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RESEARCH REPORT

Functional amyloid formation in LPS activated cells from invertebrates to vertebrates

A Grimaldi[1], G Tettamanti[1], R Girardello[1], L Pulze[1], R Valvassori[1], D Malagoli[2], E Ottaviani[2], M de Eguileor[1]

[1]*Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy,*
[2]*Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/D,41125 Modena, Italy*

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Abstract

LPS stimulation provokes serious cellular stress with an increase of cytoplasmic reactive oxygen species (ROS). We have investigated, among the different cellular defenses, amyloidogenesis as common physiological response to attenuate oxidative stress. Optical and electron microscopic observations of the following LPS activated cell lines [insect (larval hemocytes, IPLB-LdFB and *Drosophila* Schneider's S2 cells); mouse (NIH3T3 embryonic fibroblasts); Human (Human Umbilical Vein Endothelial Cells (HUVEC), neutrophils, and mesenchymal stem cells] reveal that, all are characterized by irregular profiles, cytoplasmic empty vacuoles or by cisternae containing fibrillar material. The compartmentalized fibrillar material shows staining properties typical of amyloid fibrils. LPS activation leads to ROS generation, resulting in pH acidification. Stimulated cells show pink cytoplasm in May-Grünwald Giemsa differential staining, giving a gross indication of a lower intracellular pH. Moreover the activation of amyloidogenesis is also linked with an extensive production of ACTH and α-MSH in all cultured cell types. We suggest that amyloidogenesis is a common, physiological cellular response to weak ROS, starting when other anti-stress cellular systems failed to restore homeostasis. The morphological evidence and/or functional characterization of synthesized amyloid fibrils could be an early indicator of oxidative stress that may lead to a general inflammatory process.

Key Words: LPS; amyloid fibrils; ROS; ACTH axis

Introduction

Organisms/tissues/cells live successfully even if they are constantly exposed to different types of stressors. Contacts with foreign organisms/molecules, such as bacteria, fungi, parasites, or chemicals, can represent serious stress and induce protective responses (Nappi and Ottaviani, 2000; Iwasaki and Medzhitov, 2010). The defense responses, that follow *non-self* recognition belong to immunity (Nappi and Ottaviani, 2000) nonetheless the basis of stress remains the imbalance between the rate of production and clearance of reactive oxygen species (ROS) (Droge, 2002; Dowling and Simmons, 2008; Dickhout *et al.,* 2012; Pietraforte and Malorni, 2014). As far as stress response is concerned, it consists of mixed and complex responses, involving the bidirectional communication between the immune and neuroendocrine systems (Smith *et al*., 1991; Weigent and Blalock, 1995; Ottaviani and Franceschi, 1997; Wright *et al.,* 2000; Scholzen, 2004; Dores and Lecaude, 2005; Huising and Flik, 2005; Slominski *et al.,* 2005; Lovejoy and Jahan, 2006; Malagoli *et al.,* 2007, 2011; Rivest, 2010; Caruso *et al*., 2012; Falabella *et al.,* 2012; Grimaldi *et al.,* 2012a; Thyaga Rajan and Priyanka, 2012). A controlled intracellular level of ROS is fundamental in promoting various physiological functions, but high ROS concentration exerts damaging effects. The intensity of oxidative stress can stimulate proportioned intracellular responses ranging from synthesis of detoxifying molecules, housekeeping autophagy, ACTH/α-MSH loop activation, and

Corresponding author:
Magda de Eguileor
Department of Biotechnology and Life Sciences
University of Insubria
Via JH Dunant 3, 21100 Varese, Italy
E-mail: magda.deeguileor@uninsubria.it

List of abbreviations:
ACTH = adrenocorticotropin hormone; α-MSH = alpha melanocyte-stimulating hormone; LPS = lipopolysaccharide; NEP = neutral endopeptidase; RER = rough endoplasmic reticulum; ROS = reactive oxygen species; ThT = thioflavine T

amyloid-driven synthesis of melanin up to extreme point of no return such as apoptotic cell death (Cesaratto *et al.,* 2004; Kroemer *et al.,* 2010; Tillement *et al.,* 2011; Grimaldi *et al.,* 2012a, b). As a consequence, a time-sustained and excessive ROS production can be implicated in a lot of diseases such as chronic inflammation, cancer, neurodegenerative-diseases, and in senescence mechanisms (Christen, 2000; Simon *et al*., 2000; Nogueira de Sousa Andrade *et al*., 2012).

Recently several authors demonstrated in different contexts that an important immune response as the melanin synthesis is strictly linked to physiological amyloidogenesis (Berson *et al*., 2001; Fowler *et al*., 2006; Harper *et al*., 2008; Maji *et al*., 2009; Rochin *et al*., 2013; Watt *et al*., 2013).

Amyloidogenesis has usually been associated with aetiology of the neurodegenerative diseases. Even if the aggregation of proteins into amyloid fibrils is the hallmark feature of diseases (Grenwald and Riek, 2010) including Alzheimer's (AD), Parkinson's, and prion diseases it has been evidenced that amyloid is also a fundamental non-pathological protein folding retrieved from bacteria to humans (Kelly and Balch, 2003; Cherny *et al*., 2005; Chiti and Dobson, 2006; Fowler *et al*., 2006; Maury, 2009; Eisenberg and Juker, 2012; Falabella *et al.,* 2012; Grimaldi *et al*., 2012a). Nowadays, it is well-demonstrated that the ability to form amyloid structures is not an unusual feature of the small number of proteins associated with diseases but it is a general property of polypeptide chains (Kourie *et al.,* 2002; Huff *et al.,* 2003; Goldschmidt *et al.,* 2010; Schnabel, 2010, 2011). In mammalian tissues non-pathogenic amyloid structure that functions in melanosome biogenesis has been described by numerous authors (Fowler *et al.,* 2006; Maji *et al*., 2009; Rochin *et al.,* 2013). Melanosomes are subcellular organelles specialized in melanin synthesis in melanocytes and retinal epithelial cells. The melanocytes produce a glycoprotein called Pmel17 able to polymerise into amyloid fibrils, on which melanin is assembled (Berson *et al.,* 2001; Harper *et al*., 2008; Watt *et al.,* 2013). The amyloid fibrils appear to play a role in mitigating the toxicity associated with melanin formation by sequestering and minimizing diffusion of highly reactive, toxic melanin precursors out of the melanosome.

As far as invertebrates are concerned, amyloidogenesis has been implicated in a variety of developmental functions (Qiu *et al*., 1995; Coulson *et al*., 2000). Our recent data have shown the relationship between amyloid and melanin synthesis in phylogenetically distant metazoan (*viz,* cnidarians, molluscs, annelids, insects, ascidians and vertebrates) (Grimaldi *et al*., 2012a, b), confirming the physiological role of amyloid fibrils also in invertebrates.

Protostomes and Deuterostomes share the same nexus between melanin synthesis and amyloid fibril production, and the presence of melanin is indissolubly linked to amyloid scaffold.

During invertebrate pigment synthesis the amyloidogenesis is sustained by the cross-talk between immune and endocrine systems, by the redox status/cytoplasmic pH modification, and by the cleavage of pro-protein precursors. More in detail the amyloid fibril formation is accompanied by the overexpression of adrenocorticotropin hormone (ACTH), melanocyte-stimulating hormone (α-MSH), and neutral endopeptidase (NEP) (Falabella *et al*., 2012; Grimaldi *et al.,* 2012a, b). In all, several data suggest that the massive amyloid fibril formation is an ancient physiological cell response harmonically integrated with the stress response.

In the present paper we show that different types of cells from diverse organisms can generate invariant responses against an oxidative stress agent by producing amyloid fibrils and in some cases melanin. The production of amyloid fibrils acts here as an efficient and prompt method to strive ROS overproduction and to avoid severe damages that may end with apoptosis or necrosis. The highlighted relationship between amyloidogenesis and ROS production is validated here with morpho-functional experiments and it allows to surmise a new background of information on the effects of oxidative stress.

Material and Methods

All experiments were performed in four independent replicates

Cell culture

Insect hemocytes were obtained from last instar larvae of *Heliothis virescens* (Lepidoptera). Samples of hemolymph (40/60 µl per larva) were collected and transferred in Eppendorf tubes containing an equal volume of buffer MEAD. Hemocytes were pelletted by centrifugation of the hemolymph at 400*g* per 7 min at 4 °C. The hemocytes were resuspended in complete medium (Grace's medium, FBS 10 %, antibiotic-antimicotic solution 1 %, SIGMA) and were plated at concentration of $1x10^6$ cells/ml into 24-well culture plates. The IPLB-LdFB cell line derived from the fat body of the insect *Lymantria dispar* (Lepidoptera) and generously gifted by prof. Ottaviani (University of Modena and Reggio Emilia, Modena), was used. The cells were cultured in Ex-Cell 400™ medium (JRH Biosciences Ltd., Andover, UK) at 26 °C. *Drosophila* Schneider's S2 cells were cultured in Schneider's *Drosophila* medium (WVR), containing heat inactivated FBS 10 % (Sigma-Aldrich). Cells were used at 70 - 80 % confluence, in 12-well (25 mm) plates (Corning). NIH3T3 mouse embryonic fibroblast cells are established from a NIH Swiss mouse embryo (Sigma-Aldrich). These cells were cultured in DMEM medium and 10 % bovine calf serum. Human Umbilical Vein Endothelial Cells (HUVEC) extracted from human neonatal umbilical cords were cultured in EndoGRO media (warmed to 37 °C) CHEMICON (Merck, Germany). Human polymorphonuclear neutrophils freshly isolated from whole venous blood collected from healthy donors, by Ficoll-Paque PLUS (GE Healthcare, Milan, Italy) centrifugation and then separated from erythrocytes by Red Blood Cell Lysis and centrifugation. Mesenchymal stem cells derived from human adipose tissue, generously gifted by prof. Bernardini (University of Insubria, Varese), were cultured in Human Mesenchymal-LS Expansion. For each type of cells examined: $8x10^4$ cells/ml were seeded on rounded glass coverlips (12 mm diameter, treated

with or without 0.1 % gelatinase) placed into 24-well (for light microscopy observation) or in 6-well (for electron microscopy observation) plates in suitable medium. Cells were then either left undisturbed (control) or stimulated with 100ng/ml LPS (from *Escherichia coli*, serotype O55:B5, Sigma-Aldrich) for 30 min. After incubation with LPS, cells on coverslips from 24-weel plates were fixed with 4 % paraformaldehyde in PBS, while cells from 6-well plates were fixed with 4 % glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for TEM.

Light microscopy and transmission electron microscopy (TEM)

After samples were fixed at 4 °C for 2 h in 4 % glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2), the cells were pellet washed in 0.1 M Na-cacodylate buffer (pH 7.2) and post-fixed at 4 °C for 2 h with 1 % osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol series, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica). Cells were stained by May-Grünwald Giemsa differential staining. The differential staining depends on pH (alkaline pH increases blue and acid pH pink or reddish tinge in the stained specimens) thus giving a gross-identification of cytoplasmic pH showing a possible increase of ROS production). All semithin sections were observed with a light microscope Olympus BH2 (Olympus, Tokyo, Japan) and visualized pictures were acquired with a DS-5M-L1 Nikon digital camera system. Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Amyloid fibril characterization

Amyloid structures were identified according to Le Vine III (Le Vine III, 1999), by staining cells with thioflavine T and visualizing the amyloid-specific green/yellow fluorescence with an Olympus BH2 microscope (excitation wavelength 465 nm). Images were acquired with a DS-5M-L1 Nikon digital camera system.

Indirect immunofluorescence staining

Cells were incubated with PBS containing 2 % BSA for 30 min before the primary antibody incubation (4 °C over night). The presence of ACTH, and its cleavage product alpha (α-MSH), were assessed using: anti-human ACTH (1:50 dilution, Sigma-Aldrich) and anti-human α-MSH (1:50 dilution, SIGMA) polyclonal antibodies, respectively. Incubations with suitable secondary antibodies conjugated with tetramethylrhodamine (TRITC) (1:200 dilution, Jackson, Immuno Research Laboratories, West Grove, PA, USA) or alternatively conjugated to horseradish peroxidase (HRP) enzyme (Sigma-Aldrich) were performed for 1h at room temperature in a dark moist chamber. Nuclei were eventually stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). All washing and dilutions were performed with PBS and 2 % BSA. In negative control samples, primary antibodies were omitted. Coverslips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA. USA); slides were observed on Olympus BH2 microscope (Olympus). Data were recorded with a DS-5M-L1 digital camera system (Nikon). Images were combined with Adobe Photoshop (Adobe Systems, Inc.).

Results

The common morpho-functional traits occurring in cells undergoing a stress were assessed in diverse animal species. Selected types of cells, named in Material and Methods section, differ for cell lineages as well as for recognized function. Freshly withdrawn insect hemocytes, cultured larval fat body cells (IPLB-LdFB), *Drosophila* Schneider's S2 cultured embryonic cells, NIH3T3 mouse embryonic fibroblast cells, Human Umbilical Vein Endothelial Cells (HUVEC), human neutrophils, and human mesenchymal stem cells have been examined after stimulation with LPS at the shortest time generating observable morphological changes.

Our previous studies indicate that under stress conditions the insect hemocytes (Falabella *et al.,* 2012; Grimaldi *et al*., 2012a, b) and human neutrophils (Pulze *et al*., 2014) produce within endoplasmic reticulum cisternae a fibrillar material responsible for the assembling of an amyloidogenic scaffold.

Given the similarities between the fibrillar material released by hemocytes and neutrophils we investigated whether analogous responses could intervene also in LPS stimulated IPLB-LdFB cells, S2 cells, NIH3T3, HUVEC, and mesenchymal stem cells. In particular, we hypothesize that the formation of fibrillar material reflects an amyloidogenic process following cytoplasmic acidification. Both in invertebrates and vertebrates, the decrease of cytoplasmic pH promotes a bidirectional activation of stress-sensoring circuits (Weigent and Blalock, 1995; Ottaviani and Franceschi, 1997; Wright *et al.,* 2000; Scholzen, 2004; Dores and Lecaude, 2005; Huising and Flik, 2005; Slominski *et al.,* 2005; Lovejoy and Jahan, 2006; Carroll, 2008; Rivest, 2010; Caruso *et al*., 2012; Grimaldi *et al*., 2012a, b; Thyaga Rajan and Priyanka, 2012) that results in the release of stress-related molecules such as ACTH, α-MSH and NEP. In particular ACTH provokes cell shape changes and chemotaxis (Smith *et al*., 1991; Caselgrandi *et al.,* 2000; Ottaviani *et al*., 2007; Genedani *et al*., 2008; Malagoli *et al.,* 2011), until it is converted to α-MSH by NEP (Cohen *et al.,* 1996; Caselgrandi *et al*., 2000; Slominski *et al*., 2005). NEP regulates the ACTH/α-MSH balance and is also involved in the control of amyloidogenesis (Iwata *et al*., 2004; Hamaguchi *et al.,* 2006; El-Amouri *et al.,* 2008; Kubiak-Wlekly and Niemir, 2009; Meilandt *et al.,* 2009; Greenwald and Riek, 2010; Hafez *et al.,* 2011; Falabella *et al.,* 2012; Grimaldi *et al.,* 2012a, b; Saido and Leissring, 2012; Grimm *et al.,* 2013).

Morphology of control and LPS-treated cells
Hemocytes

TEM analyses (Figs 1A - C) showed that, *H. virescens* larval granulocytes, defined as the main circulating cell type involved in the defence

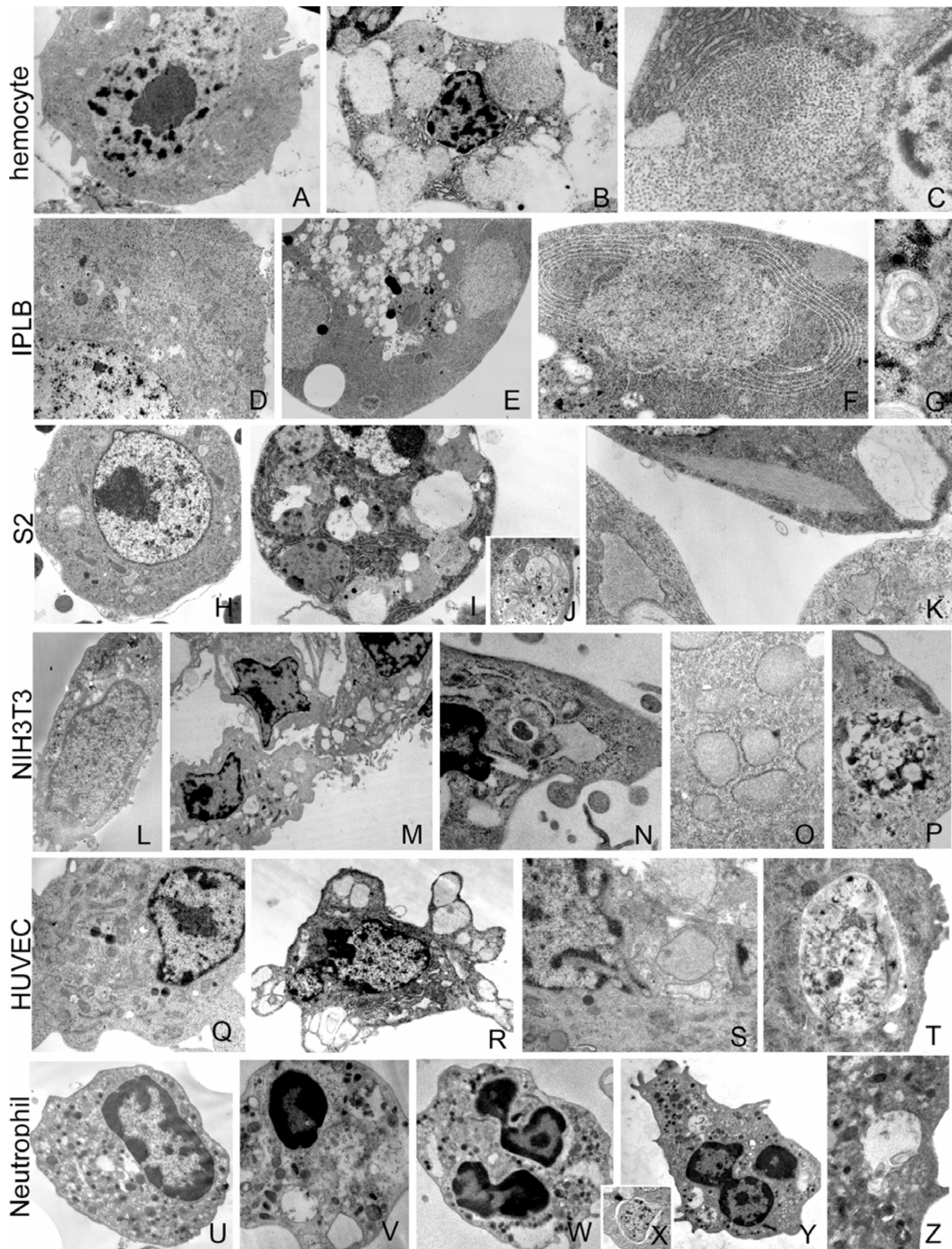

Fig. 1 Effects of LPS stimulation on cells: TEM analysis. Thin sections of unstimulated and LPS activated cells (A - Z) showing that, at short time from LPS incubation, significant morphological changes at the cytoplasmic level occur. Activated cells (B, C, E - G, I - K, M - P, R - T, V - Z), lost the roundish shape typical of control (A, D, H, L, Q, U), acquire irregular profiles. These cells exhibit the presence of dilated reticulum cisternae filled with fibrillar material (arrowheads), and of double/multiple autophagic vesicles containing organelles and cytoplasmic degraded components (O, J, P, T, X). (Scale bar : 1cm = A, 1 µm; B, 3 µm; C, 1 µm; D, 4 µm; E, 2 µm; F, 1 µm; G, 0.5 µm; H, 2 µm; I, 1 µm; J, 0.5 µm ; K, 1 µm; L, 2 µm; M, 5 µm; N, 1.3 µm; O, 0.5 µm; P, 0.8 µm; Q, 2 µm; R, 4 µm; S, 1 µm; T, 1.3 µm; U, 2 µm; V, 2 µm; W, 2 µm; X, 0.5 µm ;Y, 3 µm; Z, 1 µm).

processes of the tobacco budworm, were roundish cells characterized by cytoplasm filled with a central nucleus, RER, Golgi apparatus, mitochondria, glycogen, and few small homogenous granules (Fig. 1A) (Grimaldi *et al.,* 2012a, b). *H. virescens* granulocytes after LPS stimulation presented enlarged reticulum cisternae, occupied by fibrillar material spatially organized in respect to an electron dense body. The compartmentalized fibrillar material was located around the nucleus and hid the cytoplasmic organules (Figs 1B, C).

IPLB-LdFB cells

Electron microscopic observations of control cells revealed that IPLB-LdFB cells, from the larval fat bodies of the gipsy moth *Lymantria dispar*, were round in shape, with cytoplasm filled with nuclei, containing dispersed chromatin, small mitochondria, and abundant rough endoplasmic reticulum (Fig. 1D). LPS stimulated cells showed morphological changes only involving the RER cisternae that were dilated and filled with a massive amount of fibrillar material forming compact structured bodies (Figs 1E, F). Autophagosomes containing portions of the cytosol and membrane-like structures are evident (Fig. 1G).

Drosophila S2 cells

S2 cells display approximately a spherical morphology with a central nucleus and well distributed organules in the cytoplasm (Fig. 1H). LPS stimulation induced S2 cells to undergo a change in their phenotype: large reticulum cisternae were predominant in respect to other organules and occupied a significant portion of cytoplasm (Figs 1I, K). Stimulated cells show the presence of autophagic vacuoles enclosing cytoplasmic components (Fig. 1J).

NIH3T3 mouse embryonic fibroblast cells

Electron microscopic observations of control NIH3T3 cells showed flattened and spindle shaped cells with nucleus generally in central position. The cytoplasm contained organules and a moderately developed endoplasmic reticulum (Fig. 1L). LPS stimulated NIH3T3 cells presented well-developed pseudopodia all around the perimeter (Fig. 1M). Empty vesicles and dilated cisternae of reticulum filled with fibrillar material were visible (Figs 1N, O). Often sequestered materials (cytosol and membrane-like structures) in membrane-bound vesicles were visible (Fig. 1P).

Human Umbilical Vein Endothelial Cells (HUVEC)

Control HUVEC appeared as roundish cells with cytoplasm rich in organules (Fig. 1Q). LPS stimulated cells displayed an irregular profile due to the presence of peripheral large dilated reticulum cisternae filled with fibrillar material (Figs 1R, S). In the cytoplasm of activated cells, autophagic vacuoles, containing electron-dense elements and partially degraded material could be visible (Fig. 1T).

Human neutrophils

Resting circulating neutrophils are spheroidal cells showing an irregular, multi-lobed nucleus and cytoplasmic granules (Fig. 1U). Upon LPS administration, activated neutrophils were characterized by irregular profiles, and cytoplasmic empty vacuoles or by cisternae containing fibrillar material (Figs 1V, W, Y, Z). Autophagic vacuoles were visible (Fig. 1X).

Amyloid fibrils and ACTH/α-MSH synthesis in LPS-treated cells

The morpho-characterization of all cell types is showed in multi-panel Figure 2.

H. virescens hemocytes

Activation *via* LPS of tobacco budworm hemocytes leads to ROS generation, resulting in pH acidification evidenced by May-Grünwald Giemsa differential staining that marks a gross indication of a lower intracellular pH.

Amyloid fibril formation

The fibrillar material observed in the hemocytes of *H. virescens,* showed staining properties typical of amyloid fibrils. *H. virescens* hemocytes were positive to Thioflavine T staining, showing the typical yellow-green fluorescence, more evident in stimulated cells as compared to controls.

ACTH/α-MSH axis activation

Activation of *H. virescens* hemocytes resulted in extensive production of ACTH which, due to NEP cleavage, turned to α-MSH.

IPLB-LdFB cells

LPS stimulated IPLB cells showed pink cytoplasm in May-Grünwald Giemsa differential staining.

Amyloid fibril formation

The fibrillar material observed displayed Thioflavine T staining properties typical of amyloid fibrils.

ACTH/α-MSH axis activation

A significant increase in the production of ACTH and α-MSH was observed.

Drosophila S2 cells

LPS-treated cells showed an increased ROS production evidenced by reddish tinge in the stained specimens by differential May-Grünwald Giemsa staining.

Amyloid fibril formation

The fibrillar material observed in the reticulum cisternae showed specific staining of amyloid fibrils being Thioflavine T positive.

ACTH/α-MSH axis activation

The activation of stress circuits resulted in the extensive production of ACTH and α-MSH by LPS-activated S2 hemocytes.

NIH3T3 mouse embryonic fibroblast cells

LPS activated cells showed mostly pink cytoplasm as a marker of pH decrease.

Amyloid fibril formation

The observed fibrillar material showed typical staining properties of amyloid fibrils showing a yellow-green fluorescence after Thioflavine T exposure.

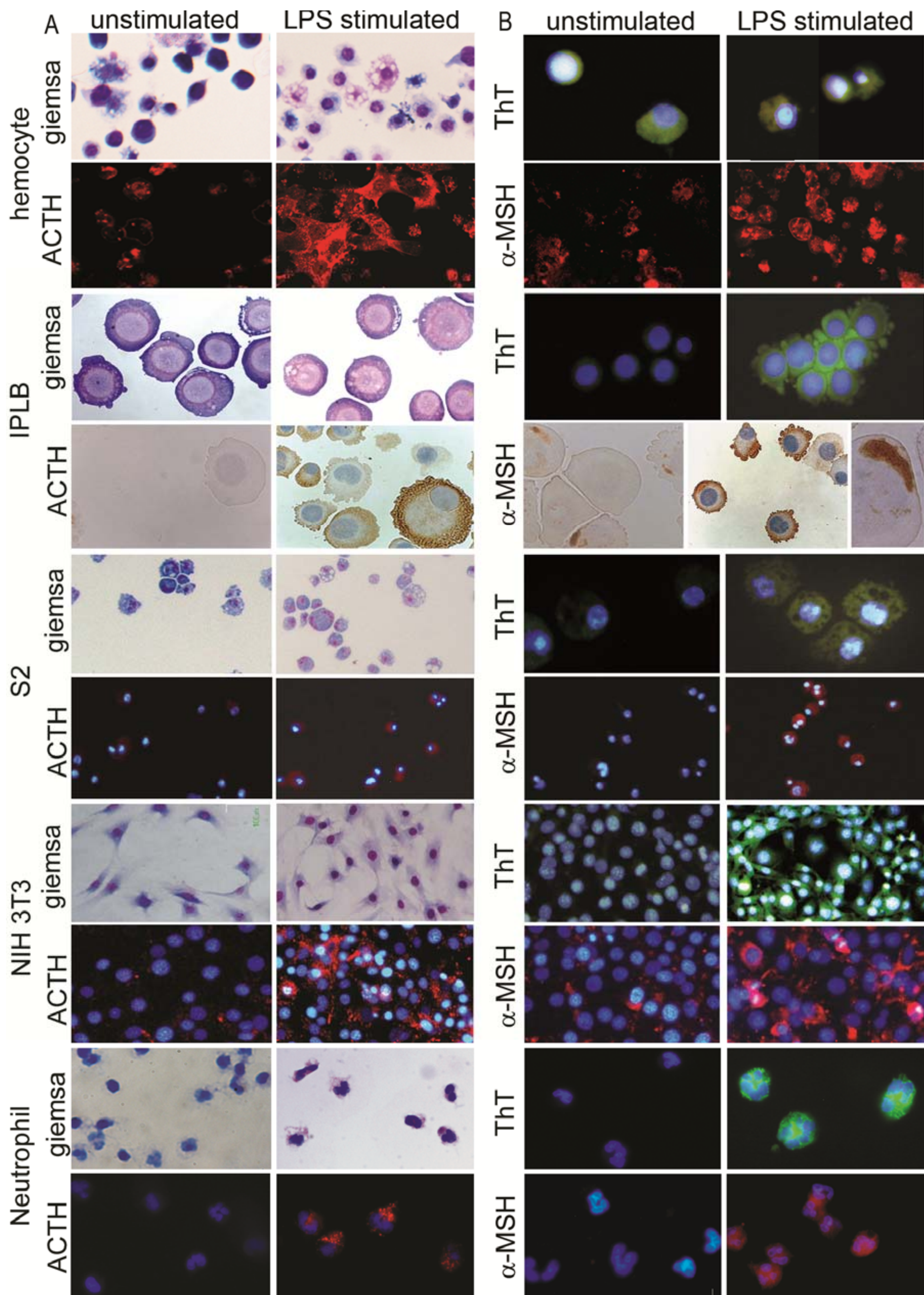

Fig 2 Characterization of LPS treated cells. Light microscopy: a gross identification of cytoplasmic acidification is obtained with differential May-Grünwald Giemsa staining (panel A). The increased ROS production responsible of cytoplasmic pH is evident by comparing unstimulated with stimulated cells (alkaline pH increases the blue tinge while acid pH the pink/reddish tinge in stained samples). Immunocytochemical characterizations for ACTH and α-MSH expressions (panel A, B) showing high levels of positivity in activated cells while in unstimulated cells the expression of melanocortin hormones is basal. Immunocytochemical evidence of amyloid fibrils, detected with thioflavin T (yellow-green brightly fluorescence), is more evident in stimulated cells as compared to controls. Nuclei are stained with DAPI and marked in brilliant blue (Panel B).

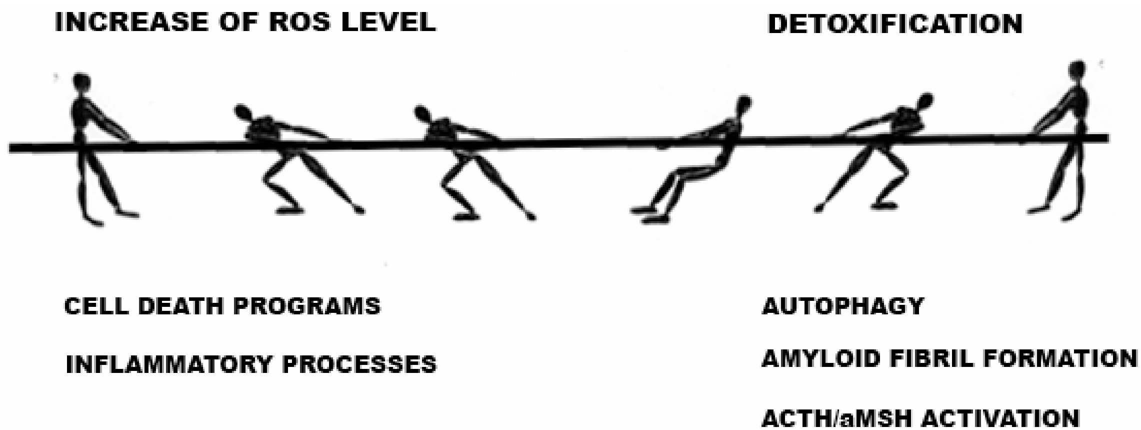

Fig 3 Schematic overview explaining the possible behaviour of cells due to LPS activation. Cells play a “tug of war” with a lot of events, to gain a dynamic *equilibium.* LPS activation of cells results in an increase of ROS levels. Autophagic processes, amyloid fibril production and the concomitant cross-talk between immune and neuroendocrine systems with an activation of stress-sensoring circuit avoid to reach the point of no return toward the cell death.

ACTH/α-MSH axis activation

An activation of stress circuits resulted in a massive presence of ACTH and α-MSH in respect to the control.

Human Umbilical Vein Endothelial Cells (HUVEC)
Amyloid fibril formation

The stimulated cells were Thioflavine T positive.

ACTH/α-MSH axis activation

Activated cells, concomitant with the increase in ACTH and *α*-MSH, have been showed.

Human neutrophils

LPS stimulated neutrophils showed pink cytoplasm in May-Grünwald Giemsa differential staining giving a gross indication of a lower intracellular pH.

Amyloid fibril formation

The fibrillar material observed in the activated cells was positive with Thioflavine T.

ACTH/α-MSH axis activation

Activation of stress circuits resulting in extensive production of ACTH and α-MSH was observed in LPS activated neutrophils.

Discussion

Basic biological principles in animals show conserved regulation of the involved processes. This is also true for cellular stress response, a complex and dynamic process that restores the homeostasis through highly conserved steps. Cellular stress conditions promote in different animal models (invertebrates and vertebrates) the same massive morphological and physiological modifications (Grimaldi *et al.,* 2012a, b). Insults, mimicking a stressful condition, provoke in all cell/tissue, detectable series of events that begin with the overexpression of ROS. Evidence presented here suggests that the imbalance of ROS levels result in amyloidogenesis and ACTH/α-MSH synthesis while other researchers have already demonstrated increased levels of inflammatory cytokines such as IL-18 (Fowler *et al*., 2006; Malagoli *et al.,* 2007; Bossù *et al.,* 2010; Alboni *et al.,* 2011; Grimaldi *et al*., 2012a).

In invertebrates amyloidogenesis is involved in immune response mediated by circulating hemocytes, whereas in vertebrates, it is combined with melanin production and packaging into melanocytes (Fowler *et al.,* 2006; Maji *et al*., 2009; Rochin *et al.,* 2013). Together with melanocortins the amyloid scaffold may template the deposition of pigment and this event gives a fundamental contribution to homeostasis restoration (Falabella *et al.,* 2012; Grimaldi *et al*., 2012a, b).

Here, we strengthen the key role of amyloidogenesis in the cellular homeostasis suggesting that the amyloid production may buffer ROS increase and attenuate oxidative stress. This may represent the primary function of amyloid fibril and is not linked to melanin synthesis. The role for amyloidogenesis has been hypothesized by several researchers in the contest of diseases such as Alzheimer’s disease (Goldsbury *et al.,* 2008; Dumont *et al*., 2009; Salminen *et al*., 2009; Millucci *et al.,* 2012; Jie *et al.,* 2013). However our findings, by studying the behaviour of different cell types in culture after incubation with LPS, indicate that the production of amyloid fibrils is an usual physiological cellular response occurring independently from melanin synthesis.

Accordingly is worth noting the common morphological pattern that follow LPS treatment in tobacco budworm hemocytes, IPLB-LdFB, S2, NIH3T3, HUVEC, human neutrophils, and human mesenchymal stem cells. When stressed by LPS these different cell lineages (mesoderm and ectoderm) all present enlarged reticulum cisternae filled with fibrillar material positive to thioflavine staining. The amyloidogenesis observed in cells from

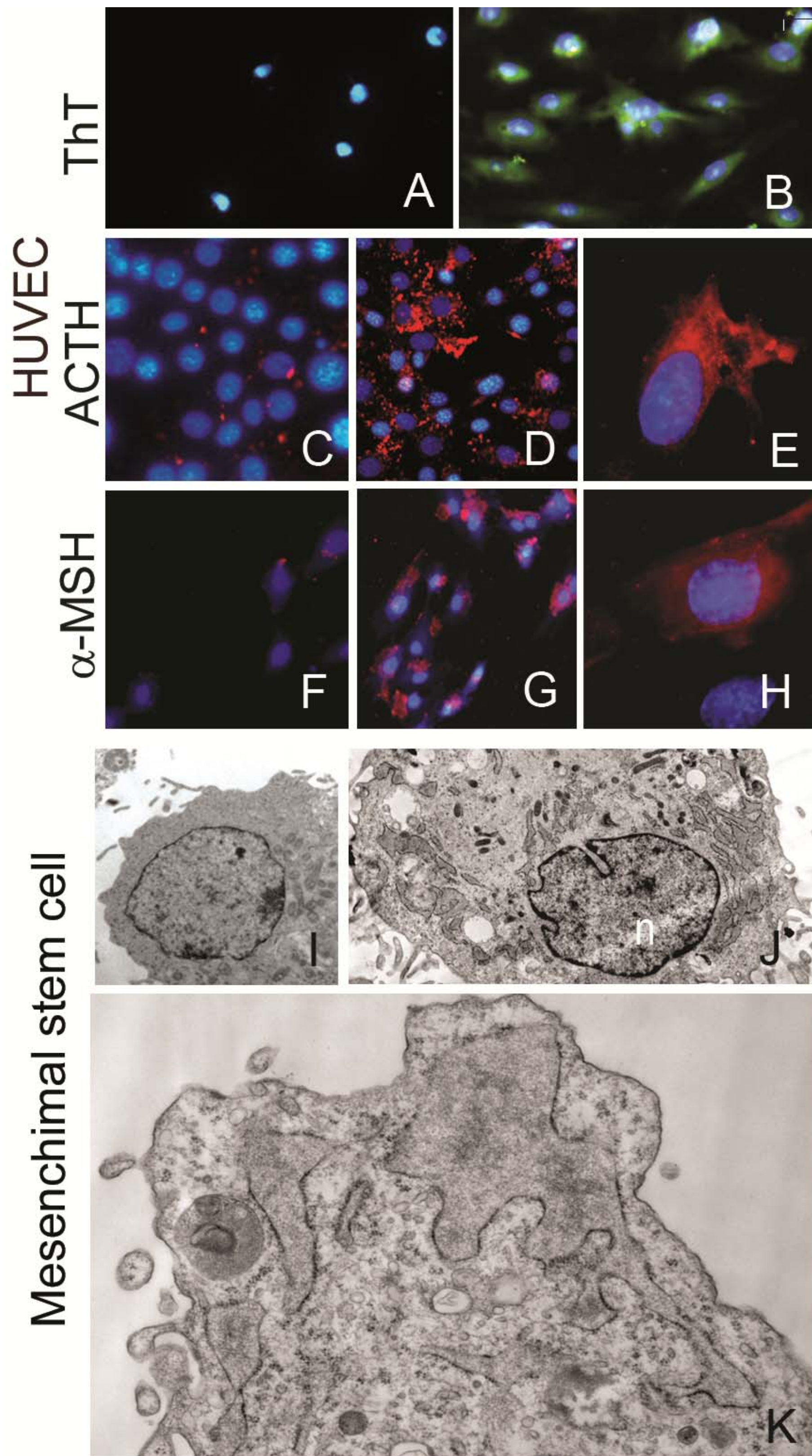

Fig 4 Predictive value of amyloidogenesis in LPS-stimulated cells. The detection of amyloidogenesis in any type of activated cells, here HUVEC (A - H) and mesenchymal stem cells (I - K) are suggested as example, gives a precocious evidence of an inflammatory process and consequence compensatory events. To validate the synthesis of amyloid fibrils in unstimulated (A)/stimulated (B) cells, and of molecules linked to stress response such as ACTH and α-MSH (C, F unstimulated cells; D, E, G, H stimulated cells) an immunocytochemical picture can be proposed. Alternatively the presence of fibrillar material in RER can be detected from an ultrastructural analysis.

various organisms occurs in parallel with the ACTH/ α-MSH increased synthesis.

The sum of evidences here presented is corroborated by the information available in literature about the spontaneous formation of amyloid fibrils as a consequence of the innate tendency of a lot of protein to form β-sheet aggregates (Kelly and Balch, 2003; Fowler *et al.,* 2006; Maury, 2009; Hye *et al.,* 2014). In accordance with our observations, amyloidogenesis, has been demonstrated to be promoted by an imbalance of the REDOX state (Christen, 2000; Slominski *et al.,* 2005; El-Amouri *et al.,* 2008; Grimaldi *et al*., 2012a, b) and regulated by several pathways that include NEP clearance activity (avoiding the fibrillar accumulation) (Tanzi *et al.,* 2004; Russo *et al.,* 2005; Meilandt *et al.,* 2009; Salminen *et al*., 2009; Nalivaeva *et al.,* 2012). The amyloid fibril synthesis is sustained by the activation of stress circuits resulting in the extensive production of melanocortin ACTH and its cleavage to α-MSH by NEP activity (Chiti and Dobson, 2006; Caruso *et al.,* 2012; Grimaldi *et al.,* 2012a, b). In these respects, NEP may function as a regulator of either amyloidogenesis or ACTH hormone levels as well (Grimaldi *et al*., 2012a, b; Pulze *et al.,* 2014).

ROS are normal products of cellular metabolism, maintained at low levels in healthy cell. When ROS production increases and persists at heavy load in the cytoplasm, protective mechanisms come into action and several antioxidant molecules and detoxifying enzyme are synthesized. We suggest that on the basis of the ROS levels, anti-stress mechanisms and consequent cellular events can vary over a wide range, from protective autophagy and amyloidogenesis up to apoptotic or necrotic cell death (Fig. 3).

Autophagy was often present in the cells here examined and probably induced by redox stress (Cecconi and Levine, 2008; He and Klionsky, 2009; Kroemer *et al*., 2010; Perrotta *et al.,* 2011; Scherz-Shouval and Elazar, 2011). In this context, autophagy has to be considered as a protective and, from an energetic point of view, “positive” mechanism because it allow to recycle the demised organelles and damaged cytoplasm. When autophagy is not sufficient to keep ROS levels under control, *i.e.,* ROS levels are too high, transient amyloidogenesis may be promoted (Esposito *et al.,* 2006; Dumond *et al.,* 2009). In this case the result of buffering oxidative damages can be reached with higher energetic cost that include amyloid fibril synthesis and their subsequent cleavage due to NEP action. Apoptotic or necrotic cell demise only intervenes when the ROS level exceeds restoration cell capacity.

As a protective and detoxificant event, amyloidogenesis seems to be an ancient process, widely distributed in different cell types and evolutionary distant animals. Not surprisingly, this ancient process has been integrated into additional physiological functions, that principally include packaging of melanin and/or the driving of pigment close to the non-self invader. In mammals neutrophils utilize amyloid fibrils during the formation of extracellular trap for harbouring and conveying various molecules against the invaders (Pulze *et al*., 2014). We speculate suggesting that the additional capabilities of amyloid fibrils could be explained considering their intrinsic properties represented by affinity for oppositely charged protein and of nucleic acid (for example melanin and DNA/RNA, respectively), thus this scaffold could function like a reversible ion exchange polymer.

As final remark, in consideration of their synthesis in the immediacy of an oxidative stress, the early detection of amyloid fibrils into the rough endoplasmic reticulum, easily evidenced by morphological observations and/or functional characterization (see for example the images referred to the LPS stressed HUVEC and mesenchimal stem cells) (Fig. 3), could be an early indicator of active networks that may lead to a general inflammatory process.

Conclusions

Amyloidogenesis is the common response of LPS-treated cells from various animals and belonging to different germ layers. Amyloid fibril formation is linked to cytoplasmic acidification and sustained to melanocortin overexpression.

Amyloidogenesis is proposed as a fundamental and ancient detoxifying event, that during evolution also acquired additional functions that include packaging of melanin and/or the driving of pigment towards a non-self invader. Amyloid fibril accumulation may lead to neurodegenerative disorders but amyloid fibril synthesis is a physiological and protective early event under oxidative stress. Amyloid fibrils could be early indicators of inflammatory processes rather than markers of established pathologies.

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