Cocktail, and mucus was collected. Cellular debris was pelleted by means of centrifugation at 45 minutes at 12,000g at 4°C. The supernatant was then diluted up to 4.5 mL of PBS in polypropylene tubes (Thinwall, 5.0 mL, 13 $\times$ 51 mm; Beckman Coulter, Indianapolis, Ind). NMDEs were then isolated by using the ultracentrifugation (UCF) procedure.

Exosome isolation with UCF

The NMDE isolation procedure from whole mucus and culture media was adapted from the UCF procedure described by Th  ry et al.¹⁸ Samples were centrifuged at 12,000g at 4°C for 45 minutes to pellet cell debris. Supernatants were diluted in PBS in polypropylene tubes (Thinwall; Beckman Coulter) and ultracentrifuged for 2 hours at 110,000g at 4°C. The pellet was then resuspended in 4.5 mL of 1 $\times$ PBS. The suspension was filtered through a 0.22- μ m filter (Fisher Scientific, Pittsburgh, Pa) and collected in a fresh (Thinwall, 5.0 mL, 13 $\times$ 51 mm; Beckman Coulter) tube. The filtered suspension was then centrifuged for 70 minutes at 110,000g at 4°C. The supernatant was collected, and the pellet was resuspended in the buffer necessary for downstream application.

Exosome isolation using OptiPrep density gradient

The exosome purification procedure was adapted from the OptiPrep Density Gradient (OptiPrep Density Gradient Medium; Sigma-Aldrich, St Louis, Mo) procedure described by Greening et al.¹⁰ Supernatants were ultracentrifuged for 2 hours at 110,000g at 4°C, and the pellet was resuspended in 2.2 mL of 1 $\times$ PBS (750 μ L was saved for exosomal yield and purity ELISAs). OptiPrep solutions were diluted in 0.25 mol/L sucrose and 10 mmol/L Tris-HCl (pH 7.5; Sucrose, BioUltra, and TRIS hydrochloride, PharmaGrade; Sigma Aldrich). A discontinuous gradient was formed by layering 3 mL of 40% OptiPrep (wt/vol), 3 mL of 20% OptiPrep (wt/vol), 3 mL of 10% OptiPrep (wt/vol), and 2 mL of 5% OptiPrep (wt/vol) in polypropylene tubes (Thinwall, 13.5 mL, 16 $\times$ 76 mm; Beckman Coulter). Of the resuspended pellet, 1.2 mL was then layered on top of the 5% OptiPrep (wt/vol). The density gradient and sample were then ultracentrifuged at 110,000g for 18 hours at 4°C. After UCF, 1-mL fractions were collected. Exosome-positive fractions were diluted with 3.5 mL of PBS (Wash 1) and ultracentrifuged for 3 hours at 110,000g at 4°C, and the supernatants were saved. These fractions were resuspended in 3.5 mL of PBS and then washed a second time for 1 hour at 110,000g and 4°C, and the supernatants were saved. Pellets were resuspended and used for yield and purity studies along with the corresponding supernatants.

NMDE yield and purity evaluation

Whole mucus, UCF-purified exosomes, OptiPrep Density Gradient-purified exosomes, and corresponding supernatants were subjected to commercially available ELISAs: calnexin (MyBioSource, San Diego, Calif) and CD81 and/or CD63 (ExoELISA; System Biosciences, Palo Alto, Calif) ELISAs to quantify the purity and exosome yields. All values were normalized to total protein concentration within the same sample by using a Micro BCA Protein Assay Kit (Pierce, Rockford, Ill).

Transmission electron microscopy of NMDEs

The transmission electron microscopy procedure was adapted from Th  ry et al.¹⁸ Exosomes were lyophilized and fixed for 1 hour at room temperature in 2% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (Electron Microscopy Sciences, Hatfield, Pa). Five microliters of the exosome were absorbed onto Formvar/carbon-coated electron microscopy grids (Electron Microscopy Sciences) for 20 minutes. After absorption, the grids were rinsed in PBS 3 times and then transferred to PBS/50 mmol/L glycine (Sigma-Aldrich) for 4 washes. Grids were blocked in 5% BSA (Fisher Scientific) in 1 $\times$ PBS (buffer) for 10 minutes at room temperature. The grids were incubated at 4°C overnight in the primary antibody (1:25, Purified Mouse Anti-Human CD81 Clone JS-81; BD Biosciences, San Jose, Calif) diluted in 1% BSA buffer. The grids were then rinsed in 0.1% BSA buffer and then 0.5% BSA buffer 6 times each.

A bridging antibody (AffiniPure Goat Anti-Mouse IgG [H+L]; Jackson ImmunoResearch, West Grove, Pa) diluted in 1% BSA was incubated at room temperature for 30 minutes to enhance staining. Then the secondary Protein G antibody (1:20 in 1% BSA buffer, EM Grade, 10 nm; Electron Microscopy Services) in 5% BSA buffer was applied for 1 hour at room temperature and rinsed 8 times with 1 $\times$ PBS. The grids were incubated in 1% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (Electron Microscopy Services) for 5 minutes. After rinsing 8 times in deionized water, grids were contrasted in uranyl-oxalate solution (pH 7; Electron Microscopy Services) for 5 minutes. The grids were blotted on filter paper and air-dried before imaging. The exosomes were observed with an FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, Ore) at an accelerating voltage of 100 kV interfaced with an AMT XR41 digital CCD camera (Advanced Microscopy Techniques, Woburn, Mass) for digital TIFF file image acquisition. Purified Mouse IgG1, κ Isotype Control Clone MOPC-21 (BD Biosciences) and CD81 lysate (Novus Biologicals CD81 Overexpression Lysate [Native]; Fisher Scientific) were used as negative and positive controls, respectively.

Primary human sinonasal epithelial cell culture

The human sinonasal epithelial cell culture (HSNECC) protocol was based on previously validated techniques.¹⁹ Briefly, mucosal biopsy samples were washed and digested in Pronase (Protease from *Streptomyces griseus*, Type XIV; Sigma-Aldrich) for 90 minutes at 37°C. Cell suspensions were separated from particulate matter by means of centrifugation (950 rpm for 3 minutes) and resuspended in bronchial epithelial growth medium (BEGM; Lonza, Basel, Switzerland). Cells were plated for 2 hours on standard tissue-culture plates to remove contaminating fibroblasts. Cells were then expanded for 5 to 7 days on human collagen type IV-coated (Collagen from human placenta, Bornstein and Traub Type IV; Sigma-Aldrich) 75-cm² flasks (Corning Life Sciences, Corning, NY). Once confluent, HSNECCs were trypsinized and reseeded evenly in human collagen type IV-coated, black-walled, 96-well tissue-culture plates (Corning 96-well plates; Corning Laboratories, Corning, NY) in BEGM and grown to 80% confluence before analysis.

In vitro LPS exposure studies

LPS dose-response experiments were performed on HSNECCs grown to 80% confluence in black-walled 96-well plates. Cells were rinsed with HBSS (Life Technologies) and then exposed to cell culture medium (BEGM or Ham's F-12 Nutrient Mix; Life Technologies) and incubated at 37°C for 1 hour. After this incubation, media were collected, and cells were rinsed with HBSS and exposed to increasing doses of LPS (up to 12.5 μ g/mL from *Pseudomonas aeruginosa* 10; Sigma-Aldrich) in cell-culture media for 1 hour at 37°C. Again, after the 1-hour incubation, media were collected, and cells were rinsed with HBSS. All rinses were collected for exosome isolation. HSNECCs used for NMDE production were grown in four 75-cm² flasks and exposed to the 12.5 μ g/mL LPS dose. All *in vitro* experiments were associated with less than 20% cytotoxicity, as determined by using the ReadyProbes Cell Viability Imaging Kit (Blue/Green; Life Technologies).

In vivo LPS exposure studies

Three patients undergoing inferior turbinate removal were consented for the *in vivo* LPS exposure study. Fifty micrograms of LPS (Sigma-Aldrich) in 0.9% sodium chloride (75 μ L of a 66.7 μ g/mL LPS solution; Irrigation USP; Hospira, Lake Forest, Ill) was applied to a PVA sponge (Medtronic) and placed against the anterior head of the inferior turbinate for 15 minutes. An identical sponge containing only 75 μ L of 0.9% sodium chloride was applied at the same site on the contralateral side. Mucus was simultaneously collected bilaterally from the posterior inferior turbinate with a dry PVA sponge over 15 minutes. After isolation, NMDE secretion from both sides was quantified by using CD81 (Systems Bioscience, Palo Alto, Calif) ELISAs and normalized to the total protein concentration within the same sample by using a Micro BCA Protein Assay Kit (Pierce).

Interrogation of NMDE secretion through the TLR4 pathway

HSNECCs were seeded in 96-well plates overnight. After rinsing with HBSS, cells were pretreated with the established TLR4 inhibitors SP600125 (10 μ mol/L; EMD Millipore, Burlington, Mass), SC514 (100 μ mol/L; EMD Millipore), or LPS from *Rhodobacter sphaeroides* (20 μ g/mL; InvivoGen, San Diego, Calif) for 1 hour at 37°C. The supernatant was then collected, and cells were rinsed again with HBSS. Then the cells were treated with the inhibitors and 12.5 μ g/mL LPS for 1 hour at 37°C, and supernatants were collected. Exosome concentrations were quantified in the cell-culture supernatant and normalized to the total protein concentration within the same sample by using a Micro BCA Protein Assay Kit. All *in vitro* experiments were associated with less than 20% cytotoxicity, as determined by using the ReadyProbes Cell Viability Imaging Kit (see Fig E1 in this article's Online Repository at www.jacionline.org).

Quantification of LPS-stimulated exosomal NOS2 expression

NMDEs were isolated by using UCF from HSNECCs after exposure to media alone and media containing 12.5 μ g/mL LPS, as described above. NOS2 concentrations in purified exosome fractions were quantified by using ELISA (USCN Life Sciences, Wuhan, China) and normalized to the total number of exosomes, as determined by using a CD81 ELISA (Systems Bioscience).

Quantification of LPS-stimulated exosomal NOS2 activity

NMDEs were isolated by using UCF from HSNECCs after exposure to media alone and media containing 12.5 μ g/mL LPS, as described above. Isolated exosomes were lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) for 30 minutes at 37°C with or without the presence of NOS inhibitors. NOS2 was specifically inhibited with 1400-W (100 μ mol/L; Sigma-Aldrich), and N ω -nitro-L-arginine methyl ester hydrochloride (100 μ mol/L; Sigma-Aldrich) was used to inhibit all forms of NOS. Intrinsic exosomal NOS2 activity in each condition was then quantified by using a Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, Mich) and normalized to the total exosome concentration measured by using CD81.

SOMAscan proteomic and bioinformatic analyses

SOMAscan (SomaLogic, Boulder, Colo) analysis of NMDEs was performed by using the SOMAscan Assay Cells & Tissue Kit, 1.3k, according to the recommended protocol from the manufacturer, by using 1.7 μ g of protein per sample. Three provided kit controls and 1 no-protein buffer control were run in parallel with the samples per plate. Median normalization and calibration of the SOMAscan data were performed according to the standard quality control protocols at SomaLogic. Results from the SOMAscan assay were reported as log₂-normalized relative fluorescent units. Results from the SOMAscan assay were imported into the MetaCore Database (Thomson Reuters, Cambridge, Mass), and an enrichment analysis was performed, selecting

for proteins involved in innate immunity and NOS-related process pathways. Morpheus software (<https://software.broadinstitute.org/morpheus/>) was used to generate a Spearman correlation matrix according to hierarchical clustering. GeneMANIA software (<https://genemania.org/>) was used to confirm innate immune networks and functional associations reported by MetaCore.

***P aeruginosa* antimicrobial activity assay**

Antimicrobial activity assays were based on a published microdrop colony-forming unit method.²⁰ *P aeruginosa* PAO1 was inoculated from a -80°C freezer stock into 5 mL of tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ) that was contained in a sterile 14-mL polypropylene culture tube. The culture was grown at 37°C for 16 hours on a rotating wheel. The following day, the overnight culture was diluted 1:100 into 6 mL of fresh TSB, and the resulting culture was grown at 37°C on a rotating wheel. Once the culture reached an OD₆₀₀ value of approximately 0.6, the cells were centrifuged at 3600 rpm for 20 minutes at 4°C to pellet the cells. Cells were resuspended in 6 mL of 10 mmol/L sodium phosphate, pH 7.4 (sterilized with a 0.2- μ m filter) supplemented with 10% TSB. The cells were washed a second time in 4 mL of the same buffer. The OD₆₀₀ of the cells was adjusted to 0.5. The cells were diluted serially (10-, 10-, and 2-fold) to achieve a dilution factor of 200. Next, 10 μ L of the diluted cells were added to wells of a sterile 96-well polystyrene plate (Corning).

Ninety microliters of the NMDE isolates was added to the cells to initiate the antimicrobial activity assay, which afforded an initial CFU per milliliter of 1×10^5 . For the colistin-treated cells, 90 μ L of 2.5 μ mol/L colistin (Enzo Life Sciences, Farmingdale, NY) dissolved in sterile-filtered 10 mmol/L phosphate, pH 7.4, buffer was added to the well, and for the untreated cells, 90 μ L of 10 mmol/L sodium phosphate, pH 7.4, buffer was added to the well. The plate was incubated at 37°C with 150 rpm of shaking. After 3 hours, a 20- μ L aliquot from each well was diluted in 180 μ L of 10 mmol/L phosphate, pH 7.4, which was in a separate 96-well plate and was mixed by pipetting up and down 20 times to afford a 10^{-1} dilution. The cells were further diluted to 10^{-2} and 10^{-3} .

Five-microliter aliquots from the 10^0 , 10^{-1} , 10^{-2} , and 10^{-3} dilutions were spotted onto prewarmed TSB agar plates in triplicate (VWR, Radnor, Pa) for every treatment condition to plate the cells. After plating, spots were allowed to dry at ambient temperature for at least 30 minutes before inverting the plates. The plates were moved to a 37°C room and allowed to incubate for 14 hours before counting the colonies. Four independent NMDE sample sources were assayed in triplicate.

Exosomal protein and nucleic acid transfer studies

NMDEs were isolated from whole mucus samples and labeled by using commercially available Exo-Green carboxyfluorescein succinimidyl diacetate ester fluorescent protein label and Exo-Red Acridine Orange fluorescent RNA label ($\times 10$ magnification; Systems Biosciences), according to the manufacturer's instructions. After labeling, 25 μ L of the purified exosomes (1.25×10^9 exosomes/mL) was added to autologous HSNECCs in a black walled 96-well plate (Corning). Wells were imaged every 5 minutes for a total of 30 minutes by using a Leica DM IL LED fluorescent microscope (Leica, Buffalo Grove, Ill) and a $\times 20$ objective. The same field of view was used for all time points. Fluorescent staining intensity was quantified according to previously described techniques.²¹ Briefly, $\times 20$ fields were chosen, and images were analyzed in ImageJ software (version 1.50i; National Institutes of Health, Bethesda, Md). The cytoplasm and background areas were circled in ImageJ software by using the free-hand sections tool, and the integrated density, area of selected cell, and mean fluorescence of the backgrounds were measured by using the region of interest manager. The corrected total cell fluorescence (CTCF) was calculated as follows:

Integrated density = (Area of selected cell $\times$ Mean fluorescence of background readings).

CTCF values were averaged across fields of view.

Epithelial nitric oxide production study

HSNECCs were seeded in 96-well plates overnight for determination of NMDE-mediated enhancement of epithelial nitric oxide (NO) production.

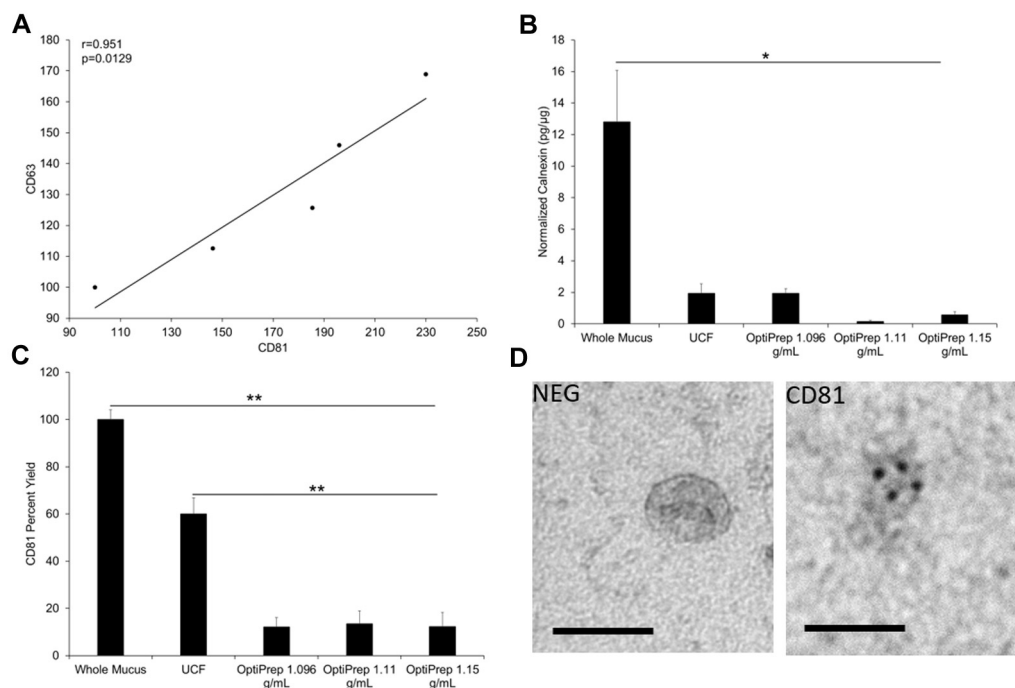


FIG 1. Characterization of NMDEs. **A**, CD81 is one of the most commonly used exosome markers and correlated strongly with CD63, another exosome-associated tetraspanin, as measured by means of ELISA (Spearman rank correlation coefficient). **B** and **C**, Exosome purity (measured by using a calnexin ELISA; Fig 1, **B**) and exosome yield after isolation by using UCF and OptiPrep Density Gradient when compared with whole mucus (Fig 1, **C**). * $P < .05$ and ** $P < .01$, 2-tailed Student t test. Data are pooled from 2 independent experiments (mean $\pm$ SEM). **D**, Transmission electron microscopic images demonstrating negative (NEG) control (bar = 100 nm for Fig 1, **A** and **B**), confirming the typical exosome size and immunogold labeling of exosome marker CD81 localizing to the exosome membrane.

After rinsing the cells 3 times with HBSS, cells were exposed to autologous LPS-stimulated (12.5 $\mu\text{g/mL}$) or unstimulated control NMDEs for 1 hour at 37°C. NO secretion was quantified by using the Nitrate/Nitrite Fluorometric Assay. All values were normalized to the total protein concentration within the same sample by using a Micro BCA Protein Assay Kit.

Statistics

Statistical analysis was performed with R software, version 3.3.0. The Shapiro-Wilk test was used for assessing normality. For nonparametric data, the Kruskal-Wallis test was used to examine statistical differences between multiple groups, and the Mann-Whitney rank sum test was used to examine statistical differences between 2 independent groups. Two-tailed Student t tests were used on parametric data. Correlations were tested by using 2-tailed Spearman rank-order correlation. Values falling outside 1.5 times the interquartile range of their respective data sets were considered outliers and indiscriminately excluded from analysis. Results were considered significant when a P value of .05 or less was obtained. All *in vitro* studies were performed in technical duplicates, with the exception of the antimicrobial assay, which was performed in technical triplicates.

RESULTS

Purification and isolation of exosomes

Multiple exosome-associated tetraspanins, including CD9, CD63, CD81, and CD82, are regularly used to quantify the presence and concentration of exosomes.⁹ Given the extremely low volumes of primary NMDE samples after purification (eg, <500 μL), we were restricted to use only a single marker for

quantification. We elected to use CD81 because this is one of the most commonly used exosome tetraspanins in the literature.⁹ Our selection of CD81 was validated by its correlation with another exosome-associated marker, CD63 (Fig 1, **A**), indicating a similar utility for both markers.

Our next step required the validation of our exosome isolation technique to maximize the yield of this relatively scarce resource without sacrificing purity. We compared the 2 most widely accepted exosome isolation methods, namely UCF alone or UCF combined with an OptiPrep Density Gradient.²² We used calnexin, an endoplasmic reticulum protein, as an established marker of sample contamination, as previously described.²³ We found no significant difference between the protein-normalized calnexin concentration in UCF-isolated exosomes (mean $\pm$ SEM, 1.19 ± 0.06 pg/ μg) versus the exosome-positive OptiPrep Density Gradient fractions (1.096, 1.11, and 1.15 g/mL and 5.7 ± 0.29 , 1.3 ± 0.074 , and 3.6 ± 1.8 pg/ μg , respectively, $P =$ not significant; Fig 1, **B**).

These results were validated by using whole mucus as a positive contamination control. As expected, the calnexin concentrations in the whole mucus fraction (12.8 ± 3.26 ng/ μg) were significantly greater than those of the UCF and OptiPrep Density Gradient fractions ($P < .05$ for all). In contrast, the total exosome yield was significantly greater in the UCF ($60.1\% \pm 6.8\%$) fraction compared with the OptiPrep Density Gradient (1.096 g/mL, $12.1\% \pm 8.1\%$; 1.11 g/mL, $13.5\% \pm 5.6\%$; and 1.15 g/mL, $12.3\% \pm 6.0\%$; $P < .01$ for all; Fig 1, **C**) when expressed as a percentage of total exosomes

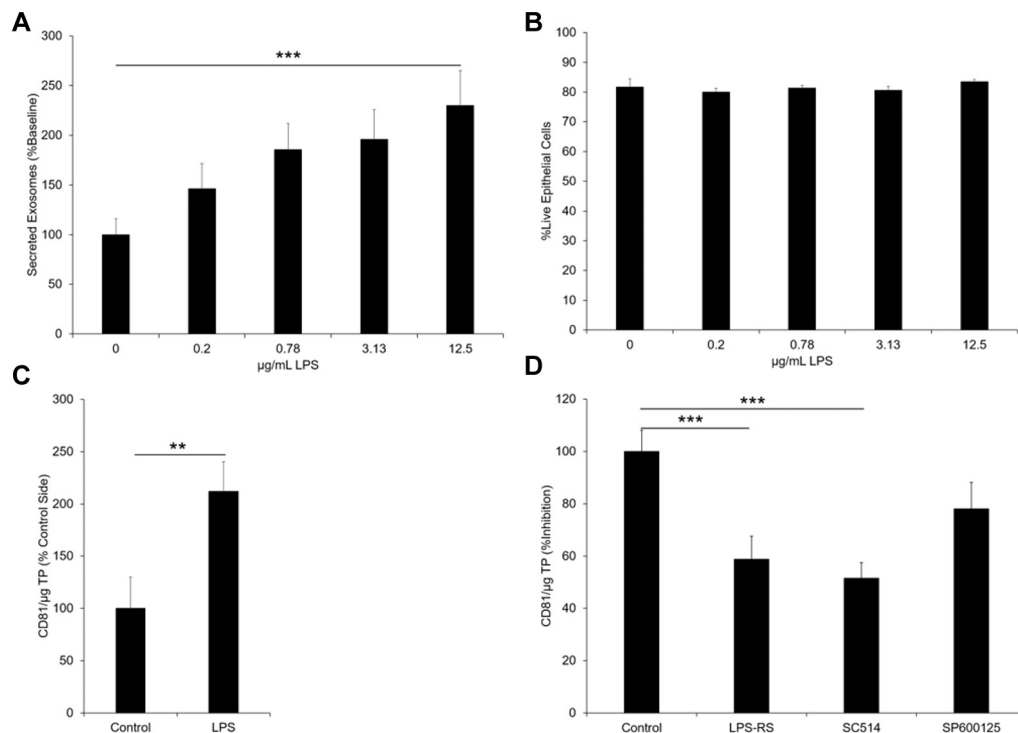


FIG 2. NMDE secretion is regulated by TLR4. **A**, *In vitro* dose response to LPS. Exosomal release, as measured by using a CD81 ELISA, significantly increases with LPS concentration when compared with baseline control. *** $P < .001$, Kruskal-Wallis test. Data represent 3 independent experiments (mean $\pm$ SEM). **B**, LPS doses used are noncytotoxic in epithelial cell culture when compared with baseline control values. P = Not significant (Kruskal-Wallis test). Data represent 3 independent experiments (mean $\pm$ SEM). **C**, *In vivo* LPS-stimulated exosome release in surgical patients when compared with control values. ** $P < .01$, 2-tailed Student *t* test. Data represent 3 independent experiments (mean $\pm$ SEM). **D**, Exosomes are released through the TLR4 and IKK2 subfamily pathway but not the mitogen-activated protein kinase/c-Jun N-terminal kinase subfamily pathway when compared with cells exposed to LPS. *** $P < .001$, Kruskal-Wallis test. Data represent 8 independent experiments (mean $\pm$ SEM).

in the parent's whole mucus sample ($100\% \pm 4.15\%$). Because the UCF method provided a greater yield with no significant sacrifice in purity, we elected to use this technique for the remainder of the study.

Finally, we used transmission electron microscopy to confirm that the UCF-purified exosome fractions contained the expected 30- to 150-nm size and spheroid exosome morphology. Immunogold imaging was then used to confirm the presence of CD81 within the exosome superstructure (Fig 1, D).

Human nasal exosome secretion is stimulated by TLR4 activation both *in vivo* and *in vitro*

Having optimized and validated the exosome isolation technique, we next attempted to determine whether human NMDE secretion could be regulated by TLR4 stimulation, as previously described.¹⁷ We used *Paeruginosa*-derived LPS to activate TLR4 because LPS is a canonical TLR4 agonist²⁴ and *Paeruginosa* represents a common nasal pathogen.²⁵

Using primary HSNECCs, we demonstrated that LPS was capable of stimulating exosome secretion in a dose-dependent manner. We found that 12.5 $\mu\text{g/mL}$ LPS more than doubled the quantity of secreted exosomes relative to the baseline control ($n = 3$, mean $\pm$ SEM: $230.0\% \pm 35.0\%$, $P < .01$; Fig 2, A) without inducing cytotoxicity using the live/dead assay (Fig 2, B).

In light of the clear LPS-dependent dose response for exosome secretion *in vitro*, we next wanted to confirm these findings *in vivo* within the healthy human nasal cavity. Therefore, we applied LPS (50 μg) to the anterior inferior turbinate in live surgical patients ($n = 3$) and collected NMDEs from the posterior turbinate. This dose was derived from safe airway LPS exposure doses previously reported in the literature.^{26,27} The total exposure time was 15 minutes to approximate physiologic MCC times,¹ and the contralateral side was exposed to saline as an internal control. Our findings confirmed the *in vitro* results in which anterior LPS exposure more than doubled the total number of exosomes released ($212.0\% \pm 28.4\%$) relative to the control side ($100.0\% \pm 30.2\%$, $P < .05$; Fig 2, C), which were then transported posteriorly through MCC.

After both *in vitro* and *in vivo* confirmation of an LPS-dependent effect on exosome secretion, we next sought to confirm that this effect was TLR4 mediated and dissect which downstream pathways were involved using established TLR4 pathway inhibitors. We found that in the presence of LPS, TLR4 inhibition through LPS from *R sphaeroides*²⁸ and inhibitor of nuclear factor κB kinase (IKK2) β inhibition through SC514,²⁹ both of which significantly abrogated the release of CD81⁺ exosomes ($n = 8$, $58.9\% \pm 8.7\%$, $P < .001$ and $51.6\% \pm 5.97\%$, $P < .001$; Fig 2, D). In contrast, there was a nonsignificant trend toward reduction of exosome secretion by using the mitogen-activated

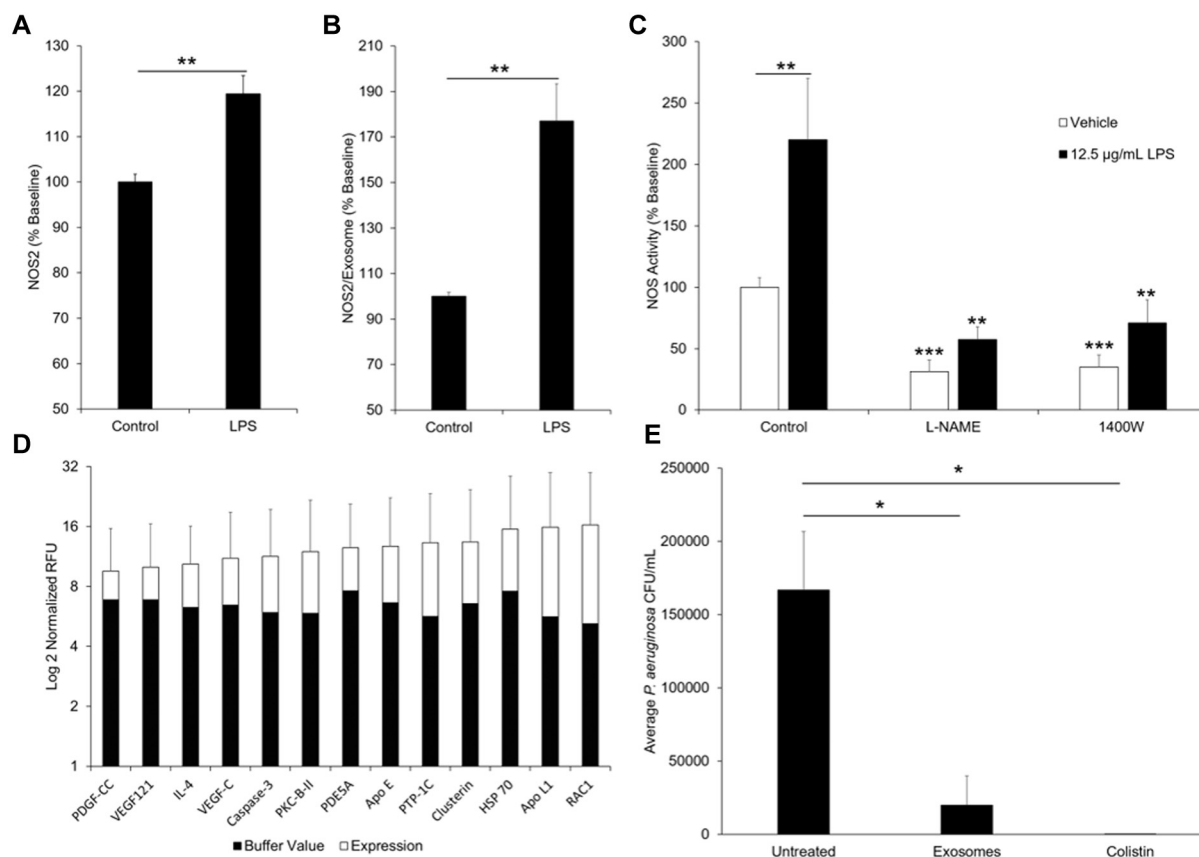


FIG 3. TLR4 stimulation promotes exosomal NOS expression and activity. **A** and **B**, LPS increases total NOS2 levels in the exosome population (Fig 3, **A**) and NOS2 levels per exosome (Fig 3, **B**) when compared with cells exposed to the baseline control. $^{**}P < .01$, Kruskal-Wallis test. Data represent 3 independent experiments (mean $\pm$ SEM). **C**, LPS exposure increases NOS activity in lysed exosomes compared with unstimulated exosomes. Activity is abrogated by *N* ω -nitro-L-arginine methyl ester hydrochloride (*L*-NAME), an inhibitor of all isoforms of NOS, and 1400-W, an inhibitor of NOS2. $^{***}P < .001$ for comparison between unstimulated conditions and $^{**}P < .01$ (Mann-Whitney rank sum test) for comparison between LPS-stimulated conditions. Data represent 4 independent experiments (mean $\pm$ SEM). **D**, Additional exosomal NO production and signaling-related proteins (by using Entrez Gene ID) among all 10 patients sampled. All are significant compared with buffer control values. **E**, Exosomes significantly decreased *P. aeruginosa* CFUs in comparison with untreated *P. aeruginosa*. Over 3 hours, colistin serves as a positive control. $^{*}P < .05$ (2-tailed Student *t* test) when compared with baseline unstimulated exosomes. Data represent 4 independent experiments (mean $\pm$ SEM).

protein kinase and c-Jun N-terminal kinase inhibitor SP600125 ($n = 8$, $78.1\% \pm 10.1\%$, $P =$ not significant; Fig 2, **D**).²⁷

TLR4 stimulation promotes nasal exosomal NOS2 expression and activity

Having confirmed that LPS stimulates exosome secretion through TLR4/IKK2, we next studied whether exosomal composition could also be modified by LPS exposure. We chose to focus on NOS2 expression and activity because bactericidal NO has been previously shown to play a critical role in human nasal innate immunity.³⁰ Using the optimized LPS dose from our *in vitro* studies (eg, 12.5 µg/mL), we first demonstrated that LPS stimulation induced a significant increase in total NOS2 expression compared with control HSNECCs, as quantified by using NOS2 ELISA ($n = 3$; $119.4\% \pm 4.0\%$ and $100.0\% \pm 1.7\%$, respectively; $P < .01$; Fig 3, **A**). This finding persisted even after normalizing the results to the total number of secreted exosomes,

suggesting that LPS induces greater NOS2 packaging within individual exosomes compared with unstimulated cells ($n = 3$; $177.1\% \pm 16.3\%$ and $100.0\% \pm 1.7\%$, respectively; $P < .01$; Fig 3, **B**). Taken together, these results suggest that LPS significantly enhances NOS2 expression through both an increase in total exosomes and increased NOS2 levels per exosome.

The next experiment explored whether this LPS-mediated increase in NOS2 expression was associated with a commensurate increase in functional activity, as measured by nitrate and nitrite levels. We found that LPS exposure significantly increased NOS activity in lysed exosomes compared with unstimulated exosomes ($n = 4$; $220.3\% \pm 50.0\%$ and $100.0\% \pm 7.7\%$, respectively; $P < .01$; Fig 3, **C**). This activity was subsequently abrogated by addition of both general NOS (*N* ω -nitro-L-arginine methyl ester hydrochloride) and NOS2-specific (1400-W) inhibitors, thereby validating that the increased nitrate/nitrite assay findings were specific for NOS2 activity. As a secondary confirmation, we then demonstrated that the NOS2/exosome levels

strongly and significantly correlated with NOS activity/exosome ($R = 0.916$, Spearman rank correlation coefficient; $P < .01$).

Although our mechanistic studies focused on the influence of TLR4-mediated exosomal NOS2 expression, we also sought to determine whether any additional proteins related to NO production and signaling could be detected within NMDEs. By using an aptamer-based proteomics platform (SOMAscan), 13 additional NO pathway-associated proteins, as defined by MetaCore, were detectable within 10 individual healthy NMDE samples at levels significantly greater than background control values (Fig 3, D).

LPS-stimulated exosomes are microbiocidal to *P aeruginosa*

Having determined that LPS stimulation increased both exosomal NOS2 expression and activity, we next studied whether these exosomes displayed intrinsic antimicrobial activity. We used *P aeruginosa* PAO1 as our model nasal pathogen²⁵ to remain consistent with the LPS source used throughout our experiments. We found that direct exposure to LPS-stimulated HSNECC-derived exosomes induced a significant reduction in *P aeruginosa* colony forming units (CFUs) to $2.01 \times 10^4 \pm 1.99 \times 10^4$ CFU/mL (mean $\pm$ SEM) relative to untreated cells ($1.67 \times 10^5 \pm 4.00 \times 10^4$ CFU/mL), which was commensurate with that of the colistin positive control (200.00 ± 0.00 CFU/mL, $P < .05$; Fig 3, E). There was no significant difference between untreated *P aeruginosa* CFUs and those exposed to unstimulated exosomes ($1.16 \times 10^6 \pm 3.56 \times 10^5$ CFU/mL). A similar but nonsignificant trend was seen after a 1-hour exposure (data not shown).

NMDEs rapidly transfer proteins to autologous epithelial cells and enhance NO production

The next set of studies explored the capability and kinetics of NMDE protein transfer to autologous nasal epithelial cells. We hypothesized that the interepithelial transport of NMDEs is mediated through mucociliary flow, and thus we temporally restricted these studies to less than 30 minutes. This time scale corresponds roughly to the maximal intranasal residence time of suspended particulates within nasal mucus before being removed by using MCC.¹

We first examined the rate of protein transfer by labeling NMDEs with Exo-Green, a validated commercially available carboxyfluorescein succinimidyl diacetate ester stain that diffuses into exosomes, is hydrolyzed to its fluorescent state, and couples to protein amino acid moieties.³¹ Exo-Green-labeled NMDEs were exposed to cultured HSNECCs and monitored for 30 minutes. Critically, only autologous exosomes were used for the exposure studies to eliminate the potential for immune cross-reactivity because exosomes have been shown to express major histocompatibility complex proteins in their membrane.³² Cellular uptake of labeled exosomal proteins was then quantified over time by calculating the CTCF, as previously described.³³ The exosomal proteins demonstrated progressive diffusion throughout the cytoplasm, which was statistically significant within 5 minutes ($n = 80$ cells, 94.8 ± 9.5 CTCF compared with 2.5 ± 0.4 CTCF for time zero, $P < .001$) and plateaued at 20 minutes (182.1 ± 15.9 , $P < .001$ when compared with baseline; Fig 4, B). To confirm these results, we then repeated the experiment using Exo-Red, a commercially available nucleic

acid-selective cationic dye, and found that transfer of labeled nucleic acids occurred over a similar time frame (Fig 4, B).

In light of the findings that LPS-stimulated exosomes have increased intrinsic NOS2 activity and are capable of transferring their protein cargo within time scales commensurate with mucociliary flow rates, we next explored whether exosome exposure could enhance NO production within an LPS-naïve recipient epithelial cell. Using the nitrate/nitrite fluorometric kit, we found that exposure to LPS-stimulated exosomes induced significantly more NO release ($n = 3$, mean $\pm$ SEM; 45 ± 23 NO fmol/TP μ g; Fig 4, C) when compared with vehicle control cells or cells exposed to unstimulated exosomes (130 ± 20 NO fmol/TP μ g, $P < .05$, and 10 ± 1.0 NO fmol/TP μ g, $P < .05$, respectively). NO was undetectable in both intact LPS-stimulated and unstimulated exosomes, confirming that the measured NO was derived from the recipient cell.

NMDEs express multiple innate immune-associated proteins

In our final experiment we extended our proteomic analysis to determine whether exosomes expressed additional innate immune-associated proteins beyond those related to NO activity, which could also be subject to interepithelial transport. We first generated a list of potential candidate proteins by performing an enrichment analysis through MetaCore for innate immunity process pathways using the SOMAscan expression data. The 31 innate immune proteins that were identified and present at levels significantly greater than background control values were then subjected to an additional correlation matrix (Fig 5, A) and GeneMANIA bioinformatics analyses (Fig 5, B). These results demonstrated that many of the proteins that exhibited strong and significant correlations in exosomal expression participated in the same predicted functional pathways, including complement activation (eg, C2, C6, and C7) and bacterial defense (eg, TLR4 and S100A9).

DISCUSSION

Despite a growing acceptance of the relative ubiquity and importance of exosomes in immune and inflammatory responses,^{10-13,34} direct *in vivo* functional studies in both health and disease have been limited by a lack of concurrent access to suitably purified samples and their tissue of origin. Therefore, the human nasal cavity provides a unique opportunity to study the contribution of exosomes to innate immunity because NMDEs and their parent epithelium can be manipulated and simultaneously harvested during routine nasal surgical procedures.

Our group has previously demonstrated that NMDEs play a critical role in the maintenance of regional inflammation in patients with chronic rhinosinusitis through interepithelial transfer of P-glycoprotein, an energy-dependent membrane efflux pump.¹³ This mechanism comports with previous studies in cancer models demonstrating both protein and nucleic acid transfer by donor exosomes to recipient cells.^{10,16,35,36} One of the most intriguing findings in our work was the fact that the transfer rate far exceeded physiologic MCC rates.¹ Concurrently, other groups have demonstrated that exosome release is regulated by PRR activation and that exosomes harbor a wide array of immunoprotective proteins.^{12,17} Taken together, these findings led us to

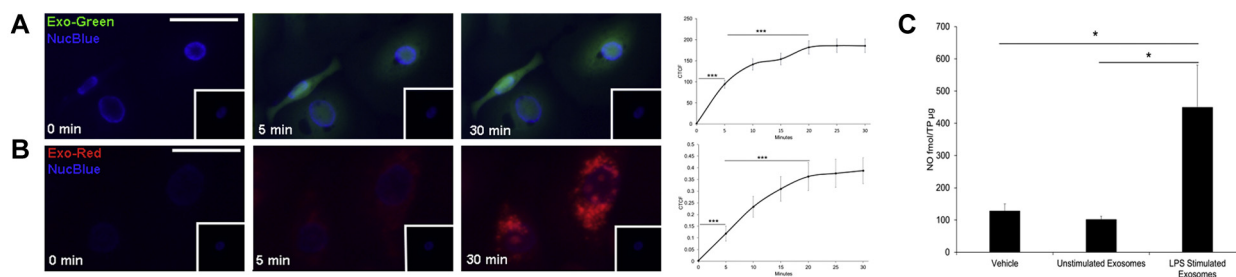


FIG 4. NMDEs rapidly transfer proteins to autologous epithelial cells and enhance NO production in primary cell culture. **A**, Interepithelial transfer of exosomal protein was visualized by using Exo-Green staining with NucBlue nuclear counterstaining. **B**, Exosomal nucleic acid transfer was visualized by using Exo-Red staining. Uptake within autologous cells was quantified by the increase in CTCTF. *** $P < .001$ (2-tailed Student t test) when comparing 0 minutes and 5 minutes, as well as between 5 and 20 minutes. Data represent greater than 80 cells (mean $\pm$ SEM). **C**, After exposure, LPS-stimulated exosomes induce significantly more NO release from autologous epithelial cells when compared with unstimulated exosomes and vehicle control. * $P < .05$ (2-tailed Student t test). Data represent 3 independent experiments (mean $\pm$ SEM).

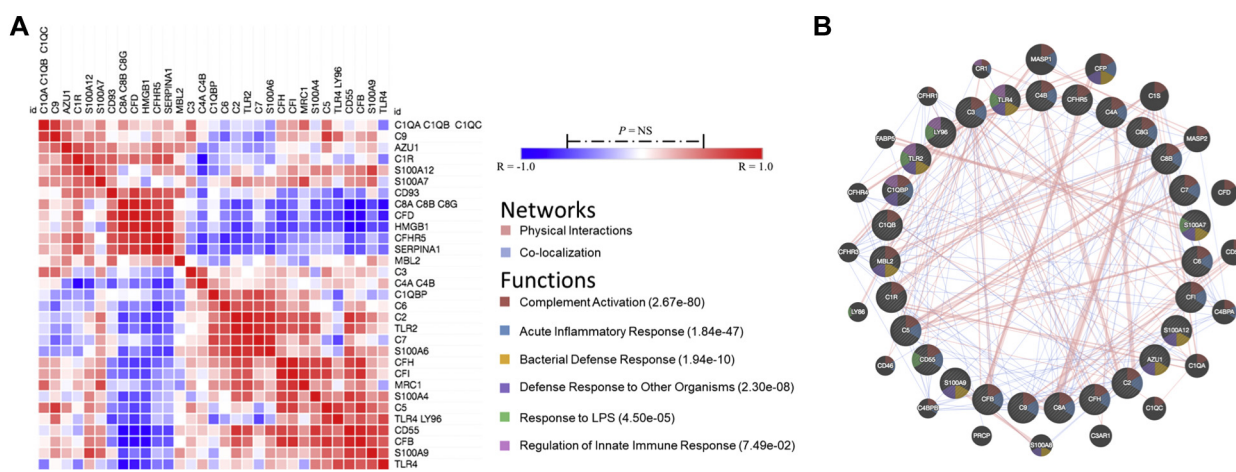


FIG 5. NMDEs express multiple innate immune proteins. **A**, Correlation matrix demonstrating significant correlations among expression levels of multiple innate immune proteins (identified by using Entrez Gene ID) within NMDEs ($n = 10$ patients). **B**, GeneMANIA (genemania.org) analysis demonstrating functional interrelationships among the same innate immune proteins as in Fig 5, A, expressed in NMDEs, as well as their associations with relevant immune and inflammatory pathway, including complement activation (eg, C2, C6, and C7) and bacterial defense (eg, TLR4 and S100A9; false discovery rate values of each functional pathway are shown in parentheses). Outer circles are associated proteins inferred from the literature.

hypothesize a novel role for exosomes in upper airway innate immunosurveillance and defense. In this conception TLR4 activation leads to a swarm-like release of exosomes, which are circulated through MCC and exert both direct antimicrobial and epithelial immunoprotective behavior.

Our direct *in vivo* and *ex vivo* mechanistic studies prohibited the use of pooled exosome samples because of the potential for immunologic cross-reactivity.³² Therefore, to maximize the limited NMDE sample volumes, our first series of experiments were designed to validate our purification method because this has been a point of controversy in previous studies.^{11,37,38} Our results confirmed that UCF provided improved yield with equivalent purity compared with previously described density gradient techniques.²²

We next used LPS derived from *P aeruginosa* to study the role of TLR4 activation in regulation of epithelial exosome secretion *in vitro*. We used only primary HSNECCs directly derived from human inferior turbinates to model *in vivo* inhaled microbe exposure. This is physiologically relevant because the anterior head of

the inferior turbinate represents the primary site of extrinsic particulate deposition given its morphologic position within the internal nasal valve.^{39,40} Similarly, we selected LPS because it is a canonical TLR4 agonist²⁸ and most closely models the *in vivo* nasal response to *Pseudomonas* species exposure, a common airway pathogen.²⁵ We found that TLR4 activation resulted in an almost immediate 2-fold increase in basal exosome secretion rates and that IKK2 is involved in the downstream signaling cascade. These results are consistent with TLR4 as an established PRR capable of rapidly recognizing and responding to the presence of pathogens through PAMP detection.^{17,41,42} Perhaps most importantly, we then found a near-identical response *in vivo* when measuring exosome release and transport to the posterior nasal cavity after anterior inferior turbinate exposure to LPS. These striking results were internally validated by using individual subject's contralateral nasal cavity as a control to eliminate any confounding variables referable to physiologic differences in basal exosome secretion rates or sensitivity to LPS exposure.

Next, we explored whether TLR4 stimulation was capable of modulating not only the release of exosomes but also their composition. Previous studies of the nasal exosomal proteome have established the presence of multiple immune-associated proteins.¹² Our proteomic analysis harmonized with these findings and identified 13 proteins associated with NO production and signaling and an additional 31 proteins associated with general innate immune function. Subsequent correlation and pathway analyses of these proteins suggested a high degree of interconnectivity and statistically significant relationships with bacterial defense-associated functional networks. We elected to focus our mechanistic analyses on exosomal NOS2 because the NOS2/NO system has been established as an important regulator of the innate immune response and has been previously shown to exhibit direct antimicrobial activity in the human nose.^{12,30,43} We found that TLR4 activation using LPS led to a nearly 2-fold increase in NOS2 expression per exosome. We then confirmed that this enhanced expression correlated with a proportionate increase in NOS2 function using a nitrate/nitrite assay and 2 independent NOS inhibitors. The final study in this series of experiments was designed to determine whether the evident increase in exosome secretion and NOS2 expression after LPS exposure was associated with a change in direct exosomal antimicrobial activity. Remarkably, we found that the microbiocidal activity of stimulated exosomes to *Paeruginosa* was commensurate with the colistin-positive control, an established anti-pseudomonal polypeptide antibiotic.⁴⁴⁻⁴⁸ Of note, although all of the individual exosome samples demonstrated this antimicrobial effect, one of the 4 samples was not statistically significant, suggesting there is some interpatient heterogeneity in this effect. The relative lack of activity seen among the unstimulated exosomes further supports our findings that TLR4 activation confers enhanced antimicrobial properties to the exosomes. The fact that this effect required a 3-hour exposure to become statistically significant might be reflective of the lower ratio of exosomes to *P aeruginosa* used in our *in vitro* assay relative to that which would be seen *in vivo*.

The next set of experiments was designed to examine a parallel mechanism of TLR4-mediated exosomal antimicrobial defense through passive epithelial immunoprotection. Using validated nonspecific amino acid fluorescent tags, we first demonstrated that exosomal proteins could be transferred to autologous HSNECCs within 5 minutes of exposure, confirming previous findings.^{13,16,36} The rapid kinetics of this transport supported our hypothesis that exosomes secreted from anterior TLR4-activated epithelial cells would have ample time to transfer their immunodefensive proteins to naive posterior nasal epithelial cells before being fully cleared into the nasopharynx.

Based on these results, we next specifically examined the exosomal capacity to enhance NO production in the recipient epithelial cell as a surrogate for the transfer of the NO-associated proteins including NOS2. We found that LPS-stimulated exosomes were capable of inducing an almost 4-fold increase in antimicrobial NO production within recipient cells compared with unstimulated exosomes.

One of the unique strengths of our approach was the ability to study the mechanisms of exosomal innate immune defense in primary autologous tissue samples and to corroborate our *in vitro* findings with highly controlled *in vivo* LPS exposure studies. This experimental design provides a significant degree of confidence in the validity and clinical relevance of our findings. However, the

relative scarcity of primary exosome samples limited the scope of some of our studies, including the ability to confirm the degree to which NO contributed to the microbiocidal effects of LPS-stimulated exosomes versus the additional exosomal AMPs identified.

Our findings provide direct *in vivo* evidence for a novel innate immunosurveillance and defense mechanism of the human upper airway. We have shown that activation of anterior nasal epithelial TLR4 by inhaled pathogens results in a doubling of basal exosome secretion rates, which in turn carry nearly twice the level of NOS2 as unstimulated exosomes. These “swarms” of exosomes not only exhibit dramatic direct microbiocidal activity but proactively arm the more posterior naive epithelial cells with immunoprotective proteins while inducing a 4-fold increase in NO production to defend against the same microbe within the advancing mucus blanket (see the Graphical Abstract). The presence of an array of additional AMPs and NO-related proteins and the ability to transfer nucleic acids within NMDEs suggest that the function of exosomes in airway immunity likely extends well beyond the results of this work and requires further study. These data also point to a potential role for nasal exosomes both in host microbiome regulation and airway therapeutic delivery.

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Key messages

- LPS stimulation increases both basal NMDE secretion and exosomal NO expression/activity by a factor of 2.
- TLR4-stimulated NMDEs confer direct anti-pseudomonal activity equivalent to colistin
- TLR4-stimulated NMDEs donate their immunoprotective cargo to naive epithelial cells through MCC and confer passive immunity to recipient epithelial cells.

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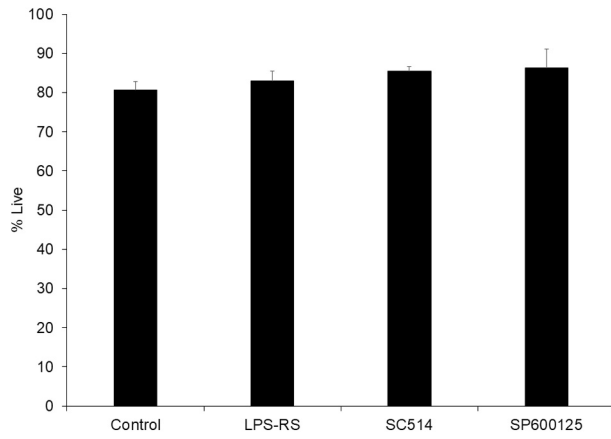


FIG E1. Exposure of LPS and TLR4 inhibitors is noncytotoxic in epithelial cell culture when compared with baseline control values. P = not significant (Kruskal-Wallis test). Data represent 3 independent experiments (mean $\pm$ SEM).