ELASTASE EXOCYTOSIS BY AIRWAY NEUTROPHILS IS DWITH EARLY LUNG DAMAGE IN CYSTIC FIBROSIS CHILDREN

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SUPPLEMENTAL MATERIAL

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SUPPLEMENTAL METHODS

Human subjects

The study was approved by our institutional ethics committees. Consent for collection of blood and bronchoalveolar lavage (BAL) samples was obtained from parents on the day of the procedure. All specimens were stored on ice after collection and during transport to the laboratory for immediate processing. Cell counts and differential for BAL specimen was performed by the clinical pathology laboratory, and culture results were reported using standard aerobic culture technique by clinical microbiology laboratories at our respective institutions.

Sample collection and processing

Blood was collected by venipuncture in K_2 EDTA tubes, and the cells were recovered as previously described (33). Bronchoscopy with BAL was performed in the right middle lobe while under general anesthetic as part of routine clinical management (28). BAL was retrieved by instillation and aspiration of three aliquots of sterile saline (1 mL/kg up to a maximum of 20 mL). The first aliquot was cultured by clinical microbiologists for the detection of pathogens using standard qualitative culture techniques. Pulmonary infection was defined as colony counts for a specific organism (excluding mixed oral flora) greater than 10^4 colony-forming units per mL. The second and third aliquots were pooled and a fraction was used to generate cytospin slides for differential leukocyte counts by the clinical pathology laboratory (28). EDTA was added to the remainder of the pooled second and third BAL fractions (2.5 mM final) to stop cell activation until processing (within an hour of collection). Cells were separated by gentle passing through an 18-gauge needle, followed by centrifugation at 800 g

for 10 minutes at 4 ºC. The supernatant was depleted of bacteria by centrifugation at 3,000 g for 10 minutes at 4 ºC, and stored at -80 °C for downstream assays, which included NE activity assays and a multiplexed assay for inflammatory mediators (IL-1α, IL-1β, IL-1RA, IL-6, IL-8, IL-10, IL-18, IFNγ, TNFα, G-CSF, GM-CSF, M-CSF, MIP-1, MCP-1, ENA-78, I-TAC, TRAIL, IP-10, VEGF, GROα).

Flow cytometry

Cells were pre-incubated for 5 minutes on ice with Fc block reagent and Zombie live/dead Aqua dye (Biolegend), followed by staining for 15 minutes on ice with antibodies against various surface markers, including CD3 (pan T-cell), CD16, CD45 (pan-leukocyte), CD63, CD66b, CXCR2, and CXCR4 (all from Biolegend); neutrophil elastase (Novus Biologicals); Siglec-8 (eosinophils), and Siglec-9 (both from R&D systems). Cells were then washed with PBS-EDTA, and fixed at 4°C with Lyse/Fix Phosflow reagent (BD Biosciences). Prior to acquisition, fixed cells were spun at 800G for 10 minutes at 4 ºC, and resuspended in PBS-EDTA. Samples were acquired on a FACS Fortessa (BD Biosciences) or on a FACS LSRII (BD Biosciences). To ensure robust acquisition of flow cytometric data on fresh samples acquired over the course of the study, we used premixes of the antibodies listed above to stain neutrophils and monocytes / macrophages, and employed a stringent calibration method with Rainbow bead-based channel standardization to provide constant output from the flow cytometers (35). This method enabled robust data acquisition on samples collected over a year in multiple sites. Fluorescence compensation and data analysis were performed using FlowJo V9.9.5 (Treestar).

Image cytometry

Cells retrieved from the BAL were fixed in Lyse/Fix Phosflow (BD Biosciences) and stored at -80 °C until use. Thawed cells were washed with PBS-EDTA, permeabilized with Perm Buffer I (BD Biosciences) and stained with DAPI (nuclear stain), cholera toxin B (CTB, to distinguish neutrophils) (31), as well as CD63 and NE. Cells were acquired on the Amnis Imagestream X Mark II (EMD Millipore), with 40x magnification and low flow rate/high sensitivity on the INSPIRE software. Brightfield was set on channels 01 and 09, while scattering was set in channel 06. Data were analyzed using the IDEAS software v6.1 (EMD Millipore).

SUPPLEMENTAL TABLES

Table E1. Details on study subjects and assays. FC: flow cytometry; IC: image cytometry; MI: multiplexed immunoassay; NA: not applicable; NE: neutrophil elastase.

Subject	Condition	Age (months)	Sex	Site	CFTR allele 1	CFTR allele 2	Posiive cultures (clinical laboratory)	PRAGMA	Assays
1	CF	23	F	Perth	G85E	unknown	*M. catarrhalis*	Yes	FC, NE, MI
2	CF	24	M	Perth	F508	F508	*Aspergillus spp, H. influenzae, P. aeruginosa*	Yes	FC, NE, MI
3	CF	24	F	Perth	F508	F508	None	Yes	FC, NE, MI
4	CF	24	M	Perth	F508	F508	*E. coli, Paecilomyces lilacinus*	Yes	FC, NE, MI
5	CF	23	F	Perth	F508	R117H	None	Yes	FC, NE, MI
6	CF	24	M	Perth	F508	1898+1G->A	None	Yes	FC, NE, MI
7	CF	11	M	Perth	F508	F508	None	Yes	FC, NE, MI
8	CF	12	M	Perth	621+1G->T	I507	*H. Influenzae*	Yes	FC, NE, MI
9	CF	11	M	Perth	F508	F508	None	Yes	FC, NE, MI
10	CF	12	F	Perth	F508	I502T	None	Yes	FC, NE, MI
11	CF	11	F	Perth	F508	F508	None	Yes	FC, NE, MI
12	CF	4	F	Perth	F508	1717-1G->A	None	Yes	FC, NE, MI
13	CF	10	M	Perth	F508	F508	*Cytomegalovirus*	Yes	FC, NE, MI
14	CF	12	F	Perth	F508	R1158X	None	Yes	FC, NE, MI
15	CF	4	M	Perth	F508	F508	None	Yes	FC, NE, MI
16	CF	4	M	Perth	F508	F508	None	Yes	FC, NE, MI
17	CF	8	F	Perth	F508	c.3718-2477C>T	*Rhinovirus*	Yes	FC, NE, MI
18	CF	3	F	Perth	F508	F508	*A. niger, C. albicans, E. coli*	Yes	FC
19	CF	39	M	Rotterdam	F508	G542X	*P. aeruginosa*	Yes	FC, NE, MI
20	CF	60	M	Rotterdam	F508	A455E	*H. influenzae*	Yes	FC, NE, MI
21	CF	62	M	Rotterdam	F508	A455E	*H. influenzae, S. aureus*	Yes	FC, NE, MI
22	CF	61	M	Rotterdam	F508	R117H-7T	None	Yes	FC, NE, MI
23	CF	61	F	Rotterdam	F508	F508	*P. aeruginosa*	Yes	FC
24	CF	61	M	Rotterdam	E60X	4015delATTT	*S. aureus*	Yes	NE, MI
25	CF	61	M	Rotterdam	F508	F508	*A. fumigatus*	Yes	FC, NE, MI
26	CF	60	F	Rotterdam	F508	F508	None	Yes	FC, NE, MI
27	CF	60	M	Rotterdam	F508	R117H	None	Yes	NE, MI
28	CF	62	F	Rotterdam	F508	R117H	None	Yes	FC, NE, MI
29	CF	61	F	Rotterdam	F508	F508	*H. influenzae, S. aureus*	Yes	FC, NE, MI
30	CF	61	M	Rotterdam	F508	DELE2,3(21KB)	None	Yes	FC, NE, MI
31	CF	12	F	Rotterdam	F508	F508	None	Yes	FC, NE, MI
32	CF	13	F	Rotterdam	Y275X	A559T	None	No	FC, NE, MI
33	CF	36	F	Rotterdam	F508	N1303K	None	No	FC, NE, MI
34	CF	62	M	Rotterdam	F508	N1303K	None	No	FC, NE, MI
35	CF	12	M	Rotterdam	F508	E60X	None	Yes	FC, NE, MI
36	CF	12	F	Rotterdam	F508	A455E	None	No	NE, MI
37	CF	60	F	Rotterdam	F508	NK1303K	None	No	FC
38	CF	61	F	Rotterdam	F508	F508	None	No	FC
39	CF	36	F	Rotterdam	F508	F508	None	Yes	FC, MI
40	CF	12	F	Rotterdam	F508	F508	None	Yes	FC, MI
41	CF	13	M	Rotterdam	R1162X	R1162X	None	No	FC, NE, MI
42	CF	12	F	Rotterdam	F508	1682dup	None	Yes	FC, MI
43	CF	35	F	Rotterdam	R117H	1857delT	None	Yes	IC
44	CF	13	F	Rotterdam	F508	F508	None	Yes	IC
45	CF	13	F	Rotterdam	F508	F508	None	Yes	IC
46	CF	13	M	Rotterdam	F508	3272-26A>G	None	Yes	IC
47	CF	37	M	Rotterdam	F508	F508	None	Yes	IC
48	CF	14	F	Rotterdam	F508	c.3407_3422del	*P. aeruginosa*	Yes	IC
49	CF	37	M	Rotterdam	F508	F508	None	Yes	IC
50	CF	36	F	Rotterdam	F508	F508	None	Yes	IC
51	CF	37	F	Rotterdam	F508	F508	*A. fumigatus*	Yes	IC
52	CF	37	F	Rotterdam	F508	F508	None	Yes	IC
53	Control	25	F	Atlanta	NA	NA	None	No	FC, NE, MI
54	Control	13	M	Atlanta	NA	NA	None	No	FC, NE, MI
55	Control	18	F	Atlanta	NA	NA	*H. influenzae, P. aeruginosa*	No	FC, NE, MI
56	Control	17	F	Atlanta	NA	NA	None	No	FC, NE, MI
57	Control	37	M	Atlanta	NA	NA	*H. influenzae*	No	FC, NE, MI
58	Control	17	M	Atlanta	NA	NA	None	No	FC, NE, MI
59	Control	12	M	Atlanta	NA	NA	None	No	FC, NE, MI
60	Control	24	F	Atlanta	NA	NA	*S. aureus*	No	FC, NE, MI
61	Control	26	M	Atlanta	NA	NA	None	No	FC, NE, MI
62	Control	25	F	Atlanta	NA	NA	*M. catarrhalis*	No	FC, NE, MI

Table E2. Absolute cell counts in BAL from CF and disease control children enrolled in the study. Shown are median [interquartile range] values for BAL counts expressed in cells/ml. Differences between CF and disease control children were assessed using the Wilcoxon rank sum test. n.s., not significant.

	CF BAL	*Disease control BAL*	*p*
Total leukocytes	$7.1x10^5$ [$2.6x10^5$ - $1.9x10^6$]	$2.3x10^5$ [$6.2x10^4$ - $4.2x10^5$]	0.0025
Macrophages	$4.9x10^5$ [$1.3x10^5$ - $1.8x10^6$]	$6.7x10^4$ [$4.1x10^4$ - $3.8x10^5$]	0.0077
Neutrophils	$7.0x10^4$ [$8.7x10^3$ - $1.7x10^5$]	$6.1x10^3$ [$1.9x10^3$ - $1.9x10^4$]	0.0044
Lymphocytes	789.4 (0.0 - $4.2x10^3$]	668.4 [93.4 - 2.2 $x10^3$]	n.s.
Eosinophils	0.0 (0.0 - 0.0)	0.0 (0.0 - 40.9)	n.s.

Table E3. Correlations of 20 selected inflammatory mediator levels measured in BALF of CF children with PRAGMA-%Dis. Indicated are respective Spearman Rho, p and q values (significance threshold of 0.05 adjusted for multiple comparisons to 0.0025 using the Bonferroni method). n.s.: not significant.

	Rho	*p*	*q*
IL-1α	-0.21	n.s.	n.s.
IL-1β	0.21	n.s.	n.s.
IL-1RA	-0.08	n.s.	n.s.
IL-6	0.25	n.s.	n.s.
IL-8	0.43	0.0135	n.s.
IL-10	0.18	n.s.	n.s.
IL-18	-0.17	n.s.	n.s.
IFNγ	-0.14	n.s.	n.s.
ENA-78	-0.08	n.s.	n.s.
GM-CSF	-0.15	n.s.	n.s.
I-TAC	0.22	n.s.	n.s.
TNFα	0.02	n.s.	n.s.
VEGF	0.21	n.s.	n.s.
G-CSF	-0.24	n.s.	n.s.
GROα	-0.04	n.s.	n.s.
IP-10	-0.07	n.s.	n.s.
M-CSF	-0.21	n.s.	n.s.
MCP-1	-0.16	n.s.	n.s.
MIP-1β	-0.05	n.s.	n.s.
TRAIL	0.09	n.s.	n.s.

SUPPLEMENTAL FIGURES

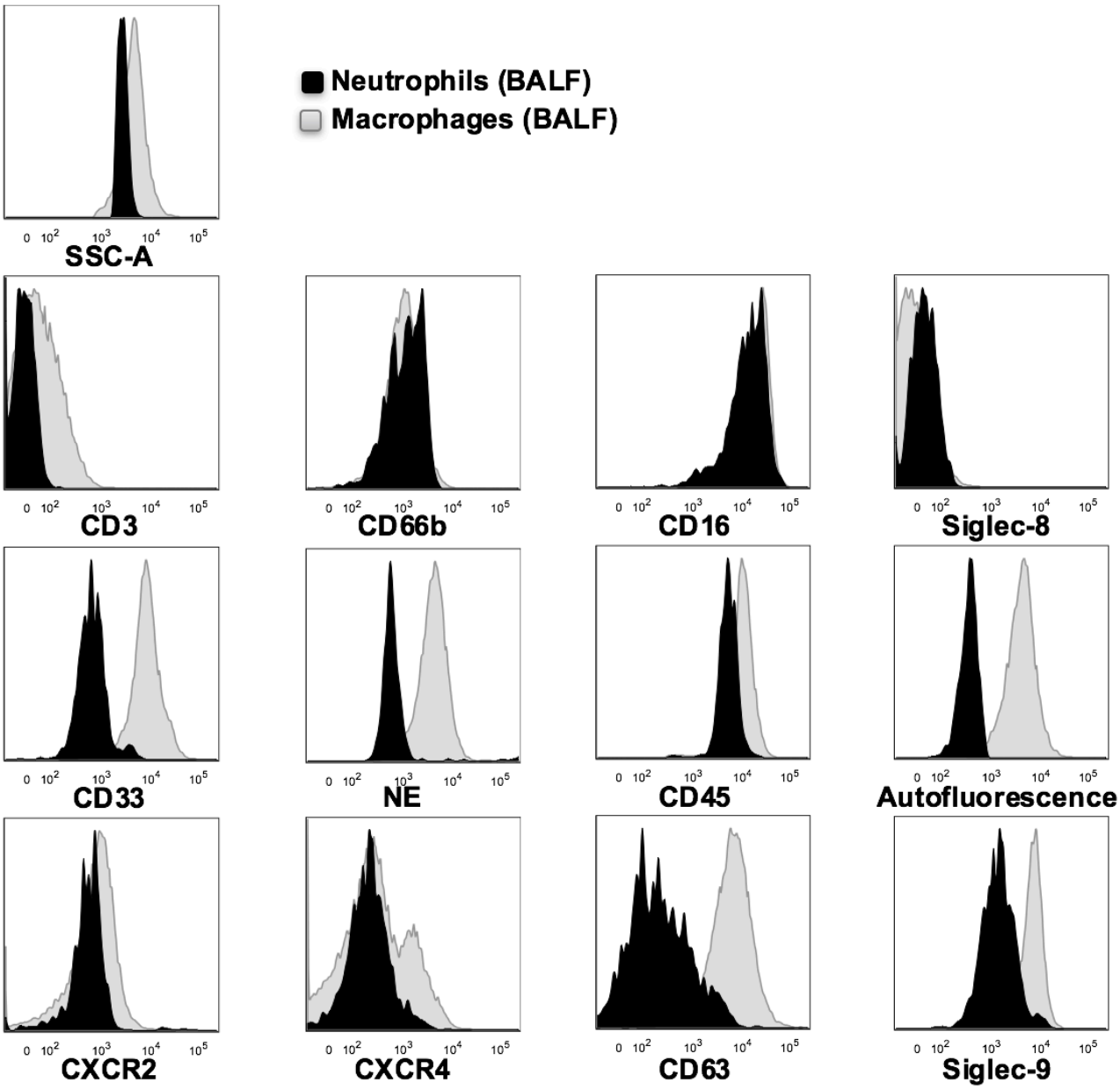

Figure E1. Multiparameter overlay of airway neutrophils and macrophages gated per our strategy. Neutrophils and macrophages from CF infant BALF (one representative patient shown here) overlap for several markers typically used to discriminate between neutrophils and monocytes in blood (e.g., CD66b, Siglec 9, CXCR2). However, CD33 and autofluorescence show expected higher expression on macrophages and allow their discrimination upon analysis.

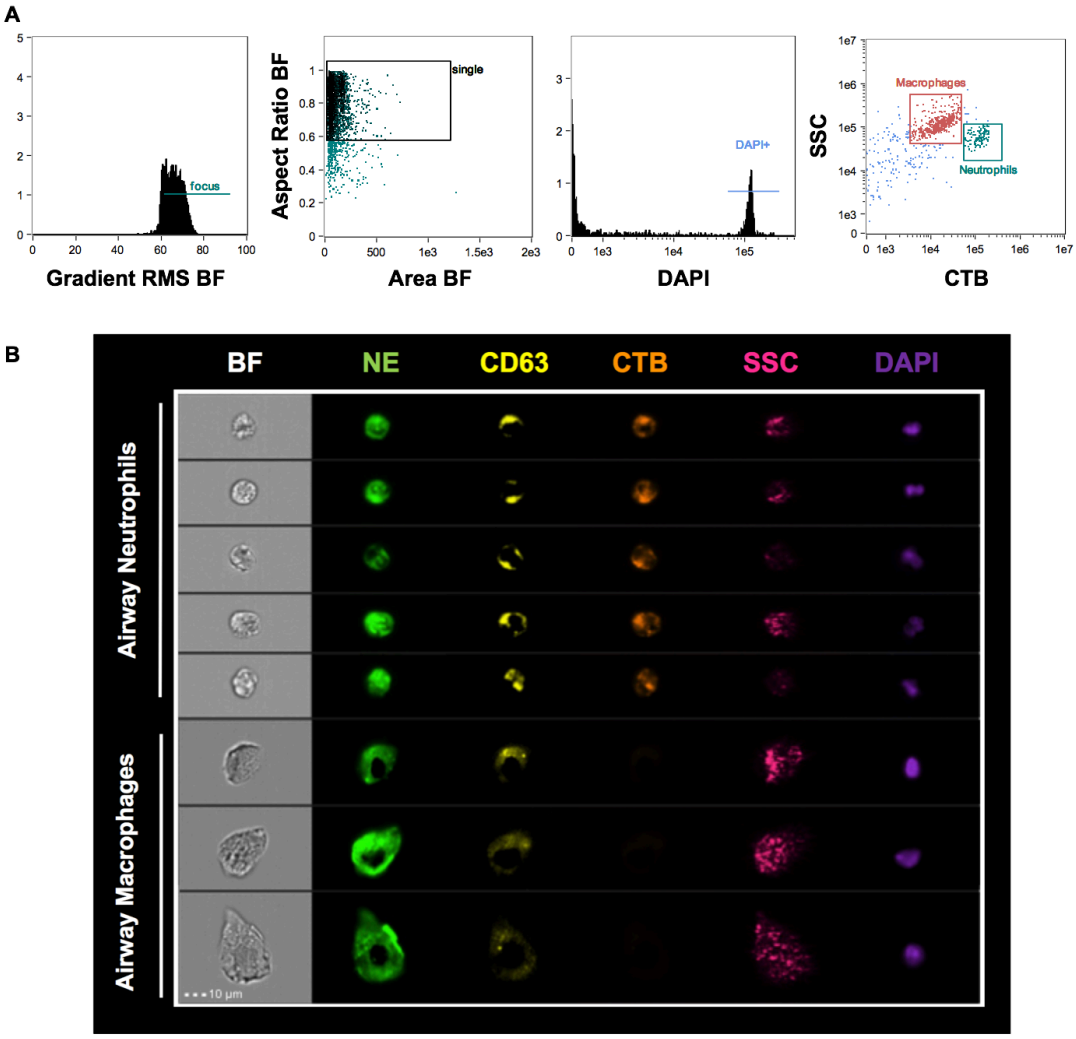

Figure E2. A. Gating strategy for image cytometry data acquisition of BAL leukocytes from CF children. From left to right: 1) Focused events were gated from the gradient RMS brightfield (BF); 2) Aspect Ratio vs. Area in the BF channel were used to gate single events; 3) Live non-apoptotic cells were gated based on staining with the nuclear dye DAPI expression (diploid); 4) Neutrophils were discriminated from macrophages based on higher level of cholera toxin B (CTB) staining and side scatter (SSC) (31). Cells contained in each gate were validated based on respective images collected during acquisition. **B.** Gated BAL neutrophils and macrophages (representative examples in top five, and bottom three rows, respectively) yield single-cell data on BF, NE, CD63, CTB, SSC, and DAPI expression (from left to right), confirming significant NE expression in both BAL subsets.