Technical notes

Evaluation of Phagocytosis Mechanism by Flow Cytometry
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1. Introduction

Innate immune response constitutes the first line of defense against invading pathogens. Phagocytosis mechanism is part of innate immune response and is, therefore, essential for organism protection. Phagocytes activity involves movement processes in response to chemotaxis stimulus, adhesion, destruction and removal of digested particles. Failures in the phagocytic activity leads to immunodeficiencies than can include bacterial and fungal chronic and recurring infections \(^{1,2}\).

Phagocytic cells (neutrophils, monocytes, macrophages, dendritic cells and mast cells) present the ability of destroying microorganisms and altered stranger and own cells. In order to develop their lytic activity, phagocytic cells have receptors specialized in recognition of stranger agents which promote activation after ligand binding. Among this receptors, it is remarkable the existence of a group of phagocytosis initiating receptors, which are characterized by their ability to recognize the immunoglobulin Fc extreme (FcR or CD16), or components derived from complement activation. Besides, these cells express a group of receptors known as pathogen-associated molecular patterns, which are characterized by their ability to identify a high number of molecules presented in most pathogens, but not in own tissues.

Phagocytosis process consists of three main stages: opsonization, phagocytosis and lysis.

- **Opsonization**: Is the process by which a pathogen is labelled for recognition and further phagocytosis. In the proteolytic pathway of Complement System, peptides with opsonin functions are generated. These peptides are deposited on the surface of microorganisms and induce phagocytosis. Also, immunoglobulins can perform as opsonins.

- **Phagocytosis**: Macrophage surrounds pathogen with its membrane, building a vacuole or phagosome. These phagosomes fusion with lysosomes, leading to a phagolysosome where destruction of microorganism takes place.

- **Lysis**: Pathogen destruction.

![Phagocytosis mechanism](image)

**Figure 1: Phagocytosis mechanism**
2. Pathogen destruction methods

Pathogen lysis or destruction methods can be oxygen dependent or independent:

**Oxygen dependent lysis intermediaries:**
Pathogen destruction through reactive oxygen species is one of the methods to measure phagocytic activity by flow cytometry. NADPH oxidase enzyme, which is present in phagosomes membrane, converts oxygen into superoxide radical (O$_2^-$), which can lead to toxic substances as hydrogen peroxide (H$_2$O$_2$), hypochlorite (ClO$^-$) or hydroxide (-OH), among others. This reaction is called respiratory burst.

The products generated as a consequence of respiratory burst have such a large toxic capacity. This way, superoxide radical and H$_2$O$_2$ are agents with large bactericide capability. In this process, myeloperoxidase (MPO), a powerful bactericide enzyme that converts H$_2$O$_2$ into hypochlorous acid (HOCl$^-$) in presence of chloride, is also involved (Figure 2).

Mutations in any gene coding for NADPH-oxidase lead to chronic granulomatous disease, which is characterized by phagocytes incapability to produce superoxide radical. This is related to recurrent bacterial and mycotic infections.

![Figure 2: Components involved in respiratory burst](image)

**Oxygen independent lysis intermediaries:**
Besides reactive oxygen species, phagocytes produce reactive nitrogen species. Nitric oxide (NO) stands out among them. NO is produced by the enzymatic activity of inducible nitric oxide synthase 2 (NOSi), which is activated in response to bacterial products, mainly after IFN-γ activity. Inside phagosomes, nitric oxide can combine with hydrogen peroxide or with superoxide leading to peroxide nitrates, which have a powerful pathogenic microorganism destructive capacity.

Besides these intermediaries, charged proteins that damage bacteria cell membrane, lysozymes that destroy bacteria cell wall, lactoferrins that remove iron from media (essential metal for bacteria) and metalloprotease and hydrolytic enzyme that digest destroyed bacterial proteins can also be produced and secreted.
3. Diseases caused by defects in phagocytosis

Patients with immunodeficiency are clinically diagnosed by recurrent infection background. The specific agent responsible for the infection can point towards which immune system part is affected. Deficiencies at innate immune system consist mainly of complement and phagocytic dysfunction and restricts our capacity to combat infections, although adaptive immune response is apparently effective.

As well as many defects related to bone marrow insufficiency, deficiencies in aggregate formation and adhesion of neutrophils to endothelial surfaces (adhesion lack) and signaling defects involving IFNγ receptor or IL-12 receptor among others (mendelian susceptibility to mycobacterial disease); the most common genetic defects related to innate immune system affect to phagocytic activity and pathogen lysis (3). All these deficiencies lead to severe chronic infections by specific pathogens (bacteria, yeast and fungi), which are resistant to standard treatments.

Respiratory burst is altered in diseases such as chronic granulomatous disease (CGD), myeloperoxidase (MPO) deficiencies and glucose-6-phosphate dehydrogenase (G6PD) deficiencies. In conclusion, this genetic alteration blocks the accumulation of an oxidative environment in phagosomes, and phagocytes are not able to efficiently destroy internal or external pathogens.

**Chronic granulomatous disease (CGD)** comprises a clinical and genetically diverse group of hereditary diseases with deficiencies in several enzymes belonging to the NADPH oxidase pathway, which generates oxygen reactive species in phagosomes, contributing to pathogen destruction (Figure 2). CGD patients suffer from pneumonia, skin, tissues and organs abscesses, suppurative arthritis, osteomyelitis, bacteremia or fungemia, and superficial skin infections, for example cellulitis or impetigo (4). On the other hand, infection persistence can develop into granulomas containing T CD4+ cells that excrete TNF and IFNγ. It is crucial to emphasize that CGD patients not only suffer from recurrent infections, but also suffer from inflammation under no infectious conditions (5).

Flow cytometry provides an indirect way of measuring the capacity of neutrophils to initiate the respiratory burst by evaluation of a fluorescent coloring (2).

**MPO deficit** is one of the most frequent hereditary disorder related to phagocytosis. Around half of the patients show a full MPO deficiency, the rest only show functional or structural alterations in the enzyme. MPO is the main component in neutrophils, and is the enzyme responsible for production of hydroxyl radical and hypochlorite using hydrogen peroxide. Hypochlorite is the most effective agent in pathogen destruction during respiratory burst (6). MPO lack is usually asymptomatic, with the exception of patients suffering from immune response conditioning diseases such as diabetes, which show susceptibility to suffer disseminated candidiasis.

This disease can be detected by flow cytometry using anti-MPO antibodies and, also in a complementary and indirect way, noticing a lower respiratory burst than in a normal control (3).

**G6PD deficiency.** G6PD enzyme act as a catalyst to generate enough NADPH in order to allow NADPH oxygenase to produce superoxide (O2-) and, therefore, enable respiratory burst to take place in phagosomes (Figure 2). When the activity of this enzyme is low, this lack is expressed by a respiratory burst decrease in phagosomes (7) and also by chronic nonspherocytic hemolytic anemia.
4. Why and when phagocytic activity should be studied

Phagocytic activity must be studied to confirm or discard defects in phagocytosis, which can lead to primary and secondary immunodeficiencies. In primary immunodeficiencies diagnosis, phagocytosis evaluation is the first election method. Clinical symptoms derived from hereditary immunodeficiencies usually appear during the first years of life, however less severe types can show up during adulthood. Without an accurate and premature therapy, these pathologies can be life threatening.

**Primary immunodeficiencies**
- Chronic granulomatous disease (defective bactericide mechanisms caused by altered function of NADPH oxidase, with an incidence of 1/200000 people; it is clinically characterized by severe infections and granulomas located in organs)
- Myeloperoxidase alterations (is characterized by candidiasis and is a relatively common alteration)
- Glucose 6-phosphate dehydrogenase deficiencies.
- Glutathione synthetase alterations (regeneration of altered NADPH)
- Chédiak-Higashi syndrome (abnormal granulocytes, degranulation alteration)
- Aberrant digestion

**Secondary immunodeficiencies**
They are caused by external agents, including drugs. Evaluation of phagocytic activity is recommended in order to monitor the patient clinical condition after treatment with the following drugs:
- Corticoid and other immunosupressive drugs
- Cytostatics (cyclophosphamide and other myelotoxic agents)
- Cytokines and growing factors (G-CSF, GM-CSF, IFNγ, TNF)
Also recommended in the following circumstances:
- After hematopoietic stem cells transplant
- In Systemic Inflammatory Response Syndrome (SIRS) cases

A poor phagocytic activity means a high risk of infection for the patient that can, by end, cause a sepsis.

Evaluation of phagocytic function in patients showing the next symptoms is recommended:
- Chronic and recurrent skin and mucosa infections (pyoderma, abscesses, impetiginous dermatitis, disseminated candidiasis, ulcerative inflammation)
- Lymphatic ganglia infections
- Gingivitis and aphthous stomatitis
- Respiratory tract infections (otitis, sinusitis, bronchitis)
- Pelvic inflammatory disease
- Osteomyelitis, meningitis
- Sepsis
- Secondary lymphadenitis after tuberculosis vaccine injection
- Infections caused by pyogenes bacteria, fungi and parasites
5. Evaluation of phagocytic activity by flow cytometry

Flow cytometry in the study of phagocytic activity of neutrophils and macrophages shows some advantages over other methods; it can evaluate phagocytic activity in relatively small samples, which is important in newborn and infants. It is a fast method and allows evaluation of biochemical and functional characteristics simultaneously \(^2\)(8)(9).

**Phagocytosis targets in flow cytometry evaluation:** usually Candida sp. or Escherichia sp. bacteria are used for phagocytosis induction, but it has also been found induction by other targets such as zymosan, fungi or polystyrene spheres. These particles or microorganisms can be opsonized with serum before incubation with phagocytes in order to achieve a more efficient phagocytosis, however, in presence of high concentration of targets and also during long incubation periods, opsonization-independent phagocytosis is promoted.

**Flow cytometry phagocytosis detection methods.** There are plenty of methods and protocols, some of the easiest are:
- Incubation of FITC labeled bacteria with phagocytes and, after phagocytosis process, addition of ethidium bromide in order to label the non-phagocyted organisms. FITC is able to excite ethidium bromide contained in the non-phagocyted microorganisms, and this will produce red emission. In the meanwhile, green emission from FITC coming from phagocyted microorganisms will also be detected \(^5\).
- FITC labeled bacteria are incubated with the sample. In order to differentiate between the free fluorescence material and the material bound to the membrane of the phagocyted material, a solution that inhibits fluorescence of non-phagocyted rests needs to be added after incubation period.

**Detection of defects in respiratory burst in granulocytes.** This method is used at diagnosis in chronic granulomatous disease. It is based on detection of oxidative products after incubation of phagocytic cells with bacteria able to promote respiratory burst. After phagocytosis, granulocytes activate NADPH-oxidase, giving intermediary reactive products that are able to oxidize some substances added in the incubation [for example, dihydrorhodamine-123 (DHR123) that leads to rhodamine-123, which can be detected by flow cytometry] \(^5\).
6. References


7. van Bruggen R, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. *Blood* 2002; **100**: 1026–1030.

