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MINIREVIEW

I'm Infected, Eat Me! Innate Immunity Mediated by Live, Infected Cells Signaling To Be Phagocytosed

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ABSTRACT Innate immunity against pathogens is known to be mediated by barriers to pathogen invasion, activation of complement, recruitment of immune cells, immune cell phagocytosis of pathogens, death of infected cells, and activation of the adaptive immunity via antigen presentation. Here, we propose and review evidence for a novel mode of innate immunity whereby live, infected host cells induce phagocytes to phagocytose the infected cell, thereby potentially reducing infection. We discuss evidence that host cells, infected by virus, bacteria, or other intracellular pathogens (i) release nucleotides and chemokines as find-me signals, (ii) expose on their surface phosphatidylserine and calreticulin as eat-me signals, (iii) release and bind opsonins to induce phagocytosis, and (iv) downregulate don't-eat-me signals CD47, major histocompatibility complex class I (MHC1), and sialic acid. As long as the pathogens of the host cell are destroyed within the phagocyte, then infection can be curtailed; if antigens from the pathogens are cross-presented by the phagocyte, then an adaptive response would also be induced. Phagocytosis of live infected cells may thereby mediate innate immunity.

KEYWORDS phagocytosis, phagoptosis, infection, immunity, virus, intracellular bacteria

Mammalian cells can be infected by a variety of pathogenic agents, including bacteria, viruses, fungi, protozoa, and prions. Intracellular niches within host cells are attractive for many such pathogens, providing the metabolic building blocks and protection from host immune surveillance that are often essential for their propagation, not to mention the obligate reliance of viral infections on the host cell's genetic and/or translational machinery. Such infections can overrun host cells, exploiting their resources to spread from one cell to another and between organisms, harming or killing their hosts. To limit this, the mammalian innate immune system can detect such infections and attempt to eliminate or clear the pathogen as quickly as possible, independently of any adaptive immune response that may eventually develop. If this fails, the infected cell may trigger its own cell death. However, while this stops infection of the dying cell, it may aid spread to other cells. Here, we hypothesize and review the evidence that infected cells may, in some circumstances, directly induce phagocytosis of themselves without undergoing cell death, thereby being eaten alive and mediating pathogen clearance. This hypothesis is outlined in Fig. 1.

PATHOGEN DETECTION

Infections can be detected when pathogen-associated molecular patterns (PAMPs) activate any of a variety of host cell pathogen recognition receptors (PRRs) (1). PAMPs represent structural motifs common to many pathogens, which are therefore useful for the immune system to detect, including bacterial lipopolysaccharide (LPS), flagellin, and viral genetic material. This drives an innate immune response, including upregulation of complement factors, release of microbiocidal agents, cytokine signaling, and

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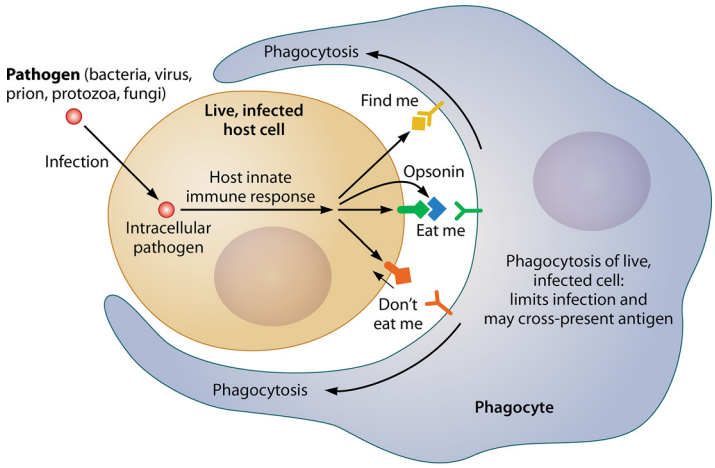

FIG 1 Outline of the hypothesis that phagocytosis of live infected cells contributes to immunity. Pathogen infection of host cells triggers an innate response that may include release of find-me and eat-me signals, binding opsonins, and downregulating don't-eat-me signals, resulting in host phagocytes phagocytosing the live infected cell, thereby killing the pathogen and limiting infection.

the activity of natural killer cells and phagocytes. Some of these innate responses are essential prior to development of adaptive responses (2). Cell surface PRRs, including toll-like receptors (TLRs) and Dectins, detect extracellular PAMPs and usually instigate general proinflammatory signaling within and between cells (3–5). In contrast, intracellular PRRs sense intracellular PAMPs, indicative of more serious intracellular invasion. Thus, these RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and AIM2-like receptors (ALRs) drive more dramatic cellular events, including cell death. Pathogens generally stimulate multiple PRRs and indeed other pathways too, giving multiple potential outcomes for the cell (3–5).

HOST CELL DEATH INDUCED BY INFECTION

Infection may induce cell death of the host cell as a protective response to deprive pathogens of intracellular niches and curtail their replicative cycles (6). We briefly outline host cell death induced by infection here for the purpose of comparison to our hypothesis. Apoptosis is mediated by caspases and Bcl-2 homologous proteins and causes nuclear condensation, membrane blebbing, and cell shrinkage. Cellular functions are shut down, and phosphatidylserine (PS) exposure is used to signal for phagocytic engulfment in a process known as efferocytosis (7). Apoptosis and other forms of cell death have been hypothesized to be intrinsically antimicrobial by killing the infected host cell and thereby limiting replication of the pathogen and, in some cases, killing the pathogen (6). However, the subsequent engulfment of infected cells by phagocytes is now considered the main cause of microbial death instead, via acidification, reactive oxygen species (ROS), and enzymatic degradation within phagolysosomes (8–10). In contrast to most host cells, phagocytes are professional killers, armed with dedicated pathogen-killing mechanisms, including NADPH oxidase, inducible nitric oxide synthase, and peroxidases (10). Thus, apoptosis itself, as opposed to the subsequent phagocytosis, may have limited antimicrobial activity. In both mice and *Drosophila*, inhibition of phagocytosis exacerbates viral infections, indicating that phagocytosis (rather than apoptosis) of infected cells is central to viral immunity (9, 11).

In addition, apoptosis is normally strongly anti-inflammatory and inhibits antigen presentation by phagocytes, suppressing both innate and adaptive responses to pathogens (12); though, it should be noted that such apoptosis can be proinflammatory under certain circumstances (13, 14). Furthermore, apoptotic cells often detach from the extracellular matrix and from other cells and breakup into soluble apoptotic bodies that may spread

infection (15–17). Thus, apoptosis may be the opposite of what is required to fight an infection.

In contrast to apoptosis, necrotic forms of cell death, such as necroptosis and pyroptosis, are inherently lytic and induce rupture of the plasma membrane prior to phagocytosis of the cell. They therefore promote inflammation through release of cytosolic proinflammatory mediators and damage-associated molecular patterns (DAMPs). Necrotic death of the cell protects against infection by depriving the pathogen of its host cell, and pyroptosis in particular may trap microbes within the cell corpse or release peptides that directly kill bacteria (18, 19). However, lytic cell death may also promote spread of infection by releasing the live pathogen (20, 21). For example, mycobacterial infections can result in both programmed and secondary (subsequent to apoptosis) necrosis of infected cells, thus allowing lytic release and spread of the mycobacteria (22, 23). For this reason, many pathogens actively induce host cell necrosis themselves through production of proteases, phospholipases, and cytolysins in order to leave the host cell (24).

Thus, some pathogens encourage and exploit host cell death, while other pathogens instead block it, indicating that host cell death may limit their infections (25–28). However, clearing pathogen-infected cells prior to host cell death is potentially better than afterwards, as it may act sooner, avoid dispersal of the pathogen, be more likely to kill the pathogen, invoke less aggressive inflammation than necrotic cell death, and enable the cross-presentation of microbial antigens at earlier stages of infection.

PHAGOCYTOSIS OF LIVE CELLS

It was thought that host cells were only phagocytosed when dead or dying. However, it is now clear that host cells can be phagocytosed alive in a wide variety of contexts, including macrophage phagocytosis of viable neutrophils, neurons, and tumor cells (29–33). This generally results in death of the engulfed cell, resulting in a type of cell death we have termed "phagoptosis," cell death due to phagocytosis (34).

This raises the possibility that infected cells are eaten alive, as alluded to in a recent excellent review on macrophage phagocytosis (35). Below, we will review the evidence that this occurs and that infected cells release find-me signals, eat-me signals, and opsonins, which induce this phagocytosis. These signals regulate the phagocytosis of live and dead host cells as well as pathogens (36–38).

Analogous to our hypothesis that innate immunity against infection is partly mediated by phagocytosis of live infected cells is the relatively recent discovery that innate immunity against cancer is partly mediated by phagocytosis of live cancer cells by host phagocytes (29–31). This has led to increasing interest in the field of phagocytosis of live cells and the development of multiple experimental treatments promoting host phagocytosis of live cancer cells (39). In addition, it has been found that certain cancer treatments promote antigen presentation by the phagocytes engulfing the cancer cell (40). Thus, there is a clear precedent for our hypothesis that innate immunity against infection is partly mediated by phagocytosis of live infected cells and that this might promote an adaptive response via antigen presentation.

FIND-ME SIGNALS

Find-me signals are chemoattractants released by cells to guide phagocytes to their location, facilitating their engulfment, and include nucleotides and chemokines (37).

ATP is released as a "danger" signal by injured, stressed, or infected cells, either passively due to cellular damage or actively through mechanisms such as pannexin hemichannels or vesicular exocytosis (41, 42). For example, HeLa, COS-7, and T84 cells infected with *Escherichia coli* release ATP (43) via Toll-like receptor-mediated exocytosis, and this ATP stimulates macrophage phagocytosis and reduces bacterial loads *in vivo* (44). Macrophages infected by *Leishmania donovani* also released ATP, but via pannexin-1 channels (45). Finally, one notable study found that in the brain, live herpesvirus-infected neurons release ATP, which recruits microglia that then phagocytose the

live infected cells and limit the infection (46). This study directly demonstrated our hypothesis.

The nucleotide UDP is another find-me signal that is also released from *E. coli*-infected mice and lipopolysaccharide-treated macrophages via gap junctions, which reduces bacterial loads *in vivo* (47, 48). Vesicular stomatitis virus infection of macrophages causes similar release alongside upregulation of the UDP receptor P2Y6, leading to reduced viral infection in mouse models; infection was decreased by addition of UDP and increased by P2Y6 knockout *in vitro* and *in vivo* (49). Chemotaxis to UDP-releasing macrophages may in some cases be mediated by the chemokine MCP-1 (48). Notably, data from other studies show that UDP released from stressed cells may also stimulate phagocytosis of such cells (50, 51).

Alongside nucleotides, chemokines are another extremely common class of find-me signal. The CC chemokines macrophage inflammatory protein-1α (MIP1α) and macrophage chemoattractant protein-1 (MCP-1) are upregulated before cell death in macrophages infected by influenza, hepatitis C virus, and the bacterium *Orienta tsutsugamushi* (52–54). Additionally, other infections such as by influenza and human rhinovirus have been shown to elicit CXCL10 release from live host epithelial and alveolar type II cells, which may guide macrophage chemotaxis (55–57). Other chemokines known to recruit macrophages include CXCL8/12, CCL3/4/13/19/21/24/25, and XCL2 (57).

Thus, find-me signals can be released by live infected cells, thereby attracting phagocytes. However, it should be appreciated that different host cell types differ in their intrinsic capacity to detect pathogens and release find-me signals such as chemokines in response. For example, though many cell types will be capable of chemokine release (and as discussed here, they may be likely to activate this when infected), other populations simply will not be. In contrast, other signals that regulate phagocytosis, such as certain eat-me signals and don't-eat-me signals as we will now discuss, are likely to be observed more ubiquitously given their broader physiological importance to most, if not all, cell types.

EAT-ME SIGNALS: PHOSPHATIDYLSERINE AND CALRETICULIN

An eat-me signal is a signal on a cell inducing a phagocyte to phagocytose the cell. The most well-studied such signal is the membrane phospholipid phosphatidylserine. Though usually confined to the internal face of the plasma membrane by ATP-dependent flippases, once externalized by scramblases, phosphatidylserine can be bound by phagocytic receptors on phagocytes to induce phagocytosis. Phosphatidylserine exposure was once thought to only occur during apoptosis, where caspase activity deactivates the flippases that maintain phosphatidylserine asymmetry and activates the scramblase XKR8 (7). However, phosphatidylserine exposure is now known to occur on viable cells in a variety of circumstances, such as immune activation, oxidative stress, or calcium elevation (42, 58–62). Phosphatidylserine exposure on viable cells is mediated by calcium-activated scramblases, such as transmembrane protein 16F (TMEM16F), and is reversible once the cytosolic calcium level returns to normal (63). Importantly, phosphatidylserine exposure on viable cells is sufficient to induce phagocytosis of such cells (60, 62, 64). In addition, cells can undergo reversible apoptosis, insufficient to induce cell death alone but sufficient to induce phagocytosis of the live cell (65, 66).

Live infected cells can expose phosphatidylserine too and thereby be subject to this phagocytic clearance. For example, live HIV-infected cells were shown to externalize phosphatidylserine, which induced macrophages to phagocytose these cells, mediated by the phagocytic receptor MerTK and the phosphatidylserine-binding opsonins Gas6 and protein S (67). In another demonstration of this process, infection of human cells by the bacterium *Chlamydia* was also shown to cause rapid and reversible phosphatidylserine exposure on host cells, dependent on calcium elevation and independent of apoptosis, which then induced macrophages to phagocytose the live infected cells (68). In yet another example, infection of mouse brain with adenovirus (modified as a vector) was shown to cause phosphatidylserine exposure on live brain cells, with subsequent phagocytosis by microglia of

the infected cells observed *in vivo* using 2-photon imaging (69). Mechanistically, this was via calcium activation of phospholipid scramblase 1 (PLSCR1), with the consequent phosphatidylserine exposure on infected cells inducing microglial phagocytosis via the phagocytic receptor MerTK, resulting in clearance of the infected cells. PLSCR1 is known to be induced by viral infection and to mediate the antiviral response of cells to many different viruses by multiple mechanisms (70, 71); the study by Tufail and colleagues (69) may add induction of phosphatidylserine exposure and subsequent live infected cell phagocytosis to that list. As a final consideration, enveloped viruses normally have phosphatidylserine on their surface and may thereby cause infected cells themselves to exhibit surface phosphatidylserine when the virus enters or leaves the host cell (72, 73).

Another well-known eat-me signal is calreticulin, which can be exposed on the surface of viable, stressed, or dying cells, and induces phagocytosis of such cells by the low-density lipoprotein (LDL) receptor-related protein (LRP1 receptor) on phagocytes (74–76). Calreticulin normally functions as a chaperone in the endoplasmic reticulum but can be released onto the cell surface, or indeed secreted, as a result of endoplasmic reticulum stress, inflammation, or infection (77–79). For example, *Mycobacterium tuberculosis* and cytomegalovirus infections cause calreticulin exposure on the surfaces of infected cells (79, 80). Inflammatory activated macrophages release calreticulin, and plasma calreticulin levels are increased in sepsis patients (30, 31, 81, 82). This extracellular calreticulin can act as an opsonin, binding both the target cell and the phagocytic receptor LRP1 on the phagocyte to stimulate engulfment (74, 83, 84). In contrast to exposed phosphatidylserine, which generally inhibits inflammation and antigen presentation, phagocytosis of calreticulin-exposed cells stimulates antigen presentation (85–87). Thus, phagocytosis of infected cells exposing calreticulin is more likely to result in cross-presentation of antigens.

DON'T-EAT-ME SIGNALS: CD47, SIALIC ACID, AND MHC-I

Counterbalancing eat-me signals, don't-eat-me signals are surface-expressed molecules which discourage phagocytosis by a potential phagocyte. The best-understood example is CD47, a plasma membrane-localized protein expressed ubiquitously on host cells which, by activating the SIRPα receptor on phagocytes, inhibits engulfment; blocking CD47 thereby increases phagocytosis of viable cells (74, 88). During malaria, *Plasmodium* parasites preferentially infect young CD47^{hi} red blood cells (89). These cells lose CD47 over time, leading to their eventual phagocytic turnover, and so infection of CD47^{hi} red blood cells allows the parasite to complete that stage of its life cycle prior to this clearance. However, the host cells fight back; infected red blood cells downregulate CD47 levels to expedite their phagocytic removal (90, 91). This is therefore an example of an infected host cell downregulating a don't-eat-me signal to enable phagocytosis of the infected cell and thereby reduce infection.

Sialic acid residues on cell surface glycoproteins and glycolipids also act as a don't-eat-me signal, whereas removal of these residues (desialylation) promotes phagocytosis of the cell (92). Infection can induce desialylation of host cells; for example, influenza infection induces a rapid decrease in surface sialic acid residues on live infected cells (93, 94); phagocytes can then phagocytose these cells (95, 96).

Major histocompatibility complex class I (MHC-I) is another don't-eat-me signal present on most healthy host cells to prevent them being phagocytosed (97). MHC-I is downregulated by many virally infected cells to prevent MHC-I-mediated antigen presentation, but this MHC-I downregulation may then promote phagocytosis of the infected host cell, providing effective immunity.

OPSONINS: MFG-E8, GAL-3, ANTIBODIES, AND COMPLEMENT

Opsonins are normally soluble extracellular proteins which, when bound to cells, stimulate phagocytes to phagocytose such opsonin-tagged cells. Opsonins often bind eat-me signals, such as phosphatidylserine, and act as transcellular bridges to phagocytic receptors on phagocytes. For example, galectin-3 can bridge target cells and

phagocytes through its carbohydrate binding domains (98–100). Galectin-3 expression and secretion are upregulated upon infection, including influenza and pneumococcal infections of epithelia (94, 101). Additionally, as noted above, live HIV-infected cells expose phosphatidylserine and bind the phosphatidylserine-binding opsonins Gas6 and protein S, which then stimulate the phagocytic receptor MerTK, resulting in phagocytosis of the HIV-infected cells by macrophages (67). MFG-E8 is yet another phosphatidylserine-binding opsonin, which can mediate microglial phagocytosis of phosphatidylserine-exposing live neurons (102). This appears to help remove prion protein-infected neurons in the brain, such that MFG-E8 knockout mice exhibit accelerated prion disease (17).

IgG antibodies are classical opsonins, which bind antigens on the pathogen and activate Fcγ receptors on the phagocyte, resulting in phagocytosis. Infected cells might also bind such antibodies, either because (i) pathogen antigens are on the host cell surface as part of the pathogen's cell cycle, for example, during entry or exit from the cell, or (ii) pathogen antigens are displayed by the host cell together with MHC-I. Antibody-dependent cellular phagocytosis (ADCP) of live infected cells is wel established and, in some cases, may mediate immunity (103–106). Whether pathogen antigens displayed by host MHC-I can bind antibodies is unclear but would require some specific mechanism to prevent it. If antibodies do bind to these displayed pathogen antigens, then they should result in phagocytosis of live infected cells.

The complement system targets infected cells, causing either lysis through formation of a membrane attack complex or opsonization of the target through deposition of opsonins C1q, C3b, iC3b, and C4b (107, 108). C1q can bind phosphatidylserine or calreticulin on host cells, and C3b binds desialylated surfaces and stimulates phagocytosis via complement receptors such as CR1, CR3, and CR4 (84, 109–111). Infected cells cause complement activation at their surface, which enables their phagocytosis by phagocytes, without host cell lysis (112–114). Exemplifying this, West Nile virus infection of neurons induces complement tagging of the neurons, resulting in complement-mediated phagocytosis of the live neurons' synapses by microglia both in culture and *in vivo* (115).

ANTIGEN PRESENTATION

Antigens from pathogen-infected cells can be cross-presented with MHC-I to T cells by dendritic cells that have phagocytosed the infected cells (116–118). Cross-presentation by dendritic cells also occurs with nonlytic virus and intracellular bacterial infections, suggesting that the death of infected cells may not be required (119). Thus, while signals from dying cells may promote cross-presentation when phagocytosed by antigen-presenting cells, these signals may also be present on infected cells (120). This is supported by the finding that incubation of live, virus-infected cells with dendritic cells leads to dendritic cell presentation of viral antigens to T cells (121, 122). Similarly, upon phagocytosis of viable neutrophils, dendritic cells can cross-present antigens from bacteria, yeast, or cancer cells that the neutrophil has previously phagocytosed (123, 124). Thus, it is possible that phagocytosis of live, infected host cells by antigen-presenting cells may result in presentation of pathogen antigens, resulting in adaptive immunity, but this would need to be tested directly. Alternatively, phagocytosis of just part of an infected cell, for example, by merocytophagy or trogocytosis, might also be used to induce adaptive immunity in a similar manner (125, 126).

RESISTANCE BY PATHOGENS

If phagocytosis of live infected cells is an important mediator of immunity, then we might expect resistance mechanisms to have developed in rapidly evolving pathogens such as viruses. Indeed, there is evidence that diverse pathogens express their own products or manipulate host gene expression to inhibit general phagocytosis (127). For example, HIV-1 encodes Tat and Nef proteins, which inhibit phagocytosis of infected cells by macrophages (128, 129), and the human cytomegalovirus (HCMV) expresses

TABLE 1 Studies directly observing phagocytosis of live infected cells by different types of macrophages

Author, year, and reference	Infectious agent	Infected cell	Phagocyte[a]
Chua et al., 2018, 67	HIV-1	$CD4^+$ T cells	Human MDM
Ayi et al., 2016, 90	*Plasmodium falciparum*	Human red blood cells	Murine and human BMDM/MDM
Baxter et al., 2014	HIV-1	Human $CD4^+$ T cells	Human MDM
Fekete et al., 2018, 46	Neurotropic herpesvirus	Murine neurons	Murine microglia
Tufail et al., 2017, 69	Adenovirus	Murine neurons	Murine microglia
Goth and Stephens, 2001, 68	*Chlamydia trachomatis*, *C. pneumoniae*	Human neutrophils	Human MDM

[a]MDM, monocyte-derived macrophages; BMDM, bone marrow-derived macrophages.

the protein UL-18, which mimics the don't-eat-me signal MHC-I α-chain to inhibit phagocytosis of the infected host cells (130). In addition, many viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), cause upregulation of CD47 or express CD47 mimics, inhibiting phagocytosis of the infected cells (39, 131–134).

CONCLUSION

We have outlined above a wide range of evidence that live, infected host cells signal to be phagocytosed and that this may contribute to limiting infection. In Table 1, we list a number of studies with direct evidence of macrophage phagocytosis of live infected cells using a range of pathogens and model systems. We hope that the explicit articulation of the hypothesis here (and illustrated in Fig. 1), with discussion of the accumulating supporting literature, will promote awareness of this potentially common innate immune mechanism and encourage its rigorous testing. Such research would involve answering the following questions. First, are host cells infected with the pathogen of interest phagocytosed alive to an extent sufficient to limit infection *in vivo*? Second, does blocking the phagocytosis of infected cells by phagocytes *in vivo* increase the spread of infection by various pathogens? Third, does phagocytosis of live infected cells lead to the presentation of pathogen antigens and effective adaptive immunity?

If the hypothesis is true, then there may be translational applications. For example, treatment with specific opsonins (or treatments that increase opsonin production) may enhance phagocytosis of infected cells. Treatments boosting phagocyte numbers or expression of specific phagocytic receptors may similarly increase such phagocytosis, as would treatments that remove or block don't-eat-me signals such as sialidase to remove sialic acid or antibodies to block CD47. Vaccines based on pathogen antigens expressed on the surfaces of host cells would be particularly efficacious and selective in removing infected cells. Moreover, targeting the phagocytosis of live infected cells may combat infections earlier than other treatments and potentially speed the adaptive immune response.

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