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RESEARCH ARTICLE

Extracellular Vesicles in Lung Health, Disease, and Therapy

CF monocyte-derived macrophages have an attenuated response to extracellular vesicles secreted by airway epithelial cells

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Abstract

Mutations in *CFTR* alter macrophage responses, for example, by reducing their ability to phagocytose and kill bacteria. Altered macrophage responses may facilitate bacterial infection and inflammation in the lungs, contributing to morbidity and mortality in cystic fibrosis (CF). Extracellular vesicles (EVs) are secreted by multiple cell types in the lungs and participate in the host immune response to bacterial infection, but the effect of EVs secreted by CF airway epithelial cells (AEC) on CF macrophages is unknown. This report examines the effect of EVs secreted by primary AEC on monocyte-derived macrophages (MDM) and contrasts responses of CF and wild type (WT) MDM. We found that EVs generally increase pro-inflammatory cytokine secretion and expression of innate immune genes in MDM, especially when EVs are derived from AEC exposed to *Pseudomonas aeruginosa* and that this effect is attenuated in CF MDM. Specifically, EVs secreted by *P. aeruginosa* exposed AEC (EV-PA) induced immune response genes and increased secretion of proinflammatory cytokines, chemoattractants, and chemokines involved in tissue repair by WT MDM, but these effects were less robust in CF MDM. We attribute attenuated responses by CF MDM to differences between CF and WT macrophages because EVs secreted by CF AEC or WT AEC elicited similar responses in CF MDM. Our findings demonstrate the importance of AEC EVs in macrophage responses and show that the Phe508del mutation in CFTR attenuates the innate immune response of MDM to EVs.

airway epithelial cells; cystic fibrosis; cytokine secretion; exosomes; macrophages

INTRODUCTION

Cystic fibrosis (CF) is a genetic disease resulting from mutations in the *CFTR* gene that encodes a chloride channel (1). Mutations in *CFTR* affect cell types in multiple organ systems. Notably, defective chloride secretion by airway epithelial cells (AEC) leads to reduced chloride secretion, reduced airway surface liquid height, impaired mucociliary clearance, and chronic bacterial lung infection. These infections lead to inflammation and progressive loss of lung function (1–4). Multiple pathogens, including *Pseudomonas aeruginosa* (*P. aeruginosa*), which colonizes up to 80% of adults with CF (5), are not cleared from the CF lung due, in part, to reduced mucociliary clearance and the inability of CF neutrophils and macrophages to kill bacteria (6–10). Although antibiotics and CFTR correctors and potentiators have improved patient outcomes, chronic bacterial lung infections remain a challenge for people with CF (11).

The lung environment contains multiple cell types, which communicate by a variety of mechanisms to maintain homeostasis. Cells such as neutrophils, macrophages, and other leukocytes, as well as epithelial cells, goblet cells, and ionocytes (12), all secrete cytokines in response to bacterial infections. For example, in response to *P. aeruginosa*, macrophages and AEC secrete monocyte chemoattractant protein-1 (MCP-1) and IL-8, which recruit neutrophils, monocytes, and macrophages to the lungs to clear the infection (13). Leukocytes and epithelial cells in the lungs also communicate with each other by secreting extracellular vesicles (EVs), a heterogeneous group of membrane-bound structures comprising exosomes and microvesicles (14). EVs deliver cargo including miRNA and proteins to target cells, affecting their behavior in diverse ways. For example, EVs mediate the innate mucosal defense and stimulate immune response to viruses, parasites, and bacteria (15, 16). EVs secreted by macrophages infected with intracellular bacteria such as *Mycobacterium* or *Legionella* activate a proinflammatory immune response in uninfected macrophages (17–19). In the airway context, EVs secreted by wild type (WT) AEC stimulate a proinflammatory response in WT macrophages (20–22). EVs can also elicit anti-inflammatory effects in target cells. EVs isolated from the serum of mice that had been fed commensal *Lactobacillus* have anti-inflammatory properties

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and decrease LPS-induced secretion of IL-6 and TNF-a by murine macrophages (23). Murine melanoma and lymphoma cell lines infected with *Mycoplasma* secrete EVs that activate B cells and induce cytokine secretion, yet inhibit T cell activation (24), highlighting the differential effect of EVs on distinct target cells.

Although EVs have emerged as novel paracrine mediators in the lung microenvironment, relatively little is known about the effects of EVs secreted by AEC on leukocytes in the context of bacterial lung infections or in the context of CF. EVs recovered from bronchoalveolar lavage fluid (BALF) are reported to contain miRNAs (~22 nucleotide noncoding RNAs) that regulate gene expression in target cells (25, 26). AEC is the main source of EVs in the lung and have been shown to play a key role in innate immune defense (27, 28). Moreover, LPS exposure significantly increases EV secretion by alveolar macrophages, neutrophils, and nasal and airway epithelial cells in a TLR4-dependent manner (29–31), and EVs secreted by AEC promote airway inflammation in people with allergies and asthma (28). However, to our knowledge, there are no studies that have examined the effect of EVs secreted by CF AEC on CF macrophages.

To address this knowledge gap, we have conducted studies to examine the effect of EVs secreted by primary human CF and WT AEC on CF and WT human MDM. In addition, we performed experiments to determine whether *P. aeruginosa* exposure alters EVs secreted by AEC and we examined the effect of those EVs on CF and WT MDM. We report that EVs secreted by AEC activated an immune response by MDM; however, the immune response was less robust in CF MDM compared to WT MDM. Although exposure of AEC to *P. aeruginosa* increased the immune response of MDM to EVs, this increase was attenuated in CF MDM. The attenuated response of CF MDM to AEC EVs may have a significant impact on chronic bacterial infection and inflammation in the CF lung.

METHODS

Culture of Human Airway Epithelial Cells

Primary airway epithelial cells (AEC, CF Phe508del homozygous or WT) were obtained from Dr. Scott Randell (University of North Carolina, Chapel Hill, NC) and cultured as previously described (32). Briefly, AEC from passages 4–8 were grown at standard cell culture conditions (37°C, 5% CO_2) in BronchiaLife basal medium (Lifeline Cell Technology, Frederick, MD) supplemented with the BronchiaLife B/T LifeFactors Kit (Lifeline) as well as 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) and subcultured at 90% confluence. The absence of mycoplasma was verified by routine checks. After expansion and before EV collection, AEC with a viability of 96%–98% were seeded at either 1×10^6 cells per T75 or 2×10^6 cells per T162 cell culture flask and grown to confluence (~5×10^6 cells for T75 and 1×10^7 cells for T162), while changing the growth medium every 2–3 days. The Dartmouth Committee for the Protection of Human Subjects has determined that the use of AEC in this study is not considered human subject research because cells are taken from discarded tissue and contain no patient identifiers.

Bacterial Culture and Exposure of Human Airway Epithelial Cells

P. aeruginosa strain PA14 was grown in lysogeny broth (LB, Invitrogen, Grand Island, NY) at 37°C and 225 rpm for 16 h. EVs secreted by *P. aeruginosa*-exposed AEC (EV-PA) were generated by exposing AEC to *P. aeruginosa* for one hour at an multiplicity of infection (MOI) of 10:1 in antibiotic-free BronchiaLife medium. After three extensive washes with PBS to remove bacteria, AEC were incubated with Gentamicin (75 μg/mL) to kill any residual *P. aeruginosa*, and supernatants with secreted EV-PA (or EV from unexposed control cells, which were also cultured with complete BronchiaLife medium containing 75 μg/mL Gentamicin) were collected 24 hr later. The culture medium volume during EV collection was 11 mL for T75 flasks and 22 mL for T162 flasks.

EV Isolation and Characterization

As suggested in a recent review on EVs in the lung (33), we followed the recommendations regarding the isolation, characterization, and functional analysis of EVs outlined in a consensus document of the International Society of Extracellular Vesicles (34). EV and EV-PA were purified from supernatants of confluent AEC cultures (passages 4–8) after 24 hr using the ExoQuick-TC EV isolation kit (#EXOTC50A-1, System Biosciences, Palo Alto, CA). Briefly, suspended cells and debris were removed from AEC supernatants by centrifugation at 3,000 *g*. Precleared supernatants (30 mL) were concentrated to 10 mL with 30K Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA), mixed with 2 mL of ExoQuick-TC polymer, and incubated overnight at 4°C. EVs were precipitated by centrifugation at 1,500 *g* for 30 min. EVs were quantified by nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern Panalytical Ltd, Malvern, UK). We assessed the purity of isolated EVs by Western blot, probing for two classic exosomal markers, TSG101 (Sigma-Aldrich) and HSP70 (Cat. no. EXOAB-Hsp70A-1, System Biosciences), as well as calnexin (Santa

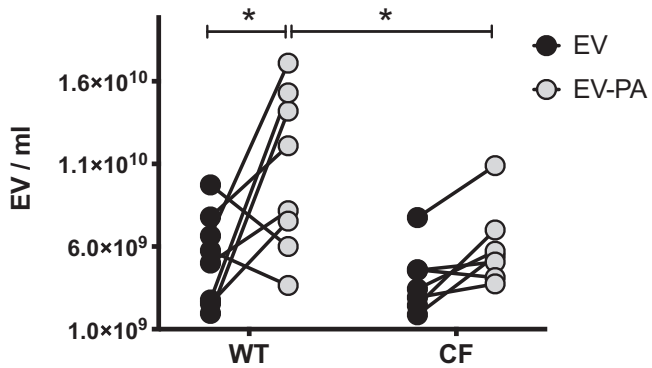

Figure 1. Quantification of EV and EV-PA secreted by WT and CF AEC. There was no significant difference in basal EV secretion by CF and WT AEC (black circles). There was a significant increase in the number of EV-PA per mL secreted by WT, but not CF, AEC after *P*seudomonas *aeruginosa* exposure (EV-PA, gray circles), with the number of EV-PA per mL significantly lower for CF AEC compared with WT AEC. Statistical significance was determined using two-way ANOVA with Sidak's multiple comparisons test, $^*P < 0.05$, $n = 8$ donors of WT AEC and 7 donors of CF AEC. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; WT, wild type.

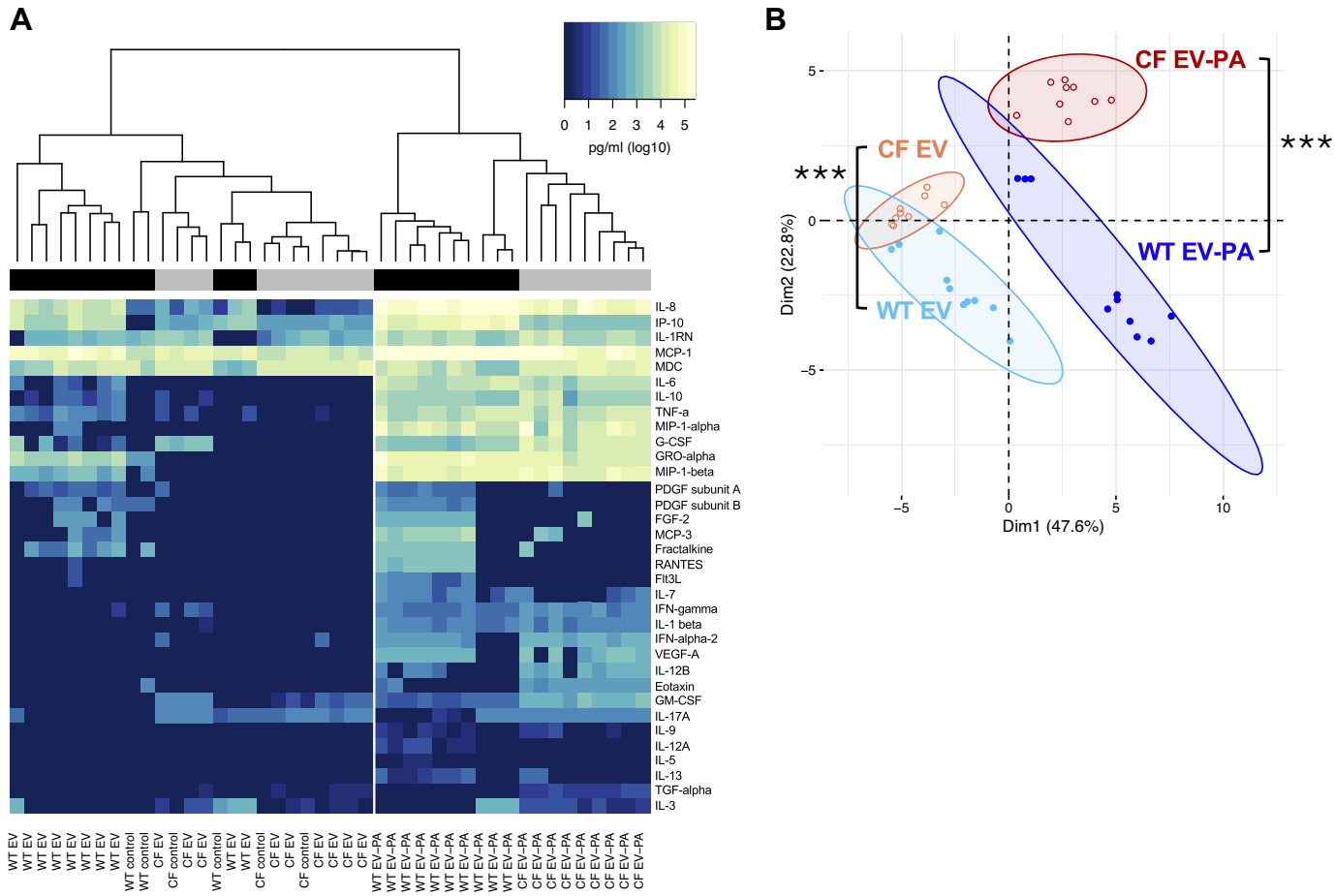

Figure 2. EV-PA and EV exposure increases cytokine secretion by MDM; however, cytokine secretion by CF MDM is less robust than cytokine secretion by WT MDM. *A*: heatmap of log10 transformed secreted cytokine concentrations (pg/mL) as measured using a human 41-Plex cytokine assay (Millipore). The rows contain 34 out of the 41 cytokines that had a median greater than 0 pg/mL in at least one of the groups. Dark blue indicates low concentrations close to 0 pg/mL, whereas the lightest yellow indicates concentrations above 100,000 pg/mL. Rows are clustered by cytokine and columns are clustered by sample similarity and the color bar below the column dendrogram indicates whether a sample was derived from CF MDM (gray) or WT MDM (black). EV exposed samples cluster together with unexposed controls and a split between CF and WT samples, whereas EV-PA exposed samples cluster separately with a clear split between CF and WT samples. *B*: principal component analysis (PCA) plot of log10 transformed data for 34 cytokines with a median greater than 0 pg/mL in at least one of the groups. The first dimension on the *x*-axis shows that 48% of variability between groups is explained by treatment (EV vs. EV-PA, Dim1), and the second dimension on the *y*-axis shows that 23% of variability is due to genotype (CF MDM vs. WT MDM, Dim2). CF MDM are depicted in shades of red using open circles, whereas WT MDM are depicted in shades of blue and filled circles. EV is shown in lighter shades of each color and EV PA in darker shades. Statistically significant differences between CF and WT MDM were calculated with permutational multivariate analysis of variance (PERMANOVA). ***$P < 0.001$, $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; MDM, monocyte-derived macrophages; WT, wild type.

Cruz Biotechnology, Dallas, TX), a marker for non-EV components. Calnexin is commonly used as a negative control to confirm the absence of contaminants such as cellular fragments or apoptotic bodies (8). Western blots experiments identified the presence of TSG101 and HSP70, but not calnexin, in EVs.

Isolation and Differentiation of Human Monocytes into MDM and Subsequent EV Exposure

This study was approved by the Institutional Review Board of Dartmouth-Hitchcock Medical Center (Protocol No. 22781). After the subjects provided written informed consent, whole blood was drawn into a heparinized syringe. CF (Phe508del homozygous) and WT monocytes from three donors each were isolated from peripheral blood as described previously (6, 35). Monocyte donor characteristics are listed in Supplemental Table S1 (see https://doi.org/10.6084/m9.figshare.13551491.v1). Monocytes were seeded at a density of 500,000 cells per well in 24-well plates and differentiated into monocyte-derived macrophages (MDM) by incubation with macrophage colony-stimulating factor (M-CSF) (100 ng/mL) for 7 days in RPMI medium supplemented with Glutamax, Gentamycin, and FBS, as described (6). MDM were switched to serum-free RPMI medium without M-CSF immediately before treatment with EV, EV-PA, or PBS as a vehicle control for 24 hr. EV and EV-PA were used at a concentration (2×10^9/mL) that has been previously measured in AEC culture supernatants and human bronchoalveolar lavage fluid (36). PBS was used as a vehicle control. After 24 hr, supernatants with secreted cytokines and total RNA were harvested from all samples.

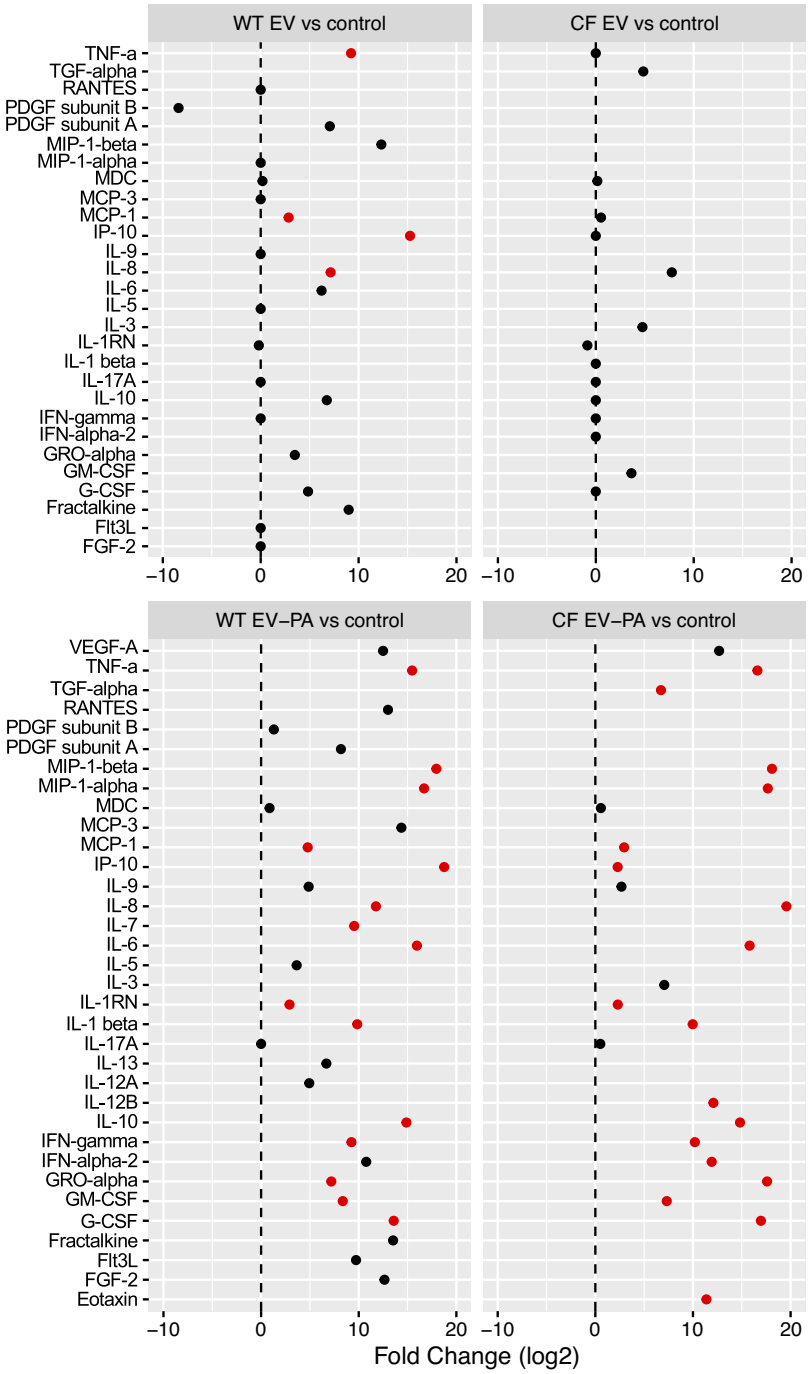

Figure 3. EV-PA induced more proinflammatory cytokine secretion by MDM than EV. Comparison of cytokine secretion by WT MDM + EV compared with control (upper left), CF MDM + EV compared with control (*upper right*), WT MDM + EV-PA compared to control (*lower left*), and CF MDM + EV-PA compared to control (lower right). Cytokines with a median greater than 0 pg/mL in at least one of the groups are listed on the *y*-axis and median log2 fold changes for the different comparisons are plotted on the *x*-axis. Data points are missing in cases where a cytokine was below detection for a given condition. A nominal value of 0.1 was added to all values in order to be able to calculate median log2 fold changes. Cytokines with $P < 0.05$ and absolute log2 fold change >1 are highlighted in red, whereas cytokines that did not reach this significance threshold are represented by black circles. Statistical significance was determined by Wilcoxon rank sum test. $n=3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; MDM, monocyte-derived macrophages; WT, wild type.

Cytokine Secretion by MDM

The effect of EV and EV-PA on cytokine secretion by CF MDM and WT MDM were measured using the MILLIPLEX MAP Human Cytokine/Chemokine 41-Plex cytokine assay (Millipore). Even though there was some donor-to-donor variability in cytokine secretion, the within-group variability was much lower than the between-group variability comparing WT to CF MDM or EV to EV-PA exposure. The mean Euclidean distance of all pairwise comparisons within groups was 4.7 ± 2.5 SD, whereas the mean Euclidean distance of all pairwise comparisons between groups was 10.3 ± 2.7 SD.

RNA-Seq of MDM

Total RNA was isolated from CF MDM and WT MDM with the miRNeasy kit (Qiagen, Germantown, MD). Sequencing libraries were prepared using the QuantSeq 3′ mRNA-seq Library Prep Kit FWD for Illumina (Lexogen, Greenland, NH) with 200 ng RNA as input. 3′ libraries were sequenced on an Illumina NextSeq500 High Output run as 75 bp single-end reads. Raw reads were processed with fastp version 0.20.0 (37) to trim off PolyA tails from the 3′ end and the first 12 bp from the 5′ end to eliminate mismatched bases introduced by the random primer used to prime the second strand

synthesis. Trimmed reads were aligned to the human reference transcriptome (version GRCh38) using Salmon version 0.14.0 (38). The R package tximport version 1.18.0 (39) was used to create gene-level counts from Salmon output. Differential gene expression analysis of RNA-seq data was performed using genewise negative binomial generalized linear models in edgeR version 3.32.0 (40). Genes with $P < 0.05$ and absolute log2 fold change >1 were considered significantly differentially expressed. Gene expression networks and functional analyses and predictions were generated through the use of Ingenuity Pathway Analysis (IPA, Qiagen, http://www.ingenuity.com) (41). Fastq files for all RNA-seq samples as well as count tables of raw and normalized aligned reads have been deposited in NCBI's Gene Expression Omnibus (42) and are accessible through GEO Series accession number GSE163095 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163095).

Statistical Analysis and Data Visualization

Data were analyzed using GraphPad Prism (version 8.4.3; San Diego, CA) and the R software environment for statistical computing and graphics version 4.0.2 (43). Statistical significance was calculated using two-way ANOVA, PERMANOVA, Wilcoxon rank sum tests, paired *t* tests, and likelihood ratio tests on genewise negative binomial generalized linear models, as indicated in the figure legends. PERMANOVA was performed with the help of the vegan package in R (44). Data were visualized and figures were created using the R packages gplots (45), ggplot2 (46), factoextra (47), and ggpubr (48) as well as Ingenuity Pathway Analysis (41).

RESULTS

P. aeruginosa Stimulates EV Secretion by WT AEC

EVs purified from the supernatants of *P. aeruginosa* exposed WT and CF AEC, or unexposed AEC, were quantified using Nanoparticle Tracking Analysis (NTA). Absolute EV/mL quantified by NanoSight is an estimate because NTA measurements include background counts from protein aggregates and dust particles. There was no significant difference between the concentration of EVs secreted by WT and CF AEC at baseline (Fig. 1). EV secretion in response to *P. aeruginosa* exposure (hereafter called EV-PA) was significantly increased in WT but not CF AEC. Moreover, EV-PA secretion by *P. aeruginosa* exposed CF AEC was significantly lower than EV-PA secretion by WT AEC (Fig. 1).

EV and EV-PA-Induced Cytokine Secretion Is Attenuated in CF MDM

To assess the ability of EVs secreted by AEC to induce cytokine secretion by macrophages, WT or CF MDM were exposed to EV or EV-PA secreted by WT or CF AEC, respectively, or exposed to vehicle alone. Secreted cytokines were measured in cell culture supernatants after 24 hr. For control MDM, there was no significant difference in the baseline cytokine secretion between CF and WT MDM for any of the cytokines measured (Supplemental Table S2; see https://doi.org/10.6084/m9.figshare.13551485.v1). Overall, samples clustered into two groups—those that were exposed to EV-PA versus those exposed to EV and unexposed controls (Fig. 2*A*). Within each group, samples clustered by genotype. EV-PA exposure elicited a robust upregulation of cytokine

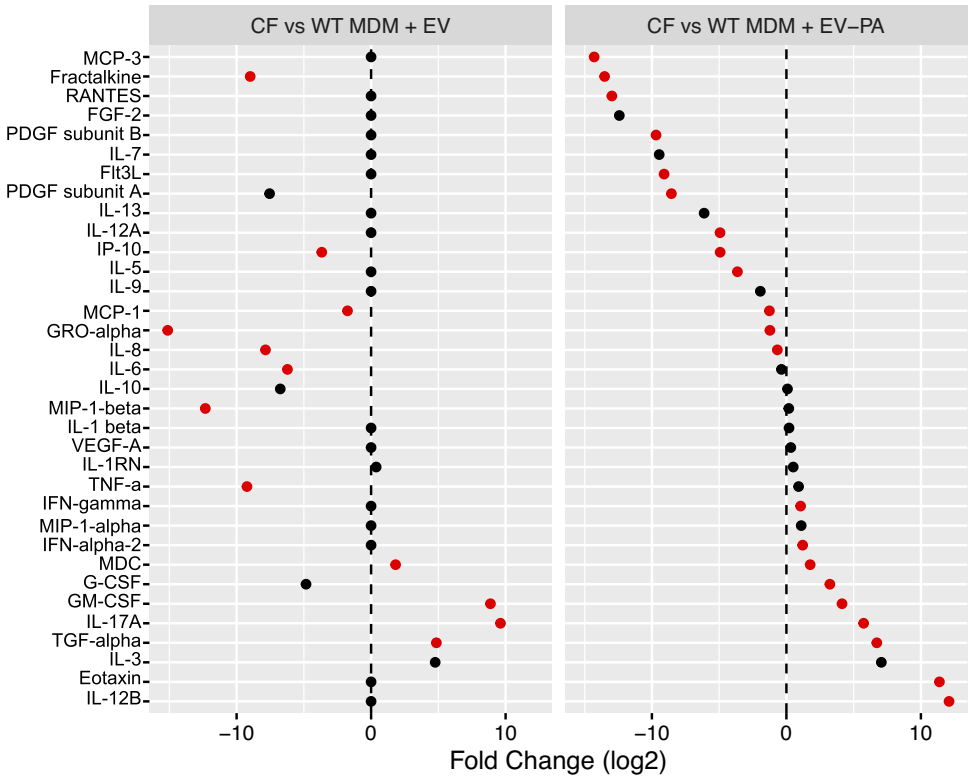

Figure 4. Attenuated CF MDM cytokine secretion in response to EV and attenuated upregulation of chemoattractants and tissue repair factors in response to EV-PA. Comparison of cytokine secretion by CF MDM versus WT MDM exposed to EV (*left*) or EV-PA (*right*). Cytokines with a median greater than 0 pg/mL in at least one of the groups are listed on the *y*-axis and median log2 fold changes for the different comparisons are plotted on the *x*-axis. A nominal value of 0.1 was added to all values in order to be able to calculate median log2 fold changes. Cytokines with FDR < 0.05 are highlighted in red, whereas cytokines that did not reach this significance threshold are represented by black circles. Statistical significance was determined by Wilcoxon rank sum test. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FDR, false discovery rate; MDM, monocyte-derived macrophages; WT, wild type.

Table 1. *Cytokines differentially secreted by CF MDM and WT MDM*

Uniprot Protein Short Name	HUGO Gene Symbol	log2 FC CF vs. WT EV	FDR CF vs. WT EV	log2 FC CF vs. WT EV-PA	FDR CF vs. WT EV-PA	Main Immune Function
IL-12B	*IL12B*	0.0	1.000	12.1	0.006	Proliferation and differentiation of Tc, NK, and LAK cells; promotes Th1 and inhibits Th2 cells
Eotaxin	*CCL11*	0.0	1.000	**11.4**	**0.002**	Eosinophil and basophil migration
IL-3	*IL3*	4.8	0.556	7.1	0.201	Proliferation and differentiation of granulocytes, monocytes, and dendritic cells
TGF-alpha	*TGFA*	**4.9**	**0.009**	**6.7**	**0.002**	Tissue repair but also fibrosis
IL-17A	*IL17A*	**9.6**	**0.002**	**5.7**	**0.002**	Activates macrophages, fibroblasts, and stromal cells
GM-CSF	*CSF2*	**8.9**	**0.002**	**4.1**	**0.002**	Maturation of dendritic cells, neutrophils, and macrophages
G-CSF	*CSF3*	−4.8	0.627	**3.2**	**0.002**	Proliferation and differentiation of granulocytes and neutrophils
MDC	*CCL22*	**1.8**	**0.016**	**1.8**	**0.002**	Th2 response, Th2-cell migration, and Treg migration
IFN-alpha-2	*IFNA2*	0.0	0.214	**1.2**	**0.002**	Inhibition of viral replication
MIP-1-alpha	*CCL3*	0.0	0.257	1.1	0.443	Macrophage and NK-cell migration, T-cell–DC interactions
IFN-gamma	*IFNG*	0.0	0.257	**1.1**	**0.007**	Inhibition of viral replication and cell proliferation
TNF-a	*TNF*	**−9.2**	**0.003**	0.9	0.535	Neutrophil chemoattractant, stimulates macrophage phagocytosis
IL-1RN	*IL1RN*	0.4	0.422	0.5	0.269	Anti-inflammatory
VEGF-A	*VEGFA*	0.0	1.000	0.3	0.280	Monocyte and macrophage chemoattractant
IL-1 beta	*IL1B*	0.0	0.422	0.2	0.316	Stimulates transmigration of phagocytes and lymphocytes
MIP-1-beta	*CCL4*	**−12.3**	**0.004**	0.2	0.930	Macrophage and NK-cell migration; T-cell–DC interactions
IL-10	*IL10*	−6.8	0.085	0.1	0.574	Anti-inflammatory
IL-6	*IL6*	**−6.2**	**0.048**	−0.4	0.269	Stimulates production of neutrophils and growth of B cells and is antagonistic to regulatory T cells
IL-8	*CXCL8*	**−7.9**	**0.002**	**−0.7**	**0.029**	Neutrophil chemoattractant
GRO-alpha	*CXCL1*	**−15.1**	**0.004**	**−1.2**	**0.017**	Neutrophil chemoattractant
MCP-1	*CCL2*	**−1.8**	**0.004**	**−1.3**	**0.014**	Inflammatory monocyte chemoattractant
IL-9	*IL9*	0.0	0.430	−1.9	0.966	Stimulates cell proliferation and prevents apoptosis
IL-5	*IL5*	0.0	0.145	**−3.6**	**0.008**	Proliferation and differentiation of eosinophiles
IP-10	*CXCL10*	**−3.7**	**0.002**	**−4.9**	**0.002**	Th1 response; Th1, CD8, NK trafficking
IL-12A	*IL12A*	0.0	1.000	**−4.9**	**0.017**	Proliferation and differentiation of Tc, NK, and LAK cells; promotes Th1 and inhibits Th2 cells
IL-13	*IL13*	0.0	1.000	−6.1	0.272	Anti-inflammatory
PDGF subunit A	*PDGFA*	−7.5	0.079	**−8.6**	**0.033**	Tissue repair
Flt3L	*FLT3LG*	0.0	0.430	**−9.1**	**0.008**	Increases the number of B and T cells by activating hematopoietic progenitors
IL-7	*IL7*	0.0	1.000	−9.5	0.083	Proliferation and differentiation of B cells, cytotoxic T cells, and NK cells
PDGF subunit B	*PDGFB*	0.0	0.079	**−9.7**	**0.008**	Tissue repair
FGF-2	*FGF2*	0.0	0.079	−12.4	0.062	Tissue repair
RANTES	*CCL5*	0.0	0.430	**−13.0**	**0.008**	Macrophage and NK-cell migration; T-cell–DC interactions
Fractalkine	*CX3CL1*	**−9.0**	**0.009**	**−13.5**	**0.029**	NK, monocyte, and T-cell migration
MCP-3	*CCL7*	0.0	0.079	**−14.3**	**0.029**	Monocyte mobilization

Thirty-four cytokines with a median greater than 0 pg/mL in at least one of the groups are ordered by median log2 fold change for CF versus WT EV-PA (column 5). *column 1* lists the uniprot protein short names, *column 2* lists the corresponding HUGO gene symbols, *column 3* contains median log2 fold changes, and *column 4* contains FDR corrected *P* values from the Wilcoxon's rank sum test for cytokine secretion by CF MDM exposed to CF EV compared with WT MDM exposed to WT EV. *Column 5* contains median log2 fold changes, and *column 6* contains FDR corrected *P* values from the Wilcoxon's rank sum test for cytokine secretion by CF MDM exposed to CF EV-PA compared with WT MDM exposed to WT EV-PA. *Column 7* lists key biological functions for each cytokine. FDR corrected *P* values > 0.05 and the associated median log2 fold changes are highlighted in bold font. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FC, fold change; FDR, false discovery rate; MDM, monocyte-derived macrophages; WT, wild type.

secretion, with distinct subsets of cytokines more strongly secreted by either WT or CF MDM (Fig. 2*A*). A subset of cytokines was also more robustly secreted by WT MDM in response to EVs compared to EV exposed CF MDM and unexposed controls. Principal component analysis revealed that 48% of variability between groups is explained by treatment (EV or EV-PA) and 23% of variability is due to genotype (WT or CF, Fig. 2*B*). For each genotype, there were significant differences in cytokine secretion in response to EV compared with EV-PA ($P < 0.001$), and within each treatment group (EV or EV-PA), there were significant differences between CF and WT MDM. As shown in Fig. 2*B*, the CF EV-PA group (red) is left-shifted toward CF EV (orange), compared with WT EV-PA (dark blue), suggesting that collectively, the response of CF MDM to EV-PA is attenuated compared with WT MDM exposed to EV-PA. Moreover, the response of CF MDM to EV is attenuated compared with WT MDM exposed to EV. Looking at changes in secretion levels of individual cytokines, the response of CF MDM to EVs was attenuated compared to WT MDM (Fig. 3). EVs elicited a moderate increase in WT MDM cytokine secretion compared with controls, with TNF-a, MCP-1, IP-10, and IL-8 reaching statistical significance ($P < 0.05$ and absolute log2 FC > 1). By contrast, EVs did not significantly increase the secretion of any cytokines by CF MDM, though there was a trend for IL-8 to increase.

When comparing macrophage cytokine secretion in response to EV-PA and EV, EV-PA (Fig. 3, *bottom*) caused a much more pronounced induction of cytokine secretion than EV (Fig. 3, *top*) for both WT and CF MDM. A direct comparison of cytokine secretion by CF versus WT MDM exposed to EV or EV-PA revealed attenuated CF MDM cytokine secretion in response to EV and reduced upregulation of chemoattractants and tissue repair factors in response to EV-PA (Fig. 4). EV-induced secretion of 8 cytokines (Fractalkine, IP-10, MCP-1, GRO-alpha, IL-6, IL-8, MIP-1-beta, and TNF-a) was significantly reduced in CF MDM compared to WT MDM, while 4 cytokines (MDC, GM-CSF, IL-17A, and TGF-alpha) were secreted at higher levels by CF MDM (Fig. 4, *left*). The upregulation of chemoattractants, such as RANTES, IP-10, GRO-alpha, MCP-1, and IL-8, and tissue repair factors PDGF subunit A and B in response to EV-PA was significantly attenuated in CF MDM compared to WT MDM, while a group of cytokines involved in immune cell activation were

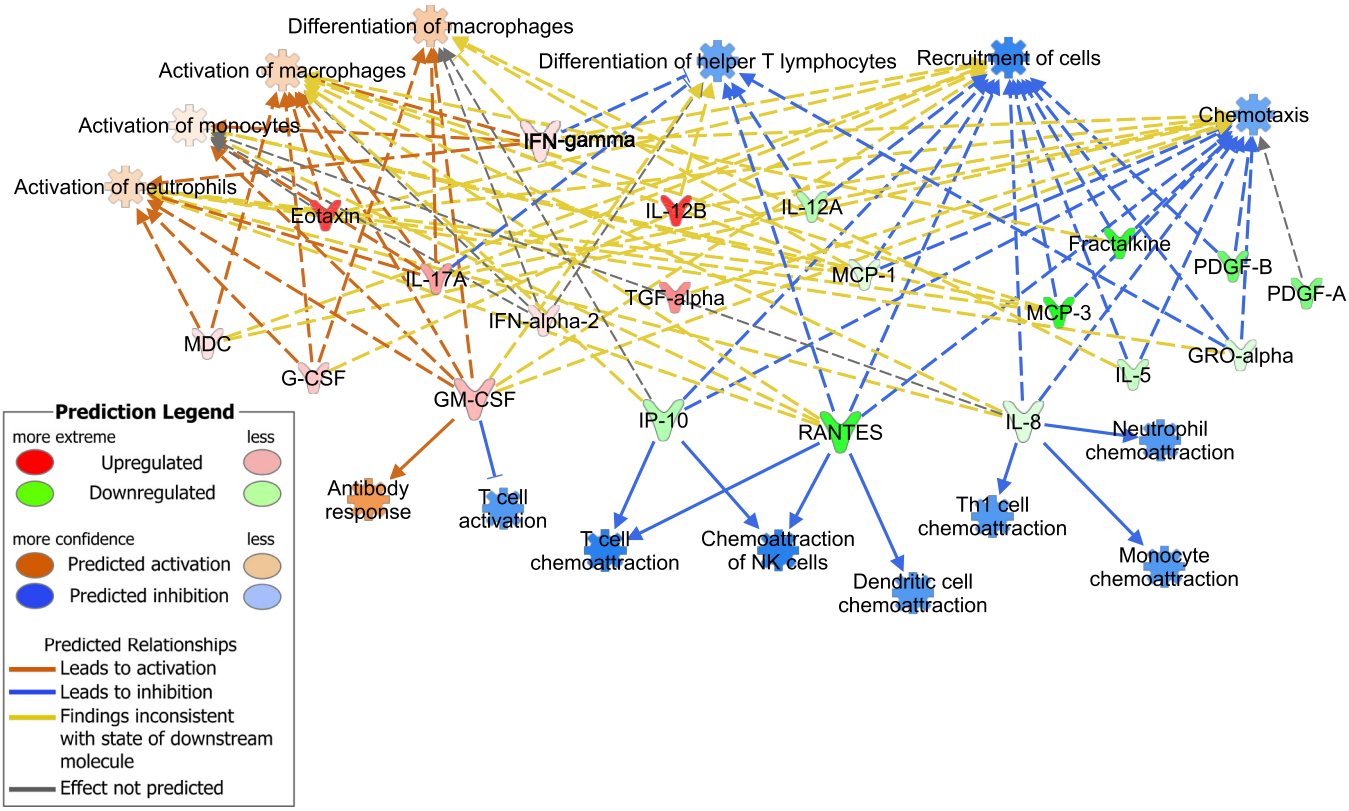

Figure 5. Differential cytokine secretion by CF MDM versus WT MDM predicts reduced chemoattraction of immune cells in response to EV-PA. Ingenuity Pathway Analysis for all cytokines that were differentially (FDR < 0.05) secreted by CF MDM exposed to CF EV-PA compared with WT MDM exposed to WT EV-PA. Cytokines that are secreted more by CF MDM compared with WT MDM are highlighted in shades of red while cytokines that are secreted less by CF MDM compared to WT MDM are shown in shades of green. Orange is predicted activation of downstream processes and functions, based on the activation status of the detected cytokines, whereas blue denotes predicted repression. Based on the overall cytokine secretion profile of EV-PA exposed CF MDM compared with WT MDM, IPA predicts increased activation of phagocytes but reduced chemotaxis and recruitment of cells. Specifically, the increased secretion of GM-CSF by CF MDM is predicted to result in increased antibody response and decreased T cell activation, whereas the reduced secretion of IP-10, RANTES, and IL-8 is predicted to lead to reduced chemoattraction of T cells, NK cells, dendritic cells, monocytes, and neutrophils. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FDR, false discovery rate; MDM, monocyte-derived macrophages; WT, wild type.

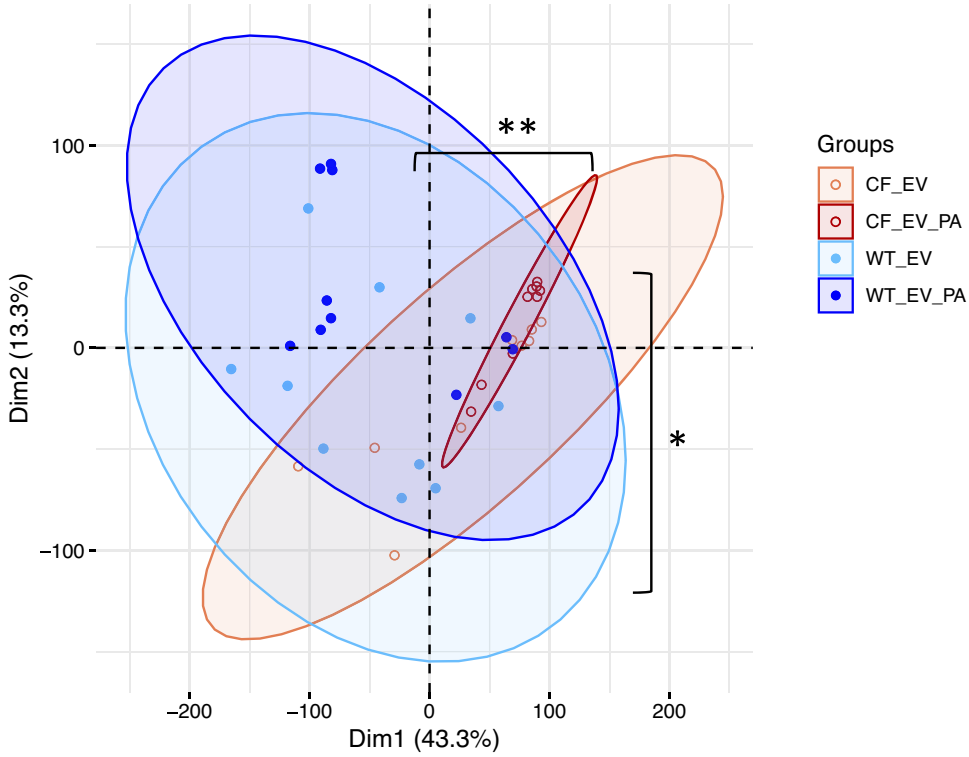

Figure 6. Significant differences between CF MDM and WT MDM mRNA expression in response to EV and EV-PA. Principal component analysis (PCA) plot of log2 transformed gene expression data for 14,370 expressed transcripts. The first dimension on the *x*-axis shows that 43% of variability between WT MDM and CF MDM is explained by treatment (EV vs. EV-PA, Dim1), and the second dimension on the *y*-axis shows that 13% of variability is due to genotype (CF MDM vs. WT MDM, Dim2). CF MDM are depicted in shades of red using open circles, whereas WT MDM are depicted in shades of blue and filled circles. EV is shown in lighter shades of each color and EV-PA in darker shades. Statistically significant differences between CF and WT MDM were calculated with permutational multivariate analysis of variance (PERMANOVA). $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; MDM, monocyte-derived macrophages; WT, wild type.

significantly increased (Fig. 4, right panel). All log2 fold changes and false discovery rate (FDR) corrected *P* values comparing cytokine secretion by CF MDM to WT MDM are listed in Table 1.

To examine the overall effect of EV-PA-induced cytokine secretion on CF versus WT MDM, we used Ingenuity Pathway Analysis (IPA) to analyze the net effect of reduced cytokine secretion by CF MDM compared to WT MDM. IPA predicted that the less robust secretion of cytokines by CF MDM will reduce chemotaxis and recruitment of immune cells (Fig. 5). The lower rate of secretion of IP-10, RANTES, and IL-8 by CF MDM compared to WT MDM is predicted to lead to reduced chemoattraction of T cells, NK cells, dendritic cells, monocytes, and neutrophils. By contrast, the higher level of secretion of IL-17A and GM-CSF by CF MDM compared with WT MDM is predicted to lead to increased activation of monocytes, neutrophils, and macrophages as well as increased antibody response but decreased activation of T cell and decreased differentiation of helper T lymphocytes (Fig. 5).

Differential Cytokine Secretion by CF MDM versus WT MDM Is Not Due to Differences between CF and WT EVs

To determine whether the differential cytokine secretion between CF and WT MDM is due to differences between EVs secreted by CF and WT AEC, rather than an inherent defect in CF MDM, we measured the cytokine secretion by CF MDM from three donors in response to EVs derived from CF and WT AEC that were either exposed or unexposed to *P. aeruginosa*. We found that there was no significant difference in cytokine secretion of CF MDM in response to EVs derived from CF or WT AEC (Supplemental Fig. S1; see https://doi.org/10.6084/m9.

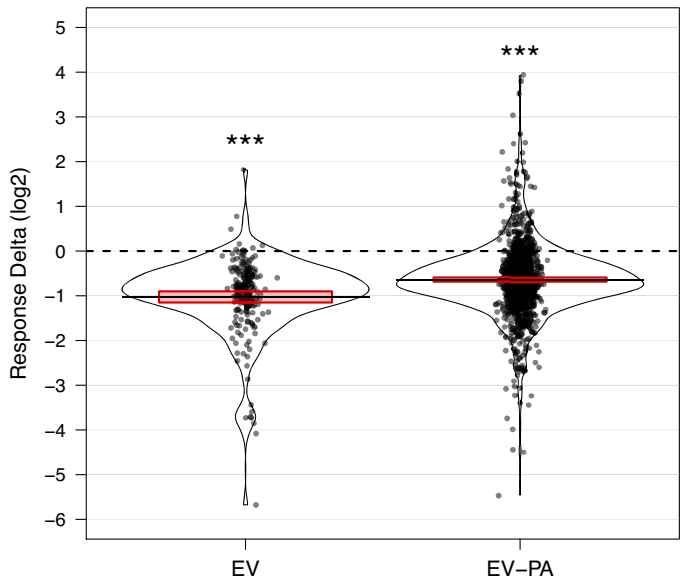

Figure 7. Transcriptomic response to EV and EV-PA is significantly attenuated in CF MDM. The absolute difference in the response of CF MDM versus WT MDM to EV or EV-PA is shown on the *y*-axis for 193 genes that were differentially expressed ($P < 0.05$ and abs. log2 FC > 1) in WT MDM in response to EV and 1,191 genes that were differentially expressed in WT MDM in response to EV-PA. The difference in the absolute fold change (=response delta) between CF and WT MDM was significantly below 0 (horizontal dashed line) for both EV and EV-PA, indicating an attenuated response by CF MDM. Red boxes depict the 95% confidence interval around the means. Statistical significance was determined using one sample *t* tests. $^{***}P < 0.001$, $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FC, fold change; MDM, monocyte-derived macrophages; WT, wild type.

figshare.13551482.v1). Samples clustered into two groups based on exposure to EV or EV-PA but did not cluster by genotype of EV-secreting AEC (Supplemental Fig. S1*A*). Sixty-five percent of the variability between groups was explained by treatment (EV vs. EV-PA), with no significant differences between EVs derived from WT or CF AEC (Supplemental Fig. S1*B*).

Gene Regulatory Networks Drive Responses of MDM to EV and EV-PA

We performed RNA-seq to assess gene expression in CF and WT MDM exposed to EV or EV-PA to elucidate differences in the response of upstream regulators of cytokine secretion, which may explain the differential cytokine secretion we observed between CF MDM and WT MDM. We found significant differences in the mRNA expression levels between CF and WT MDM exposed to EV or EV-PA (Fig. 6). 43% of the variability between CF MDM and WT MDM was explained by treatment (EV vs. EV-PA) and 13% of variability was due to genotype (CF MDM vs. WT MDM).

Genes that were induced or repressed by EV or EV-PA exposure in WT MDM were significantly less induced or repressed in CF MDM (Fig. 7), mirroring the pattern of attenuated response we previously observed for cytokine secretion. For 193 genes that were differentially expressed ($P < 0.05$ and abs. log2 FC > 1) in WT MDM in response to EVs as well as 1191 genes that were differentially expressed in WT

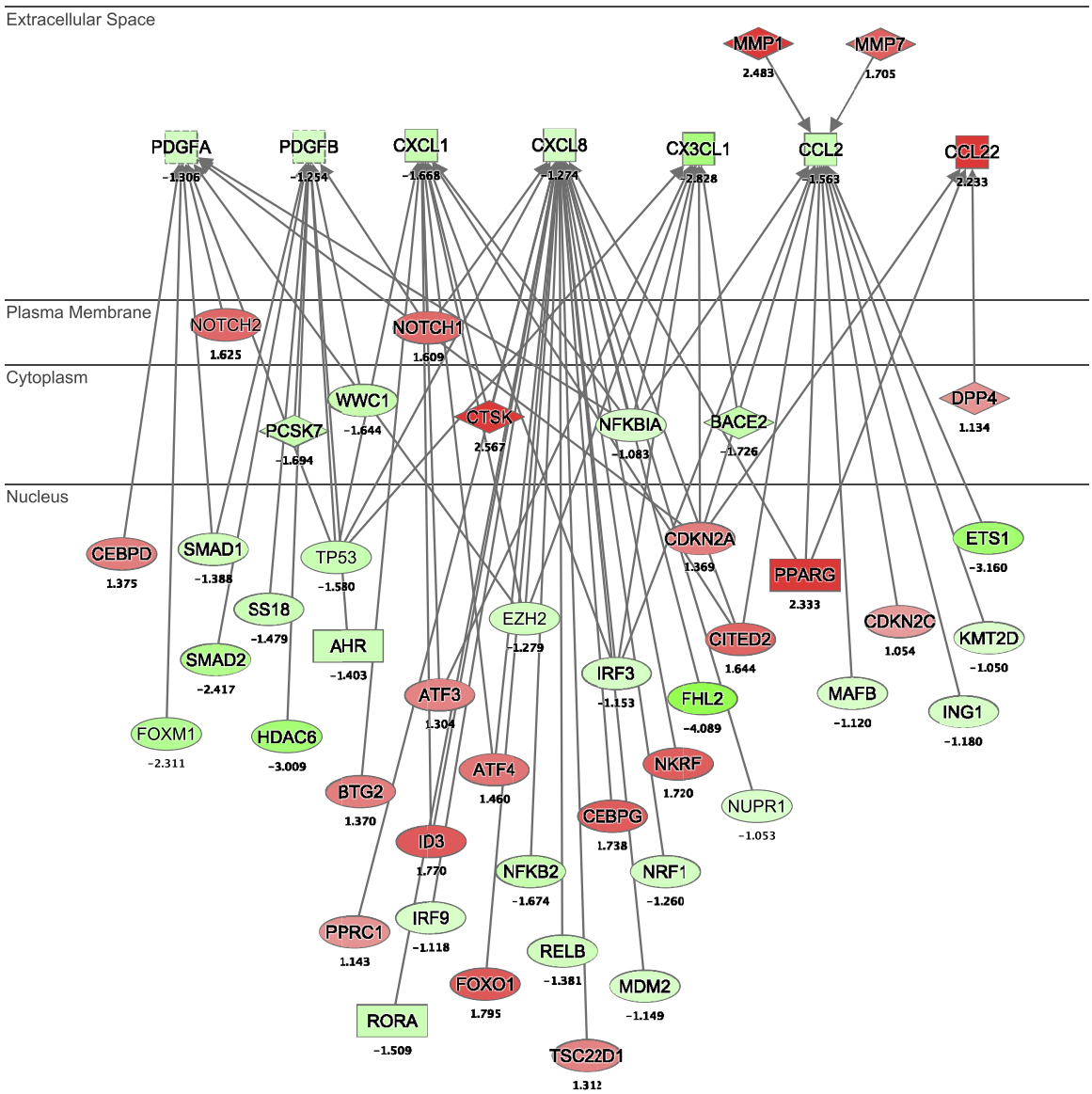

Figure 8. Changes in mRNA expression in response to EV-PA are attenuated in CF MDM. Ingenuity Pathway Analysis of seven cytokines that were detected at the mRNA level and their upstream regulators, all of which were differentially expressed ($P < 0.05$ and absolute log2 FC > 1) in EV-PA exposed CF MDM compared with EV-PA exposed WT MDM. Genes that were reduced at the mRNA level in CF MDM compared with WT MDM are shaded in green, whereas those that were expressed at higher levels in CF MDM than WT MDM are shaded in red. Expression level fold changes between CF MDM and WT MDM are listed below each gene symbol. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FC, fold change; MDM, monocyte-derived macrophages; WT, wild type.

MDM in response to EV-PA, the transcriptomic response of CF MDM was significantly attenuated. All log2 fold changes and *P* values of genes from the edgeR linear models are provided in Supplemental Table S3 (see https://doi.org/10.6084/m9.figshare.13551488.v1).

Genes encoding cytokines and upstream regulators identified by IPA were differentially expressed in EV-PA exposed CF MDM compared with EV-PA exposed WT MDM (Fig. 8). In WT MDM, EV-PA exposure led to a significant induction of transcription activators *NKFB2, RELB*, and *EZH2* and repression of negative regulators *NOTCH1* and *CITED2*, consistent with increased *CXCL1* and *CXCL8* mRNA levels. Compared with WT MDM, CF MDM showed significantly less activation of *NKFB2*, *RELB*, and *EZH2* and reduced repression of *NOTCH1* and *CITED2* in response to EV-PA, resulting in attenuated upregulation of *CXCL1* and *CXCL8*. In addition, mRNA levels of *PDGFA*, *PDGFB*, *CX3CL1*, and *CCL2* were significantly less upregulated in CF MDM compared with WT MDM and *CCL22* expression was significantly higher in CF MDM. The attenuated EV-PA mediated induction of *PDGFA*, *PDGFB*, *CXCL1*, *CXCL8*, *CX3CL1*, and *CCL2* and increased induction of *CCL22* in CF versus WT MDM are consistent with the observed differential secretion of cytokines encoded by these genes (PDGF subunit A, PDGF subunit B, GRO-alpha, IL-8, Fractalkine, MCP-1, and MDC) in response to EV-PA (Fig. 5).

For CF MDM exposed to EV-PA, we observed a significant increase in expression of the metalloproteinases *MMP1* and *MMP7* compared EV-PA exposed WT MDM (Fig. 8). The monocyte chemoattractant MCP-1 (encoded by the *CCL2* gene) is degraded by MMP1 and MMP7 (49, 50), which may explain the significantly lower levels of MCP-1 detected in CF MDM supernatants (Fig. 4).

MDM Gene Expression Differences Suggest Reduced Chemoattraction and Inflammation Resolution in CF

IPA predicted that the EV-PA induced adaptive and innate immune response is attenuated in CF MDM compared to WT MDM (Fig. 9). EV-PA exposure significantly upregulated *CXCL9* and *CCL19* in WT MDM (Fig. 9*A*). *CXCL9* encodes MIG, a major chemoattractant for macrophages, monocytes, and NK cells (51), and *CCL19* encodes a chemoattractant for

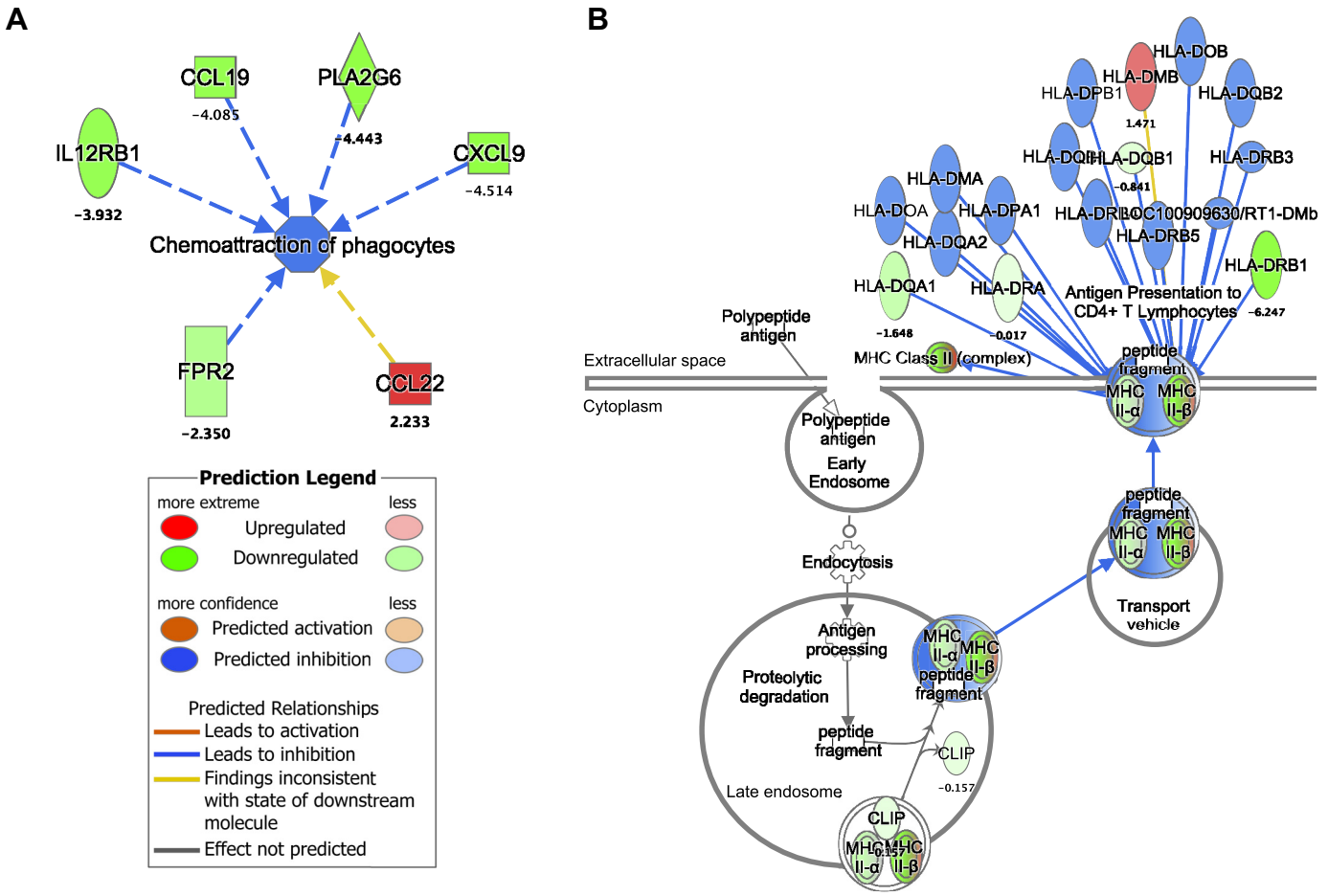

Figure 9. EV-PA induced adaptive and innate immune response of CF MDM predicted to be attenuated. Ingenuity Pathway Analysis predictions for chemoattraction of phagocytes (*A*) and MHC-II antigen presentation (*B*) with genes that were significantly upregulated in CF MDM + EV-PA compared to WT MDM + EV-PA shaded in red and those with significantly reduced expression levels in CF versus WT MDM + EV-PA shaded in green. Blue shading indicates predicted repression. Expression level fold changes between EV-PA exposed CF and WT MDM are listed below each gene symbol. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; MDM, monocyte-derived macrophages; WT, wild type.

macrophages, NK, T, B, and dendritic cells—MIP-3-beta (52). Upregulation of these cytokines in response to EV-PA is predicted to lead to increased chemoattraction of phagocytes. However, the EV-PA mediated upregulation of *CXCL9* and *CCL19* was significantly attenuated in CF MDM compared to WT MDM, along with reduced levels of *FPR2* and *PLA2G6*, which promote monocyte chemoattraction (53) and *IL12RB1*, which promotes macrophage chemoattraction (54). Taken together, Ingenuity Pathway Analysis predicts reduced chemoattraction of phagocytes based on the attenuated upregulation of chemoattracts, including *CXCL9* and *CCL19*, by CF MDM compared to WT MDM (Fig. 9*A*). In addition, the pro-resolution formyl peptide receptor 2 (*FPR2*) binds specialized pro-resolving mediators such as lipoxins and resolvins and has been previously reported to be downregulated in CF MDM (55).

We also detected significant differences in cytokine receptor gene expression between CF MDM and WT MDM. For four cytokines secreted at higher levels by EV-PA exposed CF MDM compared with WT MDM (MDC, GM-CSF, IL-12B, and IL-17A; Fig. 4, *right* and Fig. 5), there was a significant decrease in the mRNA expression level of the corresponding cytokine receptors in CF MDM (Table 2). A reduction in the expression of cytokine receptors by CF MDM may lead to an impaired response of CF MDM to cytokines, in spite of higher secretion levels. Finally, we also observed that EV-PA induction of genes involved in MHC-II antigen presentation, including *HLA-DQA1*, *HLA-DQB1*, *HLA-DRA*, *HLA-DRB1*, and *HLA-DRB5*, was significantly less robust in CF MDM relative to WT MDM (Fig. 9*B*).

DISCUSSION

This study presents the first observations of cytokine secretion and gene expression in primary WT MDM and CF MDM in response to EVs secreted by primary WT and CF AEC. We observed that EVs from unexposed and *P. aeruginosa* exposed AEC (EV-PA) elicited a less robust increase in cytokine and chemoattractant secretion by CF MDM compared to WT MDM and that the attenuated cytokine secretion was mirrored by parallel changes in mRNA expression. The observed differences in CF MDM and WT MDM did not depend on the source of the EVs, an observation consistent with the view that the diminished response is inherent in the CF MDM rather than due to any differences between EVs secreted by CF versus WT AEC. Notably, the differential response of CF MDM was only observed in the context of exposure to EV. We found no significant difference in basal cytokine secretion between CF MDM and WT MDM, consistent with a recent report that there was no difference in basal cytokine secretion by primary human MDM in which CFTR had been knocked out compared to WT MDM (56). Our data are also consistent with multiple studies demonstrating that EVs secreted by WT AEC stimulate the secretion of proinflammatory cytokines including IL-1 beta and TNF-a by WT macrophages (20–22), however, in these studies neither CF AEC nor CF MDM were examined.

Table 2. *Significantly differentially expressed cytokine receptors*

Cytokine	Receptor	log2 FC CF vs. WT	*P* Value
MDC	*CCR4*	−3.6	5.8E−12
GM-CSF	*CSF2RA*	−1.9	9.8E−09
IL-12B	*IL12RB1*	−3.9	7.3E−11
IL-17A	*IL17RC*	−0.9	6.6E−03
IL-17A	*IL17RD*	−3.2	6.0E−12
IL-17A	*IL17RE*	−1.4	7.8E−04
IFN-gamma	*IFNGR1*	1.4	1.9E−04

Receptors of cytokines that were secreted at significantly higher levels by CF MDM in response to EV-PA are mostly repressed at the mRNA level in CF MDM + EV-PA compared with WT MDM + EV-PA. Cytokines are listed in *column 1*, their matching receptors in *column 2*, the log2 fold changes in the mRNA level of each receptor in EV-PA exposed CF versus WT MDM in *column 3* and *P* values in *column 4*. Statistical significance was calculated by conducting likelihood ratio tests on a genewise negative binomial generalized linear model in edgeR. $n = 3$ donors each for WT and CF MDM exposed to EVs secreted by WT AEC from 4 donors or CF AEC from 3 donors. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; FC, fold change; MDM, monocyte-derived macrophages; WT, wild type.

Figure 10 represents a granular summary of our results. EVs secreted by WT AEC increased the secretion of IP-10, TNF-a, IL-8, and MCP-1 by WT MDM, while there was no significant increase in cytokine secretion by CF MDM in response to EVs secreted by CF AEC (Fig. 10*A*). A similar overall pattern of attenuated response was observed for EV-PA exposed CF MDM compared with WT MDM (Fig. 10*B*). EV-PA increased the secretion of chemoattractants and tissue repair factors by WT MDM and CF MDM and induced gene expression of chemoattractants *CXCL9* and *CCL19* as well as *NFKB* subunits (Fig. 10*B*). However, the response of CF MDM to EV-PA was significantly attenuated compared with WT MDM, with the exception of *MMP1* and *MMP7*, which were significantly more induced in CF MDM compared to WT MDM. In addition, upregulation of tissue repair factors such as PDGF subunit A and B, and chemoattractants was significantly attenuated in CF MDM compared to WT MDM. Taken together, the attenuated EV-PA induced chemoattractant secretion by CF MDM compared with WT MDM and the fact that EV-PA secretion by CF AEC was significantly reduced compared with WT AEC are predicted to reduce recruitment of immune cells to the CF lung. This prediction is consistent with the finding that people with CF had significantly lower numbers of neutrophils in their sputum (in spite of increased numbers of bacteria) compared to people with primary ciliary dyskinesia who also frequently suffer from bacterial lung infections (57). Previous studies have shown that outer membrane vesicles secreted by *P. aeruginosa* decrease MHC expression in WT lung macrophages, thus decreasing the immune response (58). By contrast, here we found that EV-PA upregulates MHC-II complex expression in WT MDM and that this upregulation is attenuated in CF MDM. Taken together, these observations indicate that, in vivo, the combined effect of vesicles secreted by AEC and *P. aeruginosa* on macrophages will depend on multiple factors including the relative concentration of vesicles secreted by both prokaryotic and eukaryotic cells.

To assess whether the attenuated secretion of cytokines, chemoattractants, and tissue repair factors by CF MDM are due to CF versus WT EV cargo, we exposed CF MDM to EVs derived from CF or WT AEC. However, we found no significant difference in the secretion of cytokines, chemoattractants, and tissue repair factors by CF MDM in response to

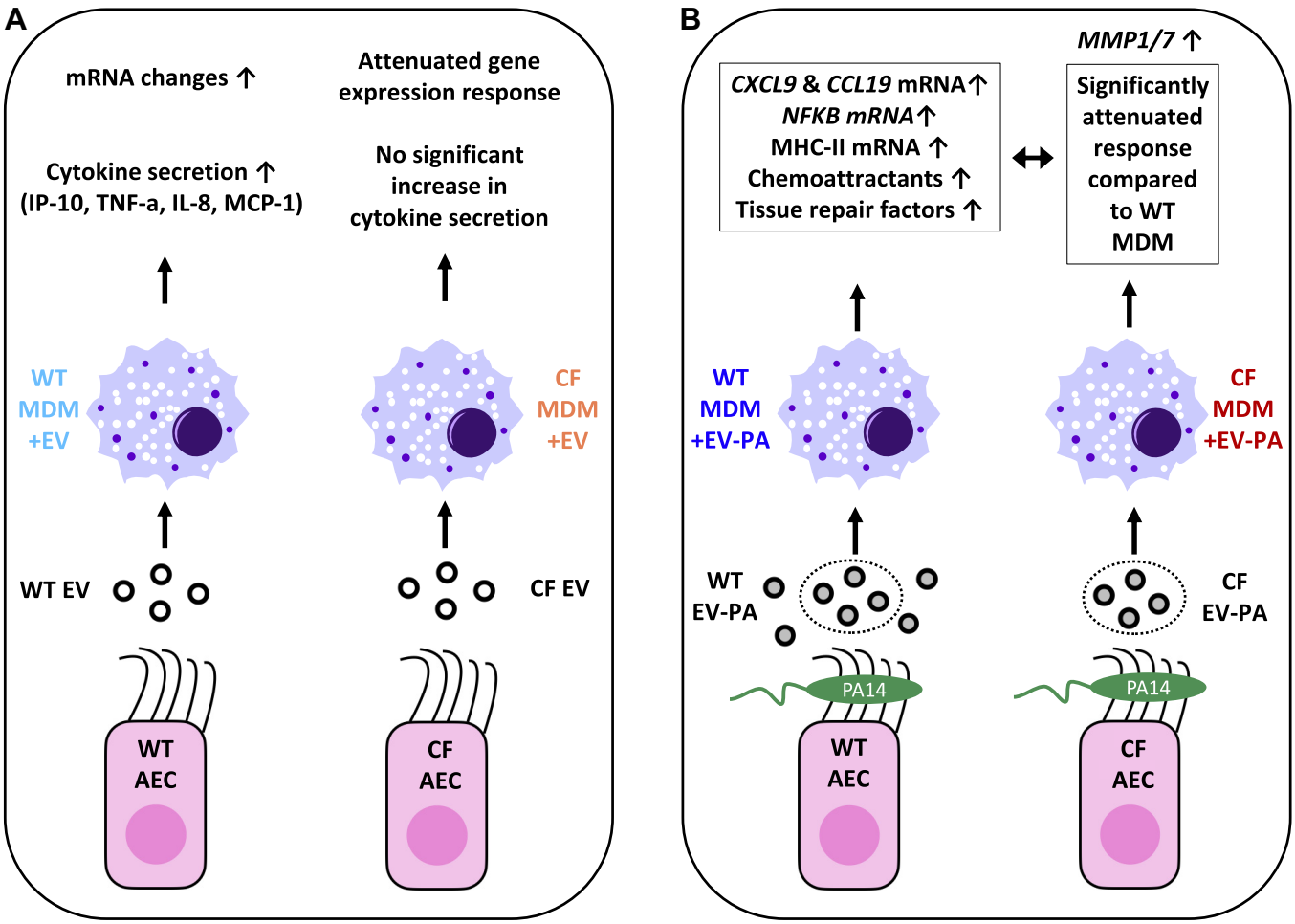

Figure 10. Summary of findings. *A*: EVs secreted by WT AEC increase secretion of IP-10, TNF-a, IL-8, and MCP-1 by WT MDM and induce differential gene expression, whereas there is no significant increase in EV-mediated cytokine secretion by CF MDM and an attenuated gene expression response. *B*: *Pseudomonas aeruginosa* (strain PA14) exposure significantly increases EV-PA secretion by WT but not CF AEC. When the same number of EV-PA, illustrated by the same number of gray dots in the dashed circles, are added to WT and CF MDM, CF MDM show an overall attenuated response at the mRNA level. CF MDM also secrete significantly less chemoattractants and tissue repair factors compared with WT MDM. AEC, airway epithelial cell; CF, cystic fibrosis; EV, extracellular vesicles; EV-PA, EVs secreted by *P. aeruginosa*-exposed AEC; MDM, monocyte-derived macrophages; WT, wild type.

EVs derived from either CF or WT AEC, at baseline or after exposure to *P. aeruginosa* (6). Hence, the attenuated response of CF MDM appears to be due to an inherent defect in the response of CF MDM to EVs rather than differences in the composition of EVs secreted by CF versus WT AEC.

Our study has several advantages and some limitations. The choice of primary AEC and primary MDM has several advantages over cell lines and monocytes including *1*) the use of primary cells from multiple donors is more representative of a population than immortalized cell lines isolated from one individual, *2*) primary MDM are more representative of macrophages in vivo, than transformed cells such as THP-1 cells, which have been highly manipulated and are likely aneuploid, and *3*) the majority of macrophages in the CF airway originated from recruited monocytes (59), making MDM a relevant cell type in the context of the CF lung. One limitation of our study is that we took a reductionist approach, examining the effects of EVs secreted by AEC on MDM, whereas the lung environment contains EVs from multiple sources including AEC, neutrophils, macrophages, and other immune cells as well as a host of cytokines and chemoattractants (1, 2, 30, 60). Thus, the response of MDM in the lung to EVs and cytokines in vivo is likely to be more complex than the response observed in this study. In addition, it is likely that MDM respond differently from tissue-resident alveolar macrophages that are embryonically derived. However, MDM are more representative of lung macrophages that are recruited in response to a bacterial infection. Another limitation of our study is the use of a laboratory strain of *P. aeruginosa* rather than a CF clinical isolate. Moreover, due to the small sample size of WT and CF MDM donors, we cannot draw conclusions about potential differences between males and females. We also did not investigate other CF genotypes besides Phe508del.

Future studies beyond the scope of this work are required to determine why CF MDM are less responsive to EVs compared to WT MDM. Although the mechanisms that mediate EV uptake and cargo delivery into acceptor cells are incompletely characterized, recent studies suggest that EV uptake is mediated by interaction with membrane proteins, receptors, sugars, or lipids, through endocytosis by clathrin-dependent and clathrin-independent pathways and by macropinocytosis, micro-

pinocytosis, and phagocytosis (61–63). Moreover, once EVs enter cells and lysosomes, cargo release may involve several mechanisms, including escape via a pH-dependent mechanism (63). Notably, all of these pathways and mechanisms are defective in CF (6, 9–11, 64, 65). Future experiments assessing all of these mechanisms, which are beyond the scope of this study, are needed to elucidate why CF MDM are less responsive to EVs than WT MDM.

In summary, our data demonstrate that EVs secreted by AEC are important players in the immune response of MDM to *P. aeruginosa* infection and that mutations in *CFTR* reduce the ability of MDM to respond to EV and EV-PA secreted by CF AEC. Our study has revealed that EV-mediated cross talk between AEC and MDM is defective in CF, which adds to the long list of disordered cell function in CF.

SUPPLEMENTAL DATA

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.13551491.v1.

Supplemental Table S2: https://doi.org/10.6084/m9.figshare.13551485.v1.

Supplemental Table S3: https://doi.org/10.6084/m9.figshare.13551488.v1.

Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.13551482.v1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.K., Z.L., T.H.H., and B.A.S. conceived and designed research; K.K., A.N., R.B., and Z.L. performed experiments; K.K. and T.H.H. analyzed data; K.K., T.H.H., A.A., and B.A.S. interpreted results of experiments; K.K. prepared figures; K.K. and B.A.S. drafted manuscript; K.K., A.N., R.B., Z.L., T.H.H., A.A., and B.A.S. edited and revised manuscript; K.K., A.N., R.B., Z.L., T.H.H., A.A., and B.A.S. approved final version of manuscript.

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