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STUDY OF THE PROCESS OF PHAGOCYTOSIS OF CORPORA AMYLACEA FROM THE CEREBROSPINAL FLUID BY HUMAN MACROPHAGES

Ferrer, Marçal;[1] Vilaplana, Jordi; Pelegrí, Carme; Riba, Marta

Department of Biochemistry and Physiology
Faculty of Pharmacy and Food Sciences
Universitat de Barcelona
Av. Joan XXIII, 27-31, 08028 Barcelona

Abstract

Corpora amylacea (CA) are granular bodies that accumulate in the human brain during aging and neurodegenerative conditions. These structures act as containers that amass waste substances from the brain and they are extruded from the central nervous system to the cerebrospinal fluid (CSF). From the CSF, they reach the cervical lymph nodes, where they can be phagocytosed by macrophages. In this work, human CSF samples and THP-1 derived macrophages were processed by immunofluorescence techniques to study the presence of phagocytosis inducers on the surface of CA, as well as macrophage surface receptors. The results suggest that CA can be phagocytosed by means of the mannose binding lectin, which attaching these bodies would cause a C3b-opsonization. Furthermore, macrophages derived from THP-1 monocytes present C3b receptors that would recognize the C3b that opsonize the CA.

Keywords: *corpora amylacea*, macrophages, phagocytosis, cerebrospinal fluid.

Resumen

Los *corpora amylacea* son unos cuerpos granulares formados por polímeros de glucosa que se acumulan en el cerebro humano durante el envejecimiento y durante procesos neurodegenerativos. Estos cuerpos son generados por los astrocitos y agrupan sustancias residuales de distintos orígenes. Recientemente, se ha descrito que los *corpora amylacea* son extruidos del cerebro hacia el líquido cefalorraquídeo y drenan mediante el sistema linfático. Recorren los vasos linfáticos hasta que llegan a los ganglios cervicales, donde podrían ser fagocitados por macrófagos. En este estudio, se ha grabado un vídeo de la fagocitosis de los *corpora amylacea* por parte de los macrófagos derivados de THP-1 utilizando la técnica del *time-lapse*, para poder visualizar las diferentes etapas del proceso de fagocitosis. En este artículo, se muestran las imágenes de los vídeos que muestran cómo los *corpora amylacea* son fagocitados, degradados y cómo el macrófago presenta los fragmentos resultantes de la digestión en su superficie como antígeno.

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

Resum

Els *corpora amylacea* són uns cossos granulars formats per polímers de glucosa que s'acumulen al cervell humà durant l'envelliment i durant processos neurodegeneratius. Aquests cossos són generats pels astròcits i agrupen substàncies residuals de diferents orígens. Recentment, s'ha descrit que els *corpora amylacea* són extruïts del

[1] Farmacèutic exercent en oficina de farmàcia. Graduat en Farmàcia. Col·laborador del grup de la Barrera Hematoencefàlica del Departament de Bioquímica i Fisiologia de la Facultat de Farmàcia i Ciències de l'Alimentació de la Universitat de Barcelona; marsalferrer@hotmail.com.

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cervell cap al líquid cefaloraquidi i drenen mitjançant el sistema limfàtic. Recorren els gots limfàtics fins que arriben als ganglis cervicals on podrien ser fagocitats per macròfags. En aquest estudi, s'ha enregistrat un vídeo de la fagocitosi dels *corpora amylacea* per part dels macròfags derivats de THP-1 utilitzant la tècnica del time-lapse, per poder visualitzar les diferents etapes del procés de fagocitosi. Es mostren les imatges dels vídeos que mostren com els *corpora amylacea* són fagocitats, degradats i com el macròfag presenta els fragments resultants de la digestió a la seva superfície com a antigen.

Paraules clau: *corpora amylacea*, macr**ò**fags, fagocitosi, *time-lapse*

1. Introduction

Corpora amylacea (CA) are granular bodies made of polyglucosan aggregates that are generated in the ageing human brain and can be found in the cerebrospinal fluid (CSF) and the cervical lymph nodes. They gather and amass waste products present in the brain, acting as containers that remove these waste products from the brain (Riba, *et al.,* 2019, 26038-48). These waste products are a consequence of the progressive degeneration that the brain suffers with ageing and with some neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's and Pick's disease (Cavanagh, 1999, 265-95).

As mentioned before, CA are mainly made of polyglucosan aggregates. These polymers are synthetized by the enzyme glycogen synthase, which is also present in CA, as some recent studies indicate (Figure 1D). Apart from these polymers that make up the structure, CA contain ubiquitin and p62, both involved in substrate degradation and the processing of waste products (Augé, *et al.,* 2018, 13525; Cissé, *et al.,* 1991, 429-33). Furthermore, CA present some neo-epitopes (NEs) that can be recognized by natural antibodies of the immunoglobulin M (IgM) isotype (Figures 1B and 1D) (Augé, *et al.,* 2017, 41807).

NEs are epitopes that emerge under pathological circumstances and are not present in healthy structures. They were first described on some polyglucosan bodies that appear with age in mouse brains. These bodies are frequently referred to as PAS granules, since they are stained with periodic acid-Schiff (PAS) staining, and have certain similarities with human CA (Augé, *et al.,* 2017, 41807).

Natural antibodies, which can act as a first line of defence against external elements, also play an important role in the maintenance of tissue homeostasis. They act on, among others, NEs that appear in situations of cellular stress and tissue damage; but also even during conventional tissue cell turnover (Vollmers, *et al.,* 2006, 755-65). They are named natural antibodies because these are antibodies that are present from birth, without previous contact with external antigens. Given that some natural antibodies recognize the NEs located on CA, it should be considered that CA may take part in a physiological cleaning system of the central nervous system (Augé, *et al.,* 2017, 41807).

Since CA's main components are polysaccharides, just as with PAS granules, they can be stained with PAS (Figures 1A and 1C). This staining technique is used to detect polysaccharides, because the periodic acid oxidizes the hydroxyl groups from the polysaccharide creating aldehydes. These aldehydes then react with the Schiff reagent, generating a purple-magenta colour (Sakai, *et al.,* 1969, 526-44).

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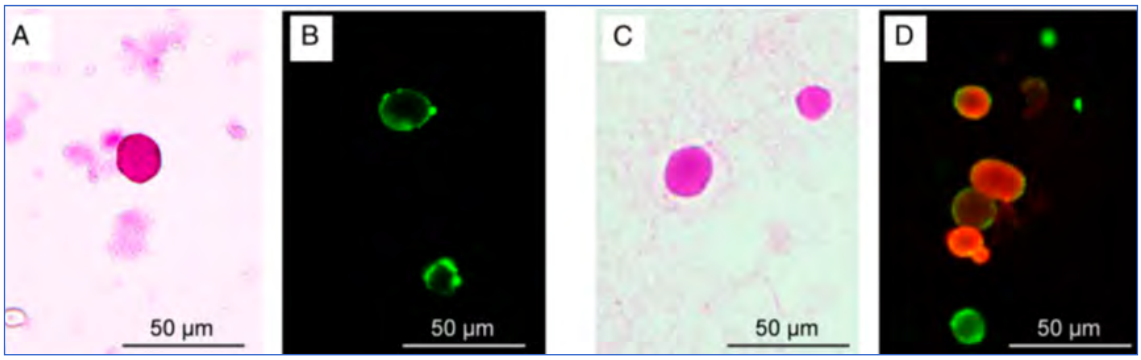

Figure 1. CA extracted from uncentrifuged CSF stained with PAS (A) and IgM_h α-NE (B). CA extracted from resuspended CSF pellets stained with PAS (C) and both α-GS and IgM_h α-NE (D, red and green, respectively). Extracted from: (Riba, *et al.,* 2019, 26038-48). CA: *corpora amylacea*; CSF: cerebrospinal fluid; GS: glycogen synthase; IgM_h α-NE: human immunoglobulin M anti-neo-epitope; PAS: periodic acid-Shiff staining.

CA are generated by astrocytes in the periventricular, perivascular and subpial regions of the brain (Cavanagh, 1999, 265-95; Sbarbati, *et al.,* 1996, 196-201). These astrocytes release the CA into the subarachnoid space, the ventricles, and the glymphatic system. In all three cases, CA end up on the CSF that drains out of the brain through the meningeal lymphatic vessels into the cervical lymph nodes. Then, inside the lymph nodes, CA could be phagocytosed by macrophages (Figure 2) (Riba, *et al.,* 2019, 26038-48). Actually, in vitro studies indicate that CA obtained from CSF are phagocytosed by THP-1-derived macrophages (THP-1') (Figure 3) (Riba, *et al.,* 2019, 26038-48).

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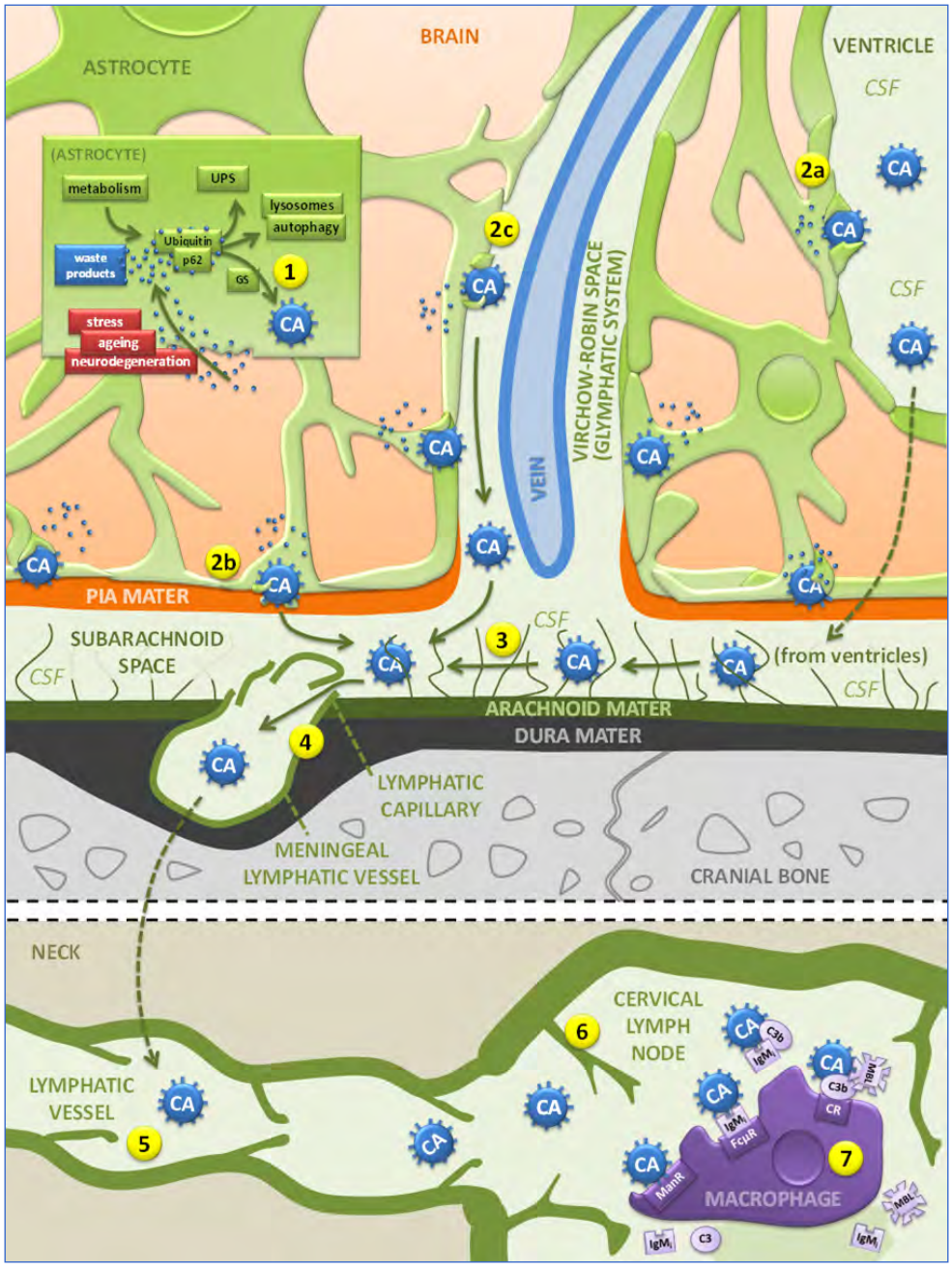

Figure 2. Illustration that represents the pathway that CA follow from their generation until they reach the macrophages on the cervical lymph nodes. CA are generated in the astrocytes (①) and are released into the CSF in the ventricular space, the subarachnoid space, or the glymphatic system (②a, b, and c, respectively). From the subarachnoid space (③), they drain through the lymphatic capillary (④) into the lymphatic vessels (⑤) until they arrive at the cervical lymph nodes (⑥) where they encounter the macrophages (⑦). Extracted from: (Riba, *et al.,* 2019, 26038-48). C3: C3 component of the complement system; C3b: C3b fragment generated from C3; CR: C3b receptor; CA: corpora amylacea; CSF: cerebrospinal fluid; FcμR: IgM Fc fragment receptor; ManR: mannose receptor; UPS: ubiquitin–proteasome system.

Macrophages, along with neutrophils, eosinophils, monocytes, dendritic cells and B-lymphocytes, make up the phagocytic cells that have an essential role in the human immune system, as they are responsible for the process of phagocytosis (Kaiser, 2020; Flannagan, *et al.,* 2012, 61-98).

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Macrophages can be found fixed inside the lymph nodes, in the spleen, or in an injured site during an inflammatory response after the monocyte differentiation. Specialized macrophages can also be found in the brain (microglia), lungs (alveolar macrophages), liver (Kupffer cells), kidneys (mesangial cells), bones (osteoclasts), and the gastrointestinal tract (peritoneal macrophages) (Kaiser, 2020).

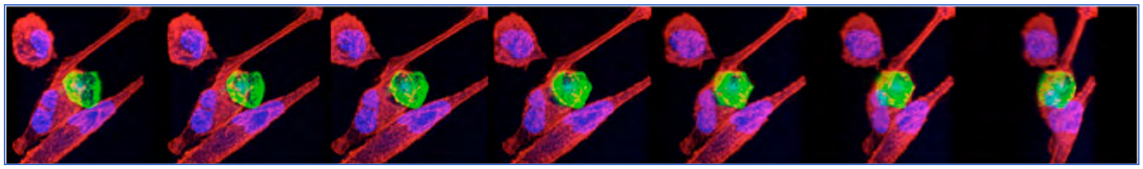

Figure 3. Sequence of images showing a macrophage phagocytosing an IgMh-opsonized CA from different points of view. The sequence was performed after a 3D reconstruction from images obtained by confocal microscopy. Phalloidin (red), fluorescent labeled α-IgMh (green), and Hoechst (blue). Extracted from: (Riba, *et al.,* 2019, 26038-48).

1.1. Phagocytosis

Phagocytosis is the process wherein a phagocyte internalizes particles larger than 0.5 μm. The phagocyte binds to the particle and draws it inward while engulfing it. Phagocytosis contributes to the first line of defence against infection and is the primary method used by the immune system to get rid of free microorganisms, infected cells, foreign bodies, apoptotic bodies and even CA. It also plays a key role in the initiation of the adaptive immune response (Kaiser, 2020; Flannagan, *et al.,* 2012, 61-98).

According to some authors (Kaiser, 2020; Flannagan, *et al.,* 2012, 61-98), phagocytosis is performed by the phagocytes following a series of steps outlined below.

1.1.1. Activation

During an infectious process, inflammatory mediators activate resting phagocytes resulting in the production of surface glycoprotein receptors by the phagocytes, increasing their ability to adhere more efficiently to the capillary walls, enabling them to squeeze out of the capillary and move to the site of infection (Kaiser, 2020). As the macrophages that phagocytose CA are bound to the lymphatic nodes, there is no need for them to gain an ability to adhere to the capillaries and to squeeze out of them.

Also, during the activation process, macrophages produce endocytic pattern-recognition receptors (Flannagan, *et al.,* 2012, 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, these structures are not involved in the process of phagocytosis of CA. On the other hand, DAMPs are endogenous molecules released from cells in response to exogenous and endogenous stressors, especially following injury or cellular death (Huang, *et al*. 2015, 3-16), and it has been observed that they can take part in some neurodegenerative diseases (Land, 2015, e157-70), so they may have a role in the phagocytosis of CA.

1.1.2. Chemotaxis

Chemotaxis is only performed by wandering macrophages, neutrophils, and eosinophils. It consists in the movement of the phagocytes towards an increasing concentration of some attractant (Rappel, 2008, 141-149). Bacterial factors, complement proteins, chemokines, fibrin split products and phospholipids released by injured cells are some of the attractants.

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CA are phagocytosed by macrophages that are bound to the lymphatic nodes so, in this case, this step is not necessary. As mentioned earlier, the CA arrive into the lymph nodes from the lymphatic vessels where they encounter the macrophages, so there is no need for the macrophages to move.

1.1.3. Attachment

The attachment step is crucial to the phagocytosis process, as engulfment is not possible without the phagocyte and the body being attached. There are two different types of attachment, as follows.

1.1.3.1. Unenhanced attachment: *this type of attachment happens when endocytic pattern-recognition receptors found on the phagocyte surface, such as scavenger receptors or mannose receptors, recognize DAMPs. The phagocyte doesn't need any external agent to recognize and attach to the body containing DAMPs.*

1.1.3.2. Enhanced attachment: *this takes place when the body that is to be engulfed is opsonized by antibodies (IgG or IgM), complement proteins like C3b produced during the complement pathways, and acute phase proteins such as mannose-binding lectin (MBL) or C-reactive protein (CRP). This type of attachment is much more specific and efficient than unenhanced attachment (Kaiser, 2020).*

In the case of CA, they contain NEs that can be recognized by natural IgM antibodies (Augé, *et al.,* 2017, 41807). In normal conditions these antibodies cannot go through the blood-brain barrier, so they can only interact with the CA once the CA have drained into the lymphatic system or if the blood-brain barrier is damaged. When IgMs opsonize the CA, they can enable macrophages to recognize and attach to them (enhanced attachment) and, consequently, phagocytose them. It was observed that THP-1' macrophages phagocytose IgM-opsonized CA (Riba, *et al.,* 2019, 26038-48). However, it was also observed that THP-1' macrophages could phagocytose non-IgM opsonized CA, which leads to the conclusion that IgM opsonization is not a requirement for phagocytosis by THP-1' (Riba, *et al.,* 2019, 26038-48).

It has also been reported that Concanavalin A (ConA) binds to CA (Liu, *et al.,* 1987, 49-60). ConA is a lectin that binds with high affinity to mannose oligomers, indicating that the CA surface can contain mannose-rich conjugates. THP-1' macrophages present mannose receptors CD206 (Genin, *et al.,* 2015, 577), indicating that THP-1' macrophages could recognise and attach to the mannose conjugates on the CA surface (unenhanced attachment), triggering phagocytosis (Riba, *et al.,* 2019, 26038-48).

A recent unpublished study also indicates that CA from CSF are opsonized with MBL and C3b. Two immunostaining assays were performed and, in both cases, CA were successfully stained with antibodies directed against the MBL and antibodies directed against C3b (Figure 4). It is therefore possible that these molecules are being recognized by macrophages, which attach to them (enhanced attachment), thus activating phagocytosis.

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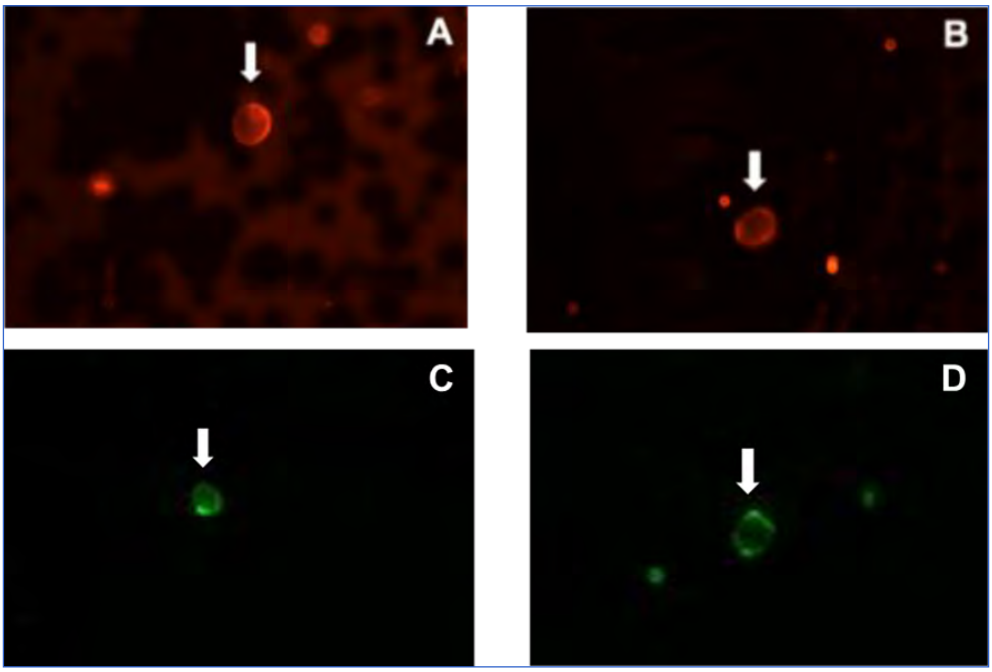

Figure 4. Images obtained by fluorescence microscopy showing CA (arrows) stained with an antibody conjugated to the TRITC fluorochrome and directed against the C3b (α-C3b-TRITC) (A and B) and α-MBL-FITC (C and D), indicating the presence of C3b and MBLα on CA (unpublished results).

1.1.4. Engulfment

Once attachment is complete, engulfment begins. Actin filaments are dynamic polymers formed by the constant association of actin monomers at one end and dissociation from the other end, a process known as treadmilling (Flannagan, *et al.,* 2012, 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, 7258-7273) (Figure 5).

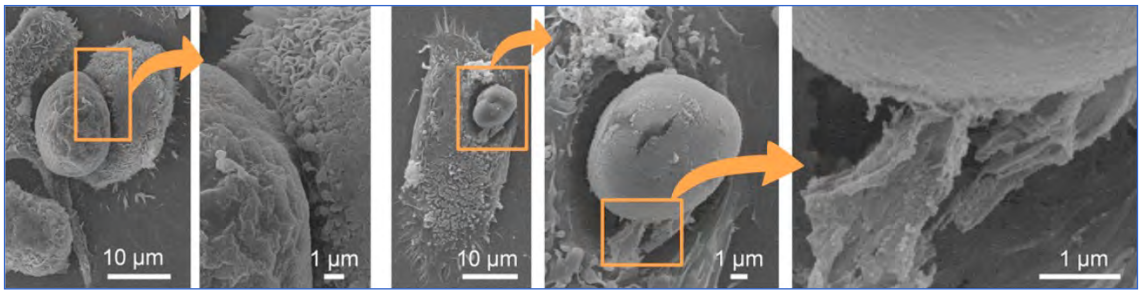

Figure 5. SEM images showing macrophages THP-1' engulfing IgM-opsonized CA obtained from CSF. Some filopodia were found emerging from macrophages and making contact with the bodies Extracted from: (Riba, *et al.,* 2019, 26038-48).

The engulfment process creates an endocytic vesicle called a phagosome. Phagosome formation around large particles entails internalization of a considerable membrane area. The plasma membrane is the main constituent of nascent and newly formed phagosomes, which are progressively remodelled by fusion with endosomal and, eventually, lysosomal compartments as the phagosomes mature (Touret, *et al.,* 2005, 157-70). The pH in the early phagosome (pH 5.4) is lower than the usual

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pH of the cell due to the action of an electron pump that bring protons into the phagosome. Also, the early phagosome becomes highly oxidative and is enriched with hydrolytic enzymes acquired from the fusing of endosomes into the phagosome membrane (Flannagan, *et al.,* 2012, 61-98). This modification of the early phagosome contents happens in preparation for the digestion process (Kaiser, 2020).

1.1.5. Digestion

As endosomes keep fusing with the membrane of the early phagosome, the early phagosome evolves into its more mature form: the late phagosome. The internal pH keeps dropping, becoming more acidic (pH 5.0), and it acquires the necessary biomolecules to get ready for the fusing of the lysosomes. Lysosomes are membranous sacs produced by the Golgi system and contain a wide range of digestive enzymes, microbicidal chemicals, and toxic radicals. The fusion between the late phagosome and the lysosomes creates the phagolysosome. The phagolysosome has an even more acidic pH than the late phagosome (pH 4.5), and is the ultimate microbicidal and degradative organelle; it can digest proteins, lipids, and carbohydrates. The relative contribution of the individual lytic mechanisms varies among the different types of phagocytes, depending on their primary function. For instance, pathogen eradication in the case of neutrophils versus antigen presentation for antigen-presenting cells (APCs) like dendritic cells and macrophages (Flannagan, *et al.,* 2012, 61-98).

1.1.6. Antigen processing and presentation

APCs play a fundamental role in the organism's adaptive immunity; they capture and process antigens in order to present them to T lymphocytes triggering its differentiation. APCs use major histocompatibility complex (MHC) molecules to present antigens, as a very important part of the processed antigens are bound to MHC when they are presented.

Antigen processing and presentation occur via two major pathways: the MHC class I and the MHC class II (Figure 6). Antigens presented by MHC class I activate CD8+ cytotoxic T lymphocytes (CTLs) to kill infected cells, whereas MHC class II-presented antigens activate CD4+ helper T lymphocytes to perform their key roles in controlling humoral, CTL-mediated and inflammatory immune responses (Sandberg, *et al.,* 2001).

While MHC class I is expressed on virtually all nucleated cells, expression of MHC class II molecules is restricted to specialized APCs, including macrophages, dendritic cells, and B lymphocytes (Sandberg, *et al.,* 2001; Bryant, *et al.,* 2002, 71-114). As CA come from the extracellular space and enter the APCs through phagocytosis, the only plausible pathway by which their antigens are processed and presented is through the MHC class II pathway.

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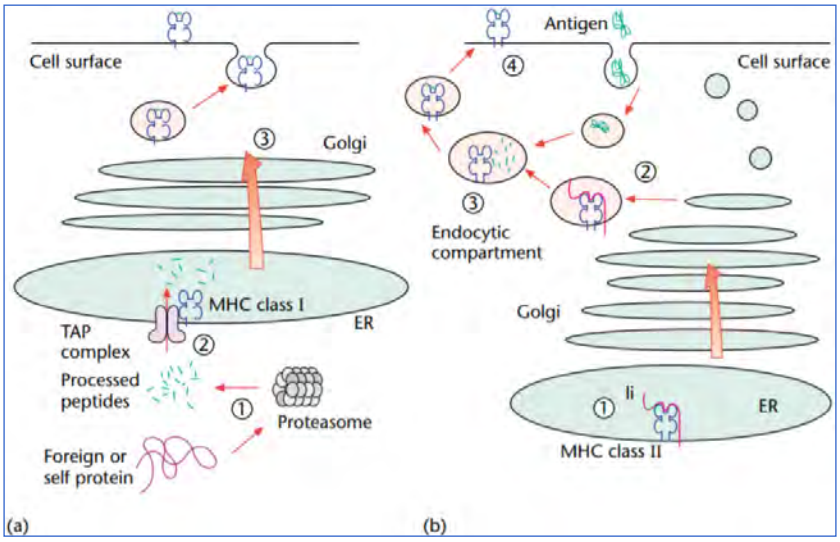

Figure 6. Schematic representation of the MHC class I and class II pathways for antigen processing and presentation. (a) The proteasome (①) processes a foreign or self protein, transforming it into peptides that can be translocated into the endoplasmic reticulum (ER) by the peptide transporter associated with antigen processing (TAP) (②). They are then loaded on MHC class I molecules and transported through the Golgi apparatus (③) to the cell surface. (b) MHC class II molecules are associated with the invariant chain (li) in the ER (①). The complex is transported through the Golgi apparatus, becoming an endocytic compartment (②) that will fuse with another endocytic compartment carrying the peptides generated from endocytosed proteins (③). The peptides are loaded into the MHC class II molecules and transferred to the cell surface (④). Extracted from: (Sandberg, et al., 2001).

Once CA have been phagocytosed, it is important to know the possible outcomes that can come out of this phagocytosis. The most predictable outcome is the degradation of the CA by the phagolysosome created during the phagocytosis. It would be interesting to observe a possible antigen presentation on the surface of the macrophage or an inflammatory response triggered by the phagocytosis. A time-lapse study could be a way of visualizing the degradation of the CA and the possible antigen presentation on the surface of the macrophage.

1.2. Time-lapse

Time-lapse photography is a technique used to capture processes that would normally appear static and slow to the human eye, making them appear more pronounced and enabling closer observation and possible study. It consists in capturing frames at a frequency which is much more spread out than the frequency used to visualize the sequence. For example, during the recording of time-lapse photography, the frequency of frame capture can be of 1 frame per minute, but when the sequence of images is played back at 1 frame per second, the process captured appears to be sped up. In other words, it enables the visualization of processes that can take hours in just a few seconds.

An overnight time-lapse study of the phagocytosis of CA could be useful in order to visualize both the degradation of the CA and the antigen presentation on the macrophage surface. This study is not aimed at the observation of the possible inflammatory response, so this outcome is out of range.

In order to visualize this process, a staining method that enables the normal macrophage physiological activity to take place would have to be used.

Carboxyfluorescein diacetate, succinimidyl ester (CFDA SE), often called CFSE, is a cell tracking reagent that passively diffuses into the cells (Figure 7) (Molecular Probes, 2001).

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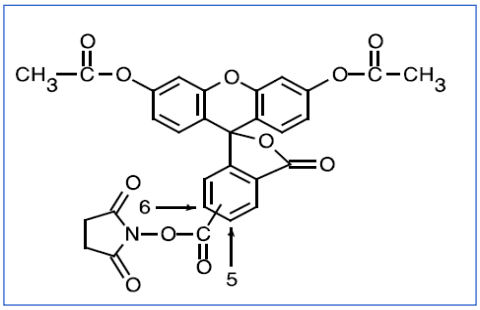

Figure 7. Structure of carboxyfluorescein diacetate, succinimidyl ester (CFDA SE). MW = 557
Extracted from: (Molecular Probes, 2001).

It is colourless and nonfluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well-retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. As it binds to intracellular macromolecules, it can be used for long term studies (Weston, *et al.,* 1990, 87-97).

2. Material and methods

2.1. Experimental design

The design of this experiment is based on the goals established previously.

1) To carry out fluorescent staining of the CA to be used in the *in vitro* studies.
2) To carry out fluorescent staining of the THP-1' macrophages to enable observation of its physiological activity *in vitro*.
3) To obtain time-lapse footage of the stained macrophages phagocytosing the stained CA.

2.2. Human CSF samples

Postmortem CSF samples from one case of a neuropathologically affected patient were obtained from the Banc de Teixits Neurològics (Biobanc Hospital Clínic - IDIBAPS, Barcelona). The samples are from a 66-year-old patient diagnosed with presenile Alzheimer's disease. The CSF samples were centrifuged at 4000 × g at 4 °C for 10 min and the pellets which were obtained were frozen and kept at -80 °C until they were used in our laboratory.

2.3. CA extraction and staining

Frozen pellets of CSF were defrosted and resuspended with phosphate buffered saline (PBS). CA were then obtained after 5 centrifugations at 700 × g for 10 min, discarding the supernatants, and resuspending the pellets with 500 µL of PBS. Thereafter, another centrifugation at 700 × g for 10 min was performed, but the pellet was resuspended with 300 µL of ConA-Rhodamine (Vector laboratories, Burlingame, USA) in PBS (1:250) (Table 1).

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TABLE 1. INFORMATION ABOUT THE CONTENT AND MAINTENANCE OF CONA-RHODAMINE.

Material	Amount	Storage	Stability
Concanavalin A (ConA), Rhodamine	1 vial, 25mg, 5mg active conjugate / mL	· 2-8 °C · Protect from light	Stable under recommended storage conditions. Avoid acidic pH.
Approximate fluorescence excitation/emission maxima: 555/580 nm			

ConA was incubated for 21 h at 4 °C with agitation. Then, another centrifugation at 700 × g for 10 min was performed, the supernatant was discarded in order to eliminate any residual ConA and the pellet was resuspended with 1000 µL of PBS. Finally, the samples were centrifugated at 700 x g for 10 min, the supernatant was discarded, and the pellet resuspended with 300 µL of supplemented RPMI. To verify the proper staining of the CA, a sample of the solution was extended on a microscope slide, it was observed under the microscope, and representative images of the staining were taken.

2.4. Cell culture and differentiation

The human monocytic leukemia cell line THP-1 provided by ATCC was cultured in RPMI 1640 medium supplemented with 10 % (v/v) of heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol, and incubated at 37 °C, 5 % CO_2 as described in: (Martínez, et al., 2013, 1920-7). Cells were subcultured at a density of 5 × 104 cells/cm^2 on µ-Slide 8 Well (Ibidi, Gräfelfing, Germany). Cells were differentiated to macrophages with 100 µM of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Darmstadt, Germany) at a concentration of 100 nmol/L in RPMI 1640 (Sigma-Aldrich) supplemented with 10 % heat-inactivated FBS (GE Healthcare Life Sciences), 50 µM β-mercaptoethanol (Sigma-Aldrich), and penicillin (100 U/mL)/streptomycin (100 µg/mL) (Life Technologies, Eugene, USA) 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 1640 for a further 3 days.

2.5. Macrophage staining

The differentiated cells were washed twice using fresh RPMI 1640 and incubated for 30 min at 37 °C with 300 µL of Vybrant® CFDA SE Cell Tracer Kit (1:2000; Molecular Probes, Inc. Eugene, USA) (Table 2) in supplemented RPMI without FBS in order to stain them. Cells were later washed using PBS and wells were refilled with 300 µl of fresh RPMI, with all supplements. During the preparatory tests, some samples of the cell culture were fixed using paraformaldehyde and observed under the microscope to verify the proper staining of the macrophages. The samples were fixed directly on the µ-Slide 8 Well and observed using an inverted fluorescence microscope with a camera attached to it in order to capture the evidence of the proper staining.

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TABLE 2. INFORMATION ABOUT THE CONTENTS AND MAINTENANCE OF THE VYBRANT® CFDA SE CELL TRACER KIT.

Material	Amount	Storage	Stability
CFDA SE (Component A) (MW = 557.47),	10 vials, 500 µg each, lyophilized powder	· ≤-20 °C · Dessicate · Protect from light · Avoid repeated freezing and thawing	When stored as directed, kit will be stable for at least 6 months.
DMSO (Component B)	1 vial of 1.3 mL		
Approximate fluorescence excitation/emission maxima: 492/517 nm, after hydrolysis.			

2.6. Phagocytosis studies

In order to prepare the sample to introduce it into the microscope for the time-lapse recording, stained macrophages were washed with supplemented RPMI and then the media was replaced by 300 µL of supplemented RPMI containing stained CA. The regions of interest (ROIs) were selected following a specific criterion based on: presence of CA, aspect of the CA (sometimes some debris can become stained with ConA and simulate the look of CA creating confusion), and the proximity of macrophages in relation to the CA that had been located. By selecting the ROIs, only the X and Y coordinates were established. X indicates the axis that goes left and right, and Y indicates the axis that goes up and down. Thus, it was also necessary to select a few Z coordinates that would indicate the depth in which each photograph would be taken. Three to four Z sections were acquired for each ROI. Once all the coordinates were selected, the recording parameters had to be defined to be able to start recording. The main parameter was the number of cycles that were going to be recorded. A cycle consists in 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 (four, in this case). In our study, the microscope took about 2 minutes to complete a cycle, which means that the frame rate of the final recording would be of 0.5 frames per minute (fpm). Once the sample was properly placed and all the necessary parameters were established, the recording process was started and the microscope was left recording overnight. The time-lapse study was performed at the Unitat de Microscòpia òptica avançada (CCiT-UB) in the Faculty of Medicine at the Universitat de Barcelona.

2.7. 3D reconstruction

Once the recording process was finalized, a series of ROIs were selected and the samples were fixed with paraformaldehyde with the intention of obtaining an additional series of images using a greater number of Z coordinates. This additional stack of images enables the elaboration of a 3D reconstruction of the aftermath of the phagocytosis process, in which it will be easier to observe the distribution of the remains of the CA inside the macrophage.

2.8. Live cell imaging

Automated multiposition live cell imaging was carried out using a Leica TCS SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with Adaptive Focus Control to keep the specimen in focus, an incubation system with set temperature (37 °C), CO_2 control, and a humidified atmosphere. Images of CFDA-SE and ConA-red were acquired sequentially line by line using 488 and 561 laser lines and detection ranges at 500-550 and 570-650, respectively. All images were acquired

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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, USA).

2.9. Preparatory test imaging

All the images from the preparatory tests were taken with a fluorescence laser and optic microscope (BX41, Olympus, Germany) and stored in TIFF format. In immunofluorescence studies, and for each staining, all images were acquired using the same microscope, laser and software settings. Image treatment and analysis were performed by means of the ImageJ program.

3. Results and discussion

The results of this work are divided into two sections: the preparatory tests, performed at the Department of Biochemistry and Physiology in the Faculty of Pharmacy and Food Sciences at the Universitat de Barcelona, and the results from the time-lapse study done at the Unitat de Microscòpia òptica avançada (CCiT-UB) in the Faculty of Medicine at the Universitat de Barcelona.

3.1. Preparatory tests

3.1.1. CA staining

As it has been reported that CA can be stained with ConA (Liu, *et al.,* 1987, 49-60), preparatory staining tests using ConA conjugated with the fluorochrome rhodamine (ConA-Rhodamine) were performed in order to ensure that ConA-Rhodamine would stain CA properly. Once the CA were obtained from the CSF and later incubated with ConA-Rhodamine, it was observed that all samples were properly stained, so it was determined that ConA-Rhodamine can successfully stain CA and that this type of staining method would be used in the time-lapse study. Figure 8 shows representative images of CA stained with ConA-Rhodamine. As shown in Figure 8, CA have a rather spherical shape, which helped to distinguish them from any kind of debris that could have been present in the sample.

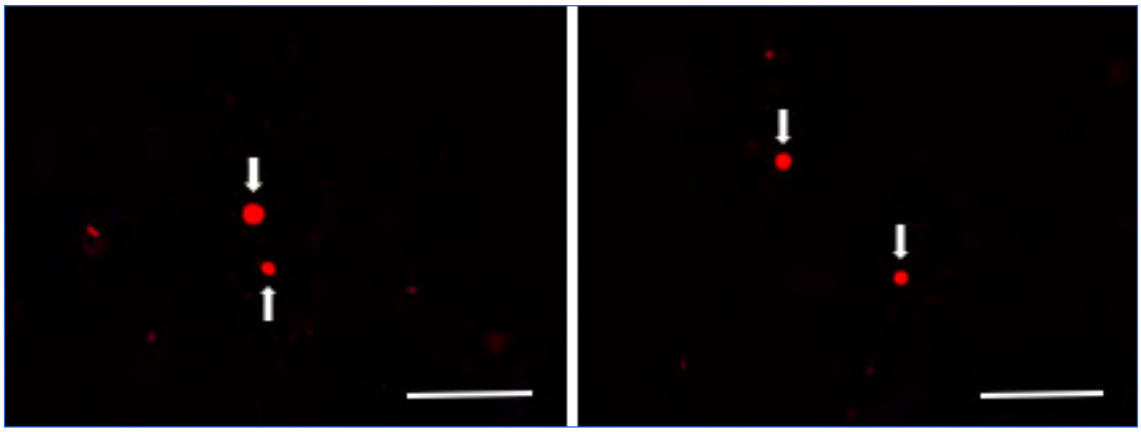

Figure 8. Images from the preparatory tests showing CA (arrows) stained with ConA-Rhodamine (red). Scale bar: 100 µm.

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3.1.2. THP-1' macrophage staining

Cell staining was tested before conducting the time-lapse study. After cell differentiation and cell staining following the protocols described in the methods section, samples were fixed and observed in the microscope. As can be seen in the representative images shown in Figure 9, the cells were successfully differentiated from monocytes into macrophages, as some macrophage features, such as an elongated shape and the presence of filopodia, can be observed. Also, they were properly stained with Vybrant® CFDA SE Cell Tracer Kit (green), so these images are proof that this is an adequate staining method to be used in the time-lapse study.

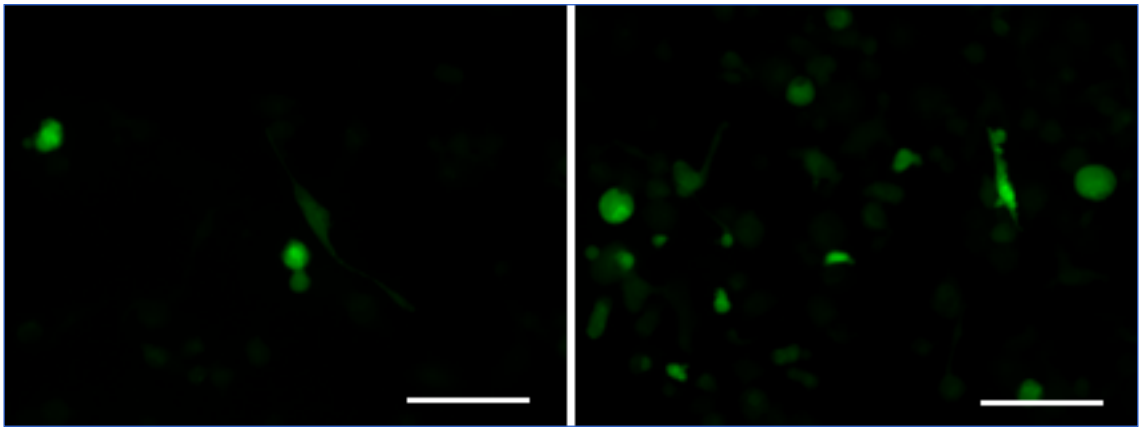

Figure 9. Images from the preparatory tests that show the differentiated macrophages stained with Vybrant® CFDA SE Cell Tracer Kit (green). Scale bar: 100 μm.

3.2. Time-lapse

The footage from the time-lapse study shows the different steps of the process of phagocytosis and its aftermath.

3.2.1. Macrophage movement

At first glance, the most noticeable event in the recording is the movement of the macrophages. As CA are not known to release any chemotactic substance, the movement of the macrophages cannot be labelled as chemotaxis. Instead, the macrophages move around phagocytosing any foreign body they encounter (Figure 10). In physiological conditions this step does not occur, as the macrophages are bound to the lymphatic nodes and cannot move and get closer to the CA, but in in vitro conditions, the macrophages are not bound to anything so they can move freely.

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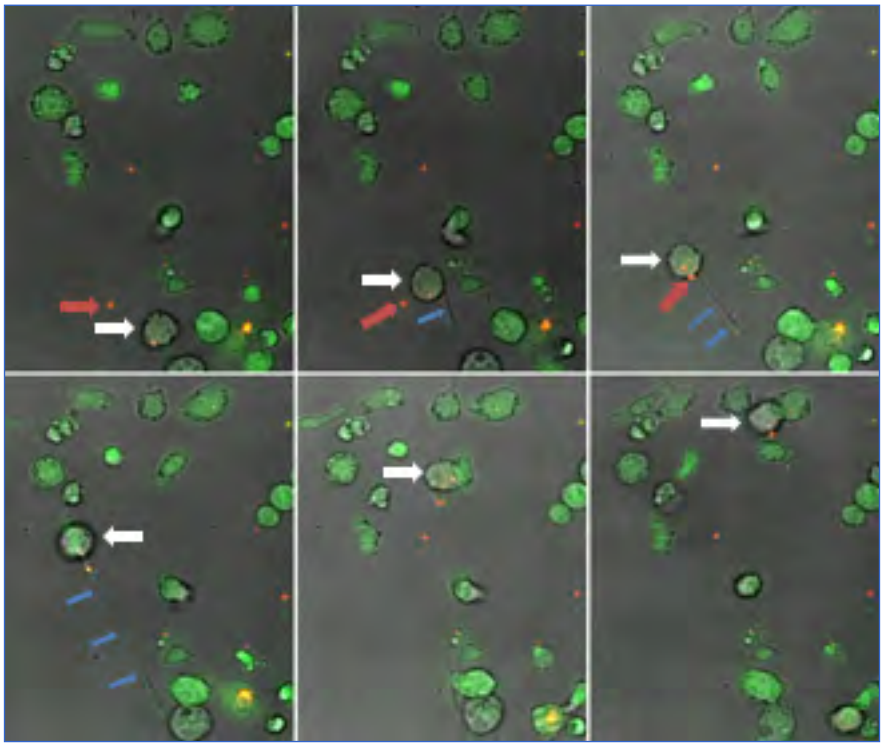

Figure 10. Series of consecutive images from the time-lapse recording that showcases the movement of a macrophage (white arrow) as it moves around and encounters CA (orange arrow). A filopodia (blue arrows) can also be seen. The curious part of this footage is that the filopodia extends from the point of origin of the macrophage to the position that the macrophage is moving to until it breaks. Elapsed time 22.08 seconds.

3.2.2. Attachment

Zooming in on a macrophage that is close to a CA, several steps of the process of phagocytosis can be observed. Firstly, a filopodium extends from the macrophage until it reaches the CA (Figure 11). This cytoplasmatic projection enables the macrophage to recognize the CA and attach to it without having to move. It can be hypothesised that this is how the macrophages capture the CA that arrive into the lymphatic nodes. Once the filopodium has attached to the CA, it pulls it towards the body of the macrophage in order to initiate the engulfment phase of the phagocytosis process.

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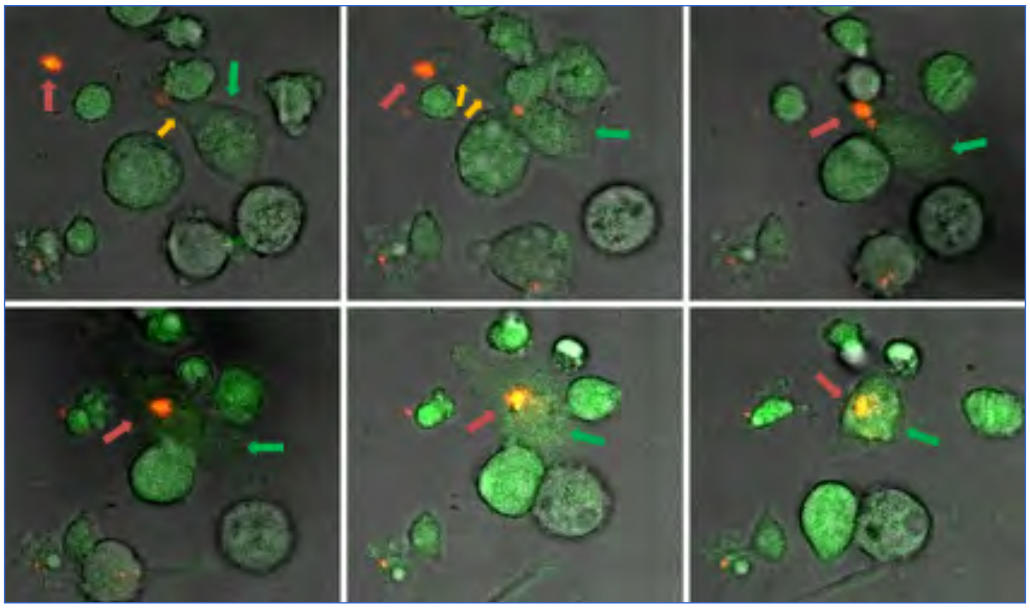

Figure 11. Series of consecutive images from the time-lapse recording that shows how the THP-1' macrophage (green arrows) extends its filopodia (yellow arrows) to reach the CA (orange arrows), and pulls it towards the body of the macrophage triggering the engulfment of the CA. Elapsed time: 23.45 seconds.

3.2.3. Engulfment and digestion

The macrophage engulfs the CA completely into its body, creating the early phagosome that will eventually turn into the late phagosome, and then into the phagolysosome once it fuses with the lysosomes. Figure 12 shows how the degradative conditions created inside the phagolysosome enable this organelle to break down the CA and start processing its remains.

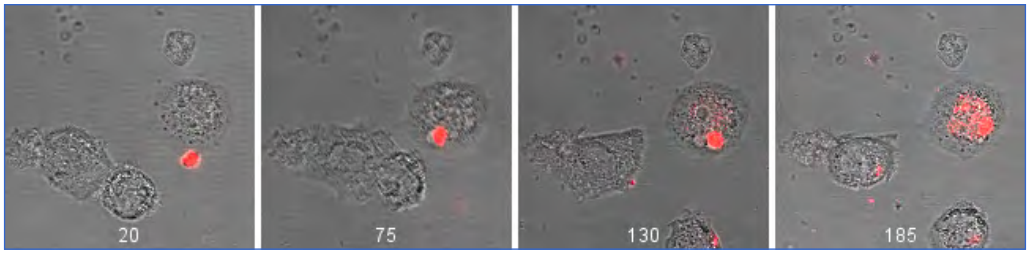

Figure 12. Sequence of bright field images showing the phagocytosis of a CA (red) by a THP-1' macrophage and the following degradation of the CA inside the macrophage. Elapsed time: 19.05 seconds.

3.2.4. Antigen presentation

After the engulfment of the CA, the macrophage can digest them and, in some cases, the macrophage acts as an APC and presents the remains of the CA on its surface. As shown in Figure 13, the macrophage that has phagocytosed and digested the CA contacts its surrounding macrophages once the antigens are presented on its surface. The macrophage contacts up to four neighbouring macrophages presenting the antigen that was obtained through the phagocytosis of the CA. In order to ensure the antigen presentation, 3D reconstructions were obtained in some cases. Figure 14 shows how the CA has been digested by the macrophage and is presenting its remains as antigens on its surface so other cells can recognize them.

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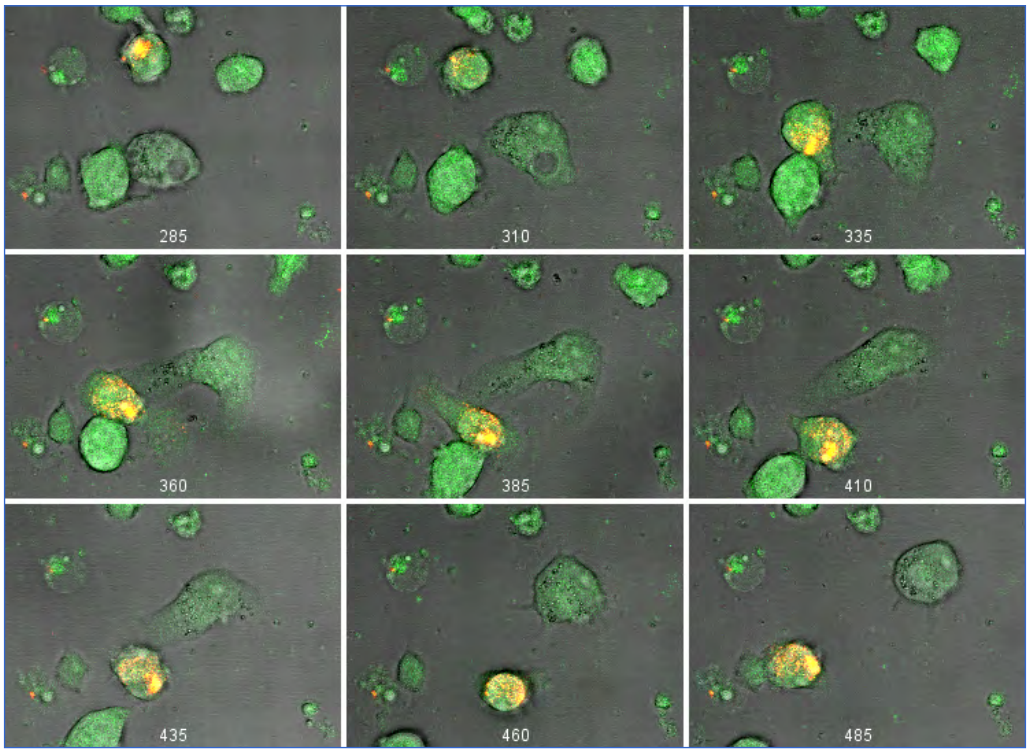

Figure 13. Series of consecutive images that follows Figure 10 and shows how the THP-1' macrophage (red arrow on the first image) digests the CA (yellow) and presents its remains to the surrounding macrophages. Elapsed time 16.45 seconds.

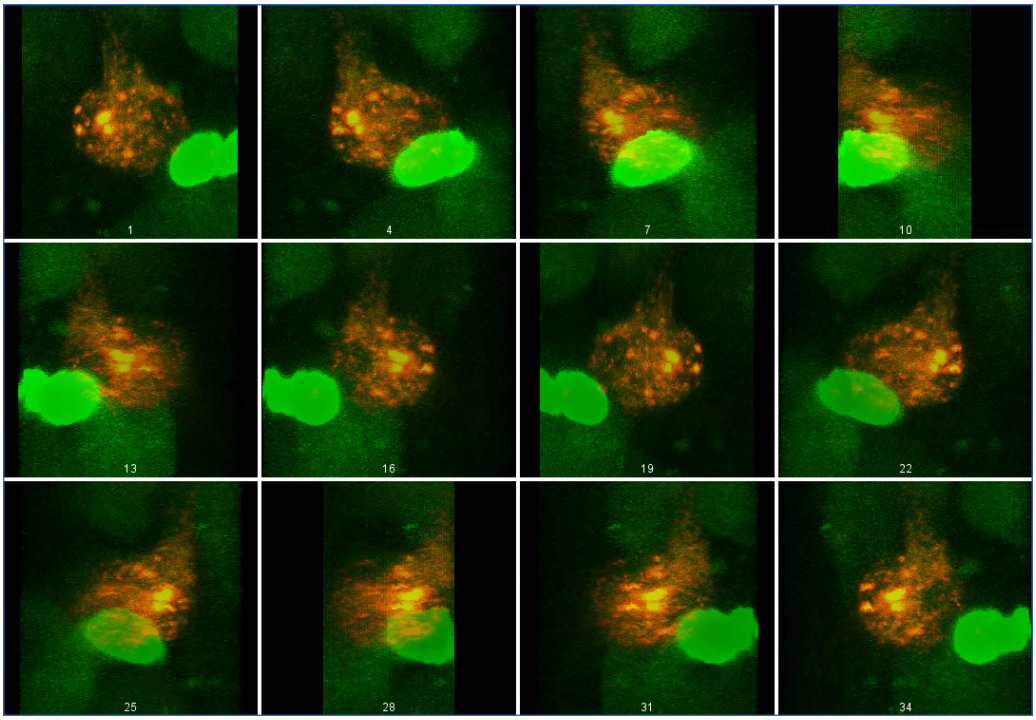

Figure 14. After the 3D reconstruction of a macrophage at the end of the process of phagocytosis, this sequence of images shows different steps of the 360° rotation of the composition and allows observation of the location of the remains of CA (red and yellow) on the surface of the macrophage.

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4. Conclusions

From the results of the experiments conducted, the following conclusions can be drawn:

- ConA-Rhodamine staining is an effective method of staining CA as it gives them a bright red colour that is easily distinguishable when observed under the microscope.
- Vybrant® CFDA SE cell tracker staining is an effective method of staining macrophages, as it gives them a bright green colour that is easily distinguishable when observed under the microscope and it does not disturb their physiological activity.
- Using both the staining methods mentioned above, the time-lapse study has been successful, as it has achieved the goal of visualizing the process of phagocytosis of a CA by a THP-1' macrophage.
- THP-1' macrophages are able to degrade the CA once they have phagocytosed them.
- Once the THP-1' macrophages have degraded the CA, they act as APCs, as they present the remains of the phagocytosed CA on their surface as antigens, while making contact with the surrounding macrophages.

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