Human neutrophil peptides and phagocytic deficiency in bronchiectatic lungs

Stefanos Voglis, Kieran Quinn, Elizabeth Tullis, Mingyao Liu, Melanie Henriques, Celia Zubrinich, Oscar Peñuelas, Holman Chan, Frances Silverman, Vera Cherepanov, Neil Orzech, Aye Aye Khine, André Cantin, Arthur S. Slutsky, Gregory P. Downey, Haibo Zhang

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Materials and Methods

HNP

Purified HNP from the sputum of CF patients were a mixture of HNP-1, -2, and -3 and confirmed by acid-urea (AU)-PAGE and mass spectroscopy (Mass Spectrometry Laboratory, Molecular Medicine Research Center, University of Toronto, Toronto, ON) as previously described (1-4). Briefly, sputum was pooled from at least 20 patients with cystic fibrosis before purification, and at least 3 different batches of purified HNP were used in the present study. Average percentage compositions from 20 batches of the purified HNP mixture were 74.1% for HNP-1, 15.2% for HNP-2, and 10.7% for HNP-3 as measured by mass spectrometry. Purified HNP were reconstituted in 0.01% acetic acid and were tested by bacterial killing and endotoxin assays before use. Synthetic HNP-1 and HNP-2 serving as controls were purchased from Sigma (St Louis, MO).

PMN isolation

PMN were isolated from bone marrow and lung lavage fluids as described below. Human PMN were isolated from peripheral blood of non-smoking, healthy donors or stable CF patients at clinic visit, and from induced sputum of healthy donors or spontaneously expectorated sputum from the same CF patients who donated blood.

A. PMN isolation from mouse bone marrow (5). C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were euthanized by a lethal dose of anesthesia and their long bones were removed. The bone cells were sedimented and layered over three gradients (47%, 59%, and 68% Percoll in Ca^{2+} free Hank's Buffered Salt Solution (HBSS). The PMN fraction was collected at the interface of the 59% and 68% layers and the cells were resuspended in RPMI (Invitrogen Inc., Burlington, ON).

B. PMN isolation from mouse lung lavage. Anesthetized mice weighing 25 - 28 g (Pallid mice deficient in α1-PI, and background strain C57BL/6J of wild type mice, Jackson Laboratories) received HNP (200 μg) or vehicle control buffer. In all animals, a volume of 30 μl of solution was instilled directly into the lung though an intratracheal angiocatheter, followed by 3 min of mechanical ventilation to equalize distribution of the solutions in the lung. The spontaneously breathing mice were kept on a heating pad to maintain constant body temperature under anesthesia with ketamine and xylazine intraperitoneally. Upon completion of a 5 hour study period, the lungs were lavaged with

cold phosphate buffered saline (PBS) by installation of three aliquots of 1 mL followed by withdrawn. The cell pellets were layered over a 3-layer gradient (47%, 59%, and 68% Percoll). Viability and cell purity of PMN populations were confirmed by cytospin preparations using Wright-Giemsa stain (Hemacolor Stain, Harleco, EM Science, Gibbstown, NJ).

C. PMN isolation from human blood (6). The platelet rich plasma was separated from the cellular fraction of anticoagulated whole blood. The cells were underlayed with 42% and 51% Percoll gradient, and the PMN-containing interface was collected and washed in the platelet poor plasma, and resuspended in HBSS for experiments. The functional integrity and quiescent state of the PMN isolated by this technique have been validated (6).

D. PMN isolation from human sputum (7-9). Sputum was induced in healthy, non-smoking subjects after inhalation of 10 mL hypertonic saline via a nebulizer (Medix ultrasonic nebulizer, Canadian Medical Products Ltd, Markham, ON) at increasing concentrations of 3, 4 and 5% for successive 7 minute periods. Following each inhalation period, subjects blew their nose, took one drink of water and expectorated from deep in the chest into a plastic specimen jar on ice.

Sputum was also collected in cold, sterile containers by a spontaneous cough sample from adult CF patients who were in a stable (non exacerbative) condition. The fresh sputum obtained from both induction and spontaneous cough was processed within 20 min by selecting portions of the expectorate that contained aggregates or “plugs” of cells. A volume (in µl) of 10% Sputolysin solution (Calbiochem, La Jolla, CA) equivalent to four times the mass of sputum plugs (in mg) was added and gently mixed by pipetting and brief vortexing. Sputolysin at the dose used had no significant effects on the expression of surface markers in the PMN isolated from induced sputum of healthy volunteers and from expectorated sputum from CF and non-CF bronchiectasis patients (**Fig E2**). After additional mixing for 15 min on a bench rocker, the sample was filtered through a 48 µm pore nylon mesh and diluted with an equivalent volume of PBS. The majority of the cell population after filtration was PMN that were mixed with a few alveolar macrophages. The cells were suspended in PBS and underlayed with a 42% and 51% Percoll gradient to separate the cell phenotypes, and the PMN-containing interface

was collected and washed in HBSS. The purity of the lung PMN suspension was confirmed by Wright-Giemsa staining.

Opsonization and labeling of beads

Polystyrene beads of 3.3 μm diameter (5 x 10^9 beads/mL of stock suspension; Bangs Laboratories, Fishers, IN) were washed and opsonized in HBSS containing 1.25 mg/mL human immunoglobulin G (IgG, Sigma-Aldrich). The beads were then washed and resuspended at a density of 5 x 10^8 beads/mL. The opsonized beads were stained by incubation in HBSS containing 3.75 μg/ml cyanine-3 (Cy3)- or fluorescein (FITC)-conjugated $F(ab')_2$ fragments of donkey anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The beads were washed in cold HBSS prior to use.

Flow cytometry

Flow cytometry was performed on a FACSCanto controlled by FACSdiva software (BD Biosciences, Mississauga, ON) to detect surface markers using mouse anti-human monoclonal anti-CR1, anti-CR3, anti-Fcγ RIII, anti-CD63 and anti-66b antibodies (Serotec Inc., Raleigh, NC), and 10,000 events were analyzed.

Phagocytosis assays

A. Flow cytometry *E. coli* assay. The phagocytosis assay followed instructions provided by the manufacturer (Phagotest; Orpegen Pharma, Carlsbad, CA). The *E. coli* was opsonized with pooled human sera.

B. Flow cytometry bead assay. PMN were stimulated with either vehicle control or HNP for 30 min while shaking at 37°C. Additional PMN were treated with an equimolar amount of α1-PI (Prolastin®, Bayer HealthCare Inc., Toronto, ON). The Prolastin was reconstituted based on its lot-specific functional activity, and stoichiometric ratios were based on average molecular weights of 3.4 kDa for HNP and 52 kDa for Prolastin. The PMN were then exposed to IgG-opsonized beads at a 1:25 ratio followed by rapid sedimentation to synchronize the interaction while shaking at 37°C to allow for phagocytosis. The PMN were then fixed in paraformaldehyde, and the cell/bead pellets were resuspended in medium containing FITC-anti-human IgG (Jackson ImmunoResearch) for 30 min to stain any remaining extracellular beads together with an allophycocyanin-labelled mouse anti-human CD45 antibody (BD Biosciences,

Mississauga, ON). The latter was used as a leukocyte marker to differentiate the cells from aggregation artifacts of beads using flow cytometry.

C. Slide bead assay. PMN were stimulated with HNP and exposed to IgG-opsonized beads. The cells were then fixed and transferred to fibronectin-coated coverslips to allow for adherence, and incubated with FITC-anti-human IgG in the presence of 5% donkey serum (Sigma) to stain the extracellular beads. The internalized beads free of FITC were counted by using confocal microscopy (LSM 510, Zeiss Canada, Toronto, ON). The total number of beads internalized per 40 cells in randomly selected fields was counted and the phagocytic index was calculated as the number of beads that were internalized per cell and was expressed as a relative change from the vehicle controls.

There were several reasons to use IgG opsonized beads in the phagocytic assays: 1) Identity, purity and quantity of the phagocytic objectives were more clearly determined by using IgG opsonized beads under confocal miscrscopy, compared to using live organisms; 2) It provides a superior tool over the use of live bacteria to differentiate internalization (unlabelled) from surface binding (i.e., FITC labelled); and 3) The assays of using IgG opsonized beads provide a 'proof-of-concept' for live bacterial assays.

Apoptotic assays

1. Flowcytometry assay

The effects of HNP on PMN viability were evaluated with an apoptosis detection kit (R&D Systems, Minneapolis, MN) that employs Annexin V-FITC to identify negatively charged phophatidylserine molecules that are characteristically found on the outer leaflet of the plasma membrane of apoptotic cells. PMN (1×10^6) were treated with 0-50 μg/mL HNP for 70 min, washed, and resuspended in 100 μl of Incubation Reagent containing 250 ng/mL Annexin V-FITC conjugate for 15 min. The reaction was blocked by the addition of 400 μl binding buffer and the cells were analyzed by flow cytometry. Unstained (autofluorescence) and single-stained compensation controls were also acquired. Results were presented as histograms by plotting cell count against FITC fluorescence intensity.

2. Confocal microscope assay

PMN were exposed to HNP for a total of 70 min as described above. The cells were in suspension for the first 40 min and placed on fibronectin-coated coverslips to

allow for attachment for the final 30 min. After washing, the cells were stained with 100 µl of Annexin V Incubation Reagent for 15 min. The cells were fixed and the coverslips were mounted on slides before confocal microscopy analysis.

HNP ELISA

The 96-well plates were coated overnight with mouse anti-human HNP-1-3 monoclonal antibody (HyCult Biotechnology, Uden, The Netherlands). After thorough washing and blocking non-specific protein binding, HNP standards and samples were added to the wells and incubated for 1 h. The wells were incubated for 1 h with 1:1,000 rabbit anti-human HNP-1-3 polyclonal antibody (Host Defence Research Centre, Toronto, ON), followed by incubation for 1 h with 1:4,000 peroxidase-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch). The 3,3',5,5'-tetramethyl-benzidine substrate (Sigma) was added and the reaction was stopped by using 1 M sulphuric acid. The absorbance was read at 450 nm.

Human neutrophil elastase assay

The concentrations of human neutrophil elastase in PMN cell culture medium were measured in the presence and absence of HNP stimulation by using a Human Elastase ELISA Kit (Hbt™, HyCult Biotechnology, Uden, The Netherlands).

Lung permeability assessment

To estimate changes of lung permeability after HNP instillation, the Evans blue dye (EBD) injection technique was employed (10). Ten mice received intratracheal instillation of either HNP or a vehicle buffer solution as a control (n = 5 each). The animals were administered 20 mg/kg EBD (Sigma Chemical Co., St. Louis, MO) by tail vein injection 30 min before the end of the 5 h experiments. The blood was collected by cardiac puncture after opening the chest. The pulmonary vessels were perfused *in situ* with 3 mL of normal saline while the lung was ventilated. EBD concentration in lung homogenates was quantified by a dual wavelength spectrophotometer at 620 nm and 740 nm, which allows for correction of contaminating heme pigments, as determined by the following formula: OD_{620} (EBD) = OD_{620} – (1.426 x OD_{740} + 0.030) (10).

Figure E1. Characterization of PMN in patients with CF and non-CF bronchiectasis. (**a**) Average number of PMN isolated from induced sputum of healthy volunteers (n = 12), the spontaneous sputum of CF (n = 35) and non-CF bronchiectasis (n = 8) patients. (**b**) PMN morphology. The PMN were isolated from blood or sputum of the healthy volunteers and the patients with CF and non-CF bronchiectasis. (**c**) Impaired phagocytosis in PMN isolated from sputum of patients with CF and non-CF bronchiectasis. The PMN were incubated with FITC-*E. coli* for 30 min at 37°C. Extracellular fluorescence was quenched with trypan blue solution. Upper panels: Representative flow cytometry data. Lower panel: The mean values of phagocytosis are expressed as percent changes of FITC-*E. coli* positive PMN isolated from blood or sputum of patients with CF and non-CF bronchiectasis in relation to PMN isolated from blood or induced sputum of healthy donors, respectively. * $p < 0.05$ *vs*. Healthy at identical conditions, respectively. † < 0.05 vs. other groups, respectively.

Figure E2. Surface expression of CD markers of PMN in healthy donors and CF patients. (**a**) Expession of CR1, CR3 and Fcγ RIII on PMN in CF. PMN isolated from blood and sputum of healthy volunteers and CF patients were fixed and stained with anti-human CR1-FITC, CR3-FITC or Fcγ RIII-FITC antibodies. Upper panels: Representative flow cytometry data. Lower panel: The mean values of FITC intensity were reported from 5, 15 and 15 subjects per group, respectively. (**b**) Blood and sputum were collected from the same healthy donors or CF patients for HNP measurement by ELISA. * $p < 0.05$ *vs*. Healthy at identical conditions, respectively.

Figure E3. Effects of HNP on PMN apoptosis. PMN isolated from healthy volunteers were treated with the indicated concentrations of HNP for 70 min on coverslips or test tubes, and apoptosis was analyzed using Annexin V-FITC staining by **A.** confocal microscopy or **B.** flowcytometry. **C.** Values of mean fluorescence intensity (MFI) from flowcytometry. n = 5 each, * $p < 0.05$ vs. 0 HNP. Note: A previous study reported that a longer duration (i.e., 72 h) is required for a low dose (i.e., 10 μg/mL) of HNP to induce cytotoxicity in CF lung epithelial cells (9).

References

1. Khine AA, Del Sorbo L, Vaschetto R, Voglis S, Tullis E, Slutsky AS, Downey GP, Zhang H. Human neutrophil peptides induce interleukin-8 production through the p2y6 signaling pathway. *Blood* 2006;107:2936-2942.

2. Porro GA, Lee JH, de Azavedo J, Crandall I, Whitehead T, Tullis E, Ganz T, Liu M, Slutsky AS, Zhang H. Direct and indirect bacterial killing functions of neutrophil defensins in lung explants. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1240-1247.

3. Syeda F, Tullis E, Slutsky AS, Zhang H. Human neutrophil peptides upregulate expression of cox-2 and endothelin-1 by inducing oxidative stress. *Am J Physiol Heart Circ Physiol* 2008.

4. Vaschetto R, Grinstein J, Del Sorbo L, Khine AA, Voglis S, Tullis E, Slutsky AS, Zhang H. Role of human neutrophil peptides in the initial interaction between lung epithelial cells and cd4+ lymphocytes. *J Leukoc Biol* 2007;81:1022-1031.

5. Kruger J, Butler JR, Cherapanov V, Dong Q, Ginzberg H, Govindarajan A, Grinstein S, Siminovitch KA, Downey GP. Deficiency of src homology 2-containing phosphatase 1 results in abnormalities in murine neutrophil function: Studies in motheaten mice. *J Immunol* 2000;165:5847-5859.

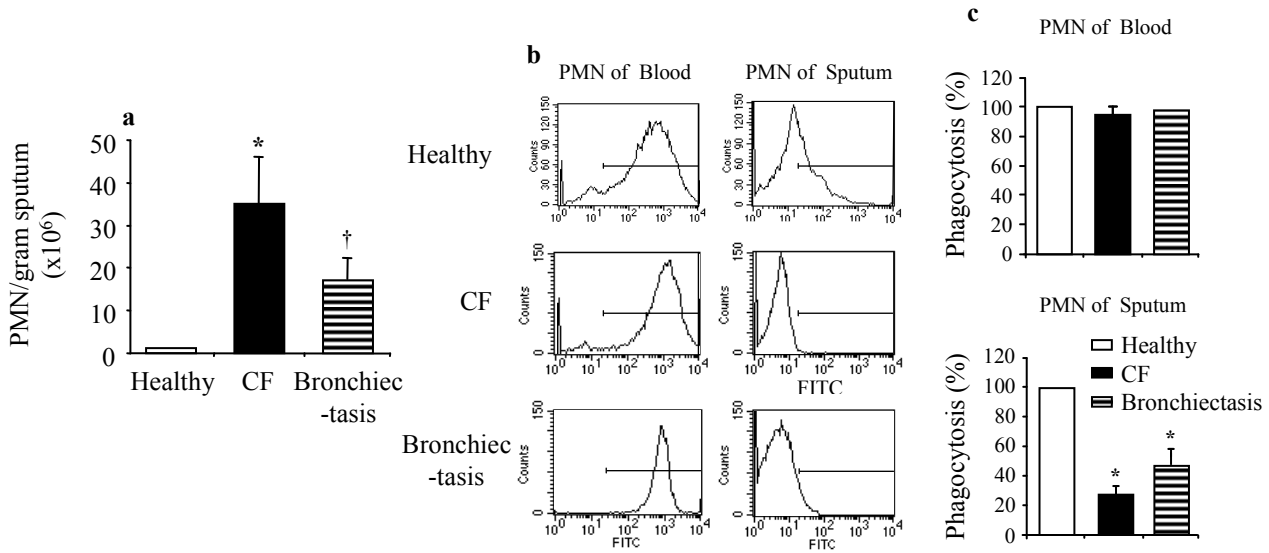
6. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB, Jr., Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 1985;119:101-110.

7. Corvol H, Fitting C, Chadelat K, Jacquot J, Tabary O, Boule M, Cavaillon JM, Clement A. Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L997-1003.

8. Reglier H, Arce-Vicioso M, Fay M, Gougerot-Pocidalo MA, Chollet-Martin S. Lack of il-10 and il-13 production by human polymorphonuclear neutrophils. *Cytokine* 1998;10:192-198.

9. Tabary O, Corvol H, Boncoeur E, Chadelat K, Fitting C, Cavaillon JM, Clement A, Jacquot J. Adherence of airway neutrophils and inflammatory response are increased in cf airway epithelial cell-neutrophil interactions. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L588-596.

10. Zhang H, Porro G, Orzech N, Mullen B, Liu M, Slutsky AS. Neutrophil defensins mediate acute inflammatory response and lung dysfunction in dose-related fashion. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L947-954.


a
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CF
Bronchiec-tasis
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PMN of Sputum
Healthy
CF
Bronchiec-tasis
Counts
FITC
c
PMN of Blood
Phagocytosis (%)
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80
60
40
20
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PMN of Sputum
Healthy
CF
Bronchiectasis
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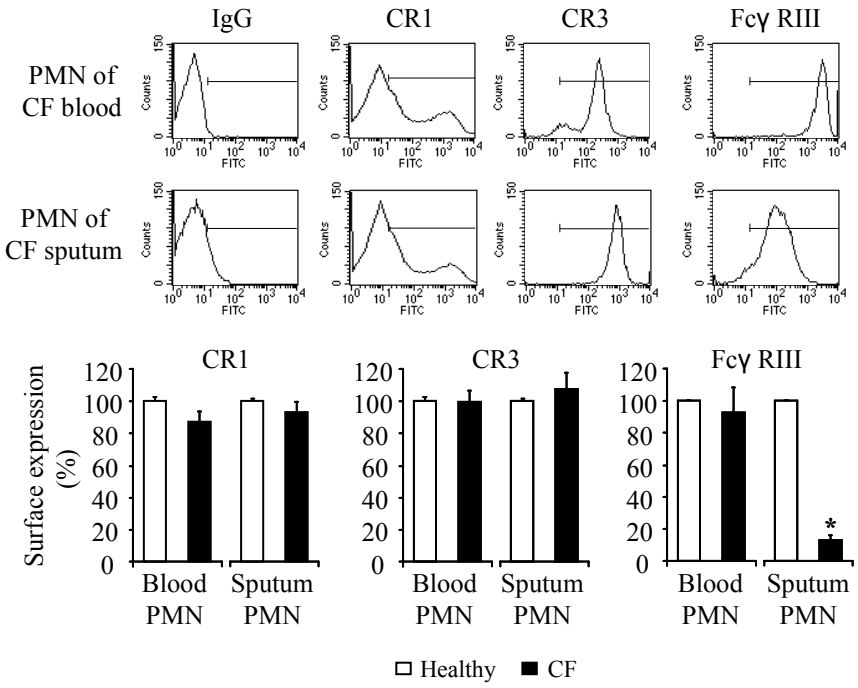
Figure E1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CR3
Fcγ RIII
PMN of CF blood
PMN of CF sputum
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FITC
Surface expression (%)
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100
80
60
40
20
0
Blood PMN
Sputum PMN
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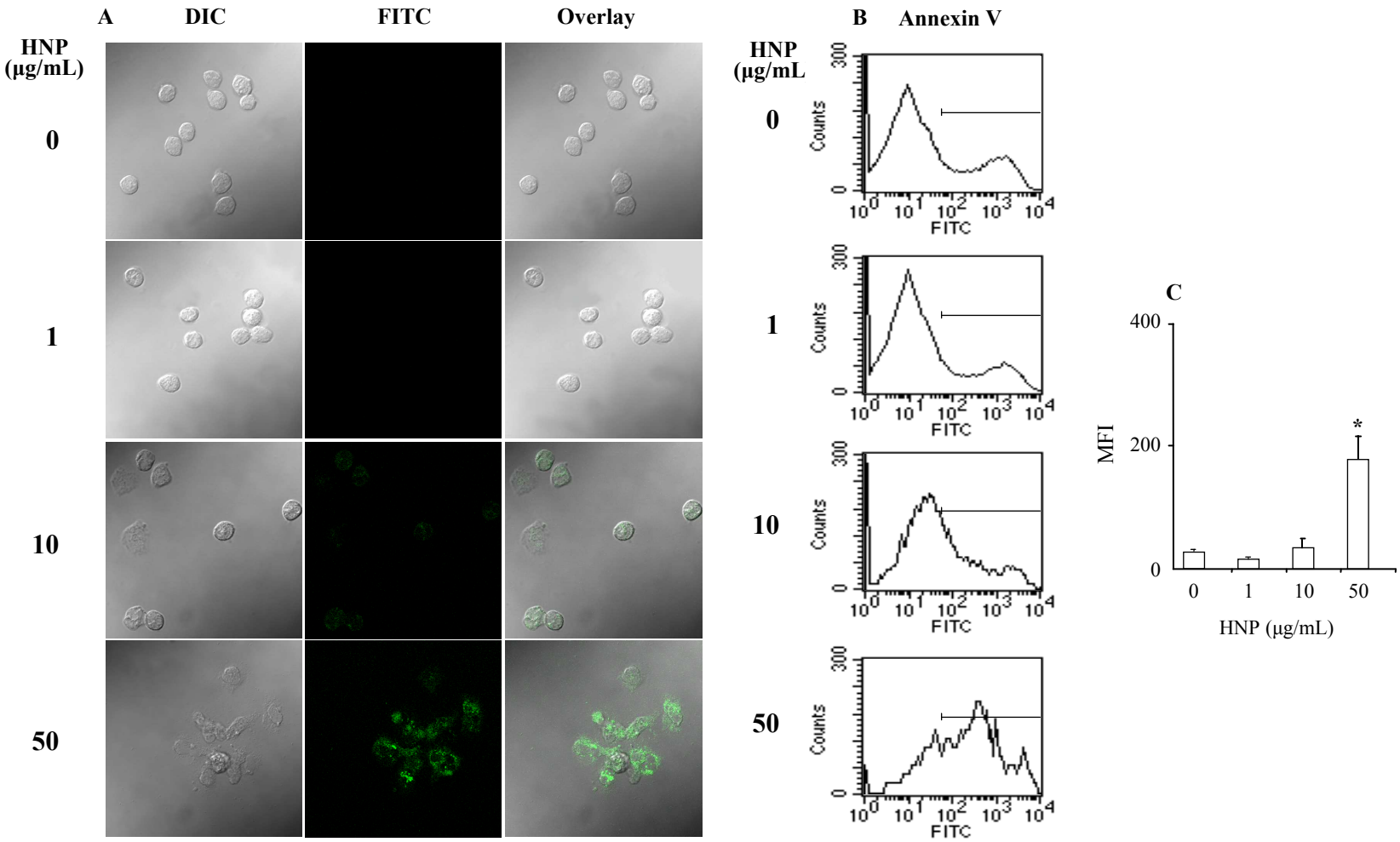
Healthy
CF

Figure E2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HNP (μg/mL)
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Figure E3