CHAPTER 1

Introduction

Olle Stendahl

Through their capacity to recognize, phagocytose and inactivate invading microorganisms, phagocytic cells have a key role in the innate immune response and host defense. During this process there is an intimate interplay between different recognition mechanisms displayed by both the host cells and the microorganisms. Understanding the complex process of phagocytosis requires insight into the mechanisms of receptor function, signal transduction, actin-based movements, membrane and vesicle trafficking, and oxidative activation, as well as how pathogens interfere with and subvert these processes. The complexity is thus in part due to the diversity of receptors capable of stimulating phagocytosis, and in part due to the capacity of different microbes to influence their own fate, as they are recognized and internalized. It is now evident that pathogens are not passive bystanders evading phagocytosis and intracellular killing, but have evolved specific means of subverting the process of phagocytosis through different mechanisms, involving inhibition of opsonization and receptor recognition, inactivation of specific GTPases, dephosphorylation, inhibition of PI-3 kinases, and actin polymerization. Studies of the pathogenicity strategies of bacteria such as *Salmonella*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *Shigella*, *Mycobacterium tuberculosis*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes* have not only shed light on microbial pathogenicity but have also been useful tools for elucidating the phagocytic process *per se*. Understanding how *Listeria* escapes from the phagosome by forming an actin-rich tail has revealed how actin polymerization is initiated and controlled. The role of *Yersinia* YopH protein as a protein phosphatase interfering with signal transduction

Phagocytosis and Bacterial Pathogenicity, ed. J. D. Ernst and O. Stendahl. Published by Cambridge University Press.

and adhesion complexes has also given us an insight into the mechanism of phagocytosis.

A primary challenge for the innate immune system is to discriminate between potential pathogens and self, utilizing a restricted number of phagocyte receptors. This challenge has been met by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eucaryotes. These motifs are essential for the invading agents, and are therefore conserved and not subjected to high mutation rates. These "pathogen-associated molecular patterns" (PAMP) include mannans from yeast, formylated bacterial peptides, lipopolysaccharides, lipoteichoic acid, peptidoglycans, CpG motifs characteristic of microbial DNA, and flagellin of invading microorganisms. Much interest has focused on the role of at least ten different Toll-like receptors (TLR), not only as specific PAMP receptors but also as modulators of the innate immune response and inflammation. An important observation in this respect is that mice resistant to endotoxin and endotoxic shock have a natural mutation in TLR4. In humans a number of polymorphic alleles of TLR4 have also been identified; one of these is associated with increased risk of septic shock, but not of other infections such as meningococcal diseases. The expression of TLR4 has also been linked to susceptibility to urinary tract infections in children. Because TLR drive the transcriptional program associated with a proinflammatory response, they play an integral part in the innate immune response. It was recently shown that TLR not only trigger proinflammatory cytokines, but also regulate phagolysosome maturation and subsequent inactivation of ingested bacteria. On the other hand, phagocytosis of apoptotic cells through phosphatidyl serine receptors does not initiate phagosomal maturation and proinflammatory cytokine production. Whether TLR and other PAMP receptors are engaged in the phagocytic process will thus determine the state of activation of phagocytic cells, and the subsequent inflammatory response.

Several inherited defects in phagocytic cells cause impairment of host defense. These observations have revealed important functions of phagocytic cells in host defense and inflammation. Since the discovery of genetic defects in NADPH-oxidase in chronic granulomatous diseases (CGD), leading to the understanding of the regulation of the respiratory burst and the function of reactive oxygen species, several genetic traits have been characterized linking phagocyte function to host defense. Leukocyte adhesion defects (LAD) led to the discovery of adhesion molecules and the understanding of integrins in adhesion and migration, and novel therapeutic approaches to inflammation. Recently, mutations in the signal transduction genes coding for the GTPase protein Rac1 have been characterized in patients with enhanced susceptibility

to infections. These rare but severe phenotypes have deepened our understanding of the function of phagocytic cells, not only in host defense but also in non-infectious inflammatory diseases. In fact, it has become evident that activation of phagocytic cells, leading to generation of reactive oxygen and nitrogen species and to apoptosis, not only plays a vital role in innate immune reaction but forms a link to adaptive immunity as well.

The purpose of this book is to present the current state of understanding of the cellular and molecular mechanisms of phagocytosis and the mechanisms used by pathogenic bacteria to avoid phagocytosis and survive extra- or intracellularly. The book will focus on mechanisms of phagocyte recognition and ingestion, describing receptor-initiated signal transduction, and how certain pathogens interfere with these events. From these reviews it is evident that there is an intimate interplay between phagocytic cell responses and pathogenic microorganisms. A proinflammatory response may be beneficial for both the host and the pathogen, depending on the site and course of infection. Future research must focus on how to control the signaling events and cell responses of neutrophil leukocytes and macrophages interacting with different pathogens. Several animal models targeting specific genes have been very useful in this respect. With new tools of molecular biology it should now be possible in humans to identify genes conferring enhanced susceptibility and resistance to infections. Because reduction of excess inflammation is a major therapeutic goal during treatment of severe infections, and phagocytic cells are important effector cells during inflammation, modulation of the innate immune response in these cells is vital. Understanding the mechanisms of receptor recognition and cross-talk, signal transduction, and intracellular processing, will facilitate new therapeutic approaches to microbe-related inflammatory diseases.

CHAPTER 2

Phagocytosis: receptors and biology

Wouter L.W. Hazenbos and Eric J. Brown

INTRODUCTION

Consumption followed by digestion has developed from a nutrition mechanism in unicellular eukaryotes into a highy regulated and indispensable mechanism of host defense against infection in mammals. *Phagocytosis* of pathogenic microorganisms by *phagocytes*, or "eating cells," is a major host defense mechanism of the innate immune system. The process of phagocytosis was first described at the beginning of the twentieth century by Elie Metchnikoff, who observed ingestion of small particles by cells from starfish larvae. Phagocytosis is generally defined as the internalization of particles with a diameter of at least 0.5 μm, such as bacteria, viruses, parasites, large immune complexes, or apoptotic cells and cell debris. Ingestion of smaller particles, such as small immune complexes or other macromolecules, occurs through a fundamentally distinct mechanism, called *endocytosis*. Phagocytosis and endocytosis are distinguishable by the importance of actin polymerization, which directs membrane motility during phagocytosis, but not endocytosis. Another distinction can be made by the presence of clathrin coats around vacuoles formed during some forms of endocytosis, but not phagocytosis (Greenberg 1986). Recently, one more distinction has been added by showing that endocytosis by IgG Fc receptors (Fcγ R), but not phagocytosis, requires ubiquitylation (Booth *et al.* 2002). Thus, the specific molecular pathways that direct the process of ingestion depend on the size of the particle. When the target particle is too large to be ingested, a process designated "frustrated phagocytosis" may occur, involving activation of pathways partially similar, but not identical, to those activated during phagocytosis. In contrast

Phagocytosis and Bacterial Pathogenicity, ed. J. D. Ernst and O. Stendahl. Published by Cambridge University Press.

to active entry by invasive pathogens, phagocytosis depends exclusively on molecular mechanisms in the phagocytic cell, while the ingested particle plays an apparently passive role.

Among the first detailed investigations of the mechanisms of phagocytosis were those performed by Cohn and Silverstein in the 1960s and 1970s (Steinman & Moberg 1994; Silverstein *et al*. 1977). Silverstein and colleagues proposed the "zipper hypothesis" to explain particle engulfment during phagocytosis. According to this hypothesis, phagocytosis occurs through initial attachment of a target via specific phagocyte receptors, followed by complete engulfment, which requires sequential and circumferential ("zipper"-like) interactions between ligands distributed around the particle and receptors on the phagocyte (Griffin *et al*. 1975, 1976). Much evidence supports this hypothesis for phagocytosis triggered by IgG receptors. It remains unclear whether complement-receptor-mediated phagocytosis uses a similar mechanism, because it is morphologically distinct and appears to occur through "sinking" of the particle into the phagocyte cytosol. It is possible that additional morphologically distinct mechanisms of phagocytosis also exist.

PHAGOCYTOSIS IN STEPS

The entire process of phagocytosis of microorganisms by phagocytes can be divided into three main steps (see Figure 2.1).

The first step involves the initial *binding* of the target particle to receptors at the phagocyte surface, a recognition process mediated by a limited number of specific receptor–ligand interactions at the contact interface. The multimeric and/or immobile nature of the ligand on the particle causes a local accumulation of relevant receptors on the phagocyte membrane at the contact interface, resulting in an enhanced local receptor concentration, which likely is critical for communication with the relevant intracellular signaling cascades. This local concentration of receptors is referred to as clustering, capping, or multimerization. Receptor clustering is a major mechanism for initiation of signal transduction across the plasma membrane. The second *activation* step involves the interaction of the cytoplasmic tails of clustered receptors with cytosolic molecules, resulting in transmission of a transmembrane signal from the ligated receptor to intracellular signaling pathways, involving both kinases and cytoskeletal proteins. Activation of these signaling components in turn results in membrane motility and initiation of a number of downstream effector functions. In the third step, i.e. the process of *entry*, pseudopod extensions are formed around and closely attached to the target particle (during zippering), or the particle sinks into the ingesting cell, leading

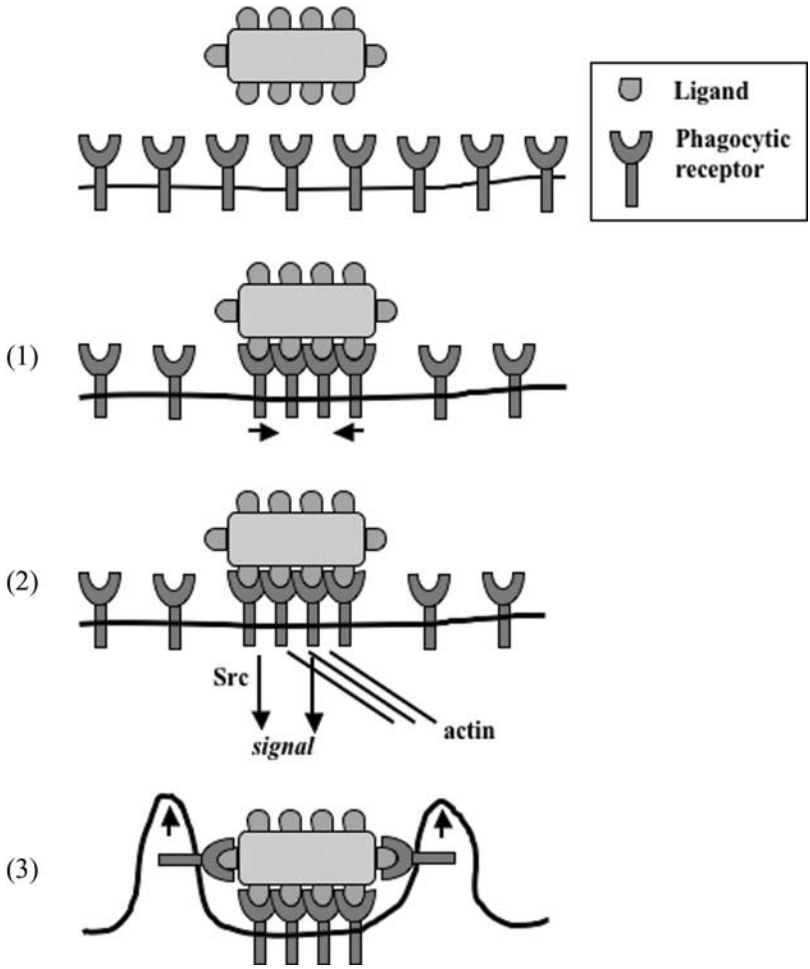

Figure 2.1 Phagocytosis in three steps. In this schematic model, phagocytosis of a ligand-coated particle by a phagocyte occurs through the following three main steps. (1) *Binding*. Unligated phagocytic receptors are normally in monomeric state and unable to signal; binding by a multimeric ligand causes receptor clustering. (2) *Activation*. Clustering of receptors facilitates their interaction with signaling molecules such as the tyrosine kinase Syk or members of the Src family, as well as cytoskeletal components including actin. This leads to cell activation and the initiation of membrane motility. (3) *Entry*. Locally increased membrane motility leads to either complete engulfment by newly formed pseudopods (as shown here) or "sinking" of the particle into the cytoplasm. This is followed by membrane fusion and closure of the phagocytic cup.

to its complete encapsulation by host cell plasma membrane. This is followed by membrane fusion events, allowing the formation of an intracellular vesicle around the particle (the *phagosome*). After a particle binds the phagocyte membrane and successfully initates a transmembrane signal, engulfment can occur quickly, i.e. within a period of one to a few minutes. In general, the process of phagocytosis is accompanied by activation of distinct downstream cellular effector functions, such as phagosome–lysosome fusion, oxidative burst, and release of antimicrobial enzymes. These latter events may facilitate killing and degradation of the ingested pathogenic microorganisms, and eventually processing and presentation of their antigens to the adaptive immune system. In addition, phagocytosis can trigger the release of vasoactive mediators and inflammatory cytokines from phagocytes, which contribute to a general activation of inflammatory and antimicrobial mechanisms at the tissue level.

It should be noted that binding of a particle to the phagocyte membrane *per se* does not necessarily lead to phagocytosis. For example, binding to complement receptors on non-activated cells alone is not sufficient to initiate phagocytosis (Wright & Silverstein 1982; Wright *et al*. 1983). IgG receptors, on the other hand, do not require cell activation prior to triggering phagocytosis. This distinction between IgG- and complement receptors is exemplified by an experiment in which both complement-opsonized pneumococci and IgG-opsonized red blood cells were bound to the surface of the same non-activated macrophages, resulting in IgG-mediated phagocytosis only (Griffin & Silverstein 1974).

PROFESSIONAL AND NON-PROFESSIONAL PHAGOCYTES

Professional phagocytes

Macrophages

Professional phagocytes are hematopoietic cells from the myeloid cell lineage: macrophages, dendritic cells, and neutrophils. Mast cells also have phagocytic potential, although the biological relevance of this is not clear. The "classic" phagocyte is the macrophage. Macrophages are part of a network of phagocytic cells present in most tissues of the body, the main function of which is to efficiently remove potential harmful particles such as microorganisms, as well as dead cells or debris. This cellular network was initially referred to by Aschoff as the reticuloendothelial system. Later, this system was re-defined by Van Furth as the mononuclear phagocyte system (MPS) (Van Furth *et al*. 1972), since it became clear that, although associated with

blood vessels, the tissue phagocytes were not of endothelial origin. The MPS concept states that macrophages in all tissues originate from common, non-lymphoid, proliferating precursor cells in the bone marrow, which enter the blood as monocytes, from where they enter the tissues and finally differentiate into macrophages (Van Furth & Cohn 1968). Their main biological function is ingestion and destruction of foreign material and subsequent processing and presentation of antigens to lymphocytes. This is a primary mechanism to link innate and acquired immunity. Tissue macrophages are minimally proliferating cells with a relatively long life-span, which has been estimated to range from four to fifteen days (Van Furth *et al*. 1985). In steady state, i.e. non-inflammatory or non-infectious conditions, there is a constitutive transit of monocytes from the bone marrow to the blood and the tissues, where they develop into macrophages. During infection, this process is significantly accelerated by local inflammatory signals, such as proinflammatory cytokines, produced by tissue macrophages and infiltrating neutrophils in response to microbial products. Inflammatory macrophages usually enter tissue within hours to a few days after the initiation of inflammation.

Dendritic cells

Dendritic cells can be of either myeloid or lymphoid origin. Recently, this cell type has received a lot of attention because of its strong antigen-presenting capacity, making it a potential target for immunotherapy. Myeloid dendritic cells are developmentally and functionally related to macrophages. They originate from myeloid progenitor cells in the bone marrow and are normally resident in most tissues. Upon activation, they migrate to the draining lymph nodes, where they present antigens to lymphocytes. Like macrophages, their main function is ingestion, destruction, and processing of infectious or foreign material, but they are more potent in presenting antigens to lymphocytes to initiate an adaptive immune response. They appear in two functional stages: an immature and a mature stage. "Maturation" of dendritic cells, resembling activation of macrophages, is a prerequisite for these cells to acquire full antigen-presenting capacity. Immature dendritic cells are very efficiently phagocytic and endocytic, but they are inefficient for antigen presentation. Dendritic cell maturation can be induced by diverse stimuli, including microbial Toll-like receptor ligands, such as lipopolysaccharide or peptidoglycan, by IgG-immune complexes, or by ligands for the TNF-receptor family (reviewed in Banchereau & Steinman 1998). Upon maturation, dendritic cells acquire increased capacity to present antigens to T cells; for example, they express high levels of MHC class II and co-stimulatory molecules, while their phagocytic and endocytic capacity weakens compared

with that of immature dendritic cells. The reason that this functional separation is necessary remains subject to speculation. Certainly, immature and mature dendritic cells are spatially separated: mature cells migrate to T-cell-rich zones in lymph nodes to present the captured antigen. Perhaps loss of phagocytic capacity is required to prevent them from ingesting the lymphocytes that they activate. In addition, the specificity of the signals that induce maturation of dendritic cells may help them to distinguish self from non-self, preventing presentation of self antigens and consequent autoimmunity.

Neutrophils

Neutrophils are cells with a shorter life span and originate from bone-marrow myeloid precursors that diverged from monocyte precursors. Neutrophils migrate faster and react more aggressively than macrophages, and are therefore more important for the first-line defense against local infection. Whereas neutrophils are more efficient in killing microorganisms than are macrophages, macrophages and dendritic cells more efficiently present pathogen-derived antigens. In response to inflammatory signals, large numbers of neutrophils enter the inflamed tissues quickly, i.e. within minutes to a few hours. Neutrophils harbor a number of cytoplasmic granules containing proteases and antimicrobial peptides. Activation of neutrophils leads to fusion of these granules with phagosomes and with the plasma membrane (degranulation), facilitating both intracellular and extracellular microbial killing. Soon after migration, activation, and killing of pathogens, neutrophils undergo programmed cell death (apoptosis) and are eventually removed by macrophages.

Mast cells

Mast cells are resident in most tissues; they are abundant in the skin and lungs. Their main function is protection against multicellular parasites, through vasoactive mediators such as histamine and serotonin, which are released by degranulation. The aberrant release of these mediators, in response to allergen-mediated crosslinking of IgE bound to mast-cell IgE receptors, is a major trigger of hypersensitivity reactions such as allergy or asthma. Mast cells can ingest particles through IgG-, IgE-, or complement receptors (Vranian *et al.* 1981; Daëron *et al.* 1993; Pierini *et al.* 1996). In addition, interaction with the glycosyl phosphatidyl inositol (GPI)-anchored protein CD48 on mast cells facilitates phagocytosis and killing of *Escherichia coli* (Malaviya *et al.* 1999; reviewed in Shin *et al.* 2000). Not much is known regarding the precise biological significance of phagocytosis by mast cells *in vivo*, because the major contribution of mast cells to the host defense against

bacterial infection is through release of the proinflammatory cytokine tumor necrosis factor (TNF)-α, which results in attraction of inflammatory neutrophils (Echtenacher *et al.* 1996; Malaviya *et al.* 1996; Jippo *et al.* 2003).

Non-professional phagocytes

Target binding

A central factor distinguishing professional from non-professional phagocytes is the unique expression of receptors for specific opsonins, e.g. IgG Fc receptors (Fcγ R) and complement receptors, on professional phagocytes. None the less, the molecular mechanisms involved in the second (activation) and third (entry) steps of phagocytosis are quite generally expressed. This principle has been elegantly demonstrated by transfection studies, in which the introduction of specific receptors into non-professional phagocytes endows them with the capacity to ingest target particles expressing the cognate ligands. For example, Chinese hamster ovary cells were able to ingest IgG-coated *Toxoplasma gondii* parasites efficiently after transfection with full-length Fcγ RIIB. Cells transfected with Fcγ RIIB lacking the intracellular tail could not ingest these IgG-coated parasites, showing that binding the target particle by itself is not sufficient and that the intracellular domain of the receptor is essential for transmembrane communication to the relevant signaling components (Joiner *et al.* 1990). Similar results were obtained by using 3T6 fibroblasts and COS cells, which were enabled to ingest IgG-coated red blood cells when transfected with a full-length human Fcγ RIIA, but not a tail-minus mutant receptor (Tuijnman *et al.* 1992), or Fcγ RIIA lacking specific cytoplasmic tail tyrosines (Mitchell *et al.* 1994). These and similar experiments are now well accepted as conclusive that non-professional phagocytes possess the relevant machinery for ingestion if they can recognize a target. None the less, they remain at odds to some extent with current paradigms of Fcγ R function, since it is generally thought that Fcγ RIIB is primarily an inhibitory rather than a phagocytic receptor (Hunter *et al.* 1998), and Fcγ RIIA is thought to require Syk kinase for phagocytic function, which is not likely to be present in 3T6 cells. Taken together these data suggest that, although non-professional phagocytes do possess the relevant machinery for ingestion, there are multiple molecular pathways unique to professional phagocytes that markedly increase the efficiency of ingestion, in addition to expression of receptors that broaden the range of pathogens recognized.

Some microorganisms express specific ligands for endogenous surface receptors on non-professional phagocytes, allowing their recognition and ingestion by these cells. These microorganisms include invasive bacteria