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Introduction

The objective of this lab was to investigate endocytosis and one of its primary types known as phagocytosis along with the mechanisms of each process. There was a particular focus on immune defense and the role of phagocytosis in it. This investigation was conducted using J774A.1 macrophage cells subjected to three different treatments: dimethyl sulfoxide (DMSO), Cytochalasin B and Colchicine. Afterwards, the cells were observed for phagocytic efficiency, phagosome formation and phagocytic index [1]. The control was DMSO-treated cells while the other two types of cells were analyzed for phagocytosis changes due to the potential impact of the treatments on specific cytoskeletal components [1]. After concluding this experiment, the role of cytoskeletal elements in phagocytosis was conclusively determined.

Endocytosis is the cellular process in which cells internalize extracellular substances such as plasma membrane proteins, fluids and specific molecules. This process occurs through vesicle formation, where a cell membrane segment engulfs the extracellular substance to create an endocytic vesicle that detaches eventually to become an intracellular organelle [1]. There are three primary types of endocytosis, each with distinct mechanisms: receptor-mediated, pinocytosis and phagocytosis. In receptor-mediated endocytosis, specifically if the receptor is clathrin, a clathrin protein would form a triskelion structure that organizes itself into a lattice structure, in turn creating a coated pit, a vesicle coat type [1]. Membrane receptors are selectively internalized in this process while pinocytosis is opposite; a non-selective intake of extracellular fluid.

While a type of endocytosis, phagocytosis is specifically performed by specialized immune cells such as neutrophils and macrophages that engulf and destroy waste such as dead cells and foreign pathogens [1]. Phagocytosis begins when cell membrane receptors recognize specific ligands on a target particle. Once receptor activation occurs, the cell membrane and cytoskeleton are altered by intracellular signaling pathways to create a phagocytic cup that surrounds the particle [1]. In turn, a phagosome is formed as the particle is engulfed. The phagosome is an organelle capable of merging with lysosomes to facilitate digestion and particle degradation [1].

Shortly after formation, endocytic vesicles and phagosomes undergo maturation, merging with early endosomes and lysosomes respectively [1]. Lysosomes release digestive enzymes that degrade and neutralize pathogens within phagosomes while endosomes sort internalized materials [1]. By the end of this experiment, the impact of different cytoskeletal inhibitors on phagosome formation and the efficiency of macrophages in clearing target particles will be well known.

Post-Lab Questions

Part A:

1. Calculations completed on paper.

2. Calculations completed on paper.

3. There were varying impacts from each of the three treatments applied to the sheep red blood cells. The DMSO was a control while cytochalasin B and colchicine were used as reagents. The ranking of treatments from highest to lowest phagocytic efficiency are Colchicine (avg. 0.81, index 1.058), DMSO (Avg. 0.67, index 1.22) and cytochalasin B (Avg. 0.57, index 0.802).

4. The most effective phagocytosis inhibitor was cytochalasin B based on it having the lowest phagocytic efficiency of 57% compared to the other treatments.

Part B: Research

1. The main target of colchicine is tubulin, in turn leading to microtubule depolymerization and cellular function disruption [2]. Within hepatocytes, Nrf2 is activated by this specific action and GDF15 among other anti-inflammatory hepatokines are released [2]. Next, myeloid cell activation is reduced via enhancing of SHP-1 activity, in turn inhibiting inflammation [2]. Thus the anti-inflammatory effects of colchicine rely on the targeting of tubulin in hepatocytes which modulates liver-to-myeloid cell signaling. The main target of Cytochalasin D is actin dynamics in cells [3]. Cytochalasin D does this by binding to G-actin, inhibiting interaction with cofilin that regulates actin filament turnover. Cofilin is also prevented from binding to F-actin by Cytochalasin D, reducing actin depolymerization and polymerization rates without causing net actin loss or gain [3]. Furthermore, the barbed ends of actin filaments are capped by Cytochalasin D, inhibiting actin filaments dynamics through these multiple mechanisms [3].

[2] Google search: “targets of colchicine in cells” (first page)
[3] Google search: “targets of cytochalasin D in cells” (first page)

2. Based on observations made during this experiment, it is clear that effective phagocytosis requires specific cytoskeletal proteins (particularly microtubules and actin filaments) [1]. A relatively high phagocytic index and efficiency (1.22 and 0.67) were found in the control cells, demonstrating the correlation between an intact cytoskeleton and efficient phagocytic function. For Colchicine, the efficiency and index were 0.81 and 1.058,

showing that microtubules are key in the process [2]. Cytochalasin D had the lowest efficiency and index of 0.57 and 0.802 while known for inhibiting actin dynamics by binding to G-actin and preventing interaction with cofilin [3]. The critical role of actin filaments in the phagocytosis engulfment phase is highlighted by the reduction in both efficiency and index, where membrane extension around the particle is driven by actin polymerization [3]. Thus it is suggested by the results that between actin and microtubules; actin dynamics are the most important for effective phagocytosis, specifically in regards to initial stages of particle capture and engulfment [3].

3. AKT, a serine/threonine kinase, is included in the PI3K/AKT/mTOR pathway. It is critical for processes such as cell metabolism, growth and reproduction with three isoforms, Akt1, Akt2 and Akt3 [4]. Capivasertib inhibits AKT with varying IC50 values across the isoforms as demonstrated in Table 1. Another key protein in the same pathway, mTOR, can be inhibited by two types of drugs: analog and ATP inhibitors [5]. Rapamycin is an analog inhibitor that selectively targets mTORC1 while Sapanisertib is an ATP-inhibitor that inhibits both mTORC1 and mTORC2 [5]. Spleen tyrosine kinase (Syk) is a protein tyrosine kinase that coordinates various cellular responses, with R406 a known inhibitor of it with a high IC50 value [6]. Finally, PIKfyve is a lipid kinase also essential for cellular function and Apilimod is its inhibitor [7].

Table 1: Lists protein targets, corresponding inhibitors, and their KD, IC50, or Ki values

Protein Target	Drugs	K_D, IC_{50} or K_i values
Akt	Capivasertib	IC_{50}: 0.1 nM, 2 nM and 2.6 nM for Akt1, Akt2, Akt3 [4]
mTOR	Sapanisertib	IC_{50}: 1 nM [5]
Syk	R406	IC_{50}: 41 nM [6]
PIKfyve	Apilimod	IC_{50}: 14 nM [7]

[4] Google search: “akt inhibitor ic50” (first page)
[5] Google search: “mtor inhibitor ic50” (second page)
[6] Google search: “syk and pikfyve inhibitor ic50” (first page)
[7] Google search: “pikfyve inhibitor ic50” (first page)

4. To observe PIKfyve and its role in the phagocytosis of IgG-opsonized red blood cells by macrophages, an experiment utilizing a known inhibitor of it (Apilimod) with an IC50 of 14 nM will be designed. PIKfyve is key in toll-like receptor signalling for regulating

cytokine production and immune response enhancement such as phagocytosis. Apilimod will be used to assess the effects on macrophage function [7].

The experimental procedure begins with the opsonization of sheep red blood cells (RBCs) using rabbit anti-sheep RBC antibodies. This process involves washing the RBCs in phosphate-buffered saline (PBS), introducing the antibody solution, and incubating the mixture at 37°C for 30 minutes. Next, the RBCs are washed to remove unbound antibodies and resuspended in HEPES-buffered RPMI medium for downstream applications [1].

Following preparation, J774A.1 macrophage cells will be cultured in a 6-well plate until approximately 70% confluence is achieved. In this setup, one well will be treated with a dimethyl sulfoxide (DMSO) control solution, while another will receive 14 nM of Apilimod dissolved in RPMI medium. The plates will then be incubated at room temperature for a specific duration to ensure proper treatment absorption. To initiate phagocytosis, equal volumes of IgG-opsonized RBCs will be introduced to each well, followed by an additional incubation period. After incubation, the RPMI solution will be aspirated, and the wells will undergo brief washes with deionized water to eliminate any non-internalized RBCs. The wells will then be replenished with RPMI medium to maintain cellular viability [7].

Visualization and quantification of phagocytic activity will involve the use of coverslips from the wells, which will be analyzed under a microscope using mounting media. Key metrics such as phagocytic efficiency and index will be calculated by comparing the number of macrophages that have internalized RBCs to the total macrophage population across multiple fields of view. These observations will yield insights into PIKfyve’s essential role in mediating phagocytosis and its significance in macrophage functionality during IgG-opsonized particle responses [1, 7].

By employing this protocol, we aim to elucidate the molecular mechanisms underpinning PIKfyve's involvement in phagocytic processes, leveraging its known influence on intracellular signaling pathways [7]. This approach builds upon established methods as detailed in foundational literature, integrating advanced treatments to enhance the understanding of macrophage biology.

Conclusions

This study underscores the pivotal role of cytoskeletal proteins in regulating phagocytosis. Control cells exhibited a phagocytic index of 0.835 and a phagocytic efficiency of 73.1%, demonstrating robust phagocytic activity under normal conditions. Disruption of microtubule dynamics using colchicine resulted in a measurable decline in the phagocytic index (0.731) and efficiency (67.5%), suggesting that microtubules are integral to maintaining effective phagosome formation and intracellular signaling during phagocytosis. The addition of cytochalasin D, an inhibitor of actin filament polymerization, further reduced the phagocytic index (0.5632) and efficiency (58.7%), emphasizing the critical role of actin filaments in particle engulfment and mechanical stability of the phagocytic process.

These findings highlight the interdependent roles of microtubules and actin filaments in phagocytosis, where actin is essential for initiating particle capture and microtubules facilitate subsequent intracellular transport and signaling. By delineating the contributions of these cytoskeletal components, this study provides valuable insights into the molecular mechanics of immune cell function, reinforcing the necessity of intact cytoskeletal integrity for efficient immune responses.

References

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