# Phagocytic removal of cells that have become unwanted: Implications for animal development and tissue homeostasis

# Phagocytic removal of cells that have become unwanted: implications for animal development and tissue homeostasis

Running title: Phagocytosis of apoptotic cells during development

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## Abstract

Cells that have become unwanted need to be promptly, selectively, and safely removed. This is made possible by apoptosis-dependent phagocytosis, in which cells unnecessary, obstructive, or dangerous to organisms are induced to undergo apoptosis so that they are earmarked for phagocytosis. The phagocytic elimination occurs so quickly that cells with hallmarks of apoptosis are barely detectable *in vivo*. The removal of particular types of cells at appropriate stages of development not only contributes to the disposal of spent cells, the creation of space for morphogenesis, and the exclusion of pathogenic or noxious cells, but seems to actively control tissue renewal, tissue remodeling, tissue function, and pathogenic state. This event thus plays an indispensable role in the maintenance of animal development and tissue homeostasis.

**Key words:** cell-cell recognition, innate immunity, morphogenesis, phagocyte, tissue homeostasis.

## Introduction

Apoptosis has been considered a physiological mode of cell death, compared to necrosis, a lytic death often seen in a pathological state (Wyllie *et al*. 1980; Ellis *et al*. 1991; Golstein & Kroemer 2007). This view is due to the fact that the permeability regulation of the plasma membrane is maintained throughout the process of apoptosis. However, cells induced to undergo apoptosis *in vitro* often become necrotic after some time, a state known as "secondary" necrosis. *In vivo*, cells are engulfed and digested by phagocytic cells immediately after the induction of apoptosis, i.e. before a shift to secondary necrosis. Therefore, apoptosis can be considered a biological phenomenon in which unnecessary cells become susceptible to phagocytosis (Gregory & Devitt 2004; Liao 2005; Nakanishi *et al*. 2009). It is unclear if necrotic cells are subjected to phagocytosis, but phagocytic elimination of lysed cells would no longer be beneficial to organisms.

Phagocytosis serves not only as an efficient mechanism for the removal of apoptotic cells in physiological states but also as a more active tactic for organisms to accomplish development and maintain tissue homeostasis. The elimination of particular types of cells at appropriate times creates space for morphogenesis, removes cells inappropriate for the establishment of tissue functions, stimulates other types of cells to differentiate or propagate, and helps phagocytic cells acquire additional activities. In fact, impairment of phagocytosis appears to lead to the development of a variety of diseases (Elliott & Ravichandran 2010; Nagata *et al*. 2010).

This article describes how the phagocytosis of apoptotic cells is implemented and contributes to the process of development and the maintenance of tissue homeostasis.

## Mechanism of apoptosis-dependent phagocytosis

*Phagocytosis as a definitive step of apoptosis*

That a cytologically abnormal cell is contained in another cell was noted even in earlier studies on apoptosis (Wyllie *et al*. 1980). A little later, it was shown *in vitro* that neutrophils become susceptible to phagocytosis by macrophages when they are induced to undergo apoptosis (Savill *et al*. 1989). The phagocytic elimination of apoptotic cells is not restricted to mammals but also found in nematodes, insects and fish. Phagocytosis is a biological event in which a cell engulfs and digests another cell to eliminate foreign materials such as pathogenic microbes (Aderem & Underhill 1999; Stuart & Ezekowitz 2005). Cells that engulf and digest other cells or macromolecules are called phagocytes, and most phagocytes are engaged in the phagocytosis of apoptotic cells. "Professional" phagocytes play a role mostly in phagocytosis, whereas non-professional or part-time phagocytes are usually responsible for biological phenomena other than phagocytosis and only exhibit phagocytic activity when needed. The former include macrophages, neutrophils, and dendritic cells, and the latter, endothelial cells, Kupffer cells of the liver, microglia of the brain, and Sertoli cells of the testis.

Phagocytic elimination seemingly occurs very fast once apoptotic cells emerge because it is difficult to detect cells with the hallmarks of apoptosis in tissues where massive apoptosis is presumed. The selectivity with which phagocytes recognize target cells is defined by changes to the surface of apoptotic cells (Lauber *et al*. 2004). Apoptotic cells seem to be earmarked for phagocytosis in the early stages of apoptosis, allowing their elimination before the permeability regulation of the plasma

membrane is lost. This helps prevent the noxious contents of apoptotic cells leaking out and damaging surrounding tissues.

*Phylogenetic conservation of apoptosis-dependent phagocytosis*

Horvitz and co-workers generated and analyzed mutant lines of the nematode *Caenorhabditis elegans* with defective phenotypes of cell death. Some mutants showed the persistent presence of dead cells, which researchers called "corpses", and the genes responsible for such a phenotype turned out to be those required for the phagocytosis of apoptotic cells (Horvitz 1999). Most genes encode intracellular proteins that constitute signaling pathways for the induction of phagocytosis. An exception is a gene named *cell death abnormal* (*ced*)-*1* coding for a single-path membrane protein, which was later shown to be a phagocytosis receptor (Zhou *et al*. 2001). All these genes possess counterparts in fruit flies, mice, and humans (Lettre & Hengartner 2006; Kinchen & Ravichandran 2007), suggesting that the final stage of apoptosis is phylogenetically conserved.

*Mechanism by which phagocytes recognize apoptotic cells*

An event for the phagocytic removal of apoptotic cells consists of four distinct steps (Fig. 1). The first step in the phagocytosis of apoptotic cells is the accumulation of phagocytes at the site of apoptosis. As neutrophils move to an area of inflammation, phagocytes are required to travel toward apoptotic cells. There are several substances, sometimes called "find-me" signals, which are released from apoptotic cells and attract phagocytes. A well-characterized find-me signal is lysophosphatidylcholine. This lipid is produced in apoptotic cells through the cleavage of an acyl chain of the

membrane phospholipid phosphatidylcholine by a phospholipase that is activated by a caspase, an apoptosis-specific protease (Lauber *et al*. 2003). Such a lipid with one acyl chain missing becomes prone to be released from the plasma membrane. A G protein-coupled receptor has been identified as a receptor for this chemoattractant (Peter *et al*. 2008). Besides this phospholipid, other substances, such as a ribosomal protein, chemokines, and nucleotides, have been reported to act as find-me signals.

The next step is the recognition of apoptotic cells by phagocytes. In general, phagocytes recognize target cells using phagocytosis receptors that specifically bind to ligands present at the surface of apoptotic cells. When phagocytes bind apoptotic cells, the phagocytosis receptors are activated so that they transmit a signal into the cytoplasm of phagocytes, which in most cases leads to a rearrangement of the actin cytoskeleton. As a result, portions of the plasma membrane of phagocytes protrude and surround the targets. Ligands for phagocytosis receptors are called markers for phagocytosis or "eat-me" signals. To date, a variety of molecules have been proposed as phagocytosis receptors and their ligands (Lauber *et al*. 2004). Unlike in the phagocytosis of bacteria, antibodies and complement components, and thus Fc receptors and complement receptors do not seem to play major roles in the phagocytosis of apoptotic cells. However, opsonin-like molecules that connect phagocytes and target apoptotic cells seem to exist. Markers for phagocytosis are generated either by the exofacial exposure of endogenous molecules or the modification of preexisting surface molecules during the process of apoptosis. The first and best-characterized marker for phagocytosis is the membrane phospholipid phosphatidylserine. This phospholipid is normally confined to the inner leaflet of the membrane bilayer but moves to the outer leaflet after the induction of apoptosis, and is exposed at the cell surface (Fadok *et al*. 1998;

Schlegel & Williamson 2001). Phosphatidylserine-mediated phagocytosis of apoptotic cells was found first in mammals (Fadok *et al*. 1992) and later in nematodes (Wang *et al*. 2007; Venegas & Zhou 2007; Züllig *et al*. 2007; Darland-Ransom *et al*. 2008), but it is unclear if insects use this mechanism. The externalization of phosphatidylserine in apoptotic cells appears to involve apoptosis-dependent changes in the activities that control the movement of phospholipids (Verhoven *et al*. 1995). Externalized phosphatidylserine is bound either by serum proteins that connect phagocytes and apoptotic cells or directly by phagocytosis receptors present at the surface of phagocytes (Table 1). Representative of phosphatidylserine-binding bridging molecules is milk fat globule-epidermal growth factor-E8 (MFG-E8) (Hanayama *et al*. 2002), which simultaneously binds phosphatidylserine on apoptotic cells and integrin present at the surface of phagocytes. There are a number of membrane-bound receptors that directly recognize and bind to phosphatidylserine. There seems no apparent similarity in primary structure among the phosphatidylserine-binding receptors, and how phagocytes use multiple receptors to recognize phosphatidylserine-exposing apoptotic cells is yet to be clarified. It is likely that utilization of engulfment-inducing pathways is defined by phagocytosis receptors not markers (Table 1, and see below).

*Signaling pathways in phagocytes leading to engulfment of apoptotic cells*

In general, there are several ways in which phagocytes engulf target cells (Aderem & Underhill 1999). Apoptotic cells are enwrapped by the extended plasma membranes of phagocytes. This change in the surface structure of phagocytes occurs through a rearrangement of the actin filament that requires the actions of small G proteins. Therefore, as the third step in the process of phagocytosis (Fig. 1), an important

function for phagocytosis receptors after receiving a signal from apoptotic cells is to activate an intracellular signaling pathway that leads to the activation of small G proteins.

Horvitz and colleagues genetically identified nematode genes involved in signaling pathways for the induction of phagocytosis. Apparently, there exist two partly overlapping pathways in *C. elegans* (Reddien & Horvitz 2004; Kinchen & Hengartner 2005; Mangahas & Zhou 2005) (Fig. 2). One pathway, involving proteins named CED-6, CED-7, and CED-10, seems to lie downstream of the phagocytosis receptor CED-1. The other pathway includes CED-2, CED-5, and CED-12. These two pathways converge at CED-10, a small G protein, which further transmits signals leading to a rearrangement of the cytoskeleton. The latter pathway, however, presumably lacks a membrane-bound phagocytosis receptor. Recent publications showed the presence of two more receptors, Frizzled (Cabello *et al*. 2010) and integrin (Hsu & Wu 2010), in *C. elegans*, and that the receptor residing furthest upstream of the CED-2/CED-5/CED-12/CED-10 pathway is most likely integrin. Of note is that the genes encoding these proteins including the receptors CED-1 and integrin are also present in other species (Lettre & Hengartner 2006; Kinchen & Ravichandran 2007) (Fig. 2). This suggests that the mechanisms by which phagocytes recognize and engulf apoptotic cells have been preserved during evolution. However, the actions of the conserved proteins could differ among species. The mammalian integrin indirectly binds to phosphatidylserine with the aid of MFG-E8, a protein that connects phosphatidylserine and integrin (Hanayama *et al*. 2002). Recently, a bridging protein called transthyretin-related family domain (TTR)-52 was found in *C. elegans*, but this protein seems to link phosphatidylserine and CED-1, not integrin (Wang *et al*. 2010).

On the other hand, Draper, a *Drosophila melanogaster* homologue of CED-1, appears to use a protein as its ligand (Kuraishi *et al*. 2009). It also remains to be shown if integrin and phosphatidylserine play roles as a receptor and an eat-me signal, respectively, in the phagocytosis of apoptotic cells in *Drosophila*.

*Fate of engulfed apoptotic cells as well as engulfing phagocytes*

Apoptotic cells are ingested being surrounded by the plasma membrane of phagocytes and form membrane vesicles called phagosomes. Subsequently, phagosomes fuse with lysosomes giving rise to phagolysosomes, and apoptotic cells are subjected to degradation by lysosomal enzymes. Once the cells are completely digested, the process of apoptosis ends. Implications of the simplest mode of apoptosis-dependent phagocytosis are as follows: the silent disappearance of unnecessary cells in itself is important; cells inappropriate for the establishment of tissue functions are eliminated; cells dangerous to organisms are removed; or the space occupied by unnecessary cells is cleared (Fig. 3). In addition, as the fourth and the last step in the phagocytosis of apoptotic cells, there are more active consequences than the mere disposal of dead cells (Figs. 1 and 3). Apoptosis-dependent phagocytosis may alter the functional properties of phagocytes leading to further biological events. Described below are detailed consequences of the phagocytic removal of apoptotic cells.

## Roles for phagocytic removal of apoptotic cells

*Silent disposal of dysfunctional cells*

Apoptosis is induced in most spent or dysfunctional cells, but the mode of cell death seems to shift to secondary necrosis if the cells are left unengulfed. In necrosis,

plasma membranes are disrupted, and cell contents including noxious substances leak out and damage surrounding tissues. It is thus important for apoptotic cells to be cleared by phagocytosis while the integrity of the plasma membrane is kept intact. When tissues or organs presumably with a high incidence of apoptosis are histochemically analyzed, evidence of apoptosis is rarely seen. This suggests that cells undergoing apoptosis are promptly eliminated by phagocytosis under physiological conditions. Therefore, apoptosis-dependent phagocytosis is a mechanism to remove spent or dysfunctional cells without collateral damage in surrounding normal tissues. This mechanism is typically observed in the renewal of tissues and organs. Most, if not all, cells that constitute the body possess their own lifespan and are periodically renewed. The elimination of spent cells is achieved through apoptosis and subsequent removal by phagocytosis. Another example of the disposal of dysfunctional cells is seen in the course of immune responses. Lymphocytes and neutrophils are destined to dye by apoptosis when they are activated and move to the secondary lymphoid tissues and sites of bacterial invasion, respectively. Lymphocytes become apoptotic unless they meet cognate antigens to further stimulate while neutrophils die after they phagocytose invading bacteria. In this way, immune cells that have fulfilled their role are made dysfunctional by apoptosis to avoid unnecessary or excess immune reactions and silently removed by phagocytosis.

*Establishment and maintenance of tissue functions*

During the expansion of the T cell repertoire, T cell clones that are unable to recognize the major histocompatibility complex expressed at the surface of thymic cells are induced to undergo apoptosis and become susceptible to phagocytosis. This process,

often referred to as “positive selection”, contrasts with the induction of apoptosis in T lymphocytes that recognize self peptides exposed as a complex with the major histocompatibility complex at the surface of antigen-presenting cells, an event called “negative selection”. This complex mechanism of the apoptosis-mediated removal of particular T cell clones contributes to the establishment of self-restricted, nonself-responsive immunity. During the development of a neural network, neurons not properly connected with other neurons or peripheral target organs are removed by apoptosis-dependent phagocytosis. Most probably, peripheral organs provide properly connected neurons with trophic factors that prevent them from undergoing apoptosis. Rendering certain neurons dysfunctional by apoptosis as well as silently eliminating them by phagocytosis is likely to be important for the establishment of neural networks.

An example of a role for phagocytosis in the maintenance of tissue functions is seen in the diurnal renewal of photoreceptor cells in the retina (Fig. 4). Outer segments of photoreceptor cells are subjected to phagocytic removal every morning by adjacent retinal pigment epithelial cells (Finnemann *et al*. 1997), and this event, sometimes called shedding of photoreceptor particles, is crucial for the durability of vision (Nandrot *et al*. 2004). Phosphatidylserine exposed at the surface of aged outer segments serves as a marker for phagocytosis, which is recognized by a complex composed of integrin and Mer, a receptor-type tyrosine kinase, with the aid of bridging MFG-E8 or directly by a scavenger receptor. The periodical phagocytosis is presumably defined by the activation of these receptors by sunlight. Another example of phagocytosis in this category occurs in the generation of gametes (Fig. 4). A substantial portion of differentiating male germ cells undergo apoptosis and are subjected to phagocytosis by Sertoli cells, a somatic type of cell present in the testis.

Sertoli cells recognize target cells using a scavenger receptor that directly binds to phosphatidylserine exposed on the surface of spermatogenic cells during apoptosis (Shiratsuchi *et al*. 1999). The elimination of apoptotic spermatogenic cells was shown to be important for the progression of spermatogenic differentiation (Nakagawa *et al*. 2005). The corpus luteum is responsible for the maintenance of early pregnancy but regresses in the absence of conception. This event is presumably required for the maintenance of the ovulatory cycle. The disappearance of corpora lutea is accomplished through apoptosis-dependent phagocytosis by macrophages that have infiltrated the ovary (Kato *et al*. 2005).

*Development and differentiation involving phagocytosis*

Phagocytic elimination of apoptotic cells creates space for changes in body shape, which is why apoptosis is often called programmed cell death by developmental biologists. Such space plays a role in morphogenesis by serving as a vacant area for the generation of new structures or the extension of pre-existing tissues, or a boundary within tissues. A typical example is found during the development of limbs (Fig. 4). In the developing footplate of the mouse, interdigital cells are induced to undergo apoptosis and engulfed mostly by infiltrating macrophages, resulting in the formation of digits (Hopkinson-Woolley *et al*. 1994). A dramatic change in body shape occurs during metamorphosis. In insects that undergo complete metamorphosis, larval structures are remodeled into adult ones at the pupal stage. Most larval tissues and organs break down and are subsequently cleared or restructured, but it is still controversial whether the programmed death of larval cells involves apoptosis or autophagic cell death, or whether dying larval cells are eliminated by phagocytosis in

*Drosophila* (Baehrecke 2003). There are several phenomena in metamorphosis that involve phagocytosis. In *Drosophila* pupae, axons of larval neurons are removed by glial cells in a manner mediated by the phagocytosis receptor Draper, leaving intact the neural cell body which later develops adult-type axons (Awasaki *et al*. 2006) (Fig. 4). This biological event, called axon pruning, is also known to occur in mammals and nematodes (Kantor & Kolodkin 2003). A similar mechanism for the remodeling of neural networks is observed with dendrites of sensory neurons of *Drosophila* (Williams *et al*. 2006). During the metamorphosis of frogs, the tadpole tail continues to be absorbed and eventually disappears. This is due to apoptosis-dependent phagocytosis: the cells constituting the tail are induced to undergo apoptosis and phagocytosed by macrophages under the control of thyroid hormone (Kerr *et al*. 1974; Nishikawa & Hayashi 1995).

During erythropoiesis in mammals, nuclei of erythrocyte precursor cells are expelled and phagocytosed by macrophages. If the digestion of incorporated nuclei in macrophages is inhibited, erythropoietic differentiation itself is severely retarded (Kawane *et al*. 2001). This could be because macrophages are not able to support the differentiation of erythrocytes without the complete digestion of engulfed materials. Cell competition, whereby one group of cells exclude another, is thought important to prevent organs from becoming larger than normal (Díaz & Moreno 2005). The involvement of apoptosis-dependent phagocytosis in cell competition in *Drosophila* was recently reported (Li & Baker 2007), but how phagocytosis actively controls this event remains to be clarified. In contrast, there seems to be a mechanism to prevent organs from becoming smaller than normal: the activation of apoptosis is associated with the induction of cell division in surrounding normal cells. This phenomenon was

first found in *Drosophila* and has been called "compensatory proliferation" (Fan & Bergmann 2008). Growth-stimulating proteins are likely to be produced in cells undergoing apoptosis depending on the "non-apoptotic function" of caspases, but the involvement of phagocytes is still possible.

*Functional changes in phagocytes after phagocytosis*

Phagocytes may undergo functional changes after accomplishing the phagocytosis of apoptotic cells. The first recorded example of this was macrophages that had phagocytosed apoptotic neutrophils: the phagocytes showed decreased production of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines (Fadok *et al*. 1998) (Fig. 5A). Later on, the transcription factors responsible, at least partly, for this action of macrophages were discovered (Kim *et al*. 2004; Mukundan *et al*. 2009). This phenomenon may mean that macrophages try to prevent unnecessary immune reactions not only by eliminating immune cells but also more actively by secreting inhibitory substances. Dendritic cells phagocytose apoptotic cells, which have been infected with microbial pathogens, and express pathogen peptides as a complex with class I molecules of the major histocompatibility complex at their surface. Such antigens are recognized by class I-restricted, CD8-positive T lymphocytes leading to the development of an antigen-specific clone of cytotoxic T lymphocytes (Albert *et al*. 1998). This is a form of antigen presentation called cross-presentation, in which antigens that are not synthesized intracellularly are obtained and used by antigen-presenting cells to stimulate cytotoxic T lymphocytes.

## Clinical relevance of apoptosis-dependent phagocytosis

A failure to remove apoptotic cells leads to the development of a pathological state. This is considered a consequence of the exposure of normal tissues and organs to the noxious contents of apoptotic cells that have been left engulfed and become necrotic (Fig. 5A). A typical example is the occurrence of inflammation, which is likely to be mediated by Toll-like receptors able to recognize endogenous host molecules in addition to microbial pathogens (Kawai & Akira 2010). Another example is an autoimmune disease such as systemic lupus erythematosus. The cause of this disease is not fully understood (Nagata *et al*. 2010), but cellular components normally kept inside cells such as DNA seem somehow to aberrantly activate the immune system (Rogers *et al*. 2009). Although a loss of expression of the genes that play roles in apoptosis-dependent phagocytosis appears to be related to some other diseases (Elliott & Ravichandran 2010), further investigation is necessary to find whether or not a causal connection exists.

Phagocytosis helps remove tumors. Cancer cells are induced to undergo apoptosis upon treatment with most chemotherapeutic agents or radiation, and are subjected to phagocytic elimination (Fig. 5B). Products of many tumor suppressor genes possess the ability to induce apoptosis in cancer cells and thus enhance phagocytosis-mediated removal of tumors. It is possible to infuse own cancer cells into patients, which have been biopsied and treated for the induction of apoptosis, aiming at the development of immunity against tumors. In cancerous tissues, a cell-in-cell cytological feature is often observed. This is caused by the “invasion”, not phagocytosis, of a cell by another cell detached from the extracellular matrix independent of apoptosis (Overholtzer *et al*. 2007). The cells that have invaded are degraded, like engulfed apoptotic cells, or proliferate and are released. It is presumed

that this phenomenon, called entosis, is a mechanism of tumor suppression. By contrast, apoptosis-dependent phagocytosis could relate to the development and expansion of tumors. The impairment of phagocytosis in cell competition may lead to the development of otherwise excluded cells into cancer cells, or tumors may utilize cell competition to create space for the invasion of surrounding tissues (Tamori *et al*. 2010) (Fig. 5B).

Host cells are often induced to undergo apoptosis upon infection with microbial pathogens. This is most likely part of a self-defense mechanism to eliminate pathogens and protect against infectious diseases. Cells infected with influenza virus are subjected to apoptosis-dependent phagocytosis by neutrophils and macrophages, and this event seems to help mitigate the pathogenicity of influenza (Hashimoto *et al*. 2007). Moreover, some microbes seem to exploit this host behavior. Hepatocytes become apoptotic when infected with the malaria parasite, but the externalization of phosphatidylserine is inhibited probably by the actions of parasite molecules (Sturm *et al*. 2006). As a result, apoptotic hepatocytes avoid phagocytosis by Kupffer cells and are detached from the liver, and consequently the parasites somehow enter the bloodstream to continue their lifecycle. Viruses with an envelope have the chance to possess phosphatidylserine at the surface, which is derived from the plasma membrane of the infected host cells undergoing apoptosis. Vaccinia virus (Ichihashi & Oie 1983; Mercer & Helenius 2008) and human immunodeficiency virus (Callahan *et al*. 2003) were shown to enter host cells by phosphatidylserine-mediated phagocytosis, leading to a dissemination of the infection. The trypanosome protozoon *Leishmania* uses a similar strategy to achieve an infection: this parasite exposes phosphatidylserine on its cell membrane and voluntarily enters macrophages (Wanderley *et al*. 2006).

*Helicobacter pylori* induce the externalization of phosphatidylserine in gastric epithelial cells and deliver an oncogenic protein in a manner dependent on phosphatidylserine (Murata-Kamiya *et al*. 2010).

## Perspective

The term "death" is confusing when used to describe the state of cells that constitute multi-cellular organisms. Unlike in unicellular organisms such as bacteria, a loss of a population of cells does not necessarily mean the termination of life in multi-cellular organisms. In fact, there has been no definition for a dying or dead cell: the term apoptosis defines not the state of dead cells but a mode of cell death. It seems more reasonable to forget about the term cell death and consider apoptosis a biological phenomenon for the effective and safe removal of cells that have become unnecessary or harmful to the body (Gregory & Devitt 2004; Liao 2005; Nakanishi *et al*. 2009). The mechanism of apoptosis-dependent phagocytosis leads us to speculate that this biological event is part of the innate immune system. The immune system mainly targets foreign cells or materials that have invaded the body. Apoptotic cells are not foreign but selves that have been altered both functionally and structurally. It is therefore possible to consider cells undergoing apoptosis as altered selves that are recognizable by the immune system (Nakanishi *et al*. 2009). This is in line with the finding that Toll-like receptors, which sense microbial substances, also recognize endogenous molecules and induce inflammation (Kawai & Akira 2010). Many pattern-recognition receptors in innate immunity recognize multiple ligands (Akira *et al*. 2006). This is likely to be true for receptors responsible for the phagocytosis of apoptotic cells as well because some receptors including CED-1/Draper, scavenger

receptors, and integrin are also engaged in the recognition of microbial pathogens. Such a property of receptors should enable the innate immune system, which is presumably equipped with only a limited number of receptors, to sense and respond to a wide variety of altered own tissues and invading microbial pathogens.

Some questions remain unanswered as to the mechanism of apoptosis-dependent phagocytosis. Although most genes responsible for phagocytosis in *C. elegans* possess counterparts in insects and mammals (Fig. 2), it still needs to be concluded whether or not two partly overlapping pathways for the induction of engulfment are conserved among species. In addition, the phylogenetic conservation of markers for phagocytosis or eat-me signals is not clear. The typical signal phosphatidylserine, which was first found in mammals, seems to play a similar role in *C. elegans* but is yet to be examined in *Drosophila*. Clarification is also necessary of whether the way phosphatidylserine is recognized is common among species (Table 1). In mammals, phosphatidylserine is bound by both bridging proteins and phagocytosis-inducing membrane receptors. In *C. elegans*, a protein that connects phosphatidylserine and CED-1 has been found (Wang *et al*. 2010), but there is no candidate receptor that directly binds to phosphatidylserine. Furthermore, a *Drosophila* homologue of CED-1 seems to recognize a protein ligand that has been relocated to the cell surface from the endoplasmic reticulum during apoptosis (Kuraishi *et al*. 2009).

Further investigation will be required before a complete picture emerges of the evolutionally conserved mechanisms and consequences of apoptosis-dependent phagocytosis.

## Conflict of interest

The authors declare that they have no conflict of interest.

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**Figure Legends**

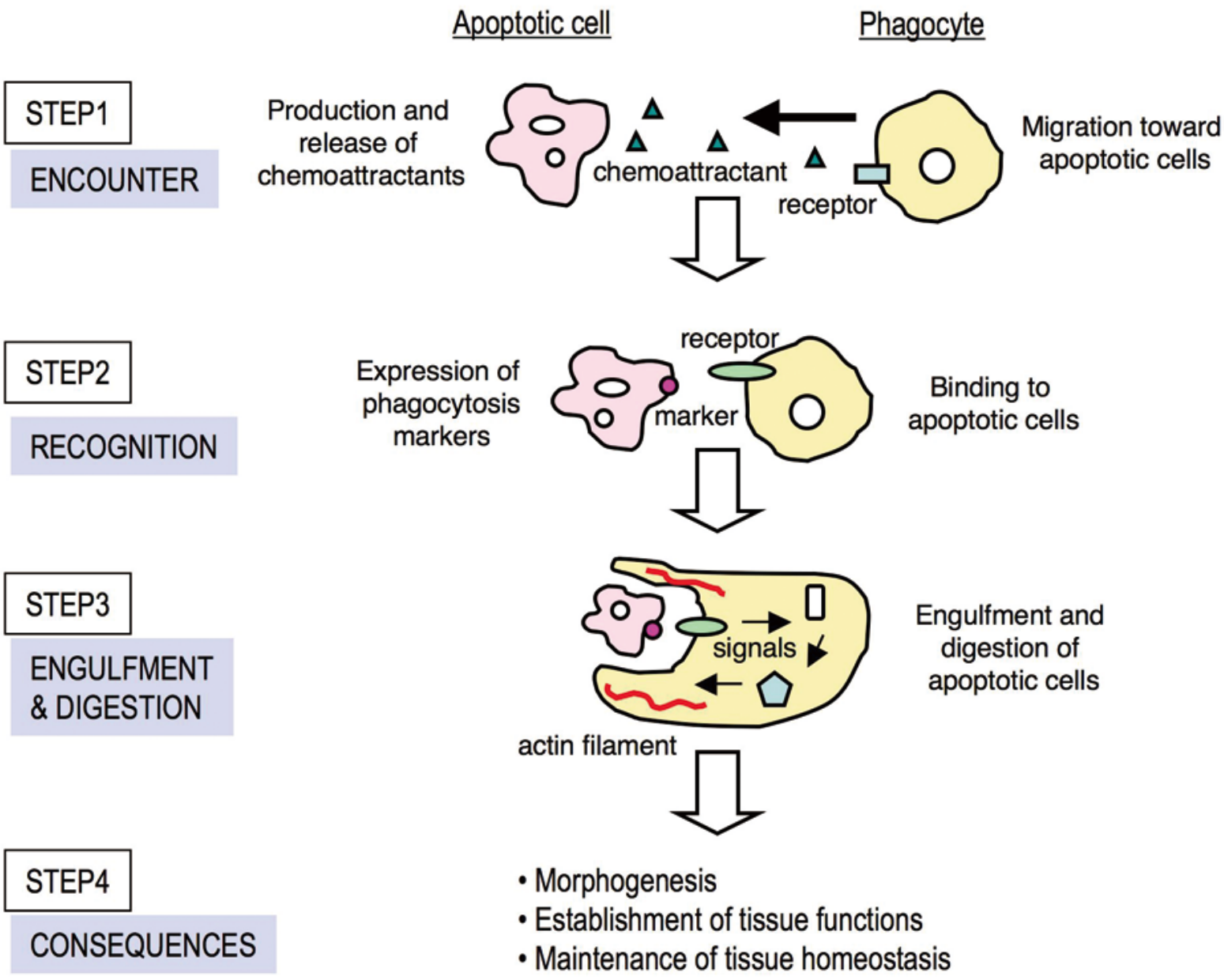
**Fig. 1.** Step-by-step view of phagocytic elimination of apoptotic cells. Cells induced to undergo apoptosis produce and release substances that attract phagocytes. Phagocytes that have moved to the site of apoptosis recognize target cells through specific interaction between markers and receptors for phagocytosis. The activated receptors transmit signals inside phagocytes so that a rearrangement of the actin cytoskeleton and the protrusion of the plasma membrane are evoked. Apoptotic cells are enwrapped in the extended membranes, incorporated into phagocytes, and digested by lysosomal enzymes. Finally, a variety of outcomes are evoked as the consequence of the removal of apoptotic cells. Refer to the text for more details.

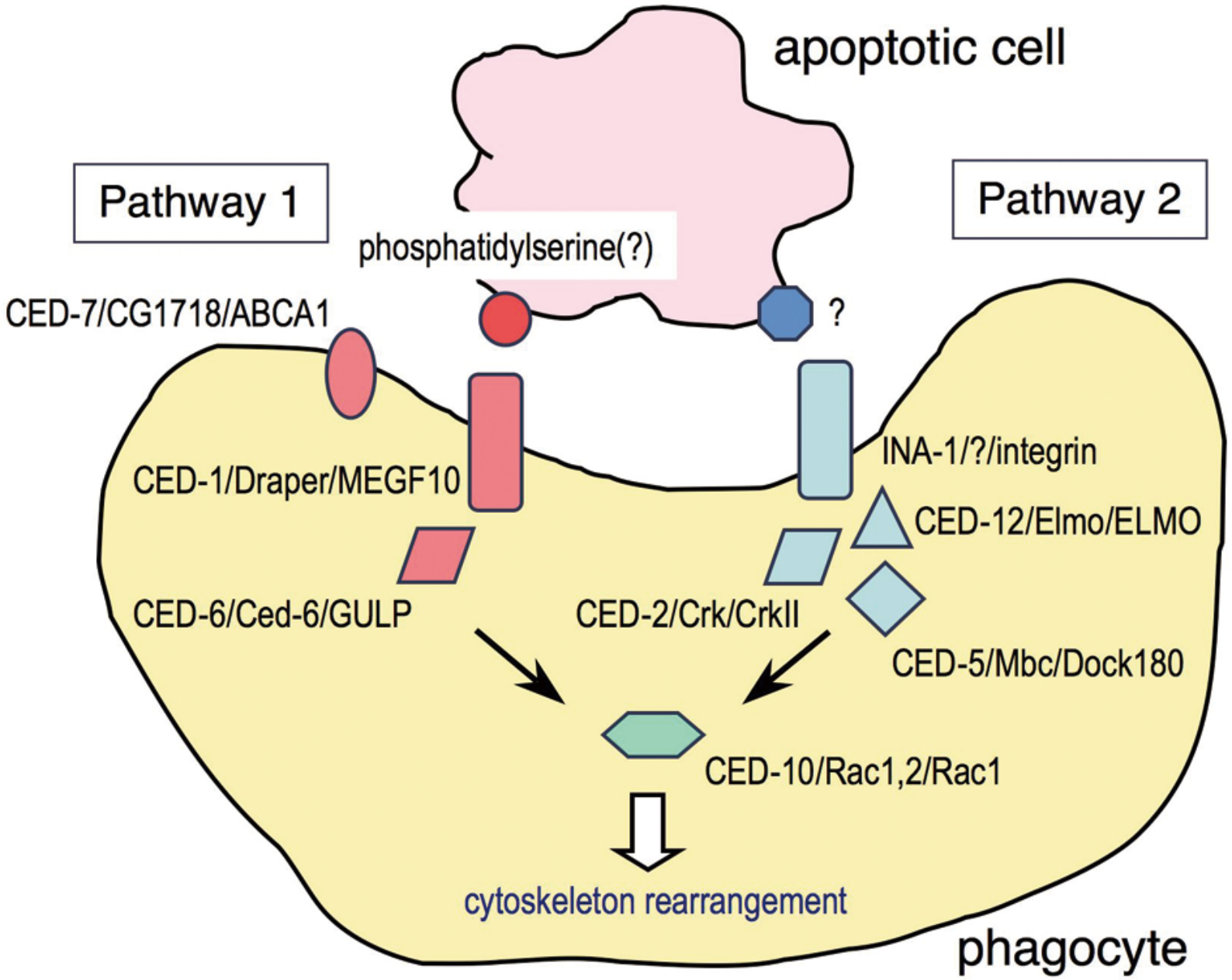
**Fig. 2.** Evolutionally conserved pathways leading to phagocytosis of apoptotic cells. Two signaling pathways for the induction of phagocytosis, which are operationally coined pathways 1 and 2, are shown. Signal mediators of *C. elegans* are indicated along with their counterparts in other species (*C. elegans*/*Drosophila*/mammals). Refer to the text for details. ABC, ATP-binding cassette; Crk, CT10 regulator kinase; Dock, dedicator of cytokinesis; ELMO, engulfment and cell motility protein; GULP, engulfment adapter protein; Mbc, Myoblast city; MEGF10, multiple epidermal-growth-factor-like motifs-10; Rac, Ras-related C3 botulinum toxin substrate.

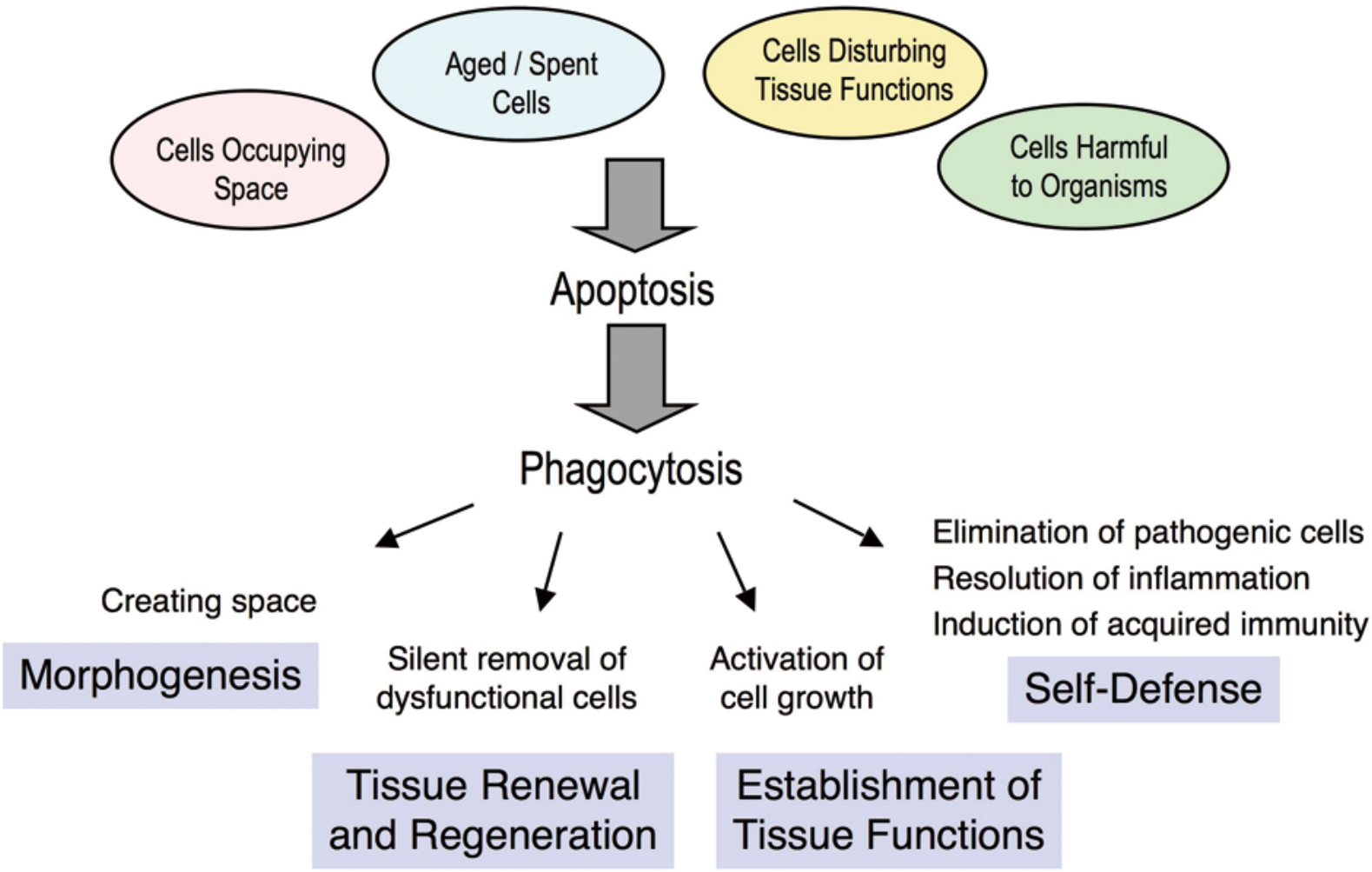
**Fig. 3.** Consequences of apoptosis-dependent phagocytosis. Various outcomes are provoked in the phagocytic elimination of apoptotic cells depending on the physiological and pathological state of organisms. Refer to the text for more details.

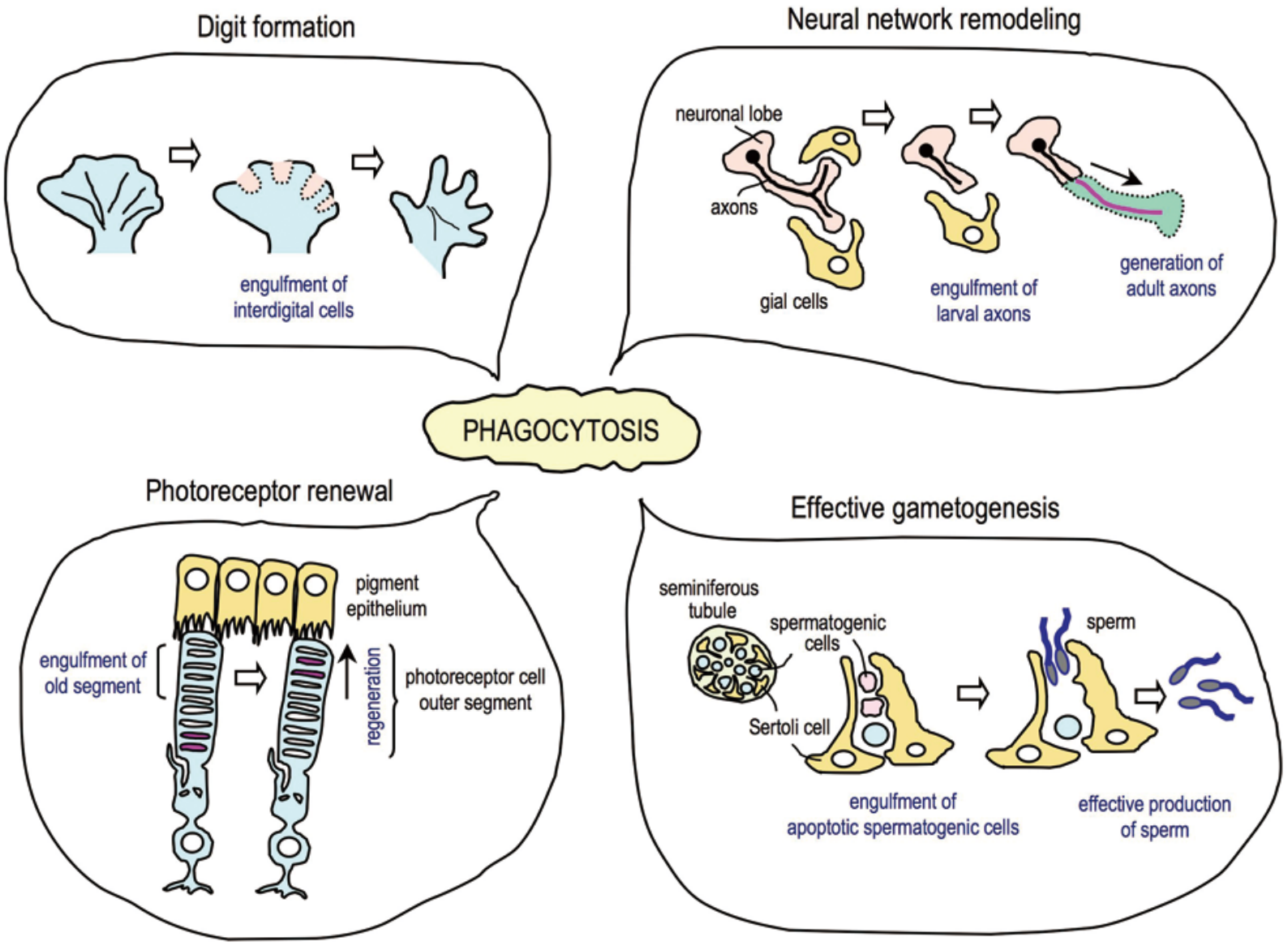
**Fig. 4.** Examples of biological events involving phagocytosis. Refer to the text for details.

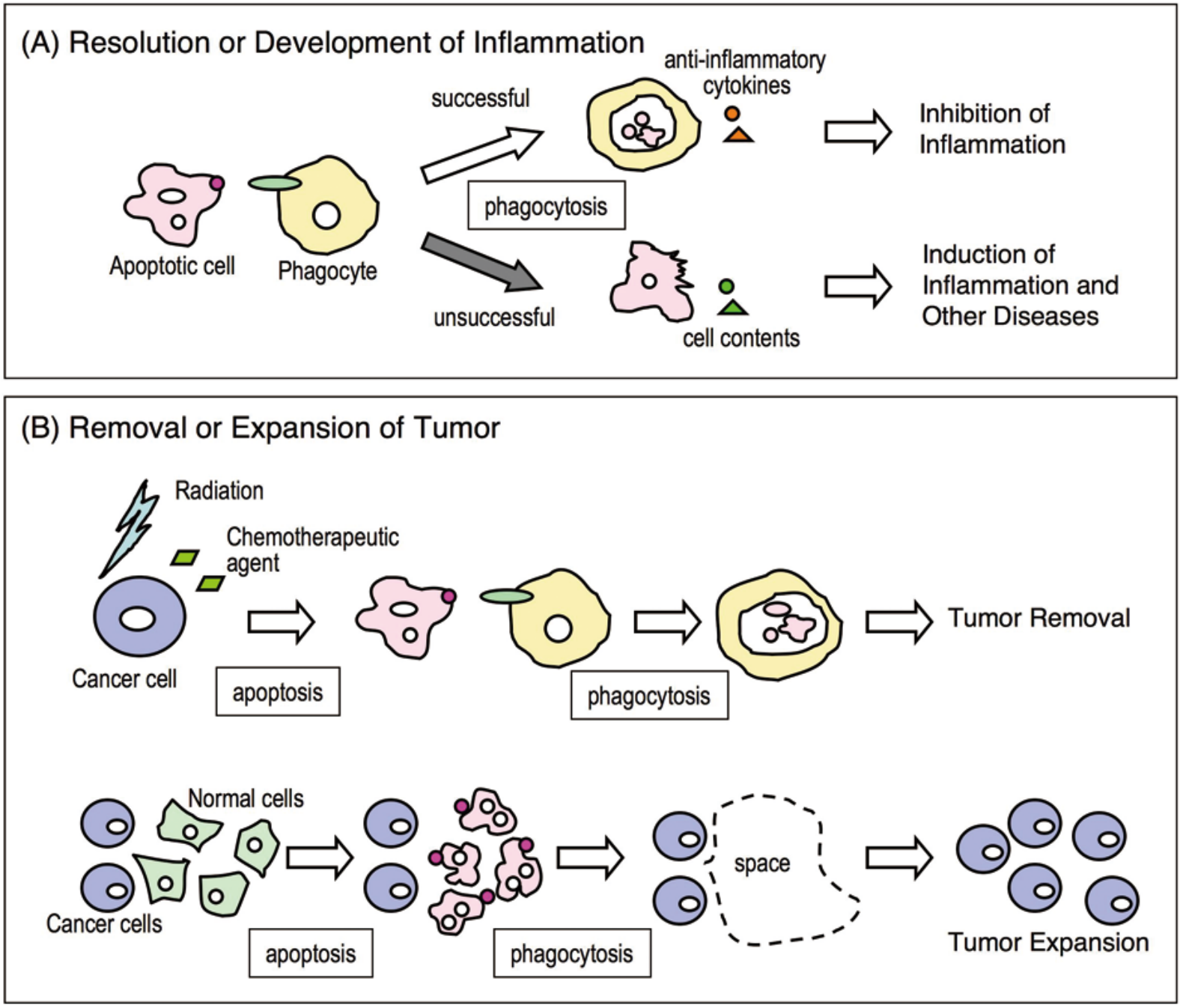
**Fig. 5.** Clinical relevance of apoptosis-dependent phagocytosis. (A) Phagocytes that have ingested apoptotic cells change an expression pattern of their genes, and in many cases produce and secrete anti-inflammatory substances to resolve inflammation. Cells left unengulfed often shift to necrosis, and endogenous noxious contents leak and damage surrounding tissues, resulting in the development of inflammation and other diseases such as an autoimmune disease. Refer to the text for more details. (B) Phagocytosis of apoptotic cells may result in both the regression and expansion of tumors. Most medical treatments induce apoptosis in cancer cells so that they become susceptible to phagocytic elimination. Some cancer cells presumably induce apoptosis in surrounding normal cells and expand using space otherwise occupied by normal tissues.


Apoptotic cell
Phagocyte
STEP1
ENCOUNTER
Production and release of chemoattractants
chemoattractant
receptor
Migration toward apoptotic cells
STEP2
RECOGNITION
Expression of phagocytosis markers
receptor
marker
Binding to apoptotic cells
STEP3
ENGULFMENT & DIGESTION
signals
actin filament
Engulfment and digestion of apoptotic cells
STEP4
CONSEQUENCES
• Morphogenesis
• Establishment of tissue functions
• Maintenance of tissue homeostasis


apoptotic cell
Pathway 1
Pathway 2
phosphatidylserine(?)
CED-7/CG1718/ABCA1
?
CED-1/Draper/MEGF10
INA-1/?/integrin
CED-12/Elmo/ELMO
CED-6/Ced-6/GULP
CED-2/Crk/CrkII
CED-5/Mbc/Dock180
CED-10/Rac1,2/Rac1
cytoskeleton rearrangement
phagocyte


Aged / Spent Cells
Cells Disturbing Tissue Functions
Cells Occupying Space
Cells Harmful to Organisms
Apoptosis
Phagocytosis
Creating space
Morphogenesis
Silent removal of dysfunctional cells
Tissue Renewal and Regeneration
Activation of cell growth
Establishment of Tissue Functions
Elimination of pathogenic cells
Resolution of inflammation
Induction of acquired immunity
Self-Defense


Digit formation
engulfment of
interdigital cells
Neural network remodeling
neuronal lobe
axons
gial cells
engulfment of
larval axons
generation of
adult axons
PHAGOCYTOSIS
Photoreceptor renewal
pigment
epithelium
engulfment of
old segment
regeneration
photoreceptor cell
outer segment
Effective gametogenesis
seminiferous
tubule
spermatogenic
cells
Sertoli cell
sperm
engulfment of
apoptotic spermatogenic cells
effective production
of sperm


(A) Resolution or Development of Inflammation
anti-inflammatory cytokines
successful
Inhibition of Inflammation
phagocytosis
Apoptotic cell
Phagocyte
unsuccessful
Induction of Inflammation and Other Diseases
cell contents
(B) Removal or Expansion of Tumor
Radiation
Chemotherapeutic agent
Cancer cell
apoptosis
phagocytosis
Tumor Removal
Normal cells
Cancer cells
apoptosis
phagocytosis
space
Tumor Expansion