

NET amyloidogenic backbone in human activated neutrophils

L. Pulze,* B. Bassani,*† E. Gini,*

P. D'Antona,* A. Grimaldi,*

A. Luini,‡ F. Marino,‡

D. M. Noonan,*† G. Tettamanti,*

R. Valvassori* and M. de Eguileor*

*Department of Biotechnology and Life

Sciences, University of Insubria, Varese, Italy,

†IRCCS MultiMedica, Scientific and Technology

Park, Milano, Italy, and ‡Center of Research in

Medical Pharmacology, University of Insubria,

Varese, Italy

Summary

Activated human neutrophils produce a fibrillar DNA network [neutrophil extracellular traps (NETs)] for entrapping and killing bacteria, fungi, protozoa and viruses. Our results suggest that the neutrophil extracellular traps show a resistant amyloidogenic backbone utilized for addressing reputed proteins and DNA against the non-self. The formation of amyloid fibrils in neutrophils is regulated by the imbalance of reactive oxygen species (ROS) in the cytoplasm. The intensity and source of the ROS signal is determinant for promoting stress-associated responses such as amyloidogenesis and closely related events: autophagy, exosome release, activation of the adrenocorticotrophin hormone/ α -melanocyte-stimulating hormone (ACTH/ α -MSH) loop and synthesis of specific cytokines. These interconnected responses in human activated neutrophils, that have been evaluated from a morphofunctional and quantitative viewpoint, represent primitive, but potent, innate defence mechanisms. In invertebrates, circulating phagocytic immune cells, when activated, show responses similar to those described previously for activated human neutrophils. Invertebrate cells within endoplasmic reticulum cisternae produce a fibrillar material which is then assembled into an amyloidogenic scaffold utilized to convey melanin close to the invader. These findings, in consideration to the critical role played by NET in the development of several pathologies, could explain the structural resistance of these scaffolds and could provide the basis for developing new diagnostic and therapeutic approaches in immunomediated diseases in which the innate branch of the immune system has a pivotal role.

Keywords: ACTH axis, amyloidogenesis, exosomes, neutrophil extracellular trap, ROS evaluation

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Correspondence: M. de Eguileor, Department of Biotechnology and Life Sciences, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy.

E-mail: magda.deeguileor@uninsubria.it

Introduction

Neutrophils, the most abundant human leucocytes, play a pivotal role in innate immunity, constituting the first cells recruited into sites of infection. In response to numerous stimuli, neutrophils migrate from the circulating blood into inflamed tissues where they employ several strategies to contain and clear infections. These strategies include phagocytosis, degranulation, the release of lytic enzymes into the phagosome or in the extracellular space and the production of reactive oxygen intermediates with anti-microbial potential [1–5]. Another neutrophil response mechanism against non-self is the production of neutrophil extracellular traps (NETs), a web-like structure for entrapping and killing bacteria, fungi, protozoa and viruses extracellularly [6–9].

The NET is formed by decondensed chromatin (DNA and histones H1, H2A, H2B, H3, H4) and is decorated by proteins from primary granules (for example, myeloperoxidase and neutrophil elastase), secondary granules (such as lactoferrin) and tertiary granules (matrix metalloproteinase 9, etc.). The NETs have been induced by internal and/or pathogen-derived molecular signals [cytokines such as interleukin (IL)-8, tumour necrosis factor (TNF)- α , high mobility group box 1 (HMGB1), lipopolysaccharide (LPS), N-formyl-methionyl-leucyl-phenylalanine (fMLP) [7–13] and pharmacological agents, such as phorbol myristate acetate (PMA) [5,7].

NETs may contribute to microbial containment by forming a scaffold functioning as a physical barrier, and enhance

anti-microbial synergy while minimizing damage to host tissues [14–16]. The formation of NETs in vertebrates has been considered a strategy linked indubitably to an innate immune response and it is a typical property shared by neutrophils, eosinophils [17], monocytes/macrophages [18] and mast cells [19].

Several authors have described that freely circulating phagocytic haemocytes in protostomes and in deuterostomes, when stimulated with LPS, show dilated reticulum cisternae filled with spatially organized fibrillar material. The objective of the accumulated material within cytoplasm is to be exocytosed and assembled into a resistant scaffold to template melanin and to drive this toxic pigment towards the non-self [20–22]. As highlighted by our previous studies, the framework of phagocytic circulating cells was made of amyloid fibrils [20–22]. Due to the similarity in the release of fibrillar structures by LPS-activated phagocytic haemocytes and LPS-stimulated neutrophils, we also hypothesized that the NET could be characterized by an amyloid structure in these human cells.

Amyloidogenesis is dependent upon cytoplasmic pH acidification due to increased reactive oxygen species (ROS), an event already described widely in activated neutrophils [2,15,23,24]. This process is also accompanied by an increase of IL-18 expression. This cytokine has been associated with amyloid deposition in vertebrates and is also linked to Alzheimer's disease [25,26], where it can be considered an early marker for amyloidogenesis and for disease progression [25].

Despite the fact that amyloidogenesis is generally associated with degenerative pathologies, amyloid fibril formation may contribute to normal physiology having different functions, such as bacterial/fungi biofilm formation, scaffolding for different molecules, regulation of melanin synthesis, epigenetic control of polyamines and information transfer in a wide range of organisms, from bacteria to mammals [22,27–31].

The bi-directional interactions between the neuroendocrine and immune systems have been examined [32,33], focusing on adrenocorticotrophic hormone (ACTH), alpha melanocyte-stimulating hormone (α -MSH) and neutral endopeptidase (NEP) production. It is now recognized that in both invertebrates and vertebrates the non-self presence is perceived and managed by the two systems, able to influence each other mutually. ACTH and α -MSH are released by the immunocompetent cells to control immune reactivity [34–36]. These factors regulate the cellular functions of the immune system locally, in an autocrine and paracrine manner, influencing the proliferation of immune mediator production and immunocyte trafficking [22,23,37].

Expression of the ACTH is regulated by cleavage to α -MSH by NEP. Further NEP is also involved in the degradation of amyloid fibrils [38,39]. Taken together, these data suggest that this stress sensor/response system occurs in neutrophils.

Materials and methods

All experiments were performed in five independent replicates.

Neutrophil purification and activation

Circulating neutrophils were isolated from venous blood or buffy coat derived from healthy donors. Briefly, blood was allowed to sediment on dextran at 37°C for 30 min (from *Leuconostoc* spp., M_r 450 000–650 000; Sigma-Aldrich, Milan, Italy). Supernatant was recovered and PMN were isolated by Ficoll-Paque PLUS (GE Healthcare, Milan, Italy) density-gradient centrifugation; contaminating erythrocytes were eliminated by hypotonic lysis for 10 min in distilled water and the following (g/l) were added: 8.25 ammonium chloride (NH_4Cl), 1.00 potassium bicarbonate (KHCO_3) and 0.04 ethylenediamine tetraacetic acid (EDTA). Cells were then washed three times in 0.15 M NaCl.

Neutrophils (8×10^4 cells/ml) were seeded on rounded glass coverslips (12 mm diameter, treated with 0.1% gelatinase) or in six-well plates in RPMI-1640 [2% fetal bovine serum (FBS)] and either unstimulated or stimulated with 100 ng/ml LPS (from *Escherichia coli*, serotype O55:B5,) for 15 or 40 min or with 5 ng/ml PMA (Sigma-Aldrich) for 15 min. Stimulated and unstimulated neutrophils were then processed for different procedures.

Light microscopy and transmission electron microscopy (TEM)

For routine TEM, neutrophils were collected in six-well plates and fixed with 4% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2).

Pellets were washed in 0.1 M Na-cacodylate buffer (pH 7.2) and post-fixed for 20 min with 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in an ethanol series, 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, the Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Intracellular ROS evaluation

Oxidative stress can induce proteins to adopt an insoluble beta-pleated sheet conformation [40], and according to numerous authors [41–43] oxidative damage appears to be the earliest event preceding amyloid fibril formation. Thus, it is important to evaluate the overproduction of ROS in relation to LPS activation responsible for amyloid fibril production [21]. According to Grimaldi and co-workers

[21,22], a gross identification of cells showing increased ROS is obtained with differential May–Grünwald–Giemsa staining (Bioptica, Milan, Italy). The different staining of cells depends upon cytoplasmic pH (alkaline pH increases blue while acid pH enhances a pink or reddish tinge in the stained specimens). Coverslips were mounted in Eukitt and slides were observed under an Eclipse Nikon microscope with $\times 50$ or $\times 100$ objectives.

ROS production and its derivation was validated by use of MitoSOXTM Red mitochondrial superoxide indicator (Molecular Probes, Eugene, OR, USA), a fluorogenic probe used to detect the degree of intracellular level of ROS obtained from mitochondrial activity. Once in the mitochondria, MitoSOXTM Red reagent is oxidized selectively by superoxide and exhibits red fluorescence. Oxidation can be detected by monitoring the increase in fluorescence. Following the manufacturer's protocol, resting and activated neutrophils were washed once in fresh medium, resuspended in phosphate-buffered saline (PBS) and incubated in the dark with 4 μM MitoSOX prepared in Hanks's balanced salt solution (HBSS)/Ca/Mg for 10 min at 37°C. Nuclei were counterstained with 4',6'-diamino-2-phenylindole (DAPI; Sigma-Aldrich). Fluorescence was determined by excitation/emission maxima of approximately 510/580 nm. Fluorescent images, visualized on a fluorescence Eclipse Nikon microscope, were acquired with a DS-5 M-L1 Nikon digital camera system.

ROS production was also evaluated by the use of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes), a fluorogenic probe used commonly to detect the overall degree of intracellular level of ROS. H₂DCFDA is a non-fluorescent compound that readily crosses cell membranes. It is hydrolyzed to 2',7'-dichlorofluorescein (DCF) within cells and becomes fluorescent when it is oxidized by ROS. Oxidation can be detected by monitoring the increase in fluorescence. Resting and activated neutrophils were incubated in the dark with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) for 30 min at 37°C; nuclei were stained with DAPI. Fluorescence was determined by excitation at 488 nm and emission at 525 nm wavelength; fluorescent images visualized on a fluorescence Eclipse Nikon microscope were acquired with a DS-5 M-L1 Nikon digital camera system.

In parallel, we also performed a quantitative experiment with the same probe. Freshly isolated cells were suspended at a concentration of 1×10^6 cells/ml in HBSS medium and then incubated for 1 h with H₂DCFDA 2 $\mu\text{mol/l}$ in the dark at 37°C; cells were then washed twice with HBSS. Fluorescence measurements were performed using a spectrofluorimeter (Perkin-Elmer LS-50B; Perkin-Elmer Instruments, Bridgeport, CT, USA). Excitation wavelength was set at 488 nm and fluorescence emission was collected at 525 nm. Intracellular ROS levels were then expressed in arbitrary units (AU) as fluorescence intensity. The overall degree of cytoplasmic ROS was tested on resting cells, on

cells after LPS activation (after 15 and 40 min) and on cells treated with PMA (after 15 min), the potent synthetic agonist.

Amyloid fibril characterization

Amyloid structures were identified according to Le Vine [44] by staining cells with thioflavin S (ThS) and visualizing the amyloid-specific green/yellow fluorescence with an Eclipse Nikon microscope. Images were acquired with a DS-5 M-L1 Nikon digital camera system.

Using the Image J software package (<http://rsbweb.nih.gov/ij/download.html>), the fluorescence ratio (expressed as integrated density) between the amyloid-associated ThS signal and the NET DNA-associated DAPI was calculated.

Amyloid fibrils were also characterized with Congo red staining, according to published methods [45], and observed under cross-polarized light with an Axioskop 2 microscope (Carl Zeiss, Jena, Germany), equipped with a MC 80 DX camera (Carl Zeiss).

Amyloid fibrils (see the next section) were also localized immunocytochemically using an antibody directed against (PMEL17), a protein that has amyloid characteristics and contributes to form fibrillar structures in mammals [46].

Immunocytochemistry for ACTH, α -MSH, NEP, interleukin 18 and Pmel17 localization

The presence of amyloid fibrils was confirmed by using the primary antibody anti-human Pmel17 (H-300) polyclonal antibody (1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The presence of ACTH and its cleavage product (due to degrading enzyme NEP), α -MSH and the proinflammatory IL-18 were assessed using the following primary antibodies: anti-human ACTH polyclonal antibody (1 : 100 dilution; Abcam, Cambridge, UK); anti-human α -MSH polyclonal antibody (1 : 100 dilution; Abcam); anti-human IL-18 polyclonal antibody (1 : 100 dilution; Abnova, Taipei City, Taiwan); and anti-human CD10/CALLA (NEP) monoclonal antibody (1 : 100 dilution; GeneTex, Hsinchu City, Taiwan). Incubations with suitable secondary antibodies conjugated with cyanin 5 (Cy5, 1 : 50 dilution; Abcam) were performed for 1 h in a dark humid chamber at room temperature. Nuclei were stained with DAPI (Sigma-Aldrich). The PBS buffer used for washing steps and antibody dilutions contained 2% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% Tween20.

In co-localization experiments, the cells were first stained with ThS (as described above) and then incubated with anti-Pmel17 antibody (as described above).

In control samples, primary antibodies were omitted and samples were treated with BSA/Tween20 containing PBS. Coverslips were mounted in CitiFluor (CitiFluor Ltd,

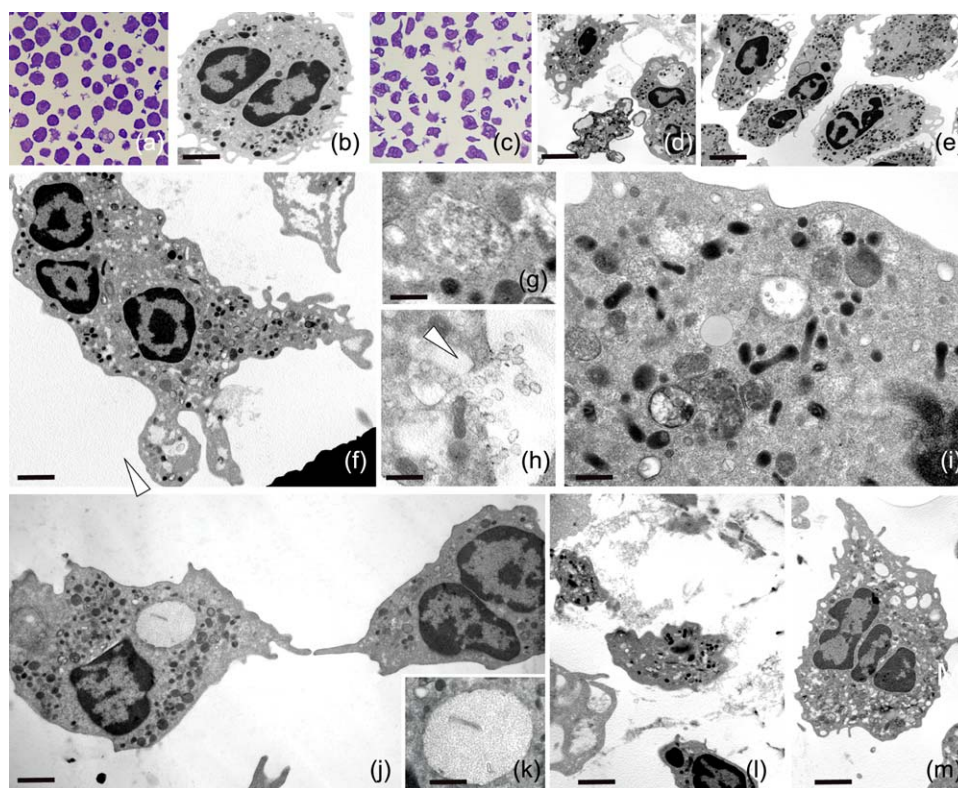


Fig. 1. Unstimulated and stimulated human neutrophils. Thin and semithin sections of resting (a,b) and activated neutrophils (c–m). The unstimulated cells (a,b) are roundish, but after stimulation (c) with both phorbol myristate acetate (PMA) (d) and lipopolysaccharide (LPS) (e–m) they lose their globoid shape. Starting from 15 min after stimulation, neutrophils show a transformed phenotype characterized by irregular profile, due to the presence of protrusions and pseudopodia (d,e). In a time lapse between 15 and 40 min from LPS activation (e–m), a series of events are detectable due to the presence of multi-vesicular bodies (f,g), released exosomes (f,h) and autophagosomes containing portions of the cytosol (i). Activated cells show large dilated reticulum cisternae (j,k) (arrowheads) filled with spatially organized fibrillar material (k) or empty vacuoles (l,m) and material among cells (m). Scale bars. (a) 6 μm ; (b) 5 μm ; (d) 9 μm ; (e) 3.5 μm ; (f) 4.5 μm ; (g) 1.9 μm .

London, UK). Slides were observed under an Eclipse Nikon microscope.

Statistical analysis

Data are presented as mean \pm standard error of the mean (s.e.m.), 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 correlations 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

Morphology and behaviour of neutrophils

In order to study the morphofunctional modifications that occur during the activation of neutrophils, we examined

these cells after stimulation with LPS at 15 min (corresponding to the starting point of the event) and 40 min according to Jaillon *et al.* [47] to observe NET formation. The rapid activation of these cells can be achieved utilizing different (natural or chemical) stressors such as LPS and PMA, respectively, with a final result of massive degranulation and NET formation [7]. Even if PMA is generally considered the most widely used stimulus to induce NET formation [48] (Fig. 1d), we preferred to focus on the activation gained with a natural (LPS) rather than a chemical (PMA) agonist. In addition, LPS provides initially slower and more reliable kinetics, allowing us to describe the modulated responses leading to NET formation step by step.

Human neutrophils, in resting condition, were spheroidal cells easily distinguishable under optical and electron microscopy by their compressed, multi-lobed nuclei and different types of granules embedded in homogeneous cytoplasm, while very few neutrophils showed cytoplasmic projections (Fig. 1a,b). Within 15 min after LPS administration, optical and TEM analysis indicated that most of the neutrophils were flattened with irregular profiles due to cytoplasmic projections and with evident multi-lobular

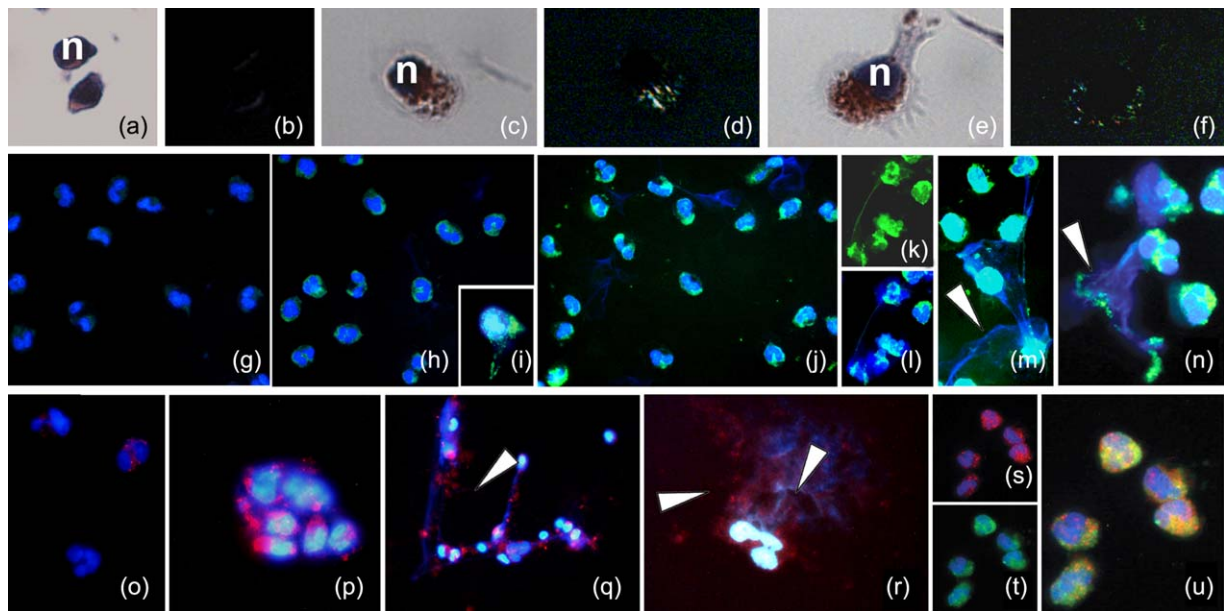


Fig. 2. Amyloidogenesis in lipopolysaccharide (LPS)-stimulated neutrophils. (a–n) Detection of amyloid fibril presence with Congo red (CR) and thioflavin S (ThS) stainings. (a–f) CR staining: unstimulated neutrophils (a,b) in comparison with LPS-stimulated cells (c–f) at the starting point of activation. The CR positive cells show the typical apple green birefringence evidencing the spotted presence of amyloid structures in the cytoplasm. Nuclei are counterstained with haematoxylin (n). (g–n) Identification of amyloid fibrils with ThS: unstimulated neutrophils (g) in comparison with stimulated cells at 15 min (h–j) and 40 min (k–n) from LPS administration. Within cells (h–j) and extracellularly (k–n), amyloid fibrils are localized by ThS bright fluorescence. Nuclei and DNA material in neutrophil extracellular traps (NETs) are stained with 4',6'-diamino-2-phenylindole (DAPI) in brilliant blue. Both single staining, for ThS and DAPI (k,l) and the two overlaid signals are proposed to better identify the presence of amyloid and DNA materials. Note the co-localization of blue (DAPI) and green (ThS) in NETs released on stimulation (arrowheads). The semi-quantitative analysis of the concomitant amyloid fibril presence (ThS) and DNA (DAPI) of NETs showed a ratio of 0.78 ± 0.03 . (o–u) (PMEL17) immunolocalization: immunofluorescence staining showing, in comparison to control (o), the increased expression of Pmel17 that is present in the cytoplasm of stimulated neutrophils (p–r) and in the NETs (q,r). DAPI (blue) staining for DNA shows distinct cores indicating nuclei, as well as diffuse patterns evidencing extracellular DNA in NETs (arrowheads). (s–u) Double-labelling of neutrophils with ThS (green) and antibody against Pmel17 (red). The co-localization of two signals (merge in yellow, u) is quite good. Nuclei (blue) are stained with DAPI.

'opened' nuclei (Fig. 1c,e). From 15 to 40 min several specific events could be detected. In the cytoplasm, the presence of multi-vesicular bodies, exocytosis events of exosomes (Fig. 1f–h) as well as premature autophagosomes, engulfing cytoplasm portions, were identified (Fig. 1i). Concomitantly, the presence of empty vacuoles or rough reticulum cisternae containing fibrillar material were observed (Fig. 1j,k).

Finally, 40 min after LPS stimulation, neutrophils were characterized by a massive cellular vacuolization accompanied, although not in all activated cells, by NET formation (Fig. 1l,m).

Amyloid fibrils production

The fibrillar material observed in the human neutrophils showed staining properties typical of amyloid fibrils [49,50]. First Congo red (CR) positivity, evaluated by the characteristic yellow-green birefringence under crossed polarization, has been used commonly to test the presence of amyloid fibrils [44]. With regard to cells in the resting

condition (Fig. 2a,b), the activated cells (Fig. 2c–f) were CR-positive, showing the typical apple-green birefringence of amyloid under polarized light (Fig. 2d,f). To confirm amyloid presence, the neutrophils were also stained by ThS (Fig. 2g–n). The characteristic bright yellow-green fluorescence, indicating amyloid presence, was visible both in cytoplasm (Fig. 2h–n) and in correspondence with the NETs (Fig. 2k–n), more readily evident in the stimulated cells compared to controls (Fig. 2g). Semiquantitative analysis of the concomitant amyloid fibril presence (ThS) and DNA (DAPI) of NETs showed a ratio of 0.78 ± 0.03 . Immunolocalization with a specific antibody directed against Pmel17, a basic mammalian protein involved in amyloidogenesis [27], further supported the presence of amyloid. In comparison to resting neutrophils, activated cells (Fig. 2o) showed increased Pmel17 expression consistent with the degree of activation (Fig. 2p–r). In particular, at approximately 40 min of stimulation, the signal, first present especially in the cytoplasm, became detectable in the NETs, typically DAPI-positive (Fig. 2q,r). In activated neutrophils, Pmel17 immunoreactivity was found

co-localized to a cytoplasmic area that is also ThS-positive (Fig. 2s–u).

Intracellular ROS evaluation

Previous studies have suggested that in different types of phagocytic/circulating cells, from invertebrates to vertebrates, amyloid scaffold formation occurs in relation to a redox status/cytoplasmic pH modification [21,22]. In addition, further information suggests that in humans, ROS influence the formation of extracellular traps (ETs) in a process termed 'ETosis' [24,51]. During the early stages of immune response, neutrophil activation, leading to ROS generation and resulting in pH acidification, is a process linked closely to NET production [15,52,53]. We showed that a growing number of LPS-stimulated neutrophils were characterized by pink cytoplasm in May–Grünwald–Giemsa differential staining (Fig. 3a–e), giving a gross indication of a lower intracellular pH.

Using the redox-sensitive dye MitoSOX and the fluorogenic probe H₂DCFDA, we detected the increased degree of the intracellular free radical production attributable to mitochondrial activity (Fig. 3f–i) and the overall degree of intracellular level of ROS (Fig. 3j–l), respectively, in relation to the administration time.

Spectrofluorimetric evaluation of the time–course of ROS generation in human neutrophils under resting conditions or stimulated with LPS in comparison with PMA validated the data of immunocytochemical localizations. In neutrophils, from the resting condition to NET formation, the ROS level concentration increased rapidly following LPS stimulation. ROS production was modulated not only to the administration time, but was also connected strictly to the type of activator (i.e. LPS or PMA).

The experimental profile of ROS levels in unstimulated neutrophils and of changes induced by LPS or PMA stimuli are shown in Fig. 3m–o. In particular, samples preincubated with LPS or PMA showed higher resting values in comparison to unstimulated samples (Fig. 3m–o).

Closely related events during NET formation: amyloidogenesis, IL-18 production and ACTH/ α -MSH axis activation

Neutrophil activation is accompanied by proinflammatory cytokine production [4]. Among the plethora of these cytokines we have examined IL-18, because it is generally known as a link with stress-associated responses and amyloid deposition [54]; LPS-activated neutrophils showed increased production in this cytokine (Fig. 4a–d).

Activation of stress circuits resulting in the extensive production of ACTH and α -MSH was also observed in LPS-activated neutrophils. The production of ACTH (Fig. 4e–h) and its cleavage product α -MSH (Fig. 4I,j) were observed by immunocytochemical localizations.

NEP is an enzyme associated with cleavage of ACTH to α -MSH [22,55–57], as well as amyloid degradation and removal [58–63].

In stimulated neutrophils, concomitant with the increase in ACTH and amyloid fibril synthesis, an increment in production of NEP (Fig. 4l–o) was also observed by immunocytochemistry both in the cytoplasm (Fig. 4m) and on NETs (Fig. 4o).

Discussion

The results presented aim to provide evidence that, after LPS stimulation, neutrophils show tuned events, culminating with the production of amyloid fibrils that, in turn, made a backbone structure for addressing reputed proteins and DNA against the non-self. The NET formation is regulated by the imbalance of reactive oxygen species in the cytoplasm.

NETs released against bacteria, fungi, viruses or protozoa have been described extensively from morphological, biochemical and molecular viewpoints [2,4,5,14–16,64] and it has long been debated whether or not these web-like structures were produced in a controlled process, rather than in a disintegration cellular event [48].

Here we provide evidence concerning the modulated events that take place during the activation phase of neutrophils showing that the LPS-stimulated cells are the reservoir of amyloid fibrillar structures that, in turn, form NETs. These fibrils show all the characteristics of amyloid: birefringence upon Congo red staining [45] and ThS positivity [44], and harbour a basic mammalian protein, Pmel17 [27,28], as validated by the co-localization of signals. Few naive neutrophils show amyloid fibrils that are compartmentalized by membranes of rough reticulum cisternae, while upon LPS challenge most of the activated neutrophils exhibit the production of amyloid material. In a sequence of events, ultrastructural and immunocytochemical analysis revealed that neutrophils start to synthesize and harbour fibrillar materials that are released from the intracellular repositories to support NET formation. We suggest that the amyloid fibrils, charged cationically, may co-operate to form the scaffold for addressing those numerous proteins and nuclear DNA, charged anionically, has already been well identified and described by several authors [4,7,8,47].

In stimulated neutrophils, the association of amyloid fibrils with NET formation is supported by several events, starting from REDOX cytoplasmic changes where ROS is immunodetected and measured quantitatively by spectrofluorimetric evaluation (Fig. 3). To strengthen the idea that amyloid production in neutrophils is a possible and common event, it is important to bear in mind that the change in cytoplasmic pH linked to ROS generation is considered as the earliest event that induces proteins to adopt a β -sheet conformation preceding the formation of amyloid

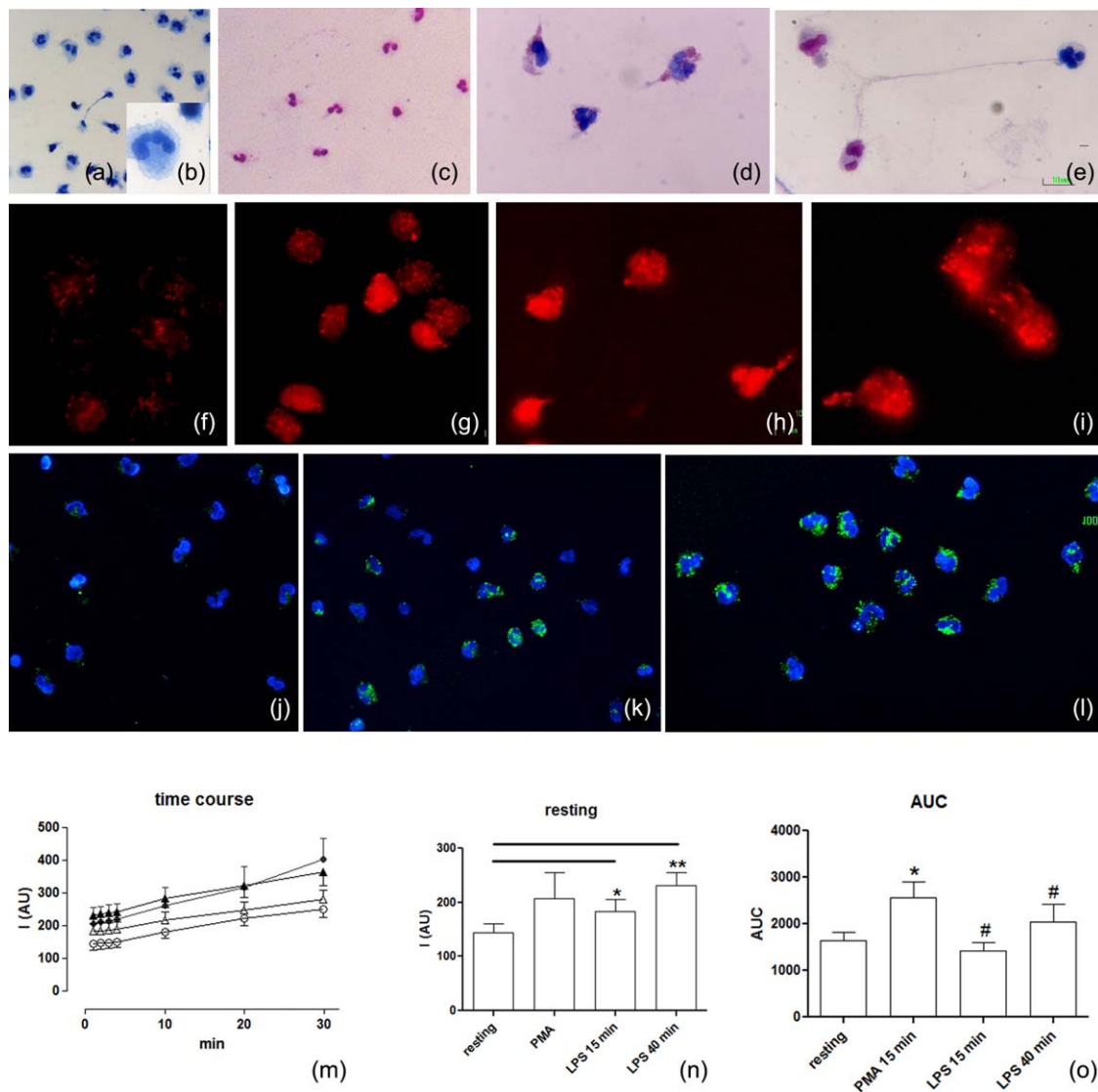


Fig. 3. Evaluation of intracellular reactive oxygen species (ROS). (a–e) Light microscopy: resting (a,b) and stimulated cells (c–e) are stained using the May–Grünwald–Giemsa technique. The differential staining of neutrophils depends on cytoplasmic pH (the pink mark indicates acid pH while the blue mark shows an alkaline pH). (f–i) ROS evaluation was obtained using fluorescence dyes: the MitoSOX red used as a selective indicator of mitochondrial superoxide and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) to detect the overall degree of cytoplasmic ROS. Most ROS (red signal) were generated in mitochondria (f–i) starting from the resting condition (f) up to 15 min (g) and 40 min (h,i) after lipopolysaccharide (LPS) stimulation. The increase of the overall degree of intracellular ROS (j–l), using H₂DCFDA, is localized by fluorescence microscopy: (j) cells in resting condition (k,l) 15 and 40 min after LPS stimulation, respectively. In neutrophils, from the resting condition to neutrophil extracellular trap (NET) formation, the concentration of cellular ROS levels increased following appropriate [LPS and phorbol myristate acetate (PMA)] stimulation on a rapid time-scale. (m) Spectrofluorimetric evaluation of the time-course of ROS generation in human neutrophils under resting conditions (empty circle), prestimulation for 15 min with PMA (filled circles) for 15 min (empty triangles) or 40 min (filled triangles) with LPS. (n) Delta variations of values measured in neutrophils under the different conditions. (o) Area under the curve (AUC) of fluorescence measured for detection of neutrophil ROS generations during the 30 min of detection. Data are presented as mean \pm standard error of at least three to five separate experiments. * $P < 0.05$ versus resting PMN; # $P < 0.05$ versus PMA.

fibrils [40,41,62]. ROS production has been reported as an absolute requisite to NET formation and has been demonstrated extensively in neutrophils stimulated by bacteria and fungi [2,5,7,8,15,19,52,53,65]. Moreover, in a recent paper, Zawrotniak and co-workers reported that the treatment of neutrophils with N-acetyl-cysteine (NAC), a

potent anti-oxidant, influences the production of NET. This drug decrease the ROS level in a dose-dependent manner [66].

ROS, despite their potential destructive activity, can be considered as a promoter of a variety of cellular processes [42,43,62]. In LPS-activated neutrophils, the 'identity' and

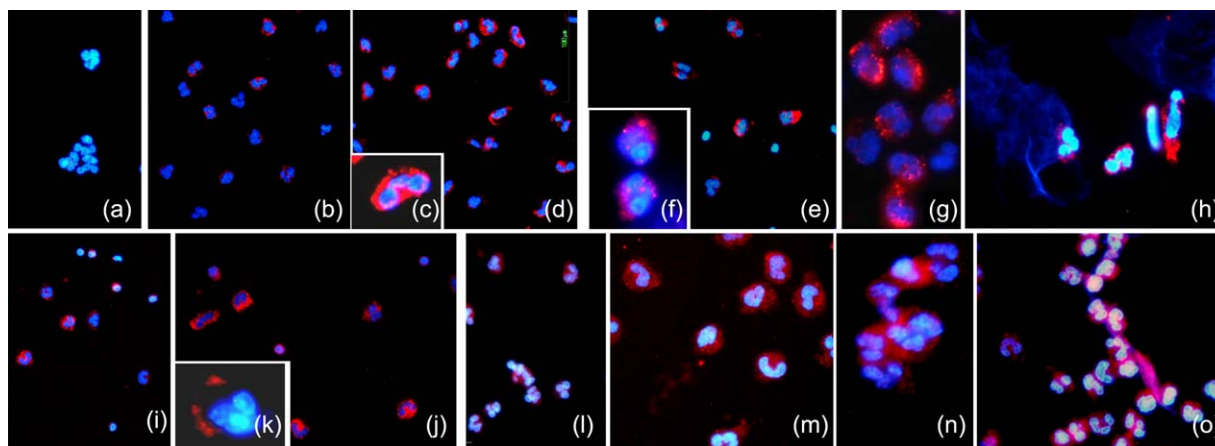


Fig. 4. Characterization of lipopolysaccharide (LPS)-stimulated neutrophils. (a–o) Immunocytochemical characterizations. Interleukin (IL)-18 (a–d), adrenocorticotrophin hormone (ACTH) (e–h), alpha melanocyte-stimulating hormone (α -MSH) (i–j) and neutral endopeptidase (NEP) (l–o) expressions show high levels of positivity in activated neutrophils (b–d, f–h, j–k, m–o), while in resting cells the expression of IL-18 (a), ACTH (e), α -MSH (i) and NEP (l) is basal.

intensity of the ROS signal are of paramount importance: the site of ROS production is initially mitochondrial (as demonstrated by analysis with Mitosox) and the ROS level is lower compared to that measured after 40 min from activation, when it can be considered cytoplasmatic (as demonstrated by H_2DCFDA positivity). The changes in ROS intensity from 15 to 40 min are important regulators and sustainers of several crucial cellular events: (i) activation of

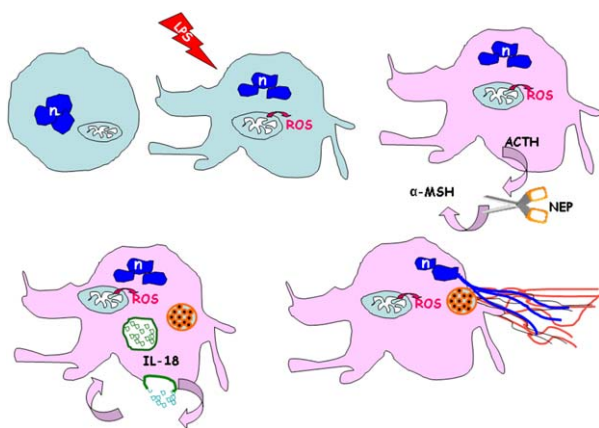


Fig. 5. Schematic overview explaining the possible behaviour of lipopolysaccharide (LPS)-activated neutrophils. LPS activation of neutrophils results in an increase of reactive oxygen species (ROS) levels. Concomitant cross-talk between immune and neuroendocrine systems induces an activation of stress-sensing circuits to produce adrenocorticotrophin hormone (ACTH), neutral endopeptidase (NEP), alpha melanocyte-stimulating hormone (α -MSH) and release of interleukin (IL)-18. In the cell-linked events, such as production of exosomes, autophagocytosis and amyloidogenesis take place. The reticulum cisternae become an amyloid fibril reservoir. Subsequently, the exocytosed amyloid lattice co-operates to form the backbone structure of NET. n = nucleus.

stress-sensing circuits to produce and release molecules including ACTH, α -MSH and NEP; (ii) promotion of the salvific autophagic process; and (iii) exocytosis of exosomes. ACTH, produced by numerous types of cells at many sites in the body [67] with autocrine/paracrine activation, can alter the immune function significantly, provoking cell shape changes and chemotaxis [38,39,68]. Furthermore, it is also recognized as a regulator of amyloidogenesis. ACTH is converted to α -MSH by NEP, resulting finally in ACTH inactivation and cessation of cell stimulation (see the schematic overview in Fig. 5). The interactions between the immune and neuroendocrine systems are quite complex [69–71]. The relationship between ACTH/ α -MSH production and melanogenesis in stressed areas such as the skin is well known [37,72–74]. In cells in which melanin synthesis, a potent immune response against stressful input, occurs pigment production is controlled by both the concentration of ACTH that depends, in turn, on the rate of its cleavage to α -MSH by NEP enzyme and by the amyloid scaffold produced physiologically to template the pigment [20–22,27]. It is important to emphasize that NEP activity is implicated not only in ending ACTH signalling, but is also critical to catabolize the amyloid fibrils; the two different systems share the same enzyme to regulate their presence. More provocative is that the ACTH/ α -MSH loop regulates immune and inflammatory reactions, but these factors are also linked to pathologies such as Alzheimer's disease, characterized by an important inflammatory component [36,50]. Apart from these immunomodulators, the dialogue between the immune and neuroendocrine systems is also mediated by numerous cytokines, as demonstrated in lymphocytes [75]. In particular, the enhanced production of IL-18, a representative proinflammatory cytokine activating innate immune responses, is also involved in vertebrate amyloidogenesis [25,26] and is generally transferred

by released exosomes [76]. Interestingly, several authors show the relationship between multi-vesicular bodies and autophagy that is considered to be a salvific process that contributes to the maintenance of cellular homeostasis [77,78]. The results presented here indicate that in LPS-stimulated neutrophils there is also a correlation between amyloidogenesis and ROS generation, change in cytoplasmic pH, enhanced expression of the ACTH/ α -MSH loop, synthesis of specific cytokine, autophagy and exosome release. These closely related partners appear to play a key role in the management of the amyloid fibrils that constitute the backbone of NET. Taken together, these interconnected responses represent primitive but potent innate defence weapons in invertebrates and vertebrates. In neutrophils, as in innate immunocompetent cells, amyloidogenesis ensues upon recognition of the non-self by pathogen-associated molecular pattern receptors (PAMPs). Amyloid fibrils from activated neutrophils in human, phagocytes/haemocytes and/or granulocytes in several invertebrates (all cellular types categorizing as circulating immunocytes) achieve the same objective of trapping, isolating the non-self, and favours killing of pathogens by acting as a vehicle to concentrate toxic products. Unlike other types of immune cells, those mentioned above do not arrive in close contact with the non-self, and their amyloid scaffold is utilized as a bridge towards the invader. Furthermore, these innate immune cells are able to optimize a primitive simple response that is assumed, from a biochemical viewpoint, to be a general mechanism to form amyloid fibrils [79].

In conclusion, in invertebrates from lower groups to primitive Chordata, stimulated circulating cells produce an amyloid scaffold to convey melanin close to the non-self, isolating it from other host tissues and focusing toxic pigment near the non-self invader [2,20–22,27]. In vertebrates, neutrophils work in an analogous manner and NETs are used to harbour and concentrate cytotoxic compounds against bacteria or fungi, thus enhancing killing effects [5,15].

As a final comment, considering the importance of NET formation that is involved directly or indirectly in the pathogenesis of different severe disorders (neutrophils are thought responsible for inflammatory profile in many diseases such as systemic lupus erythematosus, where they are not able to dismantle NETs, etc.) [80,81], the relevance of these findings (i.e. the presence of amyloid fibrils organized in a resistant scaffold) for clinical use needs consideration.

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Disclosure

The authors declare that they have no disclosures.

Author contributions

L. P., B. B., E. G., P. D. and A. L. performed the experiments. L. P., M. de E., F. M. and R. V. analysed the data. L. P., M. de E., F. M., G. T., A. G. and D. N. conceived or designed the experiments. L. P., M. de E., F. M. and R. V. wrote the manuscript. L. P. was a participant in the cellular and molecular biology doctoral program of the University of Insubria and B. B. and E. G. are participants in the biotechnology, biosciences and surgical technologies doctoral programmes.

References

- Borregaard N, Cowland J. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 1997; **89**:3503–21.
- Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* 2007; **5**:577–82.
- Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol* 2009; **30**:513–21.
- Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; **11**:519–31.
- Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ* 2011; **18**:581–8.
- Deban L, Jaillon S, Garlanda C, Bottazzi B, Mantovani A. Pentraxins in innate immunity: lessons from PTX3. *Cell Tissue Res* 2011; **343**:237–49.
- Brinkmann V, Reichard U, Goosmann C *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004; **303**:1532–5.
- Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* 2006; **8**:668–76.
- Saitoh T, Komano J, Saitoh Y *et al.* Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* 2012; **12**:109–16.
- Keshari RS, Jyoti A, Dubey M *et al.* Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PLOS ONE* 2012; **7**:e48111
- Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol* 2005; **66**:1146–54.
- Sangaletti S, Tripodo C, Chiodoni C *et al.* Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to

- myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood* 2012; **120**:3007–18.
- 13 Maugeri N, Campana L, Gavina M *et al.* Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. *J Thromb Haemost* 2014; **12**:2074–88.
 - 14 Wartha F, Beiter K, Normark S, Henriques-Normark B. Neutrophil extracellular traps: casting the NET over pathogenesis. *Curr Opin Microbiol* 2007; **10**:52–6.
 - 15 Yousefi S, Milhalache C, Kozłowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* 2009; **16**:1438–44.
 - 16 Zawrotniak M, Rapala-Kozik M. Neutrophil extracellular traps (NETs) – formation and implications. *Acta Biochim Pol* 2013; **60**:277–84.
 - 17 Yousefi S, Gold JA, Andina N *et al.* Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med* 2008; **14**:949–53.
 - 18 Chow OA, von Kockritz-Blickwede M, Bright AT *et al.* Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe* 2010; **8**:445–54.
 - 19 von Kockritz-Blickwede M, Nizet V. Innate immunity turned inside-out: antimicrobial defence by phagocyte extracellular traps. *J Mol Med* 2009; **87**:775–83.
 - 20 Falabella P, Riviello L, Pascale M *et al.* Functional amyloids in insect immune response. *Insect Biochem Mol Biol* 2012; **42**:203–11.
 - 21 Grimaldi A, Tettamanti G, Congiu T *et al.* The main actors involved in parasitization of *Heliothis virescens* larva. *Cell Tissue Res* 2012; **350**:491–502.
 - 22 Grimaldi A, Girardello R, Malagoli D *et al.* Amyloid/melanin distinctive mark in invertebrate immunity. *Invert Survival J* 2012; **9**:153–62.
 - 23 Kirchner T, Möller S, Klinger M, Solbach W, Laskay T, Behnen M. The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. *Mediators Inflamm* 2012; **2012**:849136.
 - 24 Stoiber W, Obermayer A, Steinbacher P, Krautgartner WD. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. *Biomolecules* 2015; **5**:702–23.
 - 25 Bossù P, Ciaramella A, Salani F *et al.* Interleukin-18, from neuroinflammation to Alzheimer's disease. *Curr Pharm Des* 2010; **16**:4213–24.
 - 26 Alboni S, Montanari C, Benatti C *et al.* Constitutive and LPS-regulated expression of interleukin-18 receptor beta variants in the mouse brain. *Brain Behav Immun* 2011; **25**:483–93.
 - 27 Fowler DM, Koulov AV, Alory-Jost C, Marks M, Balch W, Kelly J. Functional amyloid formation within mammalian tissue. *PLOS Biol* 2006; **4**:e6.
 - 28 Fowler DM, Koulov AV, Balch WE, Kelly JF. Functional amyloid from bacteria to humans. *Trends Biochem Sci* 2007; **32**:217–24.
 - 29 Watt B, van Niel G, Fowler DM *et al.* N-terminal domains elicit formation of functional Pmel17 amyloid fibrils. *J Biol Chem* 2009; **284**:35543–55.
 - 30 Maury CPJ. The emerging concept of functional amyloid. *J Intern Med* 2009; **265**:329–34.
 - 31 Iconomidou VA, Hamodrakas SJ. Natural protective amyloids. *Curr Protein Pept Sci* 2008; **9**:291–309.
 - 32 Weigent DA, Carr DJ, Blalock JE. Bidirectional communication between the neuroendocrine and immune systems. Common hormones and hormone receptors. *Ann NY Acad Sci* 1990; **579**:17–27.
 - 33 ThyagaRajan S, Priyanka HP. Bidirectional communication between the neuroendocrine system and the immune system: relevance to health and diseases. *Ann Neurosci* 2012; **19**:40–6.
 - 34 Scholzen TE, Luger TA. Neutral endopeptidase and angiotensin-converting enzyme – key enzymes terminating the action of neuroendocrine mediators. *Exp Dermatol* 2004; **13**:22–6.
 - 35 Ottaviani E, Malagoli D, Franceschi C. Common evolutionary origin of the immune and neuroendocrine systems: from morphological and functional evidence to *in silico* approaches. *Trends Immunol* 2007; **28**:497–502.
 - 36 Caruso C, Carniglia L, Durand D, Scimonelli TN, Lasaga M. Melanocortins: anti-inflammatory and neuroprotective peptides. In *Neurodegeneration*, eds. Martins LM. InTech, 2012:93–121.
 - 37 Slominski A, Zbytek B, Szczesniowski A *et al.* CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH. *Am J Physiol Endocrinol Metab* 2005; **288**:E701–6.
 - 38 Marr RA, Rockenstein E, Mukherjee A *et al.* Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. *J Neurosci* 2003; **23**:1992–6.
 - 39 Turner AJ, Nalivaeva NN. New insights into the roles of metalloproteinases in neurodegeneration and neuroprotection. *Int Rev Neurobiol* 2007; **82**:113–35.
 - 40 Drake J, Link CD, Butterfield DA. Oxidative stress precedes fibrillar deposits of Alzheimer's disease amyloid β -peptide (1–42) in a transgenic *Caenorhabditis elegans* model. *Neurobiol Aging* 2003; **24**:415–20.
 - 41 Christen Y. Oxidative stress and Alzheimer disease. *Am J Clin Nutr* 2000; **71**:621S–9S.
 - 42 Zhu X, Perry G, Smith MA. Two hits and you're out? A novel mechanistic hypothesis of Alzheimer disease. *Lancet Neurol* 2004; **3**:219–26.
 - 43 Chen Q, Ding Q, Keller JN. The stationary phase model of aging in yeast for the study of oxidative stress and age-related neurodegeneration. *Biogerontology* 2005; **6**:1–13.
 - 44 Le Vine H III. Quantification of β -sheet amyloid fibril structures with thioflavin T. *Methods Enzymol* 1999; **309**:274–84.
 - 45 Wu C, Scott J, Shea JE. Binding of Congo red to amyloid protofibrils of the Alzheimer $A\beta_{9-40}$ peptide probed by molecular dynamics simulations. *Biophys J* 2012; **103**:550–7.
 - 46 Harper DC, Theos AC, Herman KE, Tenza D, Raposo G, Marks MS. Premelanosome amyloid-like fibrils are composed of only Golgi-processed forms of Pmel17 that have been proteolytically processed in endosomes. *J Biol Chem* 2008; **283**:2307–22.
 - 47 Jaillon S, Peri G, Delneste Y *et al.* The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med* 2007; **204**:793–804.
 - 48 Cooper PR, Palmer LJ, Chapple ILC. Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? *Periodontology* 2000 2013; **63**:165–97.
 - 49 Vowles GH, Francis RJ. Amyloid. In: Bancroft JD, Gamble M, eds. *Theory and practice of histological techniques*. London: Churchill Livingstone, 2002:303e324.
 - 50 Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem* 2006; **75**:333–66.

- 51 Goldmann O, Medina E. The expanding world of extracellular traps: not only neutrophils but much more. *Front Immunol* 2013; **3**:420
- 52 Guimarães-Costa AB, Nascimento MTC, Froment GS *et al.* *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci USA* 2009; **106**:6748–53.
- 53 Wardini AB, Guimarães-Costa AB, Nascimento MTC *et al.* Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. *J Gen Virol* 2010; **91**: 259–64.
- 54 Sutinen EM, Pirttila T, Anderson G, Salminen A, Ojala J. Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid- β production in human neuron-like cells. *J Neuroinflammation* 2012; **9**:199–213.
- 55 Painter RG, Dukes R, Sullivan J, Carter R, Erdős EG, Johnson AR. The down-regulation of NEP activity on the cell membrane may modulate the function of these cells in inflammation. *J Biol Chem* 1988; **263**:9456–61.
- 56 Ottaviani E, Franchini A, Franceschi C. Pro-opiomelanocortin-derived peptides, cytokines and nitric oxide in immune responses and stress: an evolutionary approach. *Int Rev Cytol* 1997; **170**:79–141.
- 57 Turner AJ. Exploring the structure and function of zinc metallo-peptidases: old enzymes and new discoveries. *Biochem Soc Trans* 2003; **31**:723–7.
- 58 Iwata N, Mizukami H, Shirotani K *et al.* Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-beta peptide in mouse brain. *J Neurosci* 2004; **24**:991–8.
- 59 Wang DS, Dickson DW, Malter JS. β -Amyloid degradation in Alzheimer's disease. *J Biomed Biotechnol* 2006; **2006**:58406.
- 60 El-Amouri S, Zhu H, Yu J, Marr R, Verma IM, Kindy MS. Neprilysin: an enzyme candidate to slow the progression of Alzheimer's disease. *Am J Pathol* 2008; **172**:1342–54.
- 61 Meilandt WJ, Cisse M, Ho K *et al.* Neprilysin overexpression inhibits plaque formation but fails to reduce pathogenic A β oligomers and associated cognitive deficits in human amyloid precursor protein transgenic mice. *J Neurosci* 2009; **29**:1977–86.
- 62 Hafez D, Huang JY, Huynh AM *et al.* Neprilysin-2 is an important β -amyloid degrading enzyme. *Am J Pathol* 2011; **178**:306–12.
- 63 Saido T, Leissring MA. Proteolytic degradation of amyloid β -protein. *Cold Spring Harb Perspect Med* 2012; **2**:a006379.
- 64 Knight JS, Carmona-Rivera C, Kaplan MJ. Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. *Front Immunol* 2012; **3**:380
- 65 Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* 2005; **121**:667–70.
- 66 Zawrotniak M, Kozik A, Rapala-Kozik M. Selected mucolytic, anti-inflammatory and cardiovascular drugs change the ability of neutrophils to form extracellular traps (NETs). *Acta Biochim Pol* 2015; **62**:465–73.
- 67 Isales CM, Zaidi M, Blair HC. ACTH is a novel regulator of bone mass. *Ann NY Acad Sci* 2010; **1192**:110–6.
- 68 Ottaviani E, Franchini A, Genedani S. ACTH and its role in immune-neuroendocrine functions. A comparative study. *Curr Pharm Des* 1999; **5**:673–81.
- 69 Dores RM, Lecaude S. Trends in the evolution of the proopiomelanocortin gene. *Gen Comp Endocrinol* 2005; **142**:81–93.
- 70 Huising MO, Flik G. The remarkable conservation of corticotropin-releasing hormone-binding protein (CRH-BP) in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor of insects and vertebrates. *Endocrinology* 2005; **146**:2165–70.
- 71 Lovejoy DA, Jahan S. Phylogeny of the corticotropin-releasing factor family of peptides in the metazoa. *Gen Comp Endocrinol* 2006; **146**:1–8.
- 72 Slominski A, Wortsman J, Pisarchik A *et al.* Cutaneous expression of corticotropin-releasing hormone (CRH), urocortin, and CRH receptors. *FASEB J* 2001; **15**:1678–93.
- 73 Tsatmali M, Ancans J, Thody A. Melanocyte function and its control by melanocortin peptides. *J Histochem Cytochem* 2002; **50**:125–33.
- 74 Rousseau K, Kausar S, Pritchard LE *et al.* Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis. *FASEB J* 2007; **21**:1844–56.
- 75 Dixit VD, Parvizi N. Pregnancy stimulates secretion of adrenocorticotropin and nitric oxide from peripheral bovine lymphocytes. *Biol Reprod* 2001; **64**:242–8.
- 76 Gulinelli S, Salaro E, Vuerich M *et al.* IL-18 associates to microvesicles shed from human macrophages by a LPS/TLR-4 independent mechanism in response to P2X receptor stimulation. *Eur J Immunol* 2012; **42**:3334–45.
- 77 Fader CM, Colombo MI. Autophagy and multivesicular bodies: two closely related partners. *Cell Death Differ* 2009; **16**:70–8.
- 78 Hubbard VM, Valdor R, Macian F, Cuervo AM. Selective autophagy in the maintenance of cellular homeostasis in aging organisms. *Biogerontology* 2012; **13**:21–35.
- 79 Schnabel J. Amyloid: little proteins, big clues. *Nature* 2011; **475**: S12–4.
- 80 Hakkim A, Furnrohr BG, Amann K *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA* 2010; **107**:9813–8.
- 81 Fuchs TA, Brill A, Duerschmied D *et al.* Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci* 2010; **107**:15880–5.