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Macrophages Clean Up: Efferocytosis and Microbial Control

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Abstract

Phagocytic leukocytes, predominantly macrophages, not only ingest and destroy invading pathogens, but are charged with clearing dead and dying host cells. The process of engulfing apoptotic cells is called efferocytosis and has long been appreciated for its role in the resolution of inflammation. New evidence is emerging that efferocytosis represents a double-edged sword in microbial immunity. While efferocytosis of influenza and *Mycobacterium tuberculosis*-infected cells results in pathogen destruction, efferocytosis of *Leishmania*-infected neutrophils may promote infection. Understanding how macrophages, dendritic cells (DC) and neutrophils process pathogens encased within a dying cell could lead to the development of novel therapeutics that simultaneously suppress inflammation and promote pathogen clearance.

The Many Jobs of the Macrophage

Macrophages function primarily as phagocytes during innate immune responses, but perform diverse actions in homeostasis and disease. During infection, detection of pathogen-associated molecular patterns (PAMPs) by macrophages leads to the production of cytokines and chemokines, promoting the recruitment of other cells and triggering an immune response. By degrading pathogen-derived antigens and presenting them to T cells, macrophages can promote or perpetuate adaptive immunity [1]. Moreover, by engulfing and degrading bacteria, macrophages sterilize tissue, resolve inflammation, and prevent further stimulation of the immune system.

In addition to their roles in the immune response to infection, macrophages serve a second, possibly more important function. They are the cell type primarily responsible for efferocytosis, the process of engulfing and eliminating apoptotic cells. Apoptosis, or programmed cell death, is necessary for the development of multicellular organisms, and even in adults, thousands of cells die and are replaced every second [2]. Two key processes ensure that apoptosis is a non-inflammatory form of cell death: 1) the dying cell's contents are packaged into apoptotic bodies with intact plasma membranes [3]; and 2) the apoptotic bodies are engulfed by phagocytes. Without their swift removal via efferocytosis, apoptotic

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bodies disintegrate and release their intracellular contents. This process, known as secondary necrosis, leads to inflammation and can contribute to autoimmunity [4].

Efferocytosis: A Distinct Process

Although efferocytosis resembles phagocytosis, it is a distinct process, mediated by specific receptors, bridging molecules, and downstream signaling pathways. Phagocytosis by macrophages has been extensively reviewed [5,6]. Briefly, it is the process by which macrophages capture and engulf small particles or microbes. The engulfment of microbes relies on the signaling of Rho family GTPases, specifically RhoA, and subsequent actin cytoskeleton rearrangement [7]. The macrophage forms actin protrusions around the microbe, which fuse, resulting in complete engulfment (Figure 1). The resulting intracellular vesicle is termed a phagosome and initially possesses an environment similar to the extracellular milieu. Maturation of the phagosome is a step-wise process involving Rab GTPase-driven fusion of endosomes and lysosomes. These small organelles dock, releasing hydrolytic and catabolic enzymes. The resulting phagolysosome continues to acidify to a pH of nearly 4, leading to the destruction of the microbe [8].

The engulfment of apoptotic cells by efferocytosis more resembles macropinocytosis [9]. The receptors that recognize an apoptotic cell and mediate efferocytosis are distinct from those that mediate phagocytosis (see below), and once an apoptotic cell is engulfed, the intracellular signaling further diverges. During efferocytosis, RhoA activity is suppressed and Rac1 activity coordinates the engulfment of the apoptotic body and formation of a spacious phagosome, termed an efferosome, which surrounds the newly engulfed apoptotic cell (Figure 1). While new GTPases are being identified that have a role in efferosome maturation, the end result is similar to phagocytosis: successive lysosome fusion events deliver hydrolytic enzymes to the maturing efferosome. This together with its gradual acidification ultimately establishes harsh conditions that destroy the apoptotic cell [8,10].

Find Me, Catch Me, Eat Me

The process of efferocytosis is highly regulated and begins with the apoptotic cell. An early event during apoptosis is the exposure of phosphatidylserine (PS) to the exofacial leaflet of the phospholipid membrane (Figure 2A). Another early event is the release of chemokines [7,11], or “Find Me” signals by the dying cell that recruits macrophages to the site of cell death (Figure 2A). In response, the macrophage increases the expression of bridge molecules and receptors that act as tethers for the “Catch Me” step (Figure 2B). Finally, the “Eat Me” step occurs when the macrophage engulfs the dying cell and generates the large spacious efferosome (Figure 2C). Efferocytosis is said to be anti-inflammatory as macrophages produce IL-10, TGF- β , and PGE2 during the engulfment of apoptotic cells in model systems [12–15]. Thus, efferocytosis is important for the resolution of inflammation not only because it removes dead cells, relieves tissue congestion, and prevents release of phlogistic cellular contents, but also promotes tissue repair and wound healing [16,17].

Efferocytosis and Pathogen Clearance

Some pathogens have evolved mechanisms to survive intracellularly as a means of immune evasion. These pathogens have a remarkable diversity of virulence mechanisms that target and modify host physiological processes to persist within their host cell [18]. Host cells have a variety of ways to deal with intracellular infection, one of them being apoptosis. Activation of either the intrinsic (caspase-9-dependent) and extrinsic (caspase-8-dependent) cell death pathways can lead to apoptosis of the infected cell. Cytotoxic T lymphocytes can induce apoptosis in infected cells through exocytosis of cytotoxic granules that contain perforin and granzymes or via the Fas/FasL pathway [19–21]. Given the abundance of cell

death that accompanies intracellular infection, researchers have begun to investigate the fate of microbes inhabiting a cell that undergoes apoptosis and is engulfed by a phagocyte. Two possibilities have been described: the pathogen can be destroyed along with the engulfed apoptotic cell (host antimicrobial activity) or the pathogen can use the process of efferocytosis to disperse into new cellular hosts (“Trojan Horse” model). The intersection of efferocytosis and intracellular pathogen fate has only been studied in a few diseases. However any pathogen that relies on intracellular infection, particularly where apoptosis or cytolysis is linked to control, should be investigated for the role efferocytosis may play.

Examples of Efferocytosis and Microbial Immunity

During *Leishmania major* infection, efferocytosis of infected neutrophils may promote infection by the “Trojan Horse” model of disease. The infectious sand fly bite recruits neutrophils that rapidly phagocytose *L. major* metacyclic promastigotes and this is crucial for the establishment of infection. Parasite infection induces neutrophils apoptosis, which then attracts macrophages and DCs. In vitro models demonstrate that macrophages efferocytose these infected apoptotic neutrophils [22–25]. In vivo, DCs have also been found to engulf these infected apoptotic neutrophils [26]. Unfortunately, whether *L. major* survived after DC efferocytosis of infected neutrophils was not assessed. As it appears that *L. major* uses apoptotic neutrophils as a Trojan Horse to gain entry into its preferred terminal host cells, the macrophage, it is important to understand how the parasite survives in the efferosome.

Mycobacterium tuberculosis typically avoids inducing apoptotic death of the macrophage it infects and instead skews the death modality towards a necrotic fate, which facilitates its spread into neighboring cells and avoids immune clearance [27]. For reasons not entirely understood, some *M. tuberculosis*-infected macrophages die by apoptosis, which has long been known to be associated with suppression of bacterial growth [28,29]. *M. tuberculosis*-infected macrophages that die by apoptosis are rapidly engulfed by uninfected macrophages via efferocytosis and this process is responsible for the bactericidal effect associated with apoptosis [30]. Blocking efferocytosis both *in vitro* and *in vivo* abrogates bacterial control. *M. tuberculosis* killing post-efferocytosis is due to lysosome fusion as both the internalized apoptotic bodies and the bacteria are degraded simultaneously by the degradative enzymes delivered via the lysosomes. It is interesting to speculate why efferocytosis allows lysosome recruitment to the *M. tuberculosis*-containing phagosome while *M. tuberculosis* typically arrests phagosome maturation following phagocytosis of naked bacteria. The mechanism of efferocytic killing remains an important avenue for further research and will be discussed more in the next section. Similarly, *Streptococcus pneumoniae* infection of macrophages causes apoptosis and is linked with pathogen control [19]. This may suggest that efferocytosis is involved in control of the infection.

Mycobacterium marinum is a natural marine pathogen, and efferocytosis has similarly been described as a means to control this microbe in a zebra fish model of innate *M. tuberculosis* infection [31]. *M. marinum*-infected macrophages dying by apoptosis are engulfed by recruited neutrophils. Following efferocytosis of the *M. marinum*-infected macrophage, the neutrophil generates an NADPH-dependent oxidative burst, which kills *M. marinum* [32]. What is unknown is what is stimulating the ROS production. Oxidative burst is a mechanism used by innate cells to control pathogens; however, its upregulation is dependent upon pathogen recognition [33]. It is unknown whether an efferocytic cell senses the pathogen within the apoptotic cell. Alternatively, all cells generate an NADPH-dependent oxidative burst following apoptotic cell engulfment in response to the large ‘meal’ of oxidized lipids [34]. The cell employs many compensatory mechanisms to ensure that an apoptotic cell can be engulfed without damaging or triggering apoptosis in the efferocytic cell [35].

Influenza A induces apoptosis of pulmonary epithelial cells and in cultured cell lines. Co-culture of infected epithelial cells or in vitro cell lines with macrophages leads to efferocytosis [36]. Importantly, the macrophages were found to contain viral particles inside their efferosomes after efferocytosis. Blocking apoptosis inhibits efferocytosis of these virus-infected cells. During efferocytosis, there was a decline in viral titers, suggesting that for influenza A, efferocytosis is an antiviral mechanism. These data supports the idea that efferocytosis may have microbicidal activity against multiple pathogens [37].

How Efferocytosis Mediates Pathogen Clearance: Potential Mechanisms

Why efferocytosis leads to pathogen killing while the antimicrobial effect of phagocytosis and phagolysosome fusion is frequently circumvented is an important avenue for research. A few potential mechanisms have been put forward to explain the bactericidal effects of efferocytosis (Fig.3). Many pathogens actively interfere with phagosome maturation. In the case of *M. tuberculosis*, several bioactive lipids decorate the surface of the bacterium, which interferes with endosome and lysosome recruitment, thus arresting phagosome maturation and enabling *M. tuberculosis* to live within a non-caustic and protected vesicle [38,39]. Perhaps 'double-wrapped' behind both an apoptotic cell membrane and efferosome sequesters *M. tuberculosis*' virulence factors from finding their targets in the cytosol. Many intracellular pathogens, such as *Legionella*, *Coxiella*, and *Salmonella*, similarly rely on secreted proteins to exert control over their host cells and thus 'double-wrapping' may contribute to the clearance of other infections [40–42]. Additionally, a different subset of small GTPases regulates the maturation of an efferosome than a phagosome [8,43]. Perhaps these molecules are not the targets of pathogens' arsenal and thus efferosome maturation can continue uninterrupted with the successive acidification and destruction of pathogen and apoptotic cell alike. *Leishmania*, however, may avoid efferocytic killing [22]. *L. major* amastigotes subsist within acidic lysosomes and thus remain immune to killing via this strategy [44]. *Leishmania* promastigotes extensively remodel the phagosome in which it resides, by altering the composition of the lipid bilayer, preventing lysosome fusion and manipulating the surrounding actin cytoskeletal network [45,46]. Perhaps the mechanisms of phagosome disruption employed by *L. major* are so thorough that efferosome maturation is similarly inhibited. Furthermore, autophagy contributes to clearance of many intracellular pathogens, and LC3 and other autophagic components may localize to the efferosome [47–49]. More work is required to understand how autophagy may contribute to control following efferocytosis.

Little is known about the signaling within macrophages following uptake of an infected apoptotic cell. Efferocytosis typically evokes anti-inflammatory cascades, such as IL-10 and TGF- β production by the engulfing cell, as a means to dampen any potential self-directed immunity generated from the presence of dying cells [12,50]. Efferocytosis of infected apoptotic cells presents the phagocyte with two simultaneously conflicting signals: PAMPs that drive inflammation, and apoptotic cell ligands that trigger an anti-inflammatory program. Efferocytosis of an infected apoptotic cell by DC elicits a different response than is triggered when a macrophage engulfs an infected apoptotic cell [51]. IL-6 and TGF- β production from DCs following efferocytosis of an infected apoptotic cell drive differentiation of Th17 responses [52]. The unique signals produced by macrophages and neutrophils following infected apoptotic cell efferocytosis remain unknown.

Further Directions

The role of efferocytosis in microbial immunity is just now emerging. We envision efferocytosis as a critical part of the immune system's tool kit for pathogen destruction. Some microbes are easily killed by phagocytosis such as extracellular bacteria (Fig. 4a). For

other more virulent pathogens, the macrophage requires help from immune cells. For example, T cell cytokines activate the macrophage to enhance the expression of bactericidal functions (Fig. 4b). Finally, if infection overwhelms the macrophage, cytotoxic T cells can recognize heavily infected cells and induce them to die by apoptosis, or the infected cell may undergo apoptosis on its own because of increased stress. Either way, this now allows recruited macrophages to engulf and destroy the pathogens (Fig. 4c)

Efferocytosis, as both a means of pathogen clearance and inflammation suppression may be amenable for pharmacological targeting. In fact, the concept of promoting efferocytosis is already being considered by the atherosclerosis field as a way to alleviate plaque formation [17]. Harnessing the body's own means and mechanisms of pathogen killing and immunoregulation is an attractive way to combat disease. Especially at a time when drug resistance by bacteria and viruses is increasing, the ability to provide new treatments that augment the body's own defenses without targeting the microbe may alleviate the looming drug resistance crisis and be effective against otherwise recalcitrant pathogens.

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Highlights

- Apoptotic cell death commonly follows intracellular pathogen infection.
- Macrophages engulf and digest dying cells via the process of efferocytosis.
- Efferocytosis of an infected apoptotic cell can result in pathogen destruction
- This process represents a novel innate immune antimicrobial function

Figure 1. Efferocytosis is a distinct process from phagocytosis

In phagocytosis, increased levels of RhoA result in actin polymerization and stress fiber formation, allowing receptor-mediated uptake of microbes. Fusion of the actin projections leads to internalization of the bacteria in a phagosome, which undergoes stepwise maturation, leading to degradation of the pathogen. This process can lead to both tissue sterilization and antigen presentation. In contrast, efferocytosis results from increased levels of Rac1, causing formation of lamellipodia and membrane ruffles upon actin polymerization, leading to the internalization of infected and ‘double-wrapped’ apoptotic bodies in an efferosome. From here, the ‘double-wrapped’ pathogen can be destroyed if the efferosome undergoes acidification, similar to the process of phagolysosome fusion. However, some pathogens appear to be able to use the process of efferocytosis to disseminate and disperse.

Figure 2. Efferocytosis involves the regulated uptake and degradation of apoptotic bodies

When an infected cell undergoes apoptosis (A), the pathogen and its antigens are presumably packaged along with other intracellular contents into apoptotic bodies which have intact cellular membranes. During apoptosis, the dying cell produces “Find Me” signals, such as chemokines that recruit macrophages and other phagocytic cells to the site of the dying cell, along with the accumulation of phosphatidylserine (PS) on the exofacial leaflet. In the “Catch Me” phase, recruited macrophages express specific receptors and bridge molecules that bind to PS or other distinct ligands on the apoptotic cell (B). Binding between the apoptotic cell and the scavenger macrophage activates Rac1, leading to actin reorganization and production of projections that surround the infected apoptotic cell, ultimately surrounding and engulfing the apoptotic cell in the efferosome (C). The efferosome undergoes step-wise maturation that includes fusion with lysosomes and endosomes, leading to rapid degradation of both the apoptotic cell and the intracellular pathogen.

Figure 3. Four Proposed Mechanisms of Efferocytic Control

Following efferocytosis of an infected apoptotic body there are 4 potential mechanisms that contribute to microbial killing. (A) A microbe's secreted virulence factors, which typically penetrate the phagosome, are trapped behind additional membrane from the apoptotic cell; consequently, they cannot reach their host targets. As it has been proposed that the machinery involved in efferosome maturation is different than phagosome maturation, (B), microbial virulence factors are ineffective at inhibiting efferosome maturation. Following efferocytosis, autophagic machinery can be recruited to the nascent efferosome (C). A, B, and C all ultimately allow lysosome fusion. In (D), reactive oxygen species generated in response to efferocytosis indirectly kills the pathogen.

Figure 4. Efferocytosis is one mechanism that the host can use to control infection

Macrophages autonomous control (A) frequently happens with low virulence or extracellular pathogens following phagocytosis. For pathogens that are more adapted to the intracellular environment of the macrophage, the macrophage needs help and collaborates with T cells (B). T cell recognition of the macrophage leads to the elaboration of T cell cytokines such as IFN γ and TNF, which activate the macrophage and enhance phagolysosomal fusion but also stimulates other antimicrobial pathways, which augments the ability of the macrophage to kill intracellular bacteria. Some pathogens can overwhelm macrophage defenses. Under these conditions, the macrophage may die by apoptosis (C) if it is recognized by CTL; alternately, cell intrinsic factors may trigger an apoptotic death. Death by apoptosis ultimately allows for efferocytosis.