Impaired innate immune alveolar macrophage response and the predilection for COPD exacerbations

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ON-LINE SUPPLEMENTAL DATA

Subject criteria

Participants were over 30 years of age. Of 96 participants with COPD, nine were lost to follow-up for monitoring of exacerbations. All participants underwent clinical assessment, routine spirometry and chest x-rays. Ex-smokers had expired breath carbon monoxide of <0.02ppm (Vitalograph Breath CO Monitor, Lenexa, KS).

For all COPD participants, exclusion criteria included a forced expiratory volume at one second (FEV_1) <35%, hypercapnia and co-morbid diseases that would render bronchoscopy unsafe. Inclusion criteria were: 1) chronic bronchitis by history and/or emphysema by chest x-ray or CT; 2) absence of other lung disease, including asthma and bronchiectasis, based on clinical evaluation; 3) chest x-ray findings that were normal or compatible with COPD, but detected no other disease; 4) FEV_1/FVC ratio below the 95% lower confidence limit of normal on spirometry; 5) non-atopic by history; 6) no antibiotic or systemic steroid use for four weeks preceding enrollment. Healthy non-smokers met all inclusion criteria of the COPD group, except #1 and #4 (above) and that all had never smoked (E1).

Exacerbation criteria

Participants were followed at six week intervals by telephone contact and by six and twelve month clinic visits, at which they were evaluated for occurrence of exacerbation in the intervening period. They were questioned about episodes of increased shortness of breath, increased sputum production or change in sputum color. Exacerbations were defined as an increase in one of more of these symptoms lasting over 24 hours, necessitating evaluation and treatment by a health care provider.

Purification of alveolar macrophages

Macrophages were cultured in RPMI1640 (BioWhittaker, Walkersville, MD) and human AB-positive serum (Nabi, Miami FL). Alveolar macrophages were purified from individual BALs and seeded onto 48 well tissue culture plates (10^5 cells/ml) in RPMI1640/10% AB-positive serum, supplemented with penicillin-streptomycin (100 U/ml) (E1). After incubation (5% CO_2, 95% humidity, 37^oC) for 24 hours, non-adherent cells were removed. Remaining alveolar macrophages were consistently 98-100% esterase-positive (E1). Percent viability of alveolar macrophages (mean$\pm$SEM), by trypan blue exclusion, was 89.1$\pm$1.2, 88.7$\pm$0.9 and 89.6$\pm$.7 for Groups 1, 2 and 3 respectively ($p>0.1$).

Macrophage-bacteria incubation

Each bacterial strain was fully viable in 8% antibody-depleted serum (E2). Antibody-depleted human serum was purified by Protein G affinity chromatography (Amersham, Piscatawy, NJ) as a complement source, as previously described (E3). All three bacterial strains were obtained from sputum from COPD patients. All were associated with COPD exacerbations

and all elicited a systemic and/or mucosal antibody response to the infecting strain, following the COPD exacerbations, making these strains more relevant to clinical COPD exacerbations than laboratory or type strains. All experiments included control wells treated with *E. coli* K235 LPS (1μg/ml) (Sigma Chemical Co., St. Louis MO) or buffer diluents of each antigen.

TLR expression

Alveolar macrophages ($2x10^5$ cells/well) were adhered on Lab-Tek chamber slides (Nunc Inc., Naperville, IL). Macrophages were co-incubated with each bacterial strain (MOI-200:1). Anti-human-TLR2 and-TLR4 were purchased from eBiosciences, San Diego, CA. Cells were incubated with anti-human TLR2-FITC or anti-TLR4 (final concentration-1ug/ml) for one hour at 37^{o}C in light-protected containers. After repeated rinses with PBS to remove unbound antibody, TLR4-stained cells were incubated with FITC-F(ab')$_2$ goat anti-mouse IgG (1:50) (Invitrogen Corp, Camarillo, CA) for 30 min at 4^{o}C and then rinsed with PBS. Cells were kept protected from light and were analyzed by immunofluorescent image capture microscopy.

NF-κB activation

Because of limited numbers of alveolar macrophages, studies were performed with macrophages of ten non-exacerbation-prone and four exacerbation-prone donors. Alveolar macrophages were incubated with respiratory bacteria (NTHI, MC, SP at 1:200) or with buffer alone (3 hrs). Plated cells were removed from wells and centrifuged (5min at 500rpm; 4^{o}C). Protein content of nuclear extracts was quantitated by Lowry assay to obtain 20 ug nuclear protein from 4-8 $x10^6$ cells for each condition (E4). Cell pellets were resuspended in detergent-containing hypotonic buffer with centrifuged at 14000xg for 30sec (4^{o}C). The pellet containing

nuclear extract was resuspended and incubated on a rocking platform (150rpm) in lysis buffer (30min.), before centrifugation (14000xg for 10min). Supernatant was stored at -80^{o}C. Using reagents from manufacturer, 30ul of binding buffer followed by 20ul of nuclear extract in lysis buffer was added to each well. Control wells of buffer alone and p50 protein standards were included. Plates were sealed and incubated (one hour, room temp). After vigorous washing, 100ul of antibody to NF-κB (p50; 1:1000) was added and incubated for one hour. HRP-conjugated secondary antibody 100ul; 1:1000) was added after vigorous washing and incubated (one hr). 100ul developing solution was incubated for 1-3 min. Reactions were terminated at the appearance of color change and absorbance was read at 450nm.

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Online Supplement Table 1-Multivariate logistic regression tables - Immunologic outcomes with significant association with exacerbation status were analyzed for independent association with age and FEV_1.

IL-8 induction

Independent variable	Odds Ratio	95% CI	p value
Age	1.014	0.962-1.068	0.614
FEV_1	0.500	0.212-1.179	0.113
Log IL-8 (NTHI)	0.371	0.158-0.874	0.023

Independent variable	Odds Ratio	95% CI	p value
Age	1.025	0.972-1.080	0.363
FEV_1	0.595	0.251-1.411	0.239
Log IL-8 (MC)	0.377	0.145-0.981	0.046

Independent variable	Odds Ratio	95% CI	p value
Age	1.015	0.964-1.069	0.565
FEV_1	0.464	0.196-1.098	0.081
Log IL-8 (SP)	0.435	0.190-0.997	0.049

Independent variable	Odds Ratio	95% CI	p value
Age	1.024	0.974-1.077	0.353
FEV_1	0.533	0.227-1.251	0.148
Log IL-8 (Pam_3Cys)	0.455	0.195-1.059	0.068

Independent variable	Odds Ratio	95% CI	p value
Age	1.025	0.975-1.078	0.337
FEV_1	0.564	0.236-1.351	0.199
Log IL-8 (LPS)	0.400	0.170-0.842	0.036

Multivariate logistic regression tables (continued)

TNF-α induction

Independent variable	Odds Ratio	95% CI	p value
Age	1.031	0.979-1.085	0.252
FEV_1	0.512	0.221-1.185	0.118
Log TNF-α (NTHI)	0.804	0.646-1.000	0.0499

Independent variable	Odds Ratio	95% CI	p value
Age	1.030	0.980-1.083	0.241
FEV_1	0.515	0.224-1.184	0.118
Log TNF-α (MC)	0.851	0.694-1.045	0.123

Independent variable	Odds Ratio	95% CI	p value
Age	1.028	0.978-1.080	0.272
FEV_1	0.521	0.224-1.213	0.130
Log TNF-α (Pam_3Cys)	0.798	0.567-1.124	0.196

Independent variable	Odds Ratio	95% CI	p value
Age	1.025	0.975-1.078	0.335
FEV_1	0.451	0.187-1.087	0.076
Log TNF-α (LPS)	0.722	0.553-0.942	0.017

TLR expression

Independent variable	Odds Ratio	95% CI	p value
Age	1.006	0.971-1.042	0.735
FEV_1	0.929	0.929-0.482	0.826
TLR2 (MC)	0.691	0.625-0.764	<0.01

Independent variable	Odds Ratio	95% CI	p value
Age	0.998	0.963-1.034	0.893
FEV_1	0.582	0.304-1.111	0.101
TLR2 (SP)	0.770	0.711-0.833	<0.01

Online Supplement Table 2 - Comparison of cytokine induction results was made with measured values compared with values corrected for individual baseline cytokine values.

		Bacterial strains					
	COPD Groups	NTHI 11P6H1		*M. catarrhalis* 6P29B1		*S. pneumoniae* 25P55S1	
		Uncorrected value	Corrected for baseline	Uncorrected value	Corrected for baseline	Uncorrected value	Corrected for baseline
IL-8	Nonexacerbation-prone	2365 [4250.5]	993.0 [1848.3]	1710 [3383.8]	903.3 [2300.8]	1165 [2137.4]	270.5 [1246.0]
	Exacerbation-prone	775 [2814]	321.5 [1213.6]	870 [2586]	344.8 [1514.6]	460 [1319]	13.9 [392.5]
	Mann Whitney U p value	0.024	0.022	0.045	0.059	0.046	0.013
TNF-α	Nonexacerbation-prone	0 [19.2]	0.6 [38.9]	16.1 [515.0]	3.0 [43.9]	ND	ND
	Exacerbation-prone	0 [4.3]	0 [3.9]	7.0 [112.4]	0 [5.18]	ND	ND
	Mann Whitney U p value	0.046	0.07	0.08	0.03	ND	ND

Online Supplement Table 2 (continued)

		TLR Ligands			
	COPD Groups	LPS		Pam_3Cys	
		Uncorrected value	Corrected for baseline	Uncorrected value	Corrected for baseline
IL-8	Nonexacerbation-prone	1220 [1617]	341.0 [1082.6]	1160 [2179]	480.0 [1152.8]
	Exacerbation-prone	500 [1361]	33.4 [347.8]	640 [1440]	90.0 [519.0]
	Mann Whitney U p value	0.028	0.022	0.074	0.029
TNF-α	Nonexacerbation-prone	18.8 [91.9]	17.1 [82.8]	53.5 [119.8]	52.7 [120.1]
	Exacerbation-prone	4.7 [20.5]	4.5 [20.6]	21.3 [60.6]	21.3 [60.6]
	Mann Whitney U p value	0.015	0.015	0.049	0.050