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Polymorphonuclear Leukocyte Functions Enhanced by Chemotaxis

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Human polymorphonuclear leukocytes (PMN) migrate into tissues in response to chemoattractants, yet it is not known whether this process alters the functional capabilities of the PMN. Using recombinant human interleukin-8 (rHIL-8, 100 ng/ml) as a stimulus, we compared a population of PMN that migrated through a polyvinylpyrrolidone-coated polycarbonate filter containing 8.0 μm diameter pores with PMN stimulated in suspension. PMN were analyzed by flow cytometry according to functional and phenotypic criteria. CD11b/CD16 expression was unaltered by chemotaxis. In contrast, chemotaxis enhanced phagocytosis of E. coil, independent of opsonization with IgG. Similarly, chemotaxis increased baseline hydrogen peroxide production. We conclude that the chemotactic motion of PMN "primes" the cell for increased oxidative burst activity and augments the ability of PMN to ingest bacteria. This increased functional capability is distinct from rHIL-8 stimulation and appears to be independent of complement-and Fc-receptor expression.

Key Words: *Neutrophil, Chemotaxis, CD11b, CD16, Oxidative burst, Phagocytosis, Interleukin-8*

INTRODUCTION

The ability of PMN to migrate into tissues is thought to be a consequence of their ability to respond to chemoattractants. Various endogenous compounds have been demonstrated to have chemoattractive properties for PMN, including the complement-derived anaphylatoxin C5a (18), leukotriene B4 (8), and interleukin-8 (IL-8) (16).

Directed migration of PMN has been demonstrated in response to increasing gradient concentrations of these agents and instillation of these compounds into tissues causes accumulation of PMN (1).

Aside from stimulating directed migration, most chemotaxins are capable of altering PMN functions. For example, C5a provokes PMN degranulation (9), oxidative burst activity (13), and increases adherence of PMN to endothelium (6). Once PMN arrive at an extravascular site within tissues, other functions such as phagocytosis and killing of microorganisms are expected.

One method commonly employed to study the chemotactic motion of PMN in vitro utilizes the Boyden chamber (19). Two compartments are separated by an aqueous micropore filter through which PMN can migrate in response to gradients of stimuli between the two compartments. The Boyden chamber is used as a model for the in vivo process of diapedesis. In this study we employed this methodology to examine whether chemotactic motion, independent of stimuli, alters the functional capability of normal human PMN. We compared cells obtained from the bottom compartment of a Boyden chamber following chemotaxis stimulated with rHIL-8 under identical conditions in suspension.

The comparison of the two populations was made with assays for oxidative burst activity (31), phagocytosis of Escherichia coli (*E. coli*) organisms (17), and characterization of phenotypic expression of two common membrane peptides CD11b (15) and CD16 (7).

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MATERIALS AND METHODS

Materials

E. coli bioparticles labeled with fluorescin

isothiocyanate (FITC) were purchased from Molecular probes, Inc. (Eugene, OR). Monoclonal antibodies anti-CD11b (phycoerythrin-labeled, Leu-15) and anti-keyhole limpet hemocyanin (anti-KLH, FITC-labeled, IgG1 and phycoerythrin-labeled, IgG2a) were purchased from Becton Dickinson (Mountainview, CA). Monoclonal antibody anti-CD16 (FITC-labeled, 3G8) was purchased from AMAC (Westbrooke, ME). N-formyl-methionyl-leucyl-phenylalanine (f-MLP, Sigma Co., St. Louis, MO) was dissolved in dimethylsulfoxide and diluted with PBS for storage in 10^{-5} M aliquots at -20°C. Recombinant human interleukin-8 (rHIL-8, Genzyme Co., Cambridge, MA) was suspended as a stock in buffer at a concentration of 1.0μg/ml. 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) was suspended in 95% ethanol and diluted (1:10) with PBS to a stock concentratin of 5.0 mM. The final concentratin of ethanol exposed to cells for both was 0.01% (vol./vol.). Bovine serum albumin was purchased from Sigma Co.

Preparation of Buffer

Ten mM phosphate buffered saline (PBS), pH 7.4, was modified by addition of 2.0% (wt/vol.) bovine serum albumin, 0.6mM MgCl2, 1.0 mM CaCl2 (PCMA).

White Blood Cell Preparation

Ten ml of venous blood was obtained from healthy human volunteers in accordance with a protocol approved by the Committee on Human Research, Korea University. Blood was anticoagulated with ethylenediamine tetraacetic acid (EDTA), mixed with 140ml of lysing solution (166mM NH4Cl, 10 mM KHCO3, and 0.1 mM tetrasodium EDTA), and allowed to stand at room temperature (RT) for 5 minutes. White blood cells (WBC) were pelleted by centrifugation at 300×g for 5 minutes and the supernatant was removed by aspiration. The pellet was washed with PBS at 4°C and resuspended in PCMA buffer solution. The PMN concentration within the mixture was adjusted to 1.0×10^7/ml using a hemocytometer.

Chemotaxis

Chemotaxis was performed using a modified Boyden Chamber assay (8): the WBC mixture was placed into an upper acrylic chamber (330μl capacity) separated by a filter (polyvinylpyrrolidone (PVP)-coated polycarbonate, 8.0μm pore and 10μm thickness) (Nucleopore, Cambridge, MA) from a lower chamber (230μl capacity) containing buffer (PCMA) or chemotactic stimulus (100ng/ml rHIL-8) in PCMA. The chambers were sealed with silicone-glued rubber O-rings. PMN were aspirated from the lower chambers after incubation and resuspended in PBS at a concentration of 1.0×10^6 PMN/ml. For each experiment, cells undergoing migration were compared to cells stimulated identically in suspension. For controls, cells without stimuli and cells held at 4°C throughout were used.

Alternatively, for determining the ideal dose of chemotactic stimulus, we used a different Boyden chamber assay. The upper compartment was separated by a nitrocellulose filter (3.0μm pore, 100μm thick, Sartorius, Hayward, CA) from a lower chamber containing variable concentrations of rHIL-8, 10^{-8} M f-MLP or buffer alone. After chemotactic incubation at 37°C for 35 minutes, the filters were then removed, fixed in methanol, stained with hematoxylin, dehydrated in ethanol and cleared in xylene. The filters were examined microscopically and the response of PMN to chemotactic stimuli was recorded as the distance the "leading front" of cells migrated into the filters (μm). Ten microscopic fields in each filter were examined.

Phagocytosis

Each vial of *E. coli* bioparticles was reconstituted with 0.5ml of PBS, washed with PBS, vigorously vortexed to resuspend the particles homogeneously and counted with a hemocytometer using fluorescence microscopy. Opsonized-E. coil were prepared from FITC-labeled *E. coil* by adding an equal volume of purified rabbit polyclonal IgG (Molecular Probes) and incubating for 1.0 hr at 37°C, prior to washing and counting. After the above incubations, WBC obtained from the experimental groups described above were incubated with *E. coli* (FITC) or opsonized-*E. coli* (FITC) for 30 minutes at 37°C. For controls, cells without particles were held at 4°C for 30 min and non-stimulated cells were used to determine baseline phagocytosis. Incubations were performed by mixing 0.9ml of WBC (10^6 PMN/ml PCMA) with 0.1ml of *E. coli* or opsonized-*E. coli* (1×10^8/ml). After incubation, phagocytosis was terminated by addition of 0.5ml of cold 1.5% (wt/vol) paraformaldehyde (PFA) containing 6.0mM EDTA in PBS for flow cytometric analysis. The percentage of cells containing fluorescence and the quantity of fluorescence was measured for each sample run in duplicate.

Monoclonal Antibody Staining

Following chemotaxis, 0.9ml of WBC (10^6 PMN/ml PCMA) obtained as described above were incubated for 15 minutes at 37°C. Cells maintained at 4°C were used as controls. The incubation was terminat-

ed by addition of 0.5ml of cold 1.5% PFA (wt/vol.) containing 6.0mM EDTA. Cells were then washed with cold PBS and stained with fluorescent-labelled monoclonal antibodies specific for CD11b and CD16 by addition of 10μl of antisera to 90μl of cell suspension (30 minutes, 4°C). To assess for non-specific binding, cells were stained with antisera of identical isotypes directed against irrelevant epitopes (KLH). After, the cells were washed with PBS and resuspended in 0.5% PFA containing 2.0mM EDTA for flow cytometric analysis. Mean fluorescence was determined for each sample in duplicate.

Measurement of Intracellular H_2O_2 Production

Cells obtained following chemotaxis (1.0×10^6 PMN/ml PCMA) were incubated with 5.0μM DCFH-DA for 15 min at 37°C. Cells were then further incubated for 15 minutes at 37°C to assess baseline oxidation. After incubation, reactions were terminated by addition of 0.5ml 0.5mM NaN_3. Autofluorescence was obtained by analyzing cells held at 4°C without incubating with DCFH-DA. Cells were then subjected to flow cytometric analysis and mean fluorescence was determined for duplicate samples.

Flow Cytometric Analysis

Analysis was performed on a Becton Dickinson FACScan-equipped with an argon-ion laser emitting 15 miliwatts at 488 nm and capable of detection at 530 nm (FITC), 585 nm (PE/PI) and >650 nm (red fluorescence). The FACSan is supported by an Hewlett Packard-9000 computer (model 310) for data acquisition and analyzed with a commercial computer software package (Consort 30). Data was collected for 10,000 events after gating on PMN according to criteria of forward and right angle scatter. In the phagocytosis studies, samples were quenched by addition of 0.06 mM trypan blue, pH 4.5 to eliminate fluorescence exhibited by extracellular-bound particles.

Data Analysis

All mean data are presented as the mean ± SEM. For comparison of means with similar variance, we utilized the Student's unpaired two tailed t-test. The probability (p) of differences between groups is displayed (*) and $p<0.05$ was accepted as significant.

RESULTS

To determine the effects of rHIL-8 upon chemotaxis, we placed 1.0×10^7 PMN/ml into the upper well of a Boyden chamber separated by a filter (either PVP-coated polycarbonate, 8.0μm pore or cellulose nitrate, 3.0μm pore) from the lower chamber containing rHIL-8 or buffer alone (control).

Table 1. Yield of PMN in bottom compartment of Boyden chambers following chemotactic migration.[1]

Stimulus	n	Cell Count ($\times10^6$/ml)	
		Top Well	Bottom Well
None	6	10	0.24±0.02
rHIL-8 (100ng/ml)	9	10	2.80±0.40*

* $p<0.05$, vs no stimulus.

1 Compartments were separated by polyvinylpyrrolidone-coated polycarbonate filters (10μm thick) containing 8.0 μm pores. Chambers were incubated with PMN for 60 min at 37°C.

Table 2. Phagocytosis of FITC-labelled E. coli bioparticles by PMN.[1]

	No Chemotaxis	Cell Pre-treatment Chemotaxis
Unquenched		
Unopsonized	1.01±0.04	1.04±0.07
Opsonized	1.16±0.05	1.24±0.05
Quenched		
Unopsonized	1.11±0.03	1.33±0.05*
Opsonized	1.25±0.04	1.38±0.04*

* $p<0.05$, vs no chemotaxis.

1 Results are expressed as normalized ratio of mean fluorescence displayed by PMN populations after comparison to cells undergoing phagocytosis without pre-treatment with rHIL-8. Fluorescence was recorded before and after quenching of each sample with trypan blue to eliminate extracellular fluorescence. *E. coli* bioparticles were untreated (unopsonized) or pre-incubated with anti-*E. coli* polyclonal antisera (opsonized) as described in the methods.

Table 1 displays the PMN counts obtained from the bottom chamber after chemotaxis using PVP-coated polycarbonate 8.0μm pore filters. The PMN counts after chemotaxis were significantly increased with rHIL-8 compared to buffer alone.

Figure 1 showed the results of PMN migration through cellulose nitrate filters after stimulation by various concentrations of rHIL-8. rHIL-8 demonstrated dose-dependent chemoattraction for human PMN up to 100ng/ml concentration. At higher concentrations than 100ng/ml, PMN displayed stimulus-specific desensitization.

CD11b and CD16 expression by PMN following pre-

treatment with rHIL-8 is displayed in Figure 2. In these measurements of CD11b/CD16 expression, we noted that the mean fluorescence ratio displayed by both populations of PMN did not show any significant change in those undergoing chemotaxis compared to those not undergoing chemotaxis.

Intracellular hydrogen peroxide production by PMN is displayed in Figure 3 as the mean cell fluorescence for 2', 7'-dichlorofluorescin. PMN obtained following chemotaxis displayed increased production of H_2O_2.

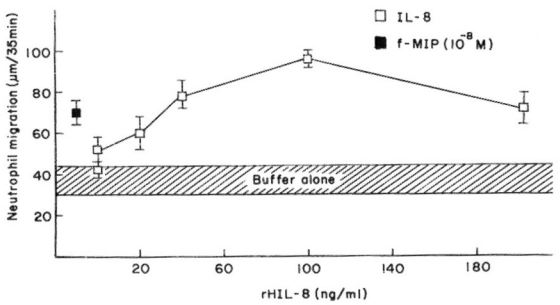

Fig. 1. Concentration dependence of rHIL-8 stimulated migration of human PMN through a nitrocellulose filter, expressed as m/35min. f-Met-Leu-Phe (f-MLP) was used as a positive control and the hatched area represents the random motion response to buffer alone.

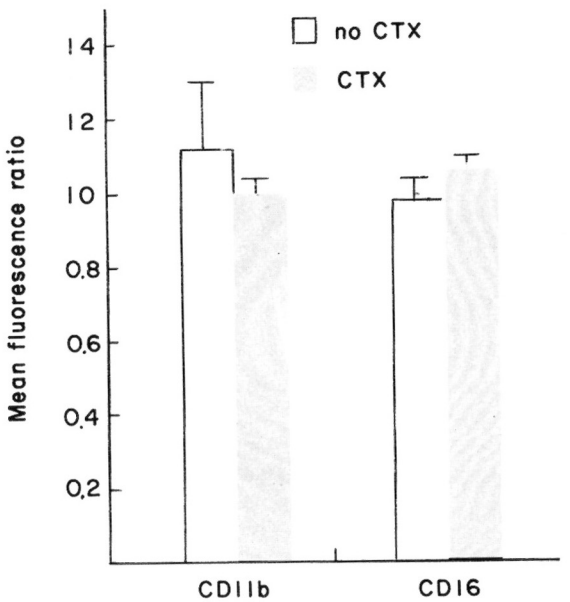

Fig. 2. Expression of CD11b and CD16 on PMN subjected to pre-treatment with no chemotaxis or chemotaxis, expressed as mean fluorescence ratio, normalized to control cells kept at 4°C.

Figure 4a demonstrates a normal representative PMN following exposure to fluorescent *E. coli*. Quenching of sample with trypan blue eliminated the fluorescence associated with membrane-bound particles and particles remaining unbound in suspension (Figure 4b).

Table 2 displays the results of phagocytosis by PMN following chemotaxis in response to rHIL-8 (100ng/ml). We compared the phagocytosis by PMN of unopsonized-and opsonized-*E. coli* with or without quenching, using trypan blue. In quenched samples, phagocytosis of opsonized-*E. coli* particles was increased as compared to unopsonized-*E. coli*, In addition, cells that underwent rHIL-8 stimulated chemotaxis had higher levels of phagocytic activity. The unquenched samples were not different when comparing two pre-treatment populations.

DISCUSSION

In this study we used an in vitro method for demon-

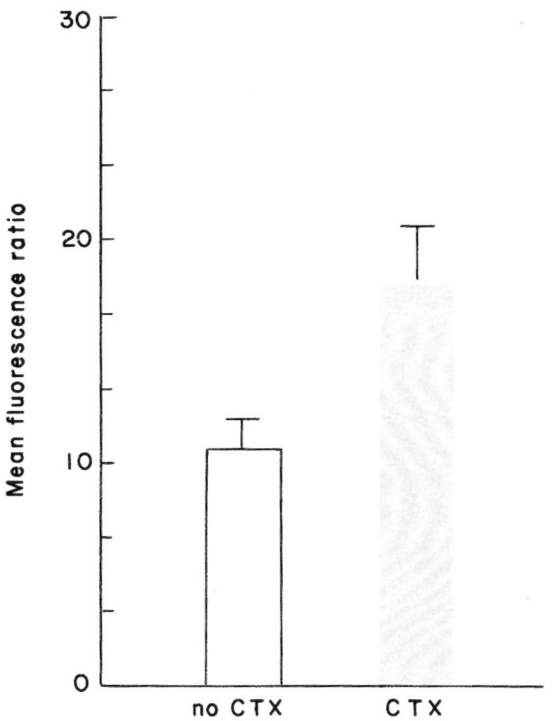

Fig.3. Oxidative burst activity of PMN which have undergone rHIL-8 stimulated chemotactic migration through a filter compared to control cells which did not undergo chemotaxis. Data is expressed as mean fluorescence ratio for intracellular 2', 7'-dichlorofluorescin, normalized to cells held at 4°C.

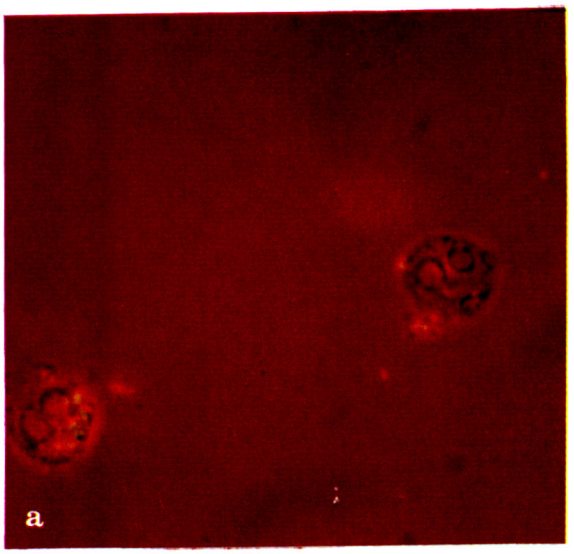

Fig. 4a. Photomicrograph of a human PMN with ingested and extracellular bound and free opsonized *E. coli*-(FITC). Magnification is 2880 times, using a combination of darkfield-and ultraviolet-illumination. The fluorescent particles appear as brightly illuminated white areas.

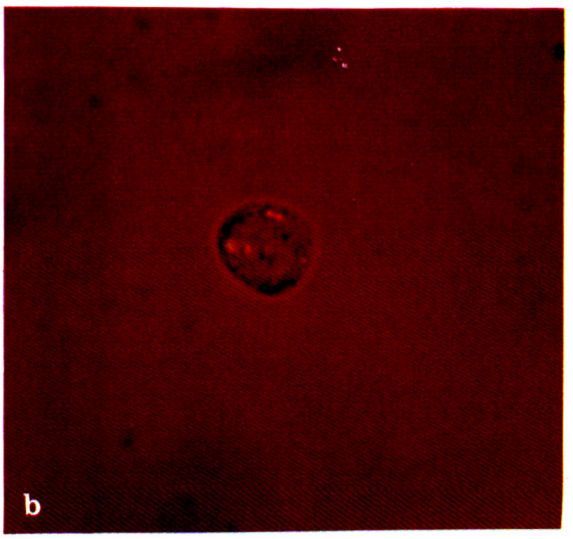

Fig. 4b. Identical conditions to Figure 4b, but fluorescence-quenching with trypan blue (0.6mM, pH 4.5) was performed to eliminate fluorescence of extracellular *E. coli*.

stration of PMN chemotaxis as a model of PMN exudation into tissues. Recovery of PMN from the bottom compartment of the Boyden chamber was utilized as a method to obtain PMN that had undergone chemotactic motion. Although this approach ignores the natural interaction of PMN with endothelial cells and the complex of proteins and other compounds found in the interstitial tissue "matrix", it serves as a model for studying the direct consequences of chemotactic motion on PMN functions.

In preliminary studies, we found that the yield of PMN in the bottom well of a Boyden chamber was dependent upon the time of incubation, the size of the pores and the thickness of the micropore filter, the actual substance the filter is made of, and the concentration gradients for chemotactic substances between the two chambers. As shown in table 1, IL-8 (100 ng/ml) stimulates a marked increase in PMN accumulation in the bottom well as compared to buffer alone, indicating that chemotaxis, not chemokinetic random motion was responsible for the passage of PMN through the filter.

The choice of IL-8 as a chemotactic agent was based on the finding that the release of this cytokine by mononuclear phagocytes and endothelial cells causes recruitment of PMN in vivo (16). We used the "leading front" method to determine the optimal concentration of commercially-obtained rHIL-8 that would stimulate PMN chemotaxis in vitro. This method employs nitrocellulose filters in order to allow for staining an "clearing" of the filters with organic solvents to facilitate microscopic reading of PMN migration in μm. For the comparison of chemotactically stimulated PMN with PMN in suspension we utilized polyvinylpyrrolidone-coated polycarbonate filters. These filters are relatively thin (10μm) and inert in aqueous solution and fail to bind neutrophils on the under surface, allowing them to "fall" from the filter back into suspension (4).

In comparison of unstimulated cells with those exposed to IL-8 demonstrated significant differences between these populations in their oxidative burst activity and expression of CD11b and CD16 (data not shown). To eliminate the effect of IL-8 stimulation, we chose cells stimulated in suspension by IL-8 under identical conditions as a control. Thus, the only difference between the two populations was the chemotactic activity of the PMN. The cells treated in suspension did not aggregate or form significant clumps when rHIL-8 was used as a stimulus.

CD11b is the alpha-chain of the integrin MAC-1 (CD11b/CD18 complex), which participates in adherence of PMN to endothelial cells and also serves as a receptor for the complement cleavage-product C3bi (11), (5). CD16 is the FcgIII-receptor for IgG commonly displayed by PMN. CD11b/CD18 is upregulated in vitro by incubation of cells at 37°C and by addition of common soluble stimuli such as C5a, tumor necrosis factor-a, and phorbol myristate acetate (14). In contrast, CD16 is shed from the surface of PMN in vitro

in response to stimulation by f-MLP (12). Clinically, patients exhibiting septic states display higher proportions of PMN that were CD11b negative (CD11b-) and CD16 negative (CD16-) in the circulating pool (2). The significance of this finding has not been explained.

We examined CD11b and CD16 expression on normal PMN following rHIL-8 stimulation (with or without chemotaxis). The expression is normalized to cells held at 4°C. No difference in the level of CD11b nor CD16 expression was observed (Fig. 2). These results indicate that the chemotactic motion was not likely to have engaged these surface receptor proteins in the process. The lack of differences in CD11b/CD16 expression for the two populations would indicate that chemotactic "priming" of other functions that we observed (i. e. phagocytosis and oxidative burst), was independent of expression of these receptors.

Oxidative burst activity was measured using a very sensitive assay for intracellular hydrogen peroxide production by cells in suspension (3). We measured baseline oxidative activity exhibited by cells in buffer alone. As shown in Figure 3, significant differences occured in the baseline level of oxidation between cells undergoing or not undergoing chemotaxis. These results indicate that chemotactic motion had a "priming" effect on baseline oxidative activity of PMN.

The phagocytosis of *E. coli* was studied by measuring the fluorescence exhibited by cells after ingesting FITC-labelled particles (17). In this assay, we compared the phagocytic activity of cells undergoing pre-stimulation with rHIL-8 with or without chemotaxis. The results were normalized for comparison of cells obtained from different donors to cells undergoing phagocytosis without pre-stimulation by rHIL-8. To eliminate the fluorescence displayed by particles either bound to the cell surface but not ingested or free in suspension, we quenched the extracellular fluorescence prior to flow cytometric measurements with trypan blue (10). Representative photomicrographs showing the effects of quenching are displayed in Figures 4a and 4b. As shown in Table 2, there was no difference between phagocytosis by the different populations of cells prior to quenching. This reflects the higher amount of extracellular fluorescence as compared to intracellular fluorescence. In contrast, quenched samples demonstrated a difference in the phagocytosis for both unopsonized and opsonized bacteria for the chemotaxis and no chemotaxis groups. These results indicate that the increased phagocytosis observed following chemotaxis was independent of opsonization by IgG, perhaps reflecting that CD16 was not different between the two populations. The increased amount of phagocytosis observed overall with use of opsonized *E. coli* would indicate that some other mechanism aside from binding to CD16 accounts for the augmentation of this function following chemotaxis.

In summary, we found that cells undergoing chemotactic motion in response to a natural endogenous chemotaxin (rHIL-8) were "primed" to a higher baseline level of oxidative burst and phagocytic activity. Our studies do not elucidate the mechanism that might explain how directed motion of cells by itself appears to stimulate cells. Such an effect on PMN would be adventageous to host defenses in those cells undergoing exudation would have a higher capability to ingest and kill microorganisms. Further studies will be required to clarify this phenomenon.

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