Edusfarm 14 *(2022), 47-65*
ISSN: 1886-6271
DOI: 10.1344/EDUSFARM2022.14.03

Rebut: 21 d'octubre de 2021
Acceptat: 8 de novembre de 2021

IN VITRO STUDY OF THE PHAGOCYTOSIS PROCESS OF HUMAN *CORPORA AMYLACEA* BY MACROPHAGES DERIVED FROM THP-1 MONOCYTES

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Abstract

Corpora amylacea (CA) are polyglucosan bodies that accumulate in the human brain during ageing and some neurodegenerative diseases. Recent studies indicate that CA gather waste substances from the brain and present some neo-epitopes that can be recognized by IgM natural antibodies and trigger an immune response. It has been reported that CA act as waste containers, since they are released from the brain into the cerebrospinal fluid and reach the cervical lymph nodes, where they might encounter macrophages. In this project, a recording of the phagocytosis of CA by THP-1-derived macrophages has been performed using the time-lapse technique. Two different staining methods have been performed in order to determine if CA are phagocytosed due to their nature or to the reactants used for staining. This works shows images of the recording in which CA are phagocytosed by macrophages and compares the results of the phagocytosis by both staining methods in order to draw some conclusions about this process.

Keywords: *corpora amylacea*, macrophages, phagocytosis, time-lapse.

Resum

Els *corpora amylacea* (CA) són uns cossos granulars formats per polímers de glucosa que s'acumulen al cervell humà durant l'envelliment i en certes malalties neurodegeneratives. Estudis recents mostren que els CA acumulen substàncies de desfeta del cervell i presenten certs neoepítops que poden ser reconeguts per anticossos naturals de l'isotip IgM i desencadenar una resposta immune. S'ha descrit que els CA són extrudits des del cervell cap als ganglis limfàtics cerebrals, on es poden trobar amb els macròfags i ser fagocitats. En aquest projecte, s'ha realitzat un enregistrament de la fagocitosi dels CA per part de macròfags derivats de THP-1 mitjançant la tècnica *time-lapse*. S'han portat a terme dos mètodes de tinció diferents per determinar si els CA són fagocitats per la naturalesa o pels reactius utilitzats per a la tinció. En aquest treball es mostren imatges dels vídeos en què els macròfags fagociten els CA i es comparen ambdues tècniques per treure'n algunes conclusions.

Paraules clau: *corpora amylacea*, macròfags, fagocitosi, time-lapse.

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Resumen

Los *corpora amylacea* (CA) son unos cuerpos granulares constituidos por polímeros de glucosa que se acumulan en el cerebro humano durante el envejecimiento, así como en ciertas enfermedades neurodegenerativas. Estudios recientes muestran que los CA acumulan sustancias de desecho del cerebro y presentan ciertos neo-epítopos que pueden ser reconocidos por anticuerpos naturales del isotipo IgM y desencadenar una respuesta inmune. Se ha descrito que los CA son extrudidos desde el cerebro hacia los ganglios linfáticos cerebrales, donde pueden encontrarse con los macrófagos y ser fagocitados. En este proyecto, se ha realizado una grabación de la fagocitosis de los CA por parte de macrófagos derivados de THP-1 mediante la técnica *time-lapse*. Se han llevado a cabo dos métodos de tinción diferentes para determinar si los CA son fagocitados por su naturaleza o por los reactivos utilizados para la tinción. En este trabajo se muestran imágenes de los vídeos en los que los macrófagos fagocitan los CA, y se comparan ambas técnicas para extraer algunas conclusiones.

Palabras clave: *corpora amylacea*, macrófagos, fagocitosis, *time-lapse*.

1. Introduction

Ageing is a physiological process characterised by a progressive loss of different functions which leads to an increased vulnerability to death. This deterioration has beendescribed as one of the main risk factors for human pathologies, including neurodegenerative diseases (López-Otin *et al.*, 2013, pp. 1194-1217). Neurodegenerative diseases are characterised by a progressive loss of neuronal function, which conducts to the death of these brain cells. To date, there is no evidence of any treatment that can stop this process definitely, only a few that have been described to halt the symptoms (Gao and Hong, 2008, pp. 357-365). One of the characteristics of ageing in human brains is the presence of different structures named *corpora amylacea* (CA), which were firstly described by J.E. Purkinje in the 19th century as some particular granular bodies in the brain of elderly patients (Catola and Achúcarro, 1906, pp. 454-469), but attention wasnot brought to their clinical implications until a few decades back (Cavanagh, 1999, pp. 265-295; Pirici and Margaritescu, 2014, pp. 33-57; Rohn, 2015, p. 3). CA are polyglucosan bodies that accumulate primarily in the periventricular, perivascular and subpial regions of the human brain during ageing and some neurodegenerative diseases. Some studies indicate that CA gather waste substances from the brain, some of them the consequence of the progressive degeneration that the brain suffers from ageing and some neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington and Pick diseases (Pirici and Margaritescu, 2014, pp. 33-57). As previously stated, CA are mostly madeof polyglucosan aggregates, which make them positive to periodic acid-Schiff (PAS) staining (figure 1A) (Riba *et al.*, 2019, pp. 26038-26048). These glucose polymers, which are the main component of the polyglucosan structure, are synthesized by the enzyme glycogen synthase which is alsopresent in CA, as recent studies point out. CA contain ubiquitin and p62 in addition to the polymers, and both molecules are involved in substrate degradation and processing of the brain waste that is collected (Augé *et al.*, 2018, p. 13525; Cissé *et al.*, 1991, pp. 429-433). Moreover, CA present some neo-epitopes (NEs) that can be recognized by natural antibodies of the immunoglobulin M (IgM) isotype (figure 1B) (Riba *et al.*, 2019, pp. 26038-26048). NEs are epitopes that arise under pathological conditions and are not present in healthy structures (Chou *et al.*, 2009, pp. 1335-1349).

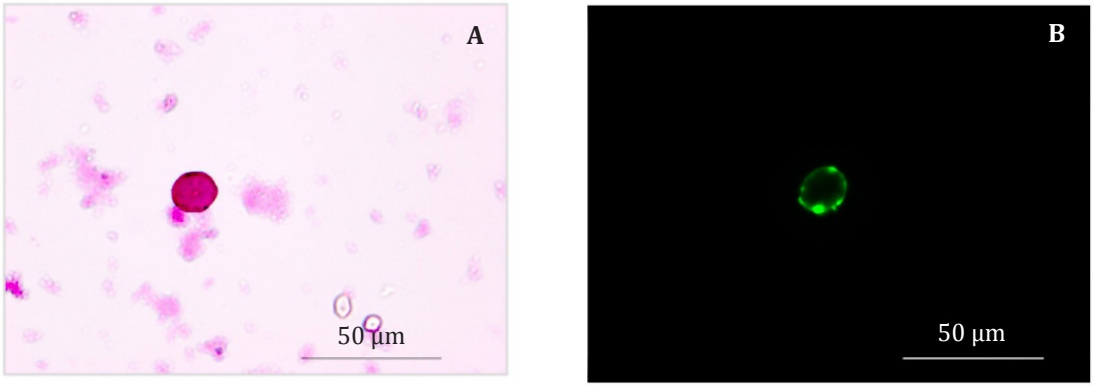

Figure 1. CA extracted from cerebrospinal fluid: CSF stained with PAS (A) and IgMs (B) (extracted from Riba *et al.*, 2019, pp. 26038-26048).

Natural IgM antibodies are generated even before birth, without exposure to external antigens, and contribute to critical innate immune functions such as the maintenance of tissue homeostasis. They act as a first line of defense against external agents and also against NEs that appear in situations of tissue damage or cellular stress. Taken into consideration the fact that some IgM natural antibodies recognize the NEs located on CA, it should be regarded that CA might take part in a physiological cleaning system of the central nervous system (CNS) (Riba *et al.*, 2019, pp. 26038-26048, Augé *et al.*, 2017, p. 41807).

Various functions have been attributed to CA but their main function, due to their location and composition, is thought to be acting as containers that remove waste products from the brain. It is well known that CA are generated by astrocytes mainly located in the periventricular, perivascular and subpial regions of the brain (Riba *et al.*, 2019, pp. 26038-26048). These astrocytes extrude the CA into the ventricles, the Virchow-Robin space, also known as the glymphatic system and the subarachnoid space. In all three cases, CA end up in the cerebrospinal fluid (CSF) that drains out of the brain through the meningeal lymphatic vessels into the cervical lymph nodes. Afterwards, inside the lymph nodes, CA could be phagocytosed by macrophages (figure 2) (Riba *et al.*, 2019, pp. 26038-26048). Recent in vitro studies point out that CA are phagocytosed by macrophages derived from THP-1 monocytes (THP-1' macrophages) (figure 3) (Riba *et al.*, 2019, pp. 26038-26048).

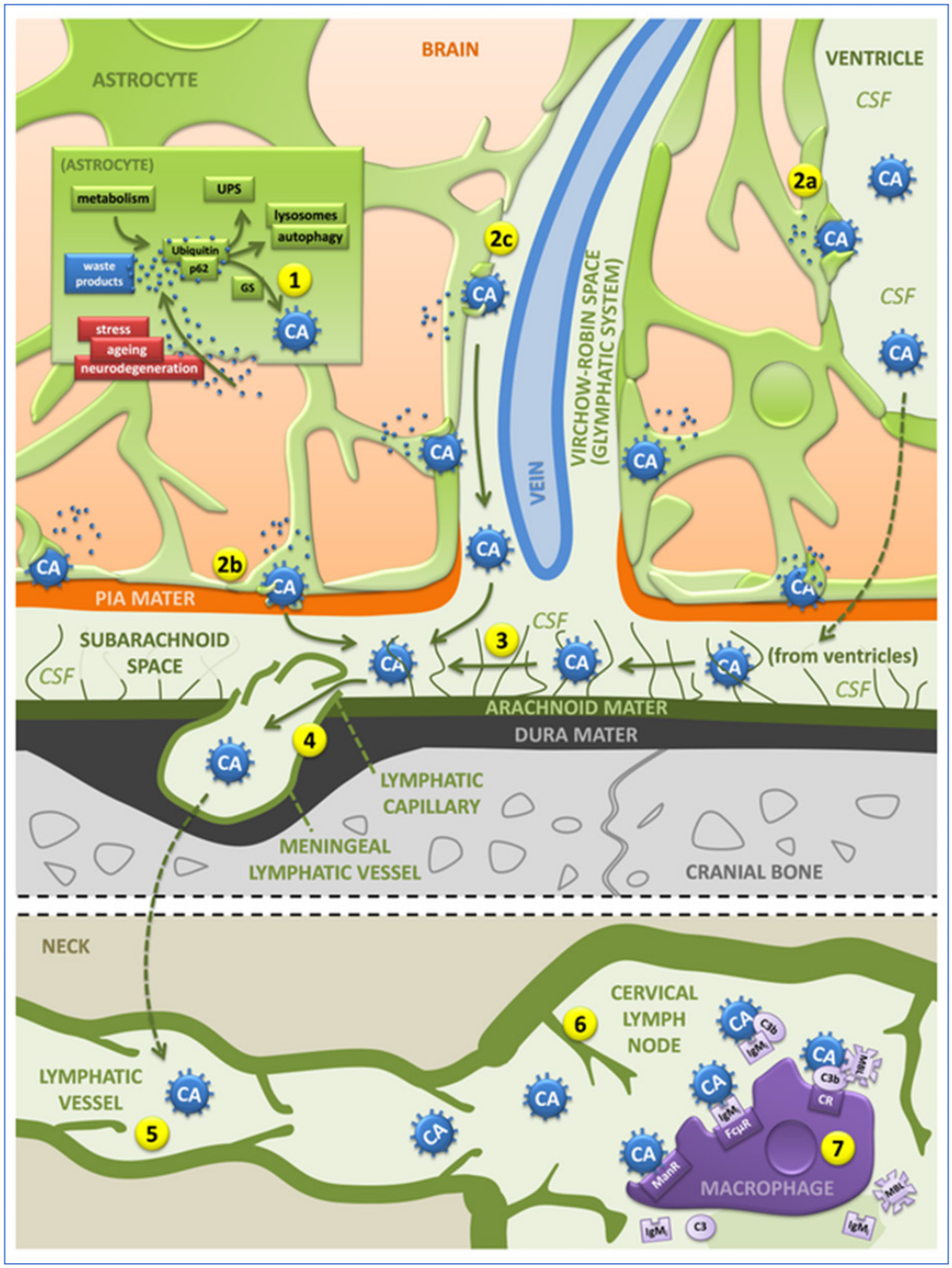

Figure 2. Representation of the pathway that CA undergo, from their generation until they encounter the macrophages in the cervical lymph nodes. CA are generated in the astrocytes (1) and are released into the CSF in the ventricular space, the subarachnoid space if the glymphatic system (2 a, b, c, respectively). From the subarachnoid space (3), they drain through lymphatic capillaries (4) into the lymphatic vessels (5) until they arrive to the cervical lymph nodes (6), where they reach the macrophages (7). Extracted from: Riba *et al.*, 2019, pp. 26038-26048. C3: C3 component of the complement system; C3b: C3b fragment generated from the split of C3; CR: C3b receptor; CA: *corpora amylacea*; CSF: cerebrospinal fluid; FcμR: IgM fragment receptor; ManR: mannose receptor; UPS: ubiquitin-proteasome system.

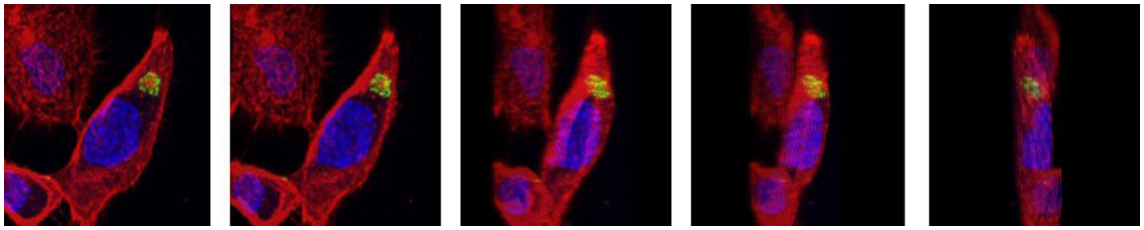

Figure 3. Sequence of images showing a macrophage phagocytosing an IgM-opsonized CA from different view-points. The sequence was performed after the 3D reconstruction from images obtained by confocal microscopy technique. Phalloidin (red), fluorescent labelled α-IgM (green) and Hoechst (blue). Extracted from Riba *et al.*, 2019, pp. 26038-26048.

Macrophages, along with neutrophils, eosinophils, monocytes, dendritic cells and B-lymphocytes, constitute the phagocytic cells, which have an essential role on the human immune system as they are responsible for the phagocytosis process, i.e., the elimination of pathogens and pathological molecules such as NEs (Kaiser, 2021; Flannagan *et al.*, 2012, pp. 61-98). These cells can be found fixed inside the lymph nodes, in the spleen or in an injured tissue during an inflammatory response after circulating monocytes differentiate into macrophages. Once differentiated, macrophages can be turned into specialized cells in different locations, to name a few examples: microglia (brain), alveolar macrophages (lungs), Kupffer cells (liver), mesangial cells (kidneys), osteoclasts (bones) and peritoneal macrophages (gastrointestinal tract) (Flannagan *et al.*, 2012, pp. 61-98).

Phagocytosis is the process in which a phagocyte internalizes particles larger than 0.5 μm. The phagocyte binds to the particle and uses its membrane to engulf it, giving rise to an internal compartment called the phagosome. This process contributes to the first line of defence against infections and is the primary method used by the immune system to eliminate microorganisms, infected cells, strange bodies and even CA. It also plays an important role in the initiation of the adaptive immune response (Flannagan *et al.*, 2012, pp. 61-98; Huang *et al.*, 2015, pp. 3-16).

Some authors state that phagocytosis process includes a series of steps (Sbarbati *et al.*, 1996, pp. 196-201; Kaiser, 2021):

1) Activation:
During an infectious process, inflammatory mediators activate circulating phagocytes leading to the production of surface glycoprotein receptors by the phagocytes, increasing their ability to efficiently adhere to the capillary walls, enabling them to go through the vessel and arrive to the site where the infection takes place.

Furthermore, during the activation process, macrophages produce endocytic pattern- recognition receptors (Flannagan *et al.*, 2012, pp. 61-98) that recognize and bind to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). As PAMPs are pathogen related structures, and CA are not pathogen related, there structures are not involved in the process of phagocytosis of CA. At the same time, DAMPs are endogenous molecules released from cells in response to different stressors, especially following an injury or cellular apoptosis (Huang *et al.*, 2015, pp. 3-16) and it has been proved that they can take part in some neurodegenerative diseases, so they might contribute to the phagocytosis of CA.

2) Chemotaxis:
Chemotaxis is only performed by circulating macrophages, neutrophils and eosinophils.It consists of the movement of phagocytes towards an increased concentration of

some attractant (Rappel and Loomis, 2009, pp. 141-149). Complement proteins, chemokines, fibrin split products and phospholipids by injured cells are some of the attractants.

CA are phagocytosed by macrophages that are bound to the lymph nodes, so in this case, this step is not necessary. As mentioned earlier, CA arrive at the lymph nodes from the lymphatic vessels where they encounter the macrophages, so there is no need for them to move (Riba *et al.*, 2019, pp. 26038-26048).

3) Attachment:

This is a crucial step in the process of phagocytosis given the fact that engulfment is not possible without the phagocyte and the body being attached. There are two different types of attachment:

- Unenhanced attachment: this type of attachment takes place when endocytic pattern-recognition receptors, such as scavenger receptors or mannose receptors, found on the phagocyte surface recognize DAMPs. There is no need for the phagocyte to interact with an external agent to recognize and attach to the body containing DAMPs.
- Enhanced attachment: this happens when the body that is to be engulfed is opsonized by antibodies of the IgG or IgM isotype, complement proteins like C3b produced during the complement pathways and acute phase proteins such as mannose-binding lectin (MBL) or C-reactive protein. This type of enhancement is much more specific and efficient than the unenhanced one (Kaiser, 2021).

As previously mentioned, CA contain NEs that can be recognized by natural IgM antibodies (Augé *et al.*, 2017, p. 41807). In physiological conditions, these antibodies cannot go through the blood-brain barrier (BBB), what means that they can only interact with CA once they have drained into the lymphatic system or if there is any damage in the BBB. When IgMs opsonize the CA, they can enable macrophages to recognize and attach to them (enhanced attachment) and consequently, phagocytose them. It has been observed that THP-1' macrophages phagocytose IgM-opsonized CA (Riba *et al.*, 2019, pp. 26038-26048). However, it was also noted that THP-1' macrophages could phagocytose non-IgM-opsonized CA. This fact draws the conclusion that IgM opsonization is not a requirement for phagocytosis by THP-1' macrophages (Riba *et al.*, 2019, pp. 26038-26048) (Figure 3).

It has also been reported that Concanavalin A (ConA) binds to CA (Liu *et al.*, 1987; pp. 49-60). ConA is a lectin that binds to mannose oligomers, meaning that the surface of CA can be shrouded by mannose-rich conjugates. THP-1' macrophages feature mannose receptors CD206 (Genin *et al.*, 2015, p. 577), indicating that THP-1' macrophages could recognize and attach to the mannose conjugates on the surface of CA (unenhanced attachment), thus triggering phagocytosis (Riba *et al.*, 2019, pp. 26038-26048). In addition, mannose can also be recognized by human MBL and act as an initiator of the lectin pathway of complement.

Previously unpublished studies show that CA from CSF are opsonized by MBL and C3b. Immunostaining assays were performed, showing that CAs were successfully stained with antibodies directed against MBL and against C3b. This suggests that these molecules are being recognized by macrophages, which attach to them (enhanced attachment) and then activate the process of phagocytosis.

Activation of the complement system through both, antibodies (classical pathway) and MBL (lectin pathway), converges in the cleavage of the C3 protein into C3a

and C3b, and the latter could opsonize CAs. In addition, C3b may be the result of a spontaneous C3b cleavage known as the alternative pathway. All of these mechanisms could trigger phagocytosis of CAs by macrophages (Figure 4) (Barnum, 1995, pp. 132-146; Sarma and Ward, 2011, pp. 227-235).

4) Engulfment:

Once the attachment step is completed, it is time for engulfment to begin. Actin filaments are dynamic polymers formed by a constant association of actin monomers at one end and dissociation from the other. This process is known as treadmilling (Flannagan *et al.*, 2012, pp. 61-98). Through polymerization and depolymerization of actin filaments, the phagocyte sends out pseudopods that encircle the body and bring it into the phagocyte (Horsthemke *et al.*, 2017; pp. 7258-7273).

This process creates an endocytic vesicle named phagosome. Phagosome formation around large particles entails internalization of considerable membrane area. Newly formed phagosomes are mainly constituted by a plasma membrane. These are progressively remodeled by fusion with endosomal and eventually lysosomal compartments as phagosomes mature (Flannagan *et al.*, 2012, pp. 61-98). The pH in the early phagosome is 5.4, which is lower than the usual pH of the cell due to the action of an electron pump that lets protons in the phagosome. Additionally, the early phagosome becomes highly oxidative and enriched with hydrolytic enzymes acquired from the fusion process of endosomes into the phagosome membrane (Flannagan *et al.*, 2012, pp. 61-98). This modification of the early phagosome contents happens in preparation for the digestion process (Kaiser, 2021).

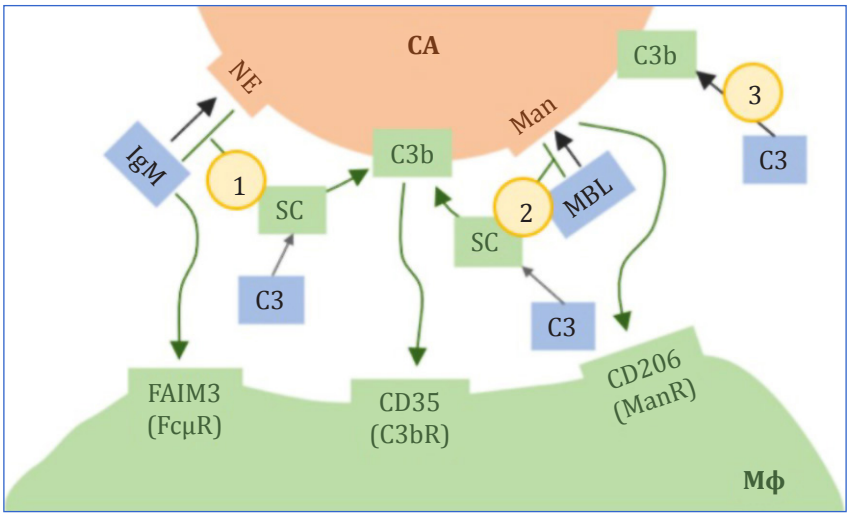

Figure 4. Representation of the different possible phagocytosis pathways that CA can undergowhen encounter macrophages. (1): Circulating IgM could recognize NE in the surface of CA and trigger the split of C3 to C3b which is the classical pathway whilst being recognized by FcμR. (2): MBL could recognize mannose in the surface of CA and activate the mannose pathway of the complement system. Mannose can be recognized by CD206 in the surface of macrophages. (3): Sudden split of C3 into C3b can happen which is known as alternative pathway. C3b opsonizes CA and these are recognized by CD35 in the surface of macrophages. NE: neo-epitope; IgM: immunoglobulin M; FAIM3/FcμR: IgM receptor in macrophages; C3: C3 component of the complement system; C3b: C3b component of the complement system; SC: complement system; Man: mannose; MBL: mannose binding lectin; CD206 /ManR: mannose receptor in macrophages (author's own elaboration).

5) Digestion:
Whilst endosomes are fusing with the early phagosome's membrane, the phagosome evolves into a more mature form: the late phagosome. The pH inside the body gets lower, about 5.0, and it enables the acquisition of the necessary biomolecules for fusing the lysosomes. Lysosomes are membranous sacks formed by the Golgi apparatus which contain a wide range of digestive enzymes, microbiocidal chemicals and toxic radicals. Once a late phagosome is fused with a lysosome, a phagolysosome is created. This phagolysosome's pH is even lower, around 4.5, and it is the ultimate microbiocidal and degradative organelle. It can digest all three types of molecules of life: proteins, lipids and carbohydrates (Flannagan *et al.*, 2012, pp. 61-98).
6) Antigen processing and presentation:
Antigen presenting cells (APCs) have a key role in the adaptative immune response, they capture and process antigens in order to present them to T lymphocytes, thus triggering its differentiation. APCs use major histocompatibility complex (MHC) molecules to present antigens given the fact that many antigens, once processed, bind to MHC molecules to be presented.

Antigen processing and presentation takes place through two main pathways: the MHC class I and the MHC class II pathway. Antigens presented by MHC class I activate CD8+ cytotoxic T lymphocytes (CTLs) to kill infected cells, whilst antigens presented by MHC class II activate CD4+ helper T lymphocytes to perform their role in controlling humoral, CTL mediated and inflammatory immune responses (Sandberg and Glas, 2001).

Despite MHC class I molecules are expressed on every nucleated cell, the expression of MHC class II molecules is restricted to specialized APCs, including macrophages, dendritic cells and B lymphocytes (Sarma and Ward, 2011, pp. 227-235; Sandberg and Glas, 2001). As there is consensus that CA come from the extracellular matrix into the APCs through phagocytosis, the only feasible pathway in which their surface antigens may be processed and presented is the MHC class II pathway (Riba *et al.*, 2019, pp. 26038-26048).

There are different possible outcomes once CA have been phagocytosed, although the most predictable one is the degradation of the CA by the phagolysosome created during the phagocytosis. The study of a possible antigen presentation on the surface of the macrophage or an inflammatory response triggered by the phagocytosis would be interesting. A time-lapse technique-based study could allow the visualization of the degradation of the CA and the possible antigen presentation of the surface of the macrophage.

Time-lapse photography is a technique used to capture processes that are static and slow for the human eye making them dynamic, thus enabling its closer observation and study. It consists of different frames captured on a much more spread out frequency used to visualize the sequence. Therefore, in matter of seconds the subject can observe processes that would normally take hours to happen (Paulson, 2018, p. 583).

An overnight time-lapse study of the process of phagocytosis of CA could be interesting to visualize the degradation of the CA and the antigen presentation on the macrophage's surface. To visualize this process, a staining method for CA and macrophages should be used. The technique is performed using a fluorescence microscopy in order to observe the processes.

In fact, previous unpublished work shows that by staining CA with ConA and performing posterior time-lapse photography, it is possible to observe the process of phagocytosis of these by macrophages (figure 5). However, Concanavalin A (ConA) is an external vegetal protein which brings up the question whether that could favor macrophages to target CA, thus phagocytosing them.

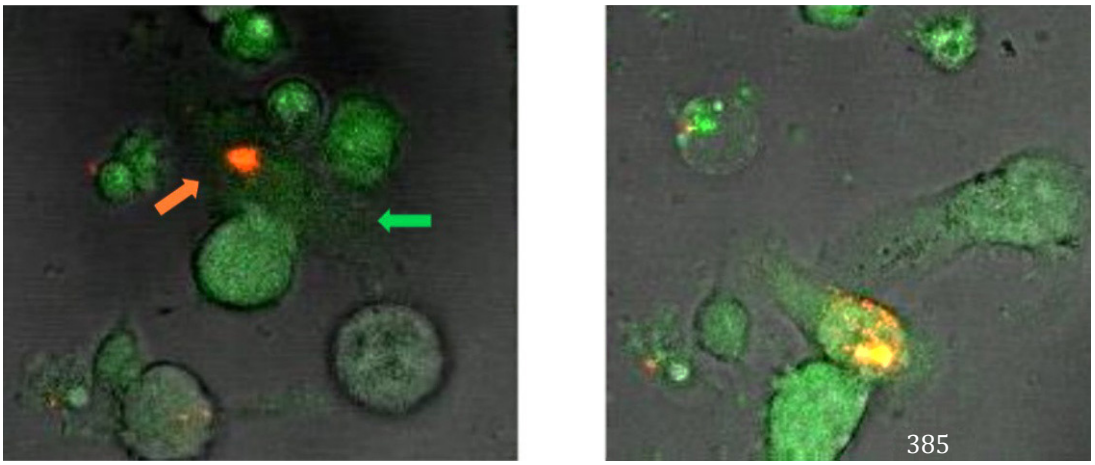

Figure 5. Images from a previously performed time-lapse that shows how THP-1 macrophages (green arrow) interact, reach, phagocytose and digest CA (orange arrow). In this case, macrophages were stained with CFDA SE (carboxyfluorescein diacetate, succinimidyl ester), cell tracker (green color) and CA were stained with ConA-Rhodamine (red color).

In order to minimize the impact that adding an external molecule could have, this project intends to carry out the same procedure, but with other reactants. In this case, the NHS ester will be used to stain CA, as it is a protein probe which conjugates to CA's proteins and makes them fluorescent.

2. Objectives:

The aim of this study is to visualize and analyze the process of phagocytosis of CA by macrophages derived from THP-1 monocytes. This main objective is partitioned into five particular objectives to be fulfilled through the development of the study:

1) To observe if CA labelled with the NHS ester attract THP-1' macrophages.
2) To observe if CA labelled with the NHS ester got phagocytosed by THP-1' macrophages.
3) To observe if the degradation of the CA labelled with the NHS ester takes place or if they remain intact.
4) To observe the processing of CA remains and the antigen presentation on the THP- 1' macrophage surface
5) To compare and to contrast the phagocytosis outcome of CA and THP-1' macrophages when using two different reactants to stain CA: ConA and NHS ester.

For the purpose of this project, a time-lapse study will be performed in which macrophages will be recorded phagocytosing CA.

3. Material and methods

3.1. Experimental design

The design of this experiment is based on the previously established objectives:

- To prepare a new fluorescent staining method for the CA to be used in in vitro studies and compare it to the previously used technique.
- To perform a fluorescent staining method for the THP-1' macrophages enabling to observe their physiological activity *in vitro*.
- To obtain time-lapse recording of stained macrophages phagocytosing stained CA.

3.2. Human CSF samples

Post-mortem CSF samples from a case affected by a neuropathological disease were obtained from the Banc de Teixits Neurològics (Biobanc-Hospital Clínic-IDIBAPS, Barcelona). The samples were from an 87-year-old patient diagnosed with Parkinson's disease. CSF was extracted with a 7 h 10 min post-mortem delay and samples were centrifuged at 4,000 × *g* at 4°C for 10 min and the pellets obtained were frozen and kept at -80ºC until used.

3.3. CA extraction and staining

Frozen pellets of CSF were defrosted and resuspended with phosphate buffered saline (PBS). The samples were then gathered and filtered through a 35 μm pore size mesh and centrifuged at 1000 x *g* for 5 min, discarding the supernatant. The pellet obtained was then resuspended with 1.5 mL of PBS and mixed by pipetting vigorously for about 50 times to break up aggregates or cell debris. The sample was transferred to an eppendorf tube and centrifuged at 1000 x *g* for 5 min, discarding the supernatant and resuspending the pellet obtained with 1 mL of PBS. The sample was then subjected to 3 sequential sedimentations letting the sample sit for 5 min under the effect of gravity, obtaining the sediment, transferring the supernatant to another eppendorf and pipetting the supernatant for about 50 times before letting it sit 5 min again. The two latter sediments were resuspended with 500 μL of PBS and gathered in an eppendorf tube. Two successive centrifugations were performed to this sample at 1000 x *g* for 5 min each, then discarding the supernatant and resuspending with 1 mL of PBS. The sample was then aliquoted in eppendorf tubes to perform the corresponding staining.

A previously mentioned, two different staining methods were performed using the same starting samples containing CA, in order to compare and discuss if previous results were a consequence of the used technique or of the mere phagocytosis process.

3.3.1. CA staining with ConA

The sample was centrifuged at 1000 × *g* for 5 min and the pellet obtained was resuspended with 500 μL of ConA-Rhodamine (Vector laboratories) in PBS (1:250). ConA was incubated 21 h at 4ºC under stirring. Next another centrifugation at 1000 × *g* for 5 min was performed, the supernatant was ruled out in order to eliminate any residual ConA and the pellet was resuspended with 1000 μL of PBS and stirred for 5 min. This washing process was repeated 3 times. Finally, the samples were centrifuged at 1000 x *g* for 5 min, the supernatant was ruled out, and the pellet was resuspended with 200 μL of supplemented RPMI (detailed in cell culture and differentiation section).

3.3.2. CA staining with the NHS ester

The sample was centrifuged at 1000 × *g* for 5 min and the pellet obtained was resuspended with 500 µL of 0.1 M sodium bicarbonate buffer (pH 8.3). In parallel, 100 µg of the amine-reactive dye Alexa Fluor (AF) 555 NHS ester (ThermoFisher Scientific) was dissolved in 10 µL of dimethyl sulfoxide (DMSO). Next the 10 µL of AF555 N- hydroxysuccinimide (NHS) ester solution was slowly added to the sample. The reaction was incubated for 1 hour at room temperature under continuous stirring. The sample was then centrifuged at 1000 x *g* for 5 min and the pellet was resuspended with 1000 µL of PBS and then stirred for 5 min. This washing process was repeated 3 times. Another centrifugation was performed at 1000 x *g* for 5 min and resuspended with 200 mL of supplemented RPMI (detailed in cell culture and differentiation section).

3.4. Cell culture and differentiation

THP-1 cells provided by ATCC were subcultured at a density of 5×10^4 cells/cm^2 on a µ-Slide 8 Well (Ibidi). Cells were differentiated to macrophages with 100 µM of phorbol 12-myristate 13- acetate (PMA; Sigma-Aldrich) at a concentration of 100 nmol/L in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences), 50 µM β-mercaptoethanol (Sigma-Aldrich), and penicillin (100 U/mL)/streptomycin (100 µg/mL) (Life Technologies) for 3 days. Differentiation of PMA- treated cells was enhanced after the initial 3-day stimulus by removing the PMA- containing media and then incubating the cells in 300 µL of fresh supplemented RPMI for a further 3 days.

3.5. Macrophage staining

The differentiated cells were washed twice using supplemented RPMI but without FBS. Cells were then incubated for 30 min at 37 °C with 300 µL of Vybrant® CFDA SE Cell Tracer Kit (1:2000; ThermoFisher Scientific) in supplemented RPMI without FBS. Next cells were washed using PBS and incubated in 300 µL of RPMI with all supplements and protected from light.

3.6. Phagocytosis studies

The time-lapse study was performed at the Unitat de Microscòpia Òptica Avançada (Scientific and Technological Centers UB [CCiT-UB]) of the Faculty of Medicine of the Universitat de Barcelona. Before performing the time-lapse studies, stained macrophages were washed with supplemented RPMI and then the media was replaced by 300 µL of supplemented RPMI containing the stained CA. The regions of interest (ROIs) were selected following specific criteria based on the presence and aspect of CA and the closeness of the macrophages in relation to the chosen CA. By selecting the ROIs, only the X and Y coordinates were established. X indicates the axis that goes horizontally, and Y indicates the axis that goes vertically. It was also necessary to establish a few Z coordinates that would indicate the depth in which each photograph would be taken. Three Z sections were pointed for each ROI. Having all the coordinates selected, the recording parameters had to be defined to start recording. The main parameter was the number of cycles that were going to be recorded. A cycle consists on the process of

capturing every picture defined by the X, Y and Z coordinates, so, in each cycle, the number of pictures captured equals the number of ROIs multiplied by the amount of Z coordinates selected for each field (3 in this case). In our study, the microscope took about 2 minutes to complete each cycle which means that the frame rate of the definitive recording would be of 0.5 frames per minute (fpm). Once the sample was placed and all the parameters were established, the recording process was started and the microscope was left recording overnight.

3.7. Live cell imaging

Automatic multi-position live cell imaging was carried out using a Zeiss LSM 880 equipped with Adaptive Focus Control to keep specimen in focus and an incubation system with temperature (37ºC), CO_2 control and a humidified atmosphere. Images of CFDA-SE and ConA-rodhamine or AF555 NHS ester dye were acquired sequentially line by line using 488 and 561 lasers lines and detection ranges at 500-550 and 570-650 respectively. All images were acquired using a Plan Apo 40x oil immersion objective lens (NA 1.1), pinhole set at 1.5 Airy units. Simultaneously, bright field images were acquired. The footage obtained was later processed using the software FIJI and ImageJ (National Institute of Health).

4. Results and discussion

4.1. Prelude

A time-lapse study has been performed using CA from CSF and macrophages from cell culture. Previous studies have proven that CA-ConA are phagocytosed following the phases: macrophage movement, attachment, engulfment, digestion and antigen presentation. ConA is a molecule found in plants, meaning is not naturally found in CA, which might favour the reactivity of macrophages against these. In this study, a protein probe, namely NHS ester, is used to stain CA without the interference of an outer molecule. The aim of this project is to study the process of phagocytosis of CA using this new probe (NHS ester) to label CA and compare it to the process that takes place when CA are stained with ConA (as previously), as well as repeating the ConA method to ensure the same conditions.

4.2. Results obtained when CA were stained with ConA

As previously stated, the experiment with ConA has been performed to enable the comparison with the new method and to make sure the same conditions are used in both cases. In this case, all phagocytosis phases have been seen when performing a time-lapse video:

4.2.1. Macrophage movement

Given the fact that the result of a time-lapse is a recording, macrophages are seen constantly moving; they wander around and move towards any strange body they may encounter. This step only occurs in in vitro conditions, as macrophages are not bound to any tissue so they can freely move, as seen in Figure 6.

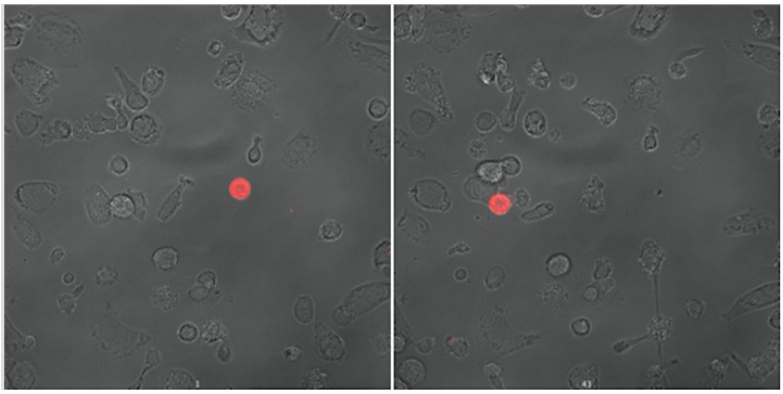

Figure 6. Series of consecutive images from the time-lapse recording that shows how a THP-1' macrophage moves towards a CA (red) to trigger the phagocytosis process.

4.2.2. Attachment

Looking closer to a macrophage next to a CA, different phases of the phagocytosis process can be seen. Firstly, a filopodium arises from the macrophage towards the CA, as seen in Figure 7. Once the filopodium has been attached to the CA, it is pulled towards the macrophage with the aim of initiating the engulfment phase of the phagocytosis process.

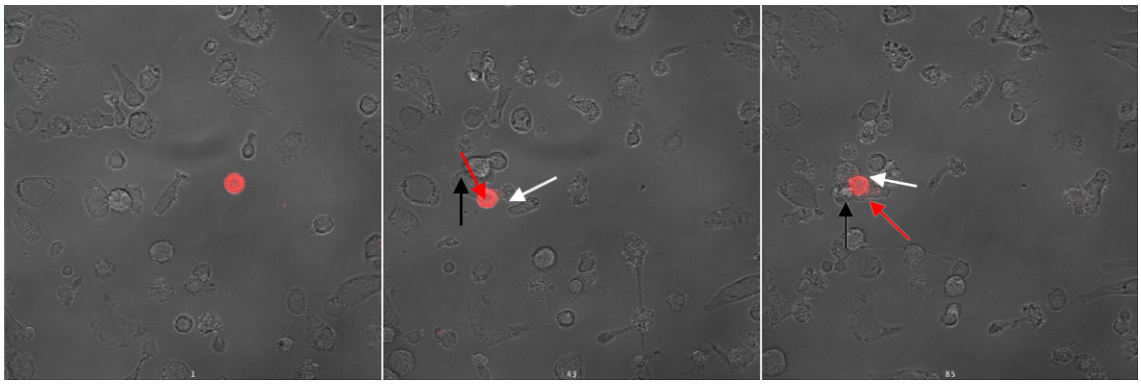

Figure 7. Series of consecutive images from the time-lapse recording that shows how the THP-1' macrophage (black arrows) extends its filopodia (white arrows) to reach the CA (red arrows) and pulls it towards the body of the macrophage so the engulfment of the CA can begin.

4.2.3. Engulfment and digestion

The macrophage engulfs the CA into its body creating an early phagosome that will eventually turn into the late phagosome, and into the phagolysosome once it merges with the lysosome. It can be noticed in Figure 8, how the degradative conditions found in the phagolysosome enable this organelle to split the CA and start processing its remains.

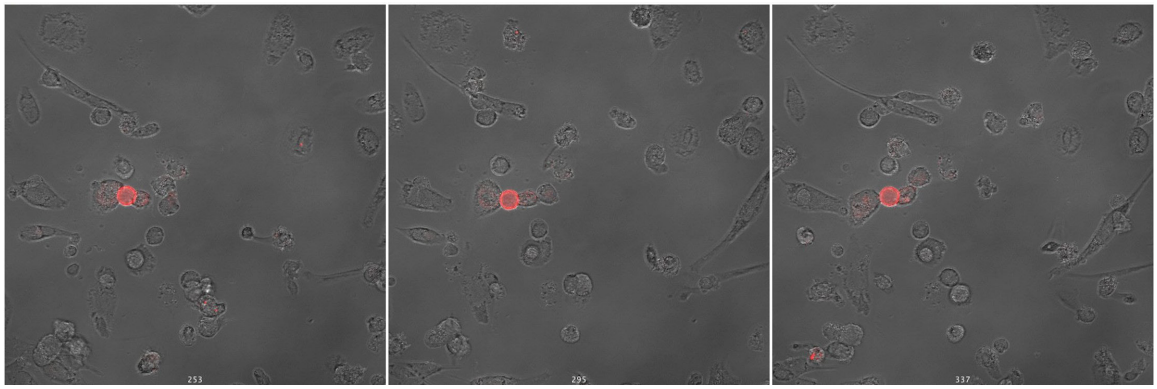

Figure 8. Series of consecutive images from the time-lapse showing the phagocytosis of a CA (red) by a THP-1' macrophage and the following degradation of the CA inside the macrophage.

4.2.4. Antigen presentation

After the engulfment of the CA, the macrophage seems to digest the particles that have been teared apart and act as an APC and place the remains of the CA on its surface. As shown in Figure 9, the macrophage that has phagocytosed and digested the CA, contacts its surrounding macrophages once the antigens are presented on its surface.

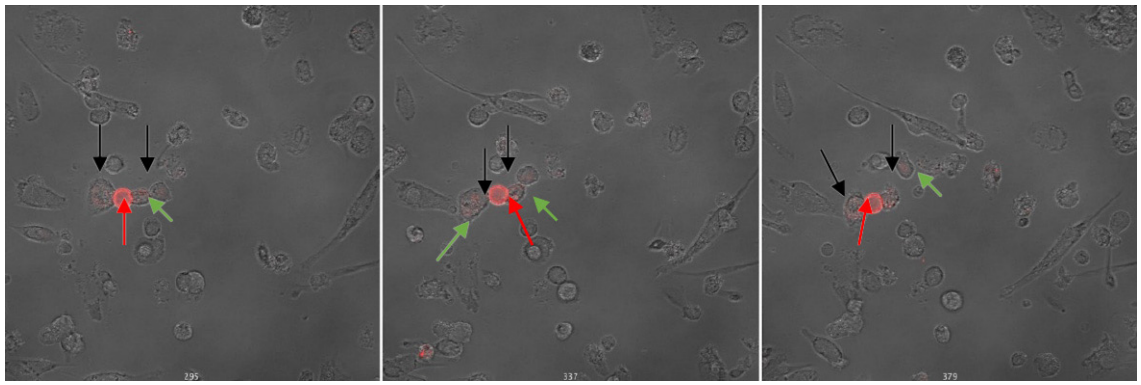

Figure 9. Series of consecutive images from the time-lapse that follows figure 10 and shows how the THP-1' macrophage (black arrow) digests the CA (red arrow) and presents its remains to the surrounding macrophages (green arrow).

It has been noted that CA are not fully digested although engulfment and internalization take place. Several particles seem to be phagocytosed by macrophages and exposed to their surface, so that macrophages can be considered APC, but the whole antigen presenting process does not seem to take place. Thus, the phagocytosis process is incomplete.

Comparing these results to previous experiments, CA in this case appear to be substantially bigger than the ones in other studies. It has already been described in an article that it seems that the smallest CA are the ones that are completely phagocytosed (Suzuki *et al.*, 2012, pp. 587-594).

4.3. Results obtained when CA were stained with the NHS ester probe

This alternative method was performed to see whether ConA somehow enhanced the phagocytosis process given the fact that it is an external vegetal protein which might favour macrophages to target CA, thus phagocytosing them. Also in this case, all phagocytosis phases have been seen performing a time-lapse video:

4.3.1. Macrophage movement

Macrophages are also seen in constant movement, moving towards any foreign body they may encounter. It has been pointed out the fact that the migrating process took longer in this case than when CA were stained with ConA, as it can be noticed in Figure 10.

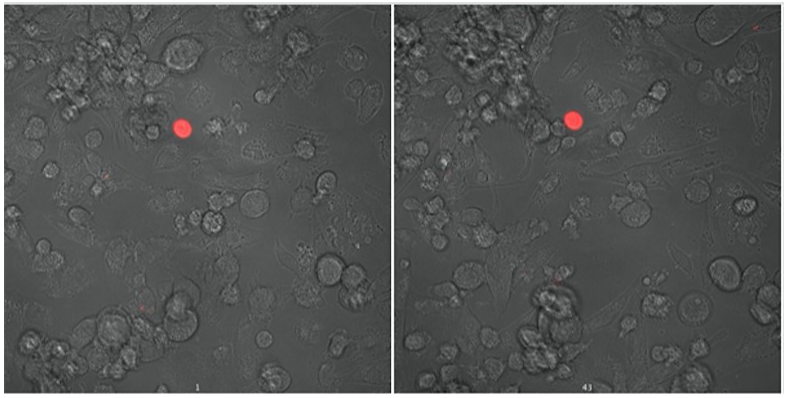

Figure 10. Series of consecutive images from the time-lapse recording that shows how the THP-1' macrophage move towards the CA (red) to trigger the phagocytosis process.

4.3.2. Attachment

Macrophages extend filopodium towards the CA, as seen in Figure 11A. Once the filopodium has been attached to the CA, it is pulled towards the macrophage with the aim of initiating the engulfment phase of the phagocytosis process. However, the process happens more slowly than in the other staining method and in this case, it is seen how some macrophages let go off the CA or they do not even attach to them, as seen in Figure 11B, so the phagocytosis process does not always take place.

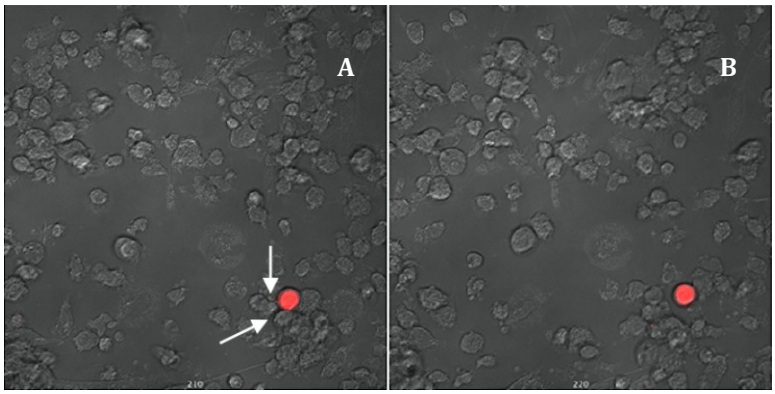

Figure 11. Series of consecutive images from the time-lapse recording that shows how the THP-1' macrophage extends its filopodia (white arrows) to reach the CA (red) and pulls it towards the body of the macrophage so the engulfment of the CA can begin (11A). Macrophages sometimes are seen letting go the CA, so phagocytosis does not take place (11B).

4.3.3. Engulfment and digestion

The CA that happen to be engulfed by macrophages are plunged into their body creating an early phagosome that will eventually turn into the late phagosome, and into the phagolysosome once it merges with the lysosome. It can be seen in figure 12, how the degradative conditions found in the phagolysosome enable this organelle to scratch little portions of CA and start processing them, although full fragmentation does not take place.

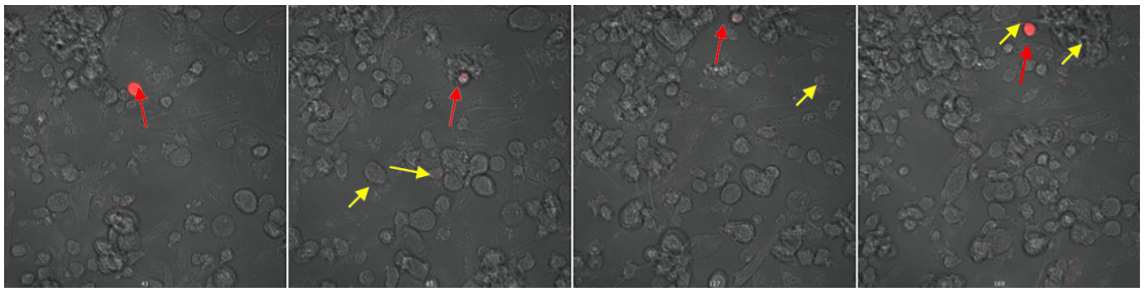

Figure 12. Series of consecutive images from the time-lapse recording that shows how a CA (red arrow) is phagocytosed by a macrophage, but afterwards is loosen. Little fragments are seen inside some macrophages (yellow arrow).

4.3.4. Antigen presentation

In this case, antigen presentation is not properly seen, some remains are seen inside macrophages, meaning they have been internalized, but it cannot be confirmed that it was antigen presentation, as seen in Figure 13.

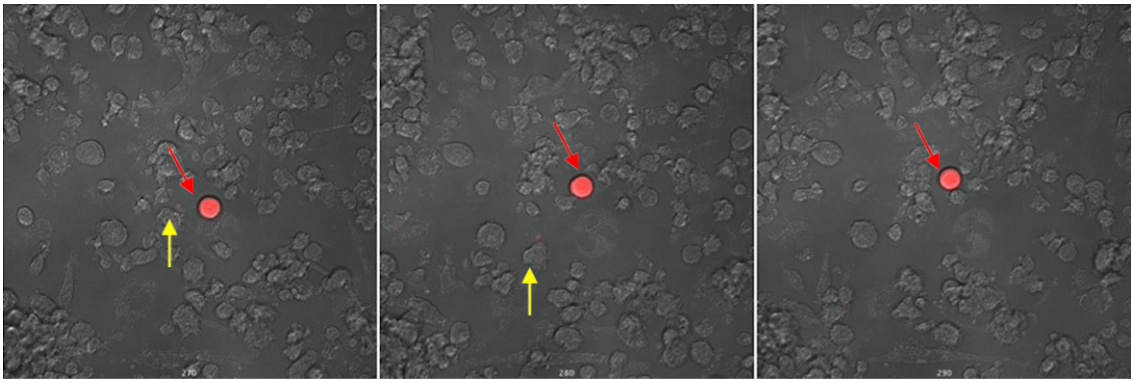

Figure 13. Series of consecutive images from the time-lapse recording that shows how a CA (red arrow) have not been fully phagocytosed (although a few particles are seen inside the macrophages marked with a yellow arrow), thus antigen presentation has not taken place.

CA give every appearance of being able to interact with macrophages due to their nature, without need of using an immunogen, which is any substance that may be specifically bound to components of the immune system, like ConA or antibodies. This leads to the idea that in vivo, once CA are extruded outside the brain, they could interact with the immune system in different points:

- In the CNS, when CA are released from the tissue (from the astrocytes) and go to the CSF, macrophages of the meninges are found and could already phagocytose them.
- Once leaving the CNS and accessing the lymphatic system, CA would reach the lymph nodes where macrophages are also found and therefore a CA-macrophage interaction would also take place.

As previously stated, CA are thought to accumulate waste substances, so the phagocytosis of CA by macrophages would be part of this end point of elimination of waste substances from the brain. However, it should be highlighted the fact that CA contain substances that come from the brain, meaning they have been extruded from the CNS (which is hard to reach by the immune system due to the BBB) and come into contact with immune cells. This fact may result in immune cells recognizing brain substances as foreign ones and trigger an immune response which may lead to an autoimmune disease.

5. Conclusions

According to the results obtained, the following conclusions can be determined:

- CA stained with the NHS ester probe attract THP-1' macrophages.
- CA stained with the NHS ester probe got phagocytosed by THP-1' macrophages.
- Regarding the degradation of the CA and the processing of their remains further studies would be needed in order to determine if these processes really take place.
- The phagocytosis outcome is very alike in both staining methods (ConA and NHS ester), so they might be considered equivalent in terms of the obtained results.
- CA, independently of the molecule they interact with, attract and interact with immune cells in vitro.

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