

## Original Article

# Production of proinflammatory mediators by human neutrophils during long-term culture

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**Abstract:** Neutrophils, usually considered short-living cells, play a key role in several inflammatory processes contributing to disease generation and progression. The present study was devised to investigate the changes occurring during long-term culture in neutrophil functions and morphology. Neutrophils were obtained from venous blood of healthy donors and cultured up to 24 h. Levels of interleukin (IL)-8, vascular endothelial growth factor (VEGF) and elastase were analysed by real time PCR and ELISA under resting and after stimulation with fMLP, LPS and IL-8. Apoptosis was measured by flow cytometry, migration by means of microscopic evaluation, reactive oxygen species (ROS) production by means of spectrofluorometry and cell morphology using optical microscopy and transmission electron microscopy. After 24 h cell number and viability was reduced with respect to 3 h of culture and number of cells in early and late apoptosis were increased. No appreciable differences were found between mRNA levels for IL-8, VEGF and elastase at the two times. Similarly elastase protein production was unchanged while on the contrary, IL-8 and VEGF protein levels were higher after 24 h. Resting and stimulated migration were unchanged up to 24 h. Values measured for spontaneous ROS generation were superimposable for the two times, fMLP-induced ROS generation was reduced at 24 h and LPS failed to increase ROS generation after 24 h. Cell morphology was preserved up to 24 h. These results indicate that neutrophils can be studied *ex vivo* even in long-term culture, although time-length of the culture affects some of their functional properties.

**Keywords:** Neutrophils, proinflammatory mediators, migration, reactive oxygen species, transmission electron microscopy

## Introduction

Polymorphonuclear leukocytes (PMN) are the major cellular arm of the innate immune system. They are among the first cells that incorporate microorganisms and damaged tissues through endocytosis, production of reactive oxygen species (ROS) and a series of proteolytic enzymes such as elastase [1, 2]. At the sites of inflammation, where the signals produced by bacteria and host cells are abundant, the initial response of neutrophils is the secretion of pro-inflammatory cytokines [3-5] and the most abundant of these is interleukin (IL)-8, which acts also as a chemoattractant factor to recruit more neutrophils at the site of injury [6].

Neutrophils are characterized by a short life, indeed they undergo to spontaneous apoptotic

process to maintain the cellular homeostasis [7, 8]. However, the lifespan is prolonged for several days by inflammatory signals. During this additional period, they release inflammatory mediators and contribute to the orchestration of the inflammatory response [9] but at present, no additional information's are available about possible functional changes occurring during this prolonged time.

Recently, the notion that these cells are short-living cells has been challenged by the results of Pillay and his collaborators, which demonstrated that in humans, *in vivo* labelling with  $^3\text{H}_2\text{O}$  under homeostatic conditions, showed an average of circulatory neutrophil lifespan of 5.4 days [10]. Similarly, in a previous study conducted in our laboratory, we showed that human PMN can be cultured up to 24 h and were able

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to respond to stimulation and releasing  $\text{Ca}^{++}$  from the intracellular stores [11].

In recent years, the role and relevance of neutrophils in health and disease has been revised and their contribution to disease progression in several pathologies has been increasingly defined [12]. For example, it was shown that neutrophils plays a key role in inflammatory diseases affecting the CNS [13, 14], like multiple sclerosis [15], or that they can contribute to pathological angiogenesis [16] through the production of key angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8 [17, 18]. On the basis of all these evidences, usually *in vitro* and *ex vivo* experiments on isolated PMN are conducted only for few hours. So far, it is difficult for example to mimics *in vitro* what happens in tissue after neutrophil invasion or to explore the long-term effects of specific drugs on their functions.

In the present study, we have investigated the changes occurring during long-term culture in circulating human neutrophils. To this end, we considered two different times: 3 hours (h, short term) and 24 h (long-term), and we have investigated cell viability, migration, ROS production and the production of three key proinflammatory mediators: IL-8, VEGF and elastase. In addition, by means of Transmission Electron Microscopy (TEM) and optical microscopy, we have analysed cell morphology.

### Materials and methods

#### *Neutrophils isolation*

Experiments were performed on buffy coats obtained by the local blood bank (Ospedale di Circolo, Fondazione Macchi, Varese, Italy). Neutrophils were isolated by standard density-gradient centrifugation as previously described [19]. Finally, cells were examined at light microscopy and no platelets or erythrocytes could be detected. Cell purity was assessed always either by light microscopic examination or by flow cytometric analysis (morphological parameters, SSC and FSC). Experiments were performed only in the conditions in which purity was higher than 95%.

#### *Cell culture*

Neutrophils were resuspended at the concentration of  $1 \times 10^7$  cells/ml in RPMI 1640 with

10% of fetal bovine serum under standard conditions as previously described [20] and incubated alone (resting) or in the presence of N-formyl-Met-Leu-Phe (fMLP; 0.1  $\mu\text{M}$ ; Sigma-Aldrich, Milano), IL-8 (10 ng/ml; Sigma-Aldrich, Milano) or Lypopolisaccharide (LPS; 1  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich, Milano) at 37°C for 3 h and 24 h. fMLP is a chemotactic peptide acting on membrane receptors that induces neutrophils activation [21]. IL-8 represents a key neutrophil product and represents also a physiological activator of these cells [22, 23]. LPS is known to induce an inflammatory response and to stimulate the production of proinflammatory mediators [24]. After incubation, cells were centrifuged (400 g, 5 min, 20°C) and pellets and supernatants were collected and stored at -80°C for subsequent analysis.

#### *Cell viability and apoptosis*

Immediately after culture, samples were centrifuged at 600  $\times g$  for 5 min at RT to remove supernatant, and washed with 1 ml of PBS. Apoptosis was evaluated by using a FITC Annexin V detection Kit I (Becton Dickinson, Milan, Italy) according to the manufacturer's instructions. Briefly, the cells were resuspended in 100  $\mu\text{l}$  of Annexin V Binding Buffer and stained with 5  $\mu\text{l}$  of FITC-conjugated Annexin V (ANX-FITC) and 5  $\mu\text{l}$  of Propidium Iodide Staining Solution (PI) for 15 min in the dark. After incubation, 250  $\mu\text{l}$  of Binding Buffer were added, samples were analyzed by BD FACSCanto II Flow Cytometer (Becton Dickinson Italy, Milano, Italy) and data were collected and elaborated using BD FACSDiva software (version 6.1.3). Neutrophils were identified based on forward-scatter (FSC) and side-scatter (SSC) properties, and at least 15.000 events were collected from each sample. Viable (ANX-/PI-), early apoptotic (ANX+/PI-) and late/necrotic (ANX+/PI+) neutrophils were identified on a biparametric plot ANX-FITC vs PI with a log scale.

#### *RNA isolation and real-time polymerase chain reaction*

Total mRNA was extracted from  $1 \times 10^6$  cells by Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany) and the amount of RNA extracted was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA)

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**Table 1.** Gene sequences assayed by real-time PCR

Gene	UniGene ID	Interrogated Sequence	Translated Protein	Exon Boundary	Assay Location	Amplicon Length	Annealing temperature (°C)	Efficiency (%)
IL-8	Hs. 00174103_m1	NM_000584.3	NP_000575.1	1-2	222	101	60	100.02
VEGF	Hs. 00900055_m1	NM_001025366.2	NP_001020537.2	3-4	1352	59	60	99.9
Elastase	Hs. 00357734_m1	NM_001972.2	NP_001963.1	3-4	402	66	60	100.06
18S rRNA	X03205.1	N.A.	N.A.	N.A.	N.A.	187	60	98.80

Data from the NCBI Reference Sequence Database (RefSeq, <http://www.ncbi.nlm.nih.gov/refseq/>).

according to the manufacturer's instructions and Real-time PCR was performed (ABI prism 7000 apparatus; Applied Biosystems) using assay-on-demand kits. Threshold cycle values (Ct1) for the genes of interest were calculated, normalized to 18S RNA (Ct2) (housekeeping) content, and finally expressed as  $2^{-\Delta Ct}$ , where  $\Delta Ct = Ct2 - Ct1$ . Primers (Applied Biosystems) are shown in **Table 1**.

### Cell migration

Cell migration was measured by the use of Boyden Chamber assay as previously described [19] and migration was quantified by means of optical microscope measuring the distance ( $\mu\text{m}$ ) from the surface of the filter to the leading front of cells. Migration was measured after 1.5 h of culture according standard procedures and our previous results [19] and after 24 h of culture in order to evaluate if long-term incubation affects this parameter.

### ROS generation

Intracellular ROS levels were assessed by use of the redox sensitive dye C-DCDHF-DA (Molecular Probes, Eugene, OR, USA) as previously described [25]. Briefly, freshly isolated, or cultured neutrophils were suspended at the concentration of  $1 \times 10^6$  cells/ml in HBSS medium and incubated with  $2 \mu\text{mol/L}$  C-DCFH-DA (1 h,  $37^\circ\text{C}$  in the dark). Cells were then washed twice with HBSS by centrifugation (400 g,  $20^\circ\text{C}$ , 5 min) and fluorescence was detected by means of a spectrofluorimeter (Perkin-Elmer LS-50B, Perkin-Elmer Instruments, Bridgeport, CT, USA), with excitation wavelength of 488 nm. Fluorescence emission was collected at 525 nm and intracellular ROS levels were finally expressed as difference ( $\Delta$ ) between resting values measured at 60 s and levels measured after 30 min monitoring. ROS levels were assayed under resting conditions and after stimulation with  $0.1 \mu\text{M}$  fMLP or  $1 \mu\text{g/ml}$  LPS.

### IL-8, VEGF and elastase production

IL-8, VEGF and elastase protein levels in supernatants (obtained from cell cultured as above described) were quantified using a sandwich-type enzyme-linked immunoadsorbent assay (QuantikineELISA; R&D System). The limits of detection were  $1 \text{ pg/ml}$  for VEGF and IL-8 and  $1 \text{ ng/ml}$  for elastase.

### Light microscopy and transmission electron microscopy of cell morphology

Isolated neutrophils were resuspended at the concentration of  $5 \times 10^6$  cells/ml in RPMI medium and incubated for 3 and 24 h under resting conditions. After the incubation, the collected pellets were fixed with glutaraldehyd 4% in  $0.1 \text{ M}$  Na-cacodylate buffer (pH 7.2).

Pellets were washed in  $0.1 \text{ M}$  Na-cacodylate buffer (pH 7.2) and post-fixed for 20 min 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol scale, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) and were observed with a light microscope (Eclipse Nikon, Amsterdam, Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol Tokyo, Japan).

### Statistical analysis

Data are presented as means  $\pm$  standard error of the mean (SEM), with n indicating the number of observations. Parametric continuous variables were compared by means of Student's *t* test. Analysis of the correlation between functional responses of neutrophils was performed by linear regression analysis (for continuous variables) and statistical significance for corre-

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**Table 2.** Cell viability (ANX-/PI-), early apoptosis (ANX+/PI-) and late apoptosis (ANX+/PI+) measured by means of cytofluorimetric evaluation with a biparametric plot ANX-FITC vs PI

	3 h			24 h		
	ANX-/PI-	ANX+/PI-	ANX+/PI+	ANX-/PI-	ANX+/PI-	ANX+/PI+
Resting	94.73±2.00	4.70±2.08	0.42±0.17	33.77±9.78 <sup>***,§</sup>	47.42±8.01 <sup>**</sup>	18.11±7.41 <sup>**</sup>
fMLP (0.1 µM)	94.99±1.49	4.17±1.40	0.57±0.25	42.11±25.44 <sup>*,§</sup>	43.58±21.82 <sup>*,§</sup>	13.76±6.84 <sup>*</sup>
IL-8 (10 ng/ml)	94.79±2.14	4.46±2.01	0.48±0.14	35.12±10.48 <sup>**,\$</sup>	46.17±8.37 <sup>**,\$</sup>	18.11±7.34 <sup>**</sup>
LPS (1 µg/ml)	94.10±1.70	3.64±1.09	1.19±0.50	60.74±16.76 <sup>*,#</sup>	27.77±14.20 <sup>*,#</sup>	10.59±3.25

PMN were cultured for 3 or 24 h under resting conditions or in the presence of 0.1 µM fMLP, 10 ng/ml IL-8 or 1 µg/ml LPS. Data are expressed as % of total cells and are means ± SE of 5 separate experiments. \*\*\* = P<0.0001, \*\* = P<0.001 and \* = P<0.05 vs 3 h; # = P<0.05 vs respective resting. § = P<0.05 vs LPS 24 h.

lations was set at P<0.05. Calculations were performed using commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com).

### Results

#### Cell viability and apoptosis

Culture of neutrophils up to 24 h, as expected, reduced the number of living cells (ANX-/PI-) with respect to 3 h of culture and increased the number of cells in early stage of apoptosis (ANX+/PI-) (**Table 2**). Cells in late stage of apoptosis (ANX+/PI+) were higher at 24 h with respect to 3 h, even if these values were never higher than 20% (**Table 2**).

No differences in the number of living, early apoptotic and late apoptotic cells were found between resting conditions or stimulation with both IL-8 and fMLP. On the contrary, stimulation for 24 h with LPS shows an increased number of living cells (ANX-/PI-) with respect to unstimulated cells or cells stimulated with fMLP or IL-8 and a reduced number of early apoptotic cells (ANX+/PI-) with respect to cells stimulated with fMLP or LPS (**Table 2**).

#### IL-8, VEGF and elastase mRNA expression and production

**IL-8:** mRNA levels under resting conditions (empty columns) were similar at both 3 and 24 h of culture, while resting protein production was higher after 24 h (**Figure 1A**). Both fMLP (left hatched columns) and LPS (filled columns) increased IL-8 mRNA and protein production. mRNA and protein levels were increased at both 3 and 24 h of culture after stimulation with fMLP and LPS and no differences were

found in the values measured at the two timing (**Figure 1A**, left). On the contrary, protein production after stimulation with both stimuli was significantly higher at 24 h compared to 3 h (**Figure 1A**, right).

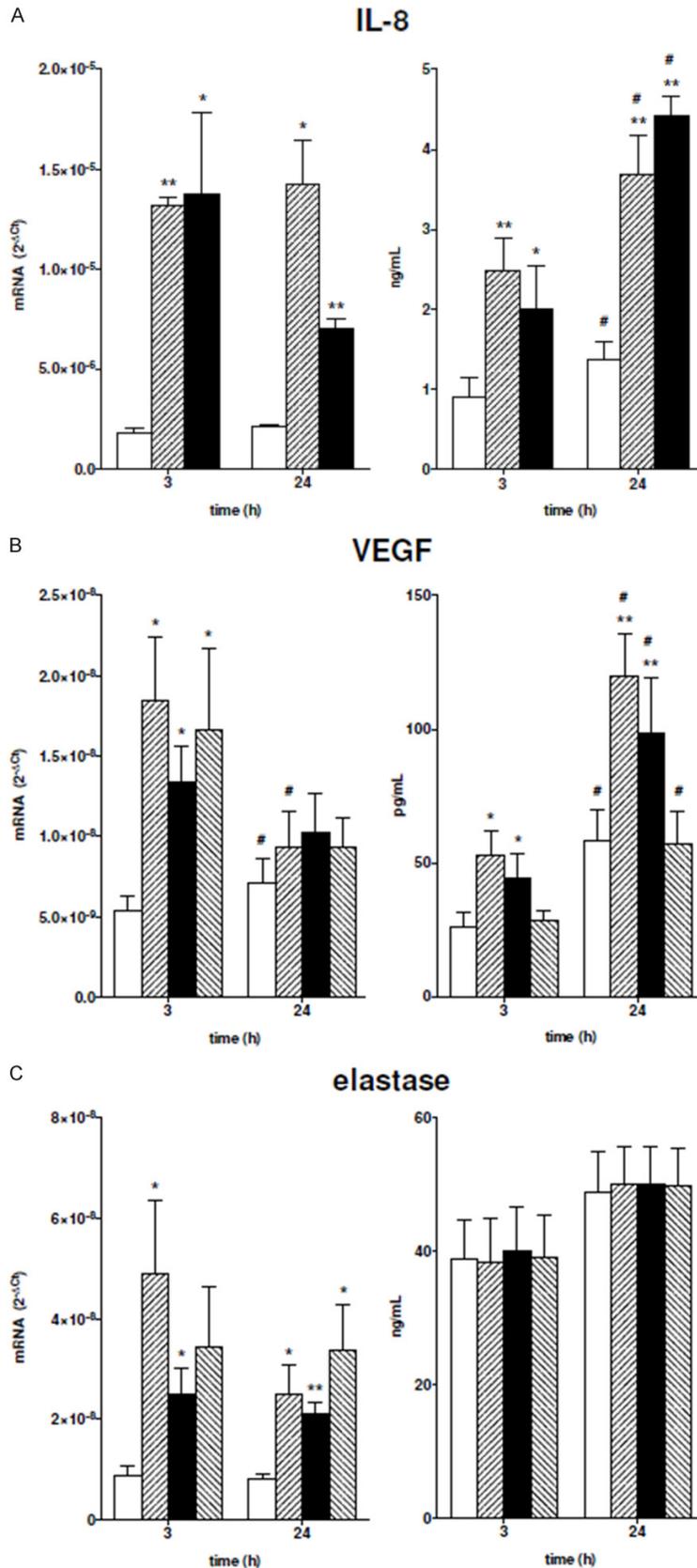
**VEGF:** mRNA and protein levels of VEGF under resting condition (empty columns) were higher at 24 h compared to 3 h (**Figure 1B**). All the stimuli increased VEGF mRNA after 3 h of culture while, on the contrary, values measured after 24 h of stimulation in all conditions were not significantly different with respect to resting values. Comparison between the two-time period showed that the values measured after stimulation were lower with respect to the values reached after 3 h, although the statistical significance was reached only for the stimulation with fMLP (left hatched columns) (**Figure 1B**).

Considering protein production, stimulation with fMLP and LPS, but not with IL-8 (right hatched columns) induced a significant increase of VEGF production after both 3 and 24 h of incubation. Comparison between the two timing showed that the values measured after 24 h were higher than values measured after 3 h of incubation (**Figure 1B**).

**Elastase:** Resting elastase mRNA levels were similar after 3 and 24 h of culture (**Figure 1C**). Treatment of cells with either fMLP (left hatched columns) and LPS (filled columns) increased elastase mRNA levels both at 3 and 24 h. IL-8 (right hatched columns) significantly increased mRNA levels only at 24 h.

Protein production was never affected by all the stimuli at both times of observations (**Figure 1C**).

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**Figure 1.** IL-8 (A), VEGF (B) and elastase (C) mRNA expression and protein production in human neutrophils in resting conditions (empty columns) and after stimulation with fMLP (0.1  $\mu$ M, left hatched columns), LPS (1  $\mu$ g/mL, filled middle columns) and IL-8 (10 ng/mL, right hatched columns). Data are means  $\pm$  SEM of n = 5-11 separate experiments. \* = P<0.05 and \*\* = P<0.01 vs resting; # = P<0.05 and ## = P<0.01 vs 3 h.

### Cell migration

Spontaneous cell migration was similar after 1.5 or 24 h culture ( $18.3 \pm 1.4$  and  $22.9 \pm 1.1 \mu$ m, respectively; P>0.05). As expected, stimulation with fMLP induced always a significant increase of neutrophil migration (1.5 h =  $26.0 \pm 2.2$ ; P<0.05; 24 h =  $32.8 \pm 1.5$ ; P<0.01 vs respective resting conditions) and no differences were observed between values measured at the two times (P>0.05).

### ROS generation

In PMN cultured for 1 and 24 h spontaneous ROS generation was unchanged. As expected, stimulation with fMLP induced a significant increase of ROS production, both after 1 and 24 h of culture, although the values measured after 24 h were lower with respect to values measured after 1 h (Table 3). In addition stimulation with LPS, significantly increased ROS generation after 1 h while did not significantly affect ROS generation after 24 h incubation (Table 3).

### Cell morphology

Optical microscopic observations showed that cells

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**Table 3.** ROS generation in PMN cultured for 1 or 24 h under resting conditions or after stimulation with 0.1  $\mu$ M fMLP or 1  $\mu$ g/ml LPS

	1 h	24 h
Resting	74.5 $\pm$ 6.4	56.9 $\pm$ 8.3
fMLP	298.8 $\pm$ 30.9**	136.2 $\pm$ 20.5**.#
LPS	90.1 $\pm$ 12.2*	55.7 $\pm$ 11.5

Data are expressed as  $\Delta$  variations (30 min FI-60 s FI) and are represented as means  $\pm$  SE of 5-16 separate experiments. \* = P<0.01 and \*\* = P<0.0001 vs respective resting conditions. # = P<0.0001 vs 1 h fMLP. For more details see Method's section.

coltured for 3 h, presented generally round shape, while on the contrary, 24 h coltured cells displayed the presence of different phenotypes (**Figure 2A** and **2D**).

Electron microscopic observations of neutrophils coltured for 3 h and 24 h (**Figure 2B, 2C, 2E, 2F**) showed the typical morphology of neutrophils under resting status: round shape and cytoplasm filled with nuclei, dispersed chromatin, well distributed organelles, granules in the cytoplasm, preserved cell membrane structure. No appreciable differences were observed at the two times of incubation (**Figure 2B, 2C, 2E, 2F**).

### Discussion

The role of neutrophils in the initiation and progression of inflammatory process is well known, but for long time the assumption was that these cells survive only few hours in tissues after leaving the circulation, even if the lifespan can be little prolonged during infection or inflammation [7-9].

In the present study, we investigated the ability of human neutrophils to survive in culture up to 24 h. As expected, during culture (after 24 h) the number of apoptotic cells significantly increased although the number of cells in the late stage of apoptosis after 24 h was not higher than 20%. Interestingly, we have shown that the number of apoptotic cells depends not only from the time of culture, but is also stimuli-sensitive. In fact, although cell viability after 1 h incubation was unchanged, on the contrary, 24 h of culture significantly reduced the number of living cells and increased the number of cells in early and late stage of apoptosis. Interestingly, after stimulation with LPS, the percentage of

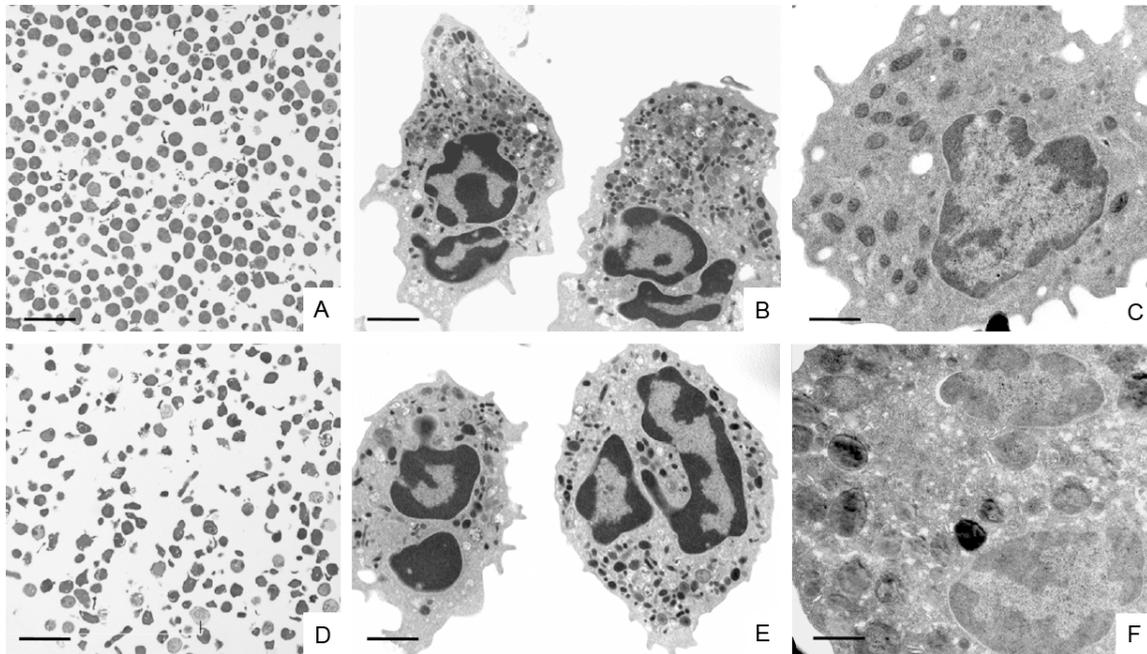
living cells was higher with respect to unstimulated cells or cells stimulated with fMLP or LPS. Similarly, the number of cells in early stage of apoptosis was reduced in presence of LPS. Interestingly, in general in all conditions tested, the number of cells in late apoptosis is never higher than 20%. Furthermore, the preserved morphology clearly evident both with optical microscopy and TEM is in line with these data.

We have also shown that migration, which represents a key step in the ability of these cells to invade tissues, was unchanged up to 24 h. Similarly, ROS generation involved in pathogen aggression and killing [26], was sustained after 24 h of culture with a significant reduction only in the stimulated values.

Interestingly, for ROS generation we showed that the stimuli employed exert different effects, as shown for apoptosis, in short-term culture with respect to long-term culture. In fact, increased ROS generation in fMLP-stimulated cells were sustained, if lower, after 24 h, while on the contrary LPS-induced increases ROS generation was not present after 24 h of culture, suggesting that these two kinds of pro-inflammatory stimuli can differently affects the short or long-term cell culture. This observation, together with the data of different effects of LPS on apoptosis (reduced apoptotic cells during LPS stimulation at 24 h), suggests that, although proinflammatory agents could affects neutrophil functions, the results can be different when the stimulation occur for long (chronic) or short time (acute).

On the basis of all these observations, we can hypothesize that, neutrophils, after the migration into inflamed tissue, through the *in loco* production of inflammatory mediators, can contribute to the orchestration and maintenance of the inflammatory status typically found in all the immune-mediated diseases. Indeed, in line with this hypothesis, the production of key mediators such as IL-8, VEGF and elastase was maintained and was higher after prolonged time of incubation and after exposition to pro-inflammatory stimuli. These data are in line with our previous observations in atherosclerotic patients, in which we have shown, that not only circulating cells but also resident cells were able to produce proinflammatory mediators [27] suggesting that neutrophils can contribute

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**Figure 2.** Semithin (A, D) and thin (B, C, E, F) sections of isolated human neutrophils cultured for 3 h (A-C) and 24 h (D-F). TEM analysis shows that no differences can be appreciated comparing the phenotypes of resting neutrophils at the two times of culture. All the cells are roundish with the typical spatial organization of the nuclei in central position. The cytoplasm is filled with numerous granules and cell membrane appears intact. Scale bars: B = 1.5  $\mu\text{m}$ ; C = 0.7  $\mu\text{m}$ ; E = 1.5  $\mu\text{m}$ ; F = 0.7  $\mu\text{m}$ .

not only to the generation, but also to the perpetration in loco of inflammatory status.

Considering that VEGF and IL-8 are potent angiogenic factors regulating vascular growth, function, and homeostasis, as well as permeability and vasodilatation [22], the observation that neutrophils are able to produce these mediators also after long time suggests a key contribution in processes characterized by intense neutrophil infiltration and neovessel formation such as in tumours and atherosclerosis [28, 29].

The presence of the proteolytic enzyme elastase within inflamed tissues is well established and elastase is considered a notable marker and inducer of inflammation [30]. To our knowledge, the present result represents the first detailed characterization of the modifications in elastase mRNA levels following exposure of human neutrophils to proinflammatory and activating stimuli such as fMLP, LPS and IL-8.

In this study, we performed for the first time the ultrafine morphology of human neutrophils after short or long time of culture. TEM evaluation clearly showed that cell architecture was

preserved suggesting that these cells are able to carry out their functions even after leaving the bloodstream from long-time.

In conclusion, the present study provided for the first time a detailed characterization of the functions of human neutrophils in response to proinflammatory stimuli, showing that these cells even after prolonged times of culture, are able to maintain the fundamental characteristics in order to perform their key functions in pathologic processes. In addition, we have shown, for the first time, that long-term culture could differently modulate some functions depending of the stimuli employed. Finally, the present study provided a detailed characterization of the human neutrophils in response to proinflammatory stimuli, showing that these cells are viable and responsive even after prolonged periods of culture, up to 24 h. However, time-length of the culture is likely to affect many responses in a function-specific fashion.

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### Disclosure of conflict of interest

None.

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