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Microglia activation and interaction with neuronal cells in a biochemical model of Mevalonate Kinase Deficiency

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To the Chief Editor, *23 November 2014*
Dr Mels Sluyser,,
Apoptosis

Dear Dr. Mels Sluyser,

we would like to submit the manuscript "Microglia activation and interaction with neuronal cells in a biochemical model of Mevalonate Kinase Deficiency" by Tricarico P.M. et al. for consideration as research article in Apoptosis.

None of the Authors has any conflict of interests regarding the matters treated in the article. All the authors have seen and approved the submission of this version of the manuscript and take full responsibility for the manuscript.

Hoping you will find our paper suitable for publication in Apoptosis, I look forward to hearing from you at your earliest convenience.

Sincerely,

Annalisa Marcuzzi

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Microglia activation and interaction with neuronal cells in a biochemical model of Mevalonate Kinase Deficiency

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Abstract

Mevalonate kinase deficiency is a rare disease whose worst manifestation, characterised by severe neurologic impairment, is called mevalonic aciduria. The progressive neuronal loss associated to cell death can be studied in vitro with a simplified model based on a biochemical block of the mevalonate pathway and a subsequent inflammatory trigger. The aim of this study was to evaluate the effect of the mevalonate blocking on glial cells (BV-2) and the following effects on neuronal cells (SH-SY5Y) when the two populations were cultured together.

In this experimental condition, glial cells underwent an evident activation, confirmed by elevated pro-inflammatory cytokines release, typical of these disorders, and a modification in morphology. Moreover, the activation induced an increase in apoptosis. When glial cells were co-cultured with neurons, their activation caused an increase of programmed cell death also in neuronal cells, but only if the two populations were cultured in direct contact.

These findings represent a preliminary step towards the understanding of the pathological and neuroinflammatory mechanisms occurring in mevalonate kinase diseases. Contact co-culture between neuronal and microglial cells appears to be a good model to study mevalonic aciduria *in vitro*, and to identify putative drugs that block microglial activation for this orphan disease.

In fact, in such a pathological condition, we demonstrated that microglial cells are activated and contribute to neuronal cell death. We can thus hypothesise that the use of microglial activation blockers could prevent this additional neuronal death.

Keywords (4-6):

Mevalonic aciduria, mevalonate kinase deficiency, microglial activation, apoptosis, lovastatin

1. Introduction

Mevalonic aciduria (MA, OMIM #610377) is a rare autosomal disorder caused by mutations in the *mevalonate kinase* gene (*MVK, 12q24.11, NM_000431*), coding for an enzyme of the cholesterol pathway known as mevalonate kinase [1] (**Figure 1**). The typical MA clinical picture in children is associated with recurrent attacks of fever, developmental delay, ataxia, dysmorphic features, failure to thrive, cataracts, and retinal dystrophy. The prognosis for MA patients is poor: more than 50% of patients die during an inflammatory crisis in infancy or early childhood, and very few survive to adolescence [2].

MA represents the most severe form of a periodic fever disease called mevalonate kinase deficiency (MKD, OMIM #260920), and its severity is mainly due to neurological involvement [3]. Cholesterol biosynthesis deregulation has already been reported as involved in several neurodegenerative diseases [4], sustaining the key role of cholesterol, also in MA.

Although the genetic defect associated with MA has already been discovered, little is known about the pathogenic mechanisms leading to severe symptoms and early death. Medical treatments are just supportive and generally ineffective. MA, indeed, is nowadays an orphan disease. The identification of specific molecular targets for the pharmacological treatment of MA strongly depends upon the comprehension of the neuroinflammatory mechanisms that occur in response to the mevalonate pathway blockade. At present, either an animal model or MA cellular models are lacking, even though a knock-out (KO) mouse for mevalonate kinase has already been developed. Homozygous KO mice, however, are non-vital, while heterozygous KO mice display a phenotype unable to fully mimic MA clinical features [5].

Recently, our research group and other authors showed that cell lines derived from both monocytes/macrophage and neurons are susceptible to statins [6-8]. Statins block the cholesterol pathway by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA), upstream the mevalonate kinase, thus mimicking some of the biochemical features found in MKD [9]. In some cell models, statins treatment triggers apoptosis: i.e., in the SH-SY5Y neuroblastoma cell line, lovastatin treatment activates programmed cell death (PCD), initiated by mitochondrial damage [7]. This evidence supports the clinical observation of severe cerebral atrophy in MA patients, characterized by a progressive decrease of the cerebellum volume due to neuronal apoptosis [10]. Both in healthy conditions and in disease, neuronal activity and brain homeostasis are regulated by microglial cells, which can be considered the macrophages of the central nervous system [12-14]. Moreover, recent findings, have shown that microglial activation can modulate the development and/or progression of many neurodegenerative disorders. Indeed, in the activated state, microglia can exhibit a double behaviour toward neurons: they can induce a protective effect and neuroprotection or, alternatively, they may cause or worsen neuron damage [15-19].

To better understand neuroinflammation occurring in MA, we evaluated the microglial activation *in vitro* and its interaction with neuronal cells. We exploited a simplified model that reproduce the cross-talk between the major players involved in neuronal damage, by co-culturing microglial and neuronal cells after simultaneous administration of lovastatin and LPS, mimicking the condition of MA patients exposed to pathogens.

In order to assess microglial activation, following the block of the mevalonate pathway and inflammatory stimulus, we measured the release of inflammatory cytokines and cell morphology changes. Moreover, to investigate the microglia behaviour towards neurons we evaluated the changes in neuronal cell apoptosis.

2. Materials and Methods

2.1. Reagents and cell lines

Lovastatin (Lova, Mevinolin from *Aspergillus terreus*) and Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 were obtained from Sigma Chemical Co. Aldrich (St. Louis, MO) and were dissolved in saline solution (Diaco SpA, Trieste, Italy).

The mouse microglial cell line (BV-2), generously provided by Dr. A. Tommasini (IRCCS "Burlo Garofolo", Trieste, Italy), was cultured in RPMI 1640 MEDIUM (Euroclone, Milano, Italy) supplemented with 10% foetal bovine serum, 2mM glutamine and penicillin streptomycin amphotericin B 1x solution (Euroclone, Milano, Italy).

Human neuroblastoma cell lines (SH-SY5Y), kindly provided by Prof. S. Gustincich (Department of Neurobiology, International School for Advanced Studied S.I.S.S.A.-I.S.A.S. Trieste, Italy) were cultured in 44.5% MEM/EBSS (Euroclone, Italy), 44.5% HAM'S/F12 (Euroclone, Italy), supplemented with 10% foetal bovine serum (FBS, Euroclone, Italy), non-essential amino acid solution 1x (NEAA, Euroclone, Italy), 2 mM glutamine and penicillin/streptomycin/amphotericin B 1x solution (Sigma Chemical Co. Aldrich St. Louis, MO).

2.2. Cell culture

2.2.1 Microglial cell line

The microglial cell line, BV-2 (2x10^5 cells/ml), seeded in 6-well plates, was cultured for two days, then treated with Lova (10μM) for 24 hours, and with LPS (1ng/ml) for further 24 hours. At the end of the incubation period, cell culture supernatants were collected for cytokine evaluation, while the cells were harvested and analyzed for the programmed cell death or mitochondrial membrane potential.

2.2.2 Co-cultures of neurons and microglia

For neuron and microglia co-cultures, SH-SY5Y and BV-2 cells were mixed at the density of 1x10^5 and 2x10^5 cells/mL, respectively, and were seeded in 6-well plates in standard contact co-culture or in transwell co-culture.

For standard contact co-cultures, microglial cells and neurons were seeded in the same well. In order to recognize the

two populations in the following analysis, prior to co-culture, microglia cells were stained with CellTrace Far Red DDAO-SE (Life Technologies, Carlsbad, CA, USA) following manufacture's instructions. Briefly, microglia cells were washed with PBS and then incubated in PBS containing the 5μM CellTrace for 15 minutes at 37°C.

For transwell co-culture, cells were cultured in two chambers separated by a semi-permeable 0.4μm membrane (Transwell plate, Corning Incorporated). Microglial cells were plated in the upper chamber at an approximate distance of 0.8 mm from the bottom of the well.

In both cases, cells were cultured for 2 days and stimulated with Lova (10μM) and subsequently with LPS (1ng/mL) for further 24 hours. At the end of the incubation period, microglia and neurons were harvested and treated for the programmed cell death assays.

2.3 Programmed cell death (PCD)

Programmed cell death was evaluated on both microglial and neuronal cells. PCD were evaluated by flow cytometry using Annexin V and Propidium Iodide staining (Apoptosis Detection Kit, Immunostep, Salamanca, Spain), as described elsewhere [6]. PCD is expressed as a percentage of Annexin V positive cells (A+). In standard contact co-culture, staining with CellTrace Far Red allowed to evaluate PCD simultaneously on BV-2 cells and on unstained SH-SY5Y.

2.4 Mitochondrial membrane potential (MMP)

MMP in microglial cells was assessed using flow cytometry by staining cells with the fluorescent dye Rhodamine 123 (Rho123; Sigma-Aldrich, St Louis, MO). After 48 hours of stimulation, cells were harvested and washed with PBS. Cells were then incubated with 1μM Rho123 at 37°C for 30 minutes in a thermostatic bath. At the end of the incubation period, cells were immediately placed in ice and washed with cold PBS. Fluorescence was acquired with a CyAn ADP analyzer and Summit software (Beckman Coulter, Brea, CA), then analyzed with FlowJo software (version 7.6, Treestar Inc., Ashland, OR). Results are expressed as the mean fluorescence intensity (MFI) of probe Rho123.

2.5 Determination of cytokines' release

The analysis of 22 cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α) was performed on culture medium of microglial cells, using magnetic bead-based multiplex immunoassays (Bio-Plex; BIO-RAD Laboratories, Milano, Italy) following manufacturer's instructions. Data from the reactions were acquired using the Bio-Plex 200 reader, while a digital processor managed data output and the Bio-Plex Manager 6.0 software presented data as Median Fluorescence Intensity and concentration (pg/ml) (BIO-RAD Laboratories, Milano, Italy).

2.6 Immunocytochemistry

BV-2 cells were placed at a density of 50,000 cells/well in 6-well plates, grown on glass coverslip, and then fixed in 4%

PFA (Sigma Chemical Co. Aldrich, St. Louis, MO). Cells were incubated in Phosphate Buffered Saline (PBS) 0.2% Triton X-100 and 1.5% normal goat serum (Euroclone, Milano, Italy) for three hours at 37°C with the β-actin (8H10D10) Mouse Antibody (Cell Signaling Technology, Beverly, MA). After rinsing, cells were incubated with the secondary antibody anti-Mouse IgG (H+L), F(ab') Fragment (Alexa Fluor 488 Conjugate, Cell Signaling Technology, Beverly, MA) in PBS 0.2% Triton X-100 and 1.5% normal goat serum (Euroclone, Milano, Italy) for one hour at 37°C. The glass coverslips were mounted using Vectashield with Dapi (Vector Laboratories, Burlingame, CA) and examined with an optical microscope Axioplan 2 (Carl Zeiss, Oberkochen, Germany).

2.7 Data analysis

All results are expressed as the mean of three different and independent experiments ± standard deviation (SD). Statistical significance was calculated using one-way analysis of variance (ANOVA) and Bonferroni post-hoc test correction in the case of multiple comparisons, using GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA).

3. Results

3.1 Modulation of PCD in microglial cells

PCD was evaluated by means of flow cytometry measuring the percentage of Annexin V positive cells (%A+, **Figure 2)**. In microglial cells, the treatment with LPS alone (18.45±9.41) did not induce a significant increase of PCD levels if compared to untreated cells (10.10±0.97) (**Figure 2**). The treatment with Lova alone (30.19±6.62) led to a significant increase in PCD values in comparison to untreated cells ($p<0.01$) (**Figure 2**). Moreover, the synergic treatment (Lova+LPS) further increased PCD (47.00±7.13) in comparison to Lova ($p<0.01$) (**Figure 2**).

3.2 Mitochondrial Membrane Potential (MMP) assay in microglial cells

To analyze mitochondria damage after the block of the mevalonate pathway in microglial cells, we assessed the impact of LPS and Lova treatments on the mitochondria membrane potential (MMP), expressed as mean fluorescence intensity (MFI) of Rho123. In **Figure 2** the effects of LPS, Lova, and Lova+LPS are shown. Treatment with LPS alone (633.6±55.4) did not significantly change MMP, while Lova alone (225.5±164.5) showed a significant decreased level of MFI in comparison with the untreated condition (589.7±155.6) ($p<0.05$). The double treatment (Lova+LPS) (104.2±75.7) further decreased MMP in comparison with the untreated condition ($p<0.01$).

3.3 Release of cytokines and chemokines by microglial cells

Out of the 22 tested cytokines and chemokines, only IL-1α, IL-1β, IL-6, TNF-α, IL-12(p40), IL-13 and G-CSF were significantly deregulated after the double treatment (Lova+LPS). In particular, Lova alone was not sufficient to significantly affect the production of any of these cytokines (IL-1α: 323.5±17.24; IL-1β: 830.3±106.7; IL-6:

93.54±15.54; TNF-α: 122.6±63.36; IL-12(p40): 89.03±43.27; IL-13: 533.5±27.77; G-CSF: 1517±234), while LPS alone only increased the production of IL-1β and G-CSF (IL-1β: 795.9±92.80; G-CSF: 2718±918.3), if compared to untreated conditions (IL-1α: 121.5±54.56; IL-1β: 515.9±99.76; IL-6: 15.28±4.03; TNF-α: 111.5±45.30; IL-12(p40): 108.2±36.91; IL-13: 561.2±48.10; G-CSF: 755±355.3). The double treatments (Lova+LPS) induced a significant increase in cytokines' secretion if compared to the untreated (IL-1α: 1142±125; IL-1β: 991.4±149.3; IL-6: 2163±409.0; TNF-α: 41832±31368; IL-12(p40): 267.9±60.83; IL-13: 870±162.3;G-CSF: 5666±716.5) (**Figure 3**).

3.4 Cell morphology

The microglial activation after Lova+LPS stimulation is also supported by immunocytochemistry. Images show that cells treated with Lova+LPS, compared to the untreated condition (**Figure 4**), assume a more amoeboid shape, which is considered as a further sign of microglia activation [20].

3.5 Microglia effect on the neuronal PCD

PCD was evaluated by flow cytometry as percentage of Annexin V positive cells. In transwell co-culture, PCD of BV-2 and SH-SY5Y was measured separately, while in contact co-culture staining of BV-2 with CellTrace far Red was exploited to distinguish the two populations during simultaneous analysis.

Neurons in standard contact co-culture with microglial cells, showed significant higher cell death after Lova+LPS treatment (%A+: 72.95±3.61) if compared with transwell co-cultured (%A+: 49.86±3.44) and neurons alone (%A+: 50.18±7.73) ($p<0.01$) (**Figure 5**). Instead, when neurons were in transwell co-culture with microglial cells, the cell viability did not change if compared with neurons alone (**Figure 5.**).

Interestingly, the triggering with LPS added to Lova-treated cells induced a significant increase of neuronal cells only in standard contact co-culture ($p< 0.05$), while it did not add any significant variation in the other experimental conditions (**Figure 5**).

4. Discussion

Due to limited information about neuroinflammation and neurodegeneration in Mevalonic Aciduria, the aim of our study was to better investigate the relationship between microglia and neurons in a simplified model that mimicked the inflammatory condition of MA. We thus exploited a well-established *in vitro* model that consisted of a biochemical block of the mevalonate pathway induced by statins [6, 21] and a subsequent inflammatory triggering with LPS to reproduce the acute phase of inflammation, typical of this pathology.

A first aim of this study was to investigate how the inflammatory *milieu* can induce an activation of the microglial cells, evaluating cytokine production, changes in shape and cell death in a model based on BV-2 microglial cells. Indeed, microglia activation is considered one of the causative factors of neuroinflammation associated to the pathophysiology

of neurodegenerative diseases. Understanding the role of microglia activation is fundamental to investigate neurodegeneration observed in MA patients.

Microglial cells represent the main cell type involved in the immune response inside the central nervous system (CNS), reason why they are also known as macrophages of the CNS.

We observed that microglial cells respond to the double treatment (Lova+LPS) in a manner comparable to systemic monocytes, analysed in previous studies [11, 22]. Thus, we assume that this increase in PCD could be due to a similarity between systemic (monocytes) and cerebral (microglia) defensive mechanisms.

In this experimental model, the increase in microglial cellular apoptosis, following Lova+LPS treatment, is closely related to mitochondrial damage. This relationship was also observed in neuronal cells in response to the blockade of the mevalonate pathway [7].

In many studies, the production of cytokines such as IL-1, IL-6 and TNF-α is the result of microglial activation [18, 19, 23, 24]. After the block of the mevalonate pathway and the inflammatory stimulus, we found a significant increase in the production of the proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α.

The increased production of these cytokines, together with microglial activation, prompts us to hypothesise their central role in the neuroinflammation seen in MKD; several works have supported this thesis reporting the involvement of these proinflammatory cytokine in MKD and MA [25, 26]. In particular, the IL-1 family is strongly supposed to play a fundamental role in MKD inflammatory processes, inasmuch as several biological therapies have successfully targeted these molecules [27, 28]. Moreover, TNF-α is one of the cytokines secreted by the activated microglia and plays a central role in the inflammatory cytokines network [24].

We also found that the Lova+LPS treatment led to an overproduction of IL-12(p40), IL-13 and G-CSF, molecules known to be involved in neurodegenerative and neuroinflammatory disorders. In particular, IL-12(p40) was seen to be produced by microglia in a mouse model of Alzheimer's disease [30, 31], while IL-13 has been shown to induce the death of activated microglia [32]. Finally, G-CSF is known to affect microglia status and this molecule, when overproduced, could induce a persistent activation of microglial cells, strictly tangled with neurodegeneration [33, 34].

The activation of microglia, after Lova+LPS treatment, is confirmed by their amoeboid shape; in fact, the change of microglial form is generally accepted as a symbol of microglial activation [20].

These preliminary results indicated that the block of the mevalonate pathway and inflammatory stimulus induces microglia activation. Microglia activation is a key event in the development and modulation of neurodegeneration in many neurodegenerative diseases.

In order to investigate the role of microglial activation in neurodegeneration, in a second set of experiments, SH-SY5Y cells (as a model of neurons) and BV-2 cells (as a model of glial cells) were cultured together, both in contact and in

transwell systems.

Our results clearly indicate that SH-SY5Y underwent an increased PCD only when cultured in contact with activated BV-2 cells. Conversely, this increase in PCD was not observed in transwell culture.

This result highlights that the direct contact between microglia and neurons is fundamental. On the contrary of what expected, activated glial cells do not induce neuroprotection, but cause an increase of neuronal apoptosis, exacerbating neuronal damage.

This work represents a step towards the understanding of the pathological and neuroinflammatory mechanisms occurring in MA. Considering the specific difficulties of obtaining a genetic model of the disease (KO mice for *MVK* are non-vital and hemyzygous littermates do not exhibit a phenotype resembling MA features) [5] and thus the impossibility of following the disease progression in the CNS, we developed a cellular model based on biochemical treatments, blocking the mevalonate pathway. Indeed, as seen in previous experiments, neuronal cells undergo PCD after Lova treatment, but LPS does not trigger, since these cells do not express an LPS receptor (i.e. TLR4) [6]. Interestingly, LPS induced an increase of neuronal PCD when added to Lova treatment only when SH-SY5Y were in standard contact co-cultured with glial cells. Besides, this condition better resembles the physiological *in vivo* condition in which neurons and glial cells are closely related. Consequently, the standard contact co-culture between neuronal and microglial cells appears to be the best model to study MA *in vitro* and identify drugs that block microglial activation. In fact, in such a pathological condition, we demonstrated that microglial cells are activated and contribute to neuronal cell death. We can thus hypothesise that the use of microglial activation blockers could prevent this additional neuronal death.

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Conflict of Interest

The authors declare that they have no competing interests.

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Figure legends

Figure 1

Schematic representation of the mevalonate pathway. Compounds used in the experiments are indicated along the pathway in bold characters: Lova (lovastatin).

Figure 2

BV-2 cells were incubated with 10μM Lova for 24h and with 1μg/mL LPS for an additional 24 hours. On the left PCD: bars represent the mean % Annexin V positive cells (A+) ± SD of three independent experiments. On the right MFI: bars represent the mean fluorescent intensity of Rhodamine 123 ± SD of 3 independent experiments. Data analyses were performed with one-way ANOVA and Bonferroni correction for multiple comparisons; *p<0.05; **p<0.01, ***p<0.001.

Figure 3

Values of cytokines and chemokines released by BV-2 cells after treatments. BV-2 cells were incubated with 10μM Lova for 24h and with 1μg/mL LPS for an additional 24 hours. Whereas Lova 10μM alone does not significantly affect the production of IL-1α, IL-1β, IL-6, TNF-α, IL-12(p40), IL-13 and G-CSF, the simultaneous administration of Lova and LPS 1ng/ml dramatically up-regulates the secretion of these molecules. This increase is statistically significant both if compared to the untreated condition and, except for IL-1β, to Lova treated cells. LPS 1ng/ml alone does not significantly affect the production of IL-1α, IL-6, TNF-α, IL-12(p40) and IL-13, instead LPS alone up-regulates the secretion of IL-1β and G-CSF. ns: not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ after One Way Anova test followed by the Bonferroni correction for multiple comparisons.

Figure 4

Representation of BV-2 cells (green: actin; blue: DAPI) either untreated (d) or treated with Lova 10μM and LPS 1ng/ml. BV-2 morphology changes after Lova+LPS treatments with increasing number of amoeboid cells (white arrows).

Figure 5

PCD in SH-SY5Y cells. Neuronal cells were incubated with 10μM Lova for 24h and with 1μg/mL LPS for an additional 24 hours. SH-SY5Y cells were cultured only, in standard contact co-culture and transwell co-culture.

%A+: bars represent the mean % Annexin V positive cells (A+) ± SD of three independent experiments; ** $p < 0.01$; after One Way Anova test followed by the Bonferroni correction for multiple comparisons.

Figure 1
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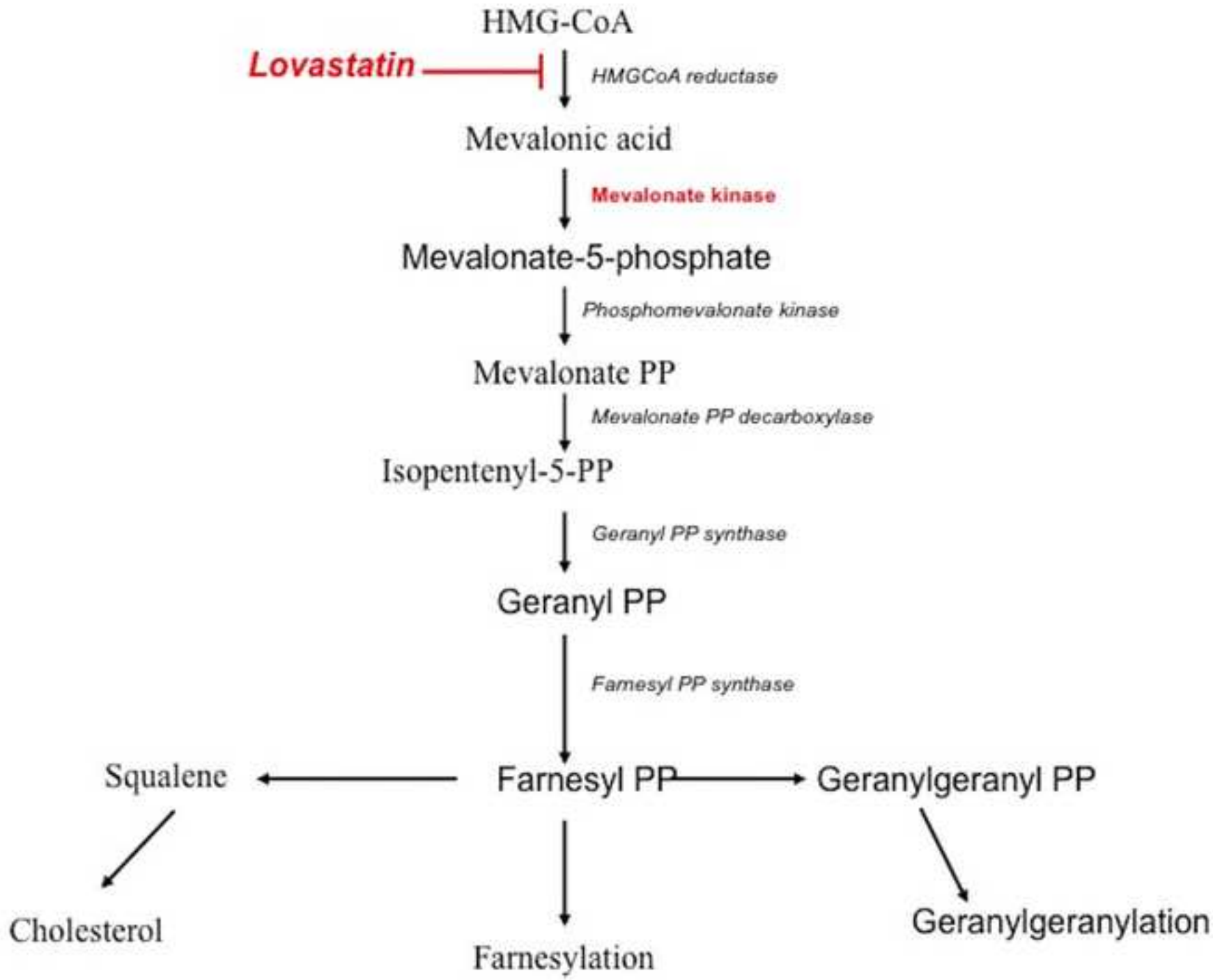

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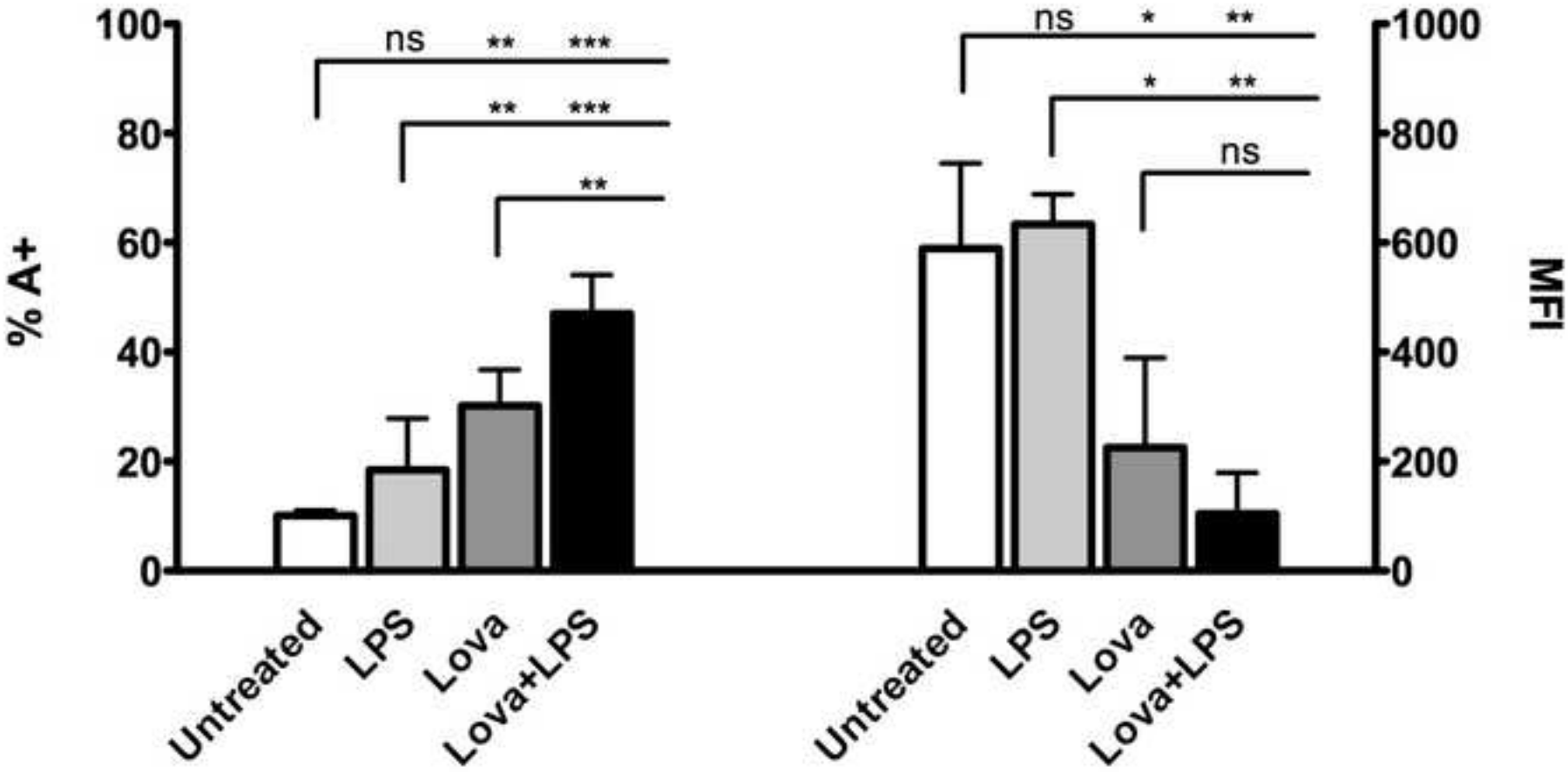

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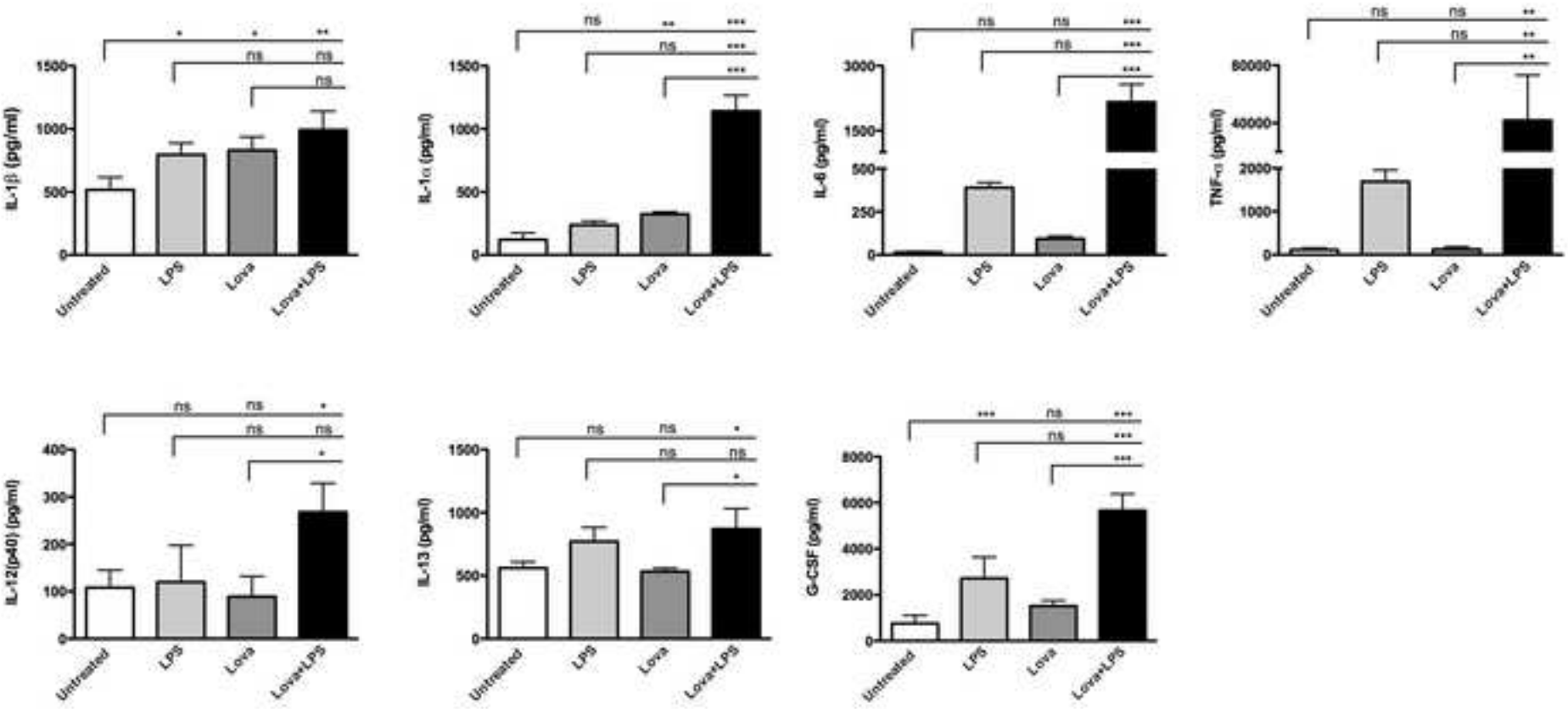

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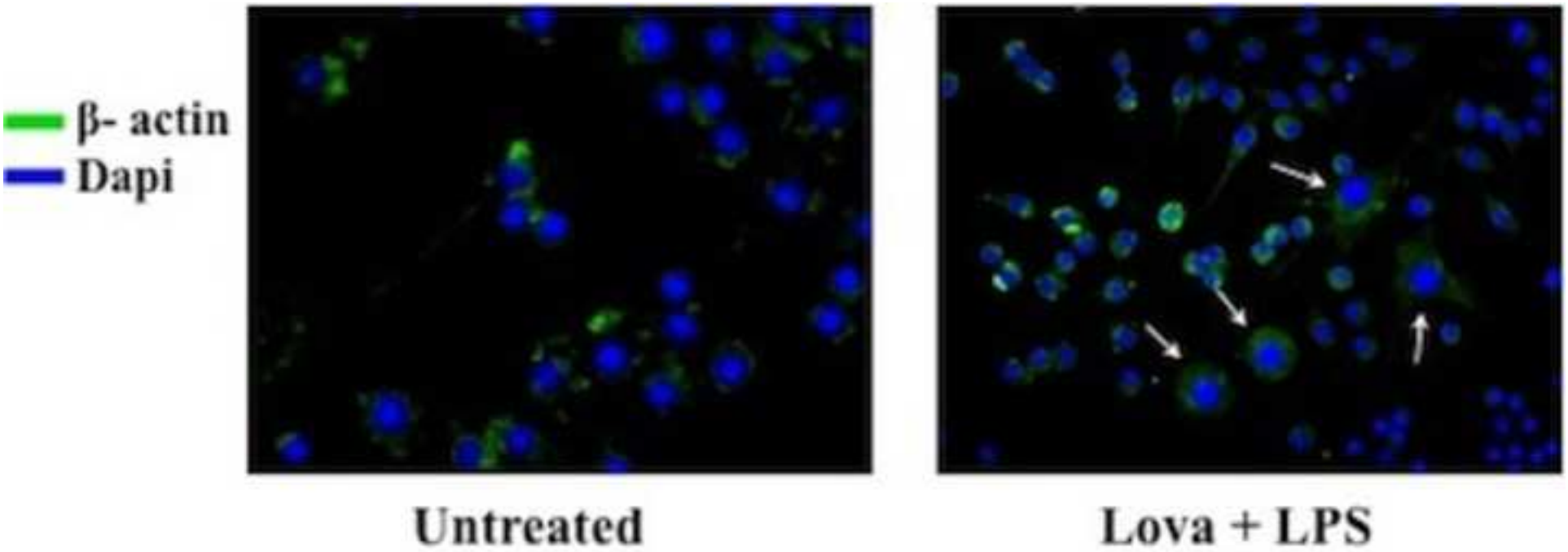

Figure 5
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