Immunology of Fungal Infections

Immunology of Fungal Infections

Edited by

Gordon D. Brown
University of Cape Town, South Africa

and

Mihai G. Netea
Radboud University Nijmegen, The Netherlands

Springer

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN 978-1-4020-5491-4 (HB)
ISBN 978-1-4020-5492-1 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springer.com

Printed on acid-free paper

Cover image reprinted from: Heinsbroek, S.E., G.D. Brown, and S. Gordon, Dectin-1 escape by fungal dimorphism. Trends Immunol, 2005. 26(7): p.352-4 with permission from Elsevier

All Rights Reserved
© 2007 Springer
No part of this work may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission from the Publisher, with the exception of any material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work.

TABLE OF CONTENTS

Preface vii

Color Plates ix

Section 1: Cells

Chapter 1: Macrophages 3
Sigrid E.M. Heinsbroek and Siamon Gordon

Chapter 2: Dendritic Cells 27
Luigina Romani

Chapter 3: The Neutrophil 51
David H. Dockrell, Emmet E. McGrath, Moira K.B. Whyte and Ian Sabroe

Chapter 4: Lymphocytes 75
Anna Vecchiarelli, Antonella Mencacci, Francesco Bistoni

Chapter 5: Other Cells: The Role of Non-Neutrophilic Granulocytes, NK and NKT Cells in Fungal Immunology 99
Jeremy C.D. Wiseman and Christopher H. Mody

Chapter 6: Genes and Gene Pathways in *Candida* Infection 131
Robert B. Ashman, Camile S. Farah and Christine Wells

Section 2: Soluble Factors

Chapter 7: Collectins and Pentraxins 151
Uday Kishore and Kenneth B.M. Reid

Chapter 8: Complement in Fungal Infections and Complement Evasion Strategies 177
Cornelia Speth, Cornelia Lass-Flörl and Reinhard Würzner

Chapter 9: Cytokines 201
Karen F. Buckland and Cory M. Hogaboam

Chapter 10: Antibodies 235
José L. López-Ribot, Rosalía Díez-Orejas and Concha Gil

Section 3: Non-Opsonic Fungal Receptors

Chapter 11: Recognition of Fungal Pathogens by Toll-Like Receptors 259
Mihai G. Netea, Jos W.M. Van der Meer and Bart Jan Kullberg

Chapter 12: Fungal β-Glucans and their Receptors 273
S. Vicky Tsoni and Gordon D. Brown

Chapter 13: Detection of Fungi by Mannose-Based Recognition Receptors 293
F. Meyer-Wentrup, A. Cambi, C.G. Figdor and G.J. Adema

Section 4: Immunity to Specific Pathogens

Chapter 14: Pneumocystis 311
Laura McKinley and Chad Steele

Chapter 15: Interactions of *Aspergillus Fumigatus* With its Host During Invasive Pulmonary Infections 331
Jean-Paul Latgé and Anne Beauvais

Chapter 16: Candida 359
Desa Lilic and Ken Haynes

Chapter 17: Immunology of Infections with Cryptococcus Neoformans 381
Pauline Ellerbroek, Anna Vecchiarelli, Andy Hoepelman and Frank Coenjaerts

Chapter 18: Histoplasma Capsulatum 407
Joshua D. Nosanchuk

Section 5: Fungal Immune Evasion Mechanisms

Chapter 19: Escape Mechanisms from the Immune Response 427
David M. Underhill

Section 6: Immune-Based Therapeutic Strategies

Chapter 20: Cytokine Treatment of Fungal Infections 443
Bart Jan Kullberg

Chapter 21: Fungal Vaccines and Vaccination: Problems and Perspectives 463
Antonio Cassone

Index **487**

PREFACE

The history of mankind has been shaped by infections, more than by war and famine together. At the same time, however, the development of society has had an equally important effect on human diseases. The emergence of agriculture, urban societies and high population densities has been proven to be crucial for the spread of pathogens, and thus human action is currently the single most important driver of infectious epidemiology. Even today, where once major killers such as poliomyelitis have been eradicated, new pathogens are appearing as result of human activity.

One such group of pathogens are the fungi, whose emergence is mainly due to modern medical practices. Fungal microorganisms, from yeasts colonizing the skin or mucosa, to molds from soil or water, are usually harmless in the context of normal host responses. However, the success of chemotherapy, as well as the AIDS pandemia, has led to immune deficiencies in a significant segment of the patient population, and the extensive use of intravenous catheters has provided a way of access for microorganisms which otherwise would find difficult to infect the host. As a result, a yeast such as *Candida* is now on the 4th place on the list of the most frequent sepsis agents, whereas infection with the mold *Aspergillus* is increasing in incidence and it is one of the most feared complications in patients with hematological malignancies. Fungal infections have thus become an important factor of morbidity and mortality, and represent an increasing burden on the medical system. An effective treatment of these infections is an absolute necessity.

We are at a cross-road in our efforts to tackle infections in general, and fungal infections in particular. While the last decennia have brought important progress in the development of more effective and safe antifungal agents, an important percentage of patients still succumb to these infections. The failure of therapy has more to do with the ineffectiveness of host defense mechanisms, than to the absence of effective antifungal agents. Therefore, combining classical antibiotic treatment with adjunctive immunotherapy would seem the logical step forward in the management of fungal infection. Until now, this goal was elusive due to the lack of proper knowledge of the immune system and its interaction with infectious microorganisms.

However, this is changing rapidly, and research done in the last 20 years has enabled us for the first time to design ways of boosting the immune system in an effective way. Discoveries such us the description of the receptors recognizing fungi, an increasing understanding of the host defense mechanisms and cell types important for host defense, as well as the ways through which fungi escape immune surveillance, are important milestones in the way towards understanding host defense to these pathogens. In the present book on the “Immunology of fungal infections” we want to provide an overview of these recent advances, and a guide for understanding the immunology to fungal infections. By asking leading experts

to present the cutting edge information in the field, as well by sharing their views on the challenges for tomorrow, we intend to provide a key source of information on the pathways through which fungi interacts with the host.

In order to respond to these aims, two approaches have been pursued: the first sections of the books present chapters on the major components of the antifungal defense (cells, soluble factors, pathogen recognition receptors), while sections in the second part of the book are devoted to the immune response to specific fungal pathogens. Special chapters deal with immune evasion mechanisms employed by the fungi, as well as with the current status of immunotherapy of fungal infections. By providing the scientific community with a comprehensive overview of the most essential aspects of fungal immunology, we believe that an important need is being addressed, and that this book will represent an principal source of information for everybody interested in this topic.

Gordon Brown and Mihai Netea

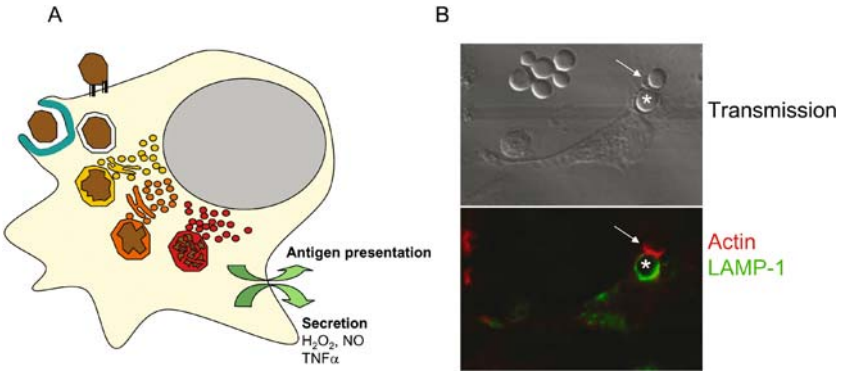


Figure 1.2. Phagocytosis A) Schematic representation of phagocytosis. Engaging of phagocytic receptors leads to particle binding and signalling for cytoskeletal rearrangements. This leads to the formation of a so called phagocytic cup that results in membrane engulfment of the particle. Once a phagosome is formed it matures by fusion and fission with early endosomes, late endosomes and lysosomes, sequentially. During this process the pH of the phagosome is lowered from 6 to around 4.5. Phagocytic signalling cascades also stimulate secretion of H_2O_2, NO and TNFα. B) *Candida albicans* phagocytosis by RAW264.7 macrophages. The cells were stained for actin with TRITC phalloidin (red) and for endosomes and lysosomes with LAMP-1 (green). The first yeast is taken up in a phagosome where the lysosomes have fused (asterisk). The second yeast is in the process of being phagocytosed and there is a clear phagocytic cup present (arrow)

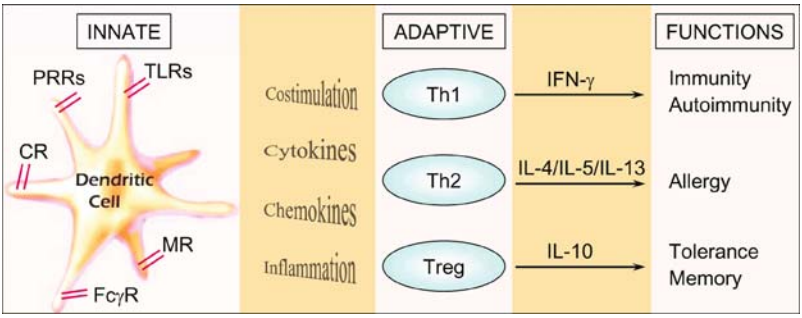


Figure 2.1. Th1/Th2/Treg polarization by dendritic cells in fungal infections. Essential to the successful removal of fungal pathogens is the early recognition of fungi by the innate immune system. Dendritic cells express numerous pathogen recognition receptors which enable them to sense distinct microbial stimuli, and they process this information and elicit distinct functional responses that induce different T-cell responses. DCs that produce IL-12 p70 stimulate protective Th1 responses. Those that produce IL-4 may yield allergic Th2 responses, and those that produce IL-10 may induce Treg implicated in tolerance and memory to fungi. PRRs, pattern recognition receptors; TLRs, Toll-like receptors, Th, helper T cells, Treg, regulatory T cells

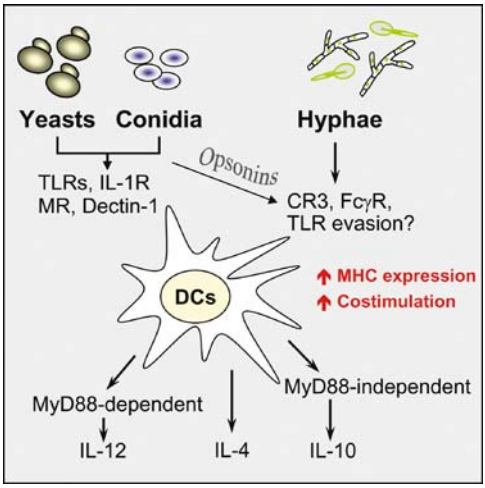


Figure 2.3. The exploitation of distinct recognition receptors in dendritic cells by the different fungal morphotypes. Dendritic cells sense fungi in a morphotype-dependent manner. The engagement of distinct receptors on dendritic cells translated into downstream signaling events that differentially affect cytokine production. The exploitation of a specific receptor invariably leads to the occurrence of a specific type of T helper cell reactivity. Fungal opsonins may subvert the receptor exploitation by fungal morphotypes. TLRs, Toll-like receptors; IL-1R, IL-1 receptor; MR, mannose receptors; CR3, complement receptor 3; FcγR, receptor for the Fc portion of immunoglobulins; MyD88, Drosophila myeloid differentiation primary response gene 88

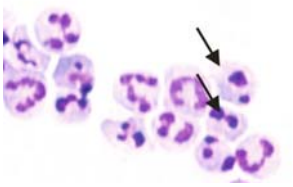


Figure 3.1. Neutrophils in culture. Healthy viable neutrophils exhibit the classical polymorphonuclear phenotype. As they age, they undergo constitutive apoptosis, with shrinking of the nucleus (pyknosis). Two apoptotic neutrophils, showing nuclear condensation, are marked by arrows

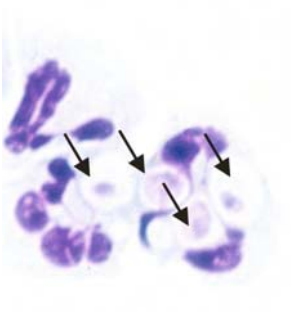


Figure 3.2. Neutrophils engulfing fungal particles. This photomicrograph shows neutrophils that have taken up particles of zymosan (derived from yeast cell walls) into phagosomes (arrowed) for destruction. The ability of the neutrophil to engulf multiple foreign particles in an attempt to neutralise infection is shown in the cell on the right

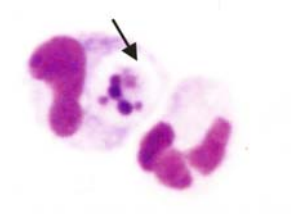


Figure 3.3. Removal of aptoptotic neutrophils. In this smear, leukocytes were isolated from the joint of a patient with rheumatoid arthritis. The two cells shown here are monocytes, one of which has engulfed an apoptotic neutrophil (arrowed), for removal in an injury-limiting fashion that is associated with downregulation of proinflammatory macrophage function

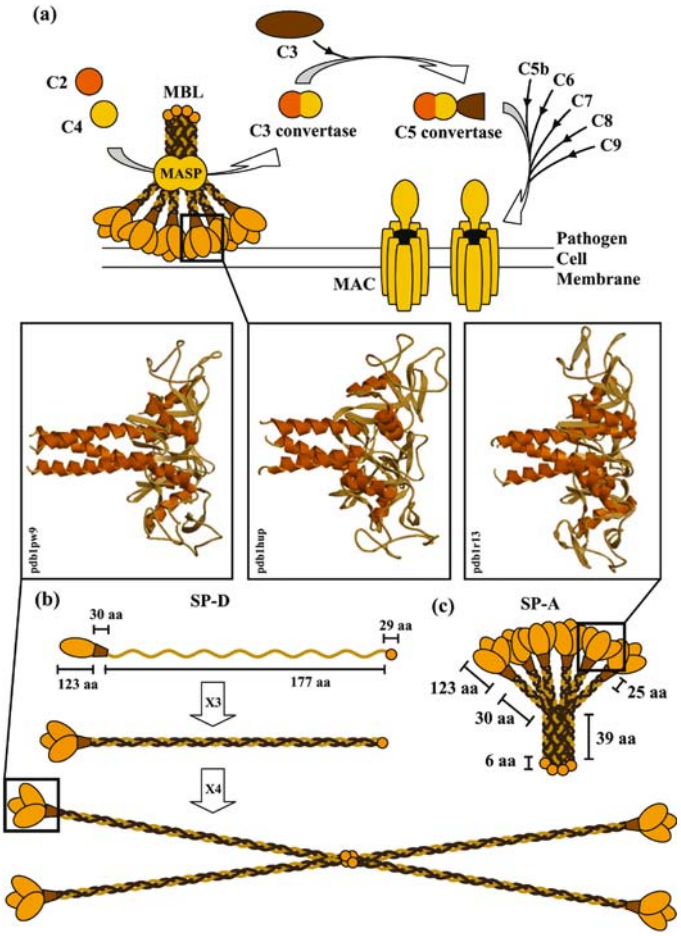


Figure 7.1. Structural organisation of human collectins. The basic polypeptide structure found in all the collectins is organised into four regions: a cysteine-containing N-terminus, a triple-helical collagen-like region composed of repeating Gly-X-Y triplets, followed by an α-helical coiled-coil neck region, and a globular CRD. This polypeptide chain undergoes trimerisation via the neck and collagen-like regions to form a trimeric structural subunit (b). Six of these trimeric subunits then undergo further assembly to yield hexameric structures in case of MBL and SP-A (a, c), although dimers, trimers, tetramers and pentamers are also found. The hexameric forms of MBL and SP-A resemble complement protein C1q in their overall organisation (C1q is only found as a hexamer of a structural subunit which is composed of three different polypeptide chains). SP-D has a tetrameric structure with four of the homotrimeric structural subunits linked via their N-terminal regions, but trimers, dimers and monomers also exist (a). Ribbon diagrams (inset) of the X-ray crystal structures of trimeric neck and CRDs of MBL, SP-A and SP-D show their predominantly β-sheet jellyroll three-dimensional structure. The primary ligand-binding sites (one per CRD) are located at the CRD surface opposite the neck region. The SP-A and SP-D illustrations are approximately to scale. MBL binding to the microbial surface via the CRDs activates the MASPs. MASP-2 cleaves C4 and C2 to generate C3 convertase (C4b2a), which cleaves C3. This leads to the complement lytic pathway, culminating in the formation of membrane attack complex (MAC) and pathogen killing (a)

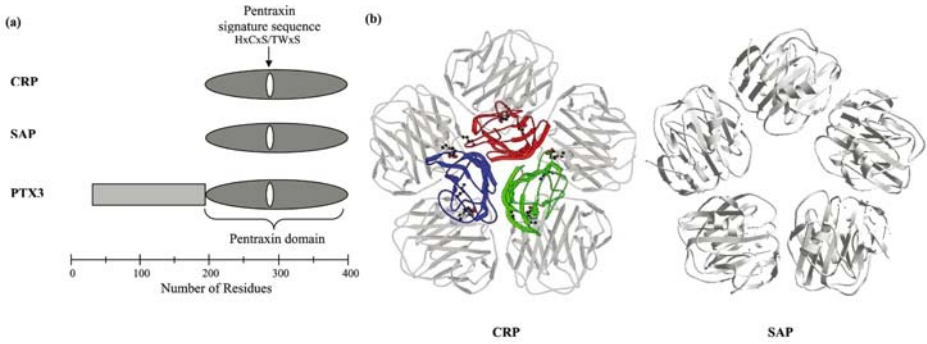


Figure 7.2. (a) Organisation of short and long pentraxins. Pentraxins are characterised by the presence in their carboxy-terminal of a 200 amino acids pentraxin domain, with an 8 amino acid long conserved pentraxin signature (HxCxS/TWxS, where x is any amino acid). The human CRP and SAP genes are located on chromosome 1q23 and are organised in two exons, the second exon encoding for the pentraxin domain. The long pentraxin, human PTX3 gene, localised on human chromosome 3 band q25, is organised in three exons separated by two introns, the third exon codes for the pentraxin domain. The mature SAP protomer is 204 amino acid long (25,462 Da) and has a pentameric structure in the presence of physiological levels of calcium (127,310 Da). In the absence of calcium, SAP consists of both pentameric and decameric forms. Each SAP protomer is glycosylated with a single N-linked biantennary oligosaccharide at Asn^{32}. Human CRP is composed of five identical nonglycosylated protomers. The PTX3 protein (40,165 Da) consists of a C-terminal 203 amino acids pentraxin-like domain (containing an N-linked glycosylation site in the C-terminal domain at Asn^{220}) and an additional N-terminal region (178 aa) unrelated to other known proteins. PTX3 protomers can assemble as decamers and higher oligomers upto 900 kDa. (b) Crystal structures of CRP and SAP. Each CRP protomer has a characteristic lectin fold composed of two layered ß sheets with a flattened jellyroll topology; five protomers are noncovalently associated to form a pentamer (115,135 Da)(Shrive et al. 1994). Ligand bound CRP or SAP can bind to C1q and activate the classical complement pathway (Nauta et al. 2003), which may be one of the mechanisms involved in enhanced phagocytosis of pathogens by phagocytic cells. The interaction between one pentameric molecule of CRP and the heterotrimeric globular domain of C1q has been shown (Kishore et al. 2004a, 2004b). The three chains of C1q (in color) are docked within the CRP pentameric structure. SAP is composed of 5 or 10 identical subunits noncovalently associated in pentameric rings interacting face to face (Emsley et al. 1994). Human SAP has a tertiary fold, which resembles that of the legume lectins like Concanavalin A. SAP protomers have a flattened ß-jelly roll topology with a single long helix folded on the top of the ß-sheet. The five subunits are arranged in a ring around a hole and are held together by hydrogen bonds and salt bridges. The decamer is stabilised by ionic interactions between the two pentamers. Each SAP subunit can bind two calcium ions, and residues involved in calcium binding are conserved. Based on molecular modelling, the PTX3 pentraxin domain has a simlar structural fold to SAP, since most of the ß-strands and the α-helical regions are conserved

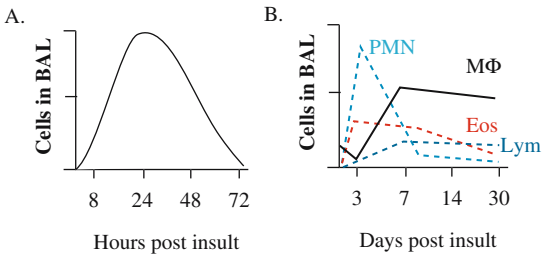


Figure 9.2. (A) The time course of total cell infiltration in the acute response to Aspergillus fumigatus occurring in the allergic airway. (B) Each leukocyte and macrophage population typically follows a distinct time course of recruitment during the chronic allergic response

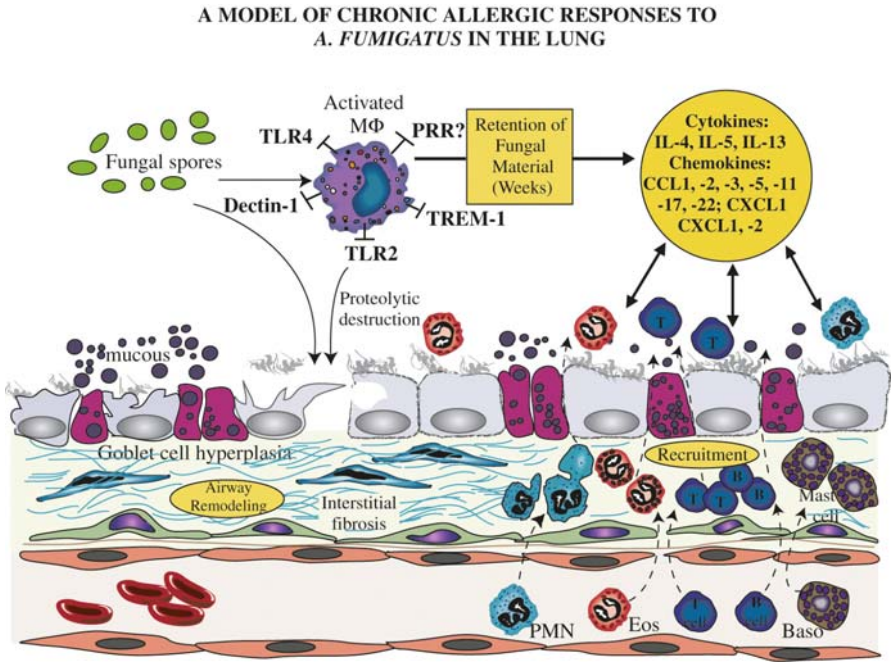


Figure 9.3. An outline of the chronic responses occurring in the allergic airway following challenge with *Aspergillus fumigatus*, featuring a central role of macrophage activation and cytokine and chemokine production

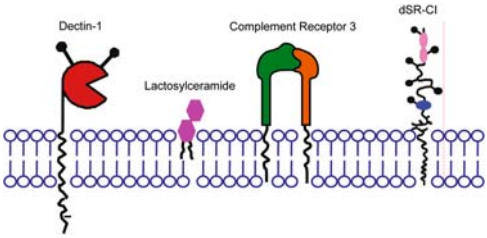


Figure 12.2. Pattern recognition receptors for β-glucans, including Dectin-1, CR3 and lactosylceramide. Also shown is SR-CI, a Drosophila scavenger receptor shown to recognise β-glucans, as the mammalian scavenger receptor(s) which recognise these carbohydrates has not been identified

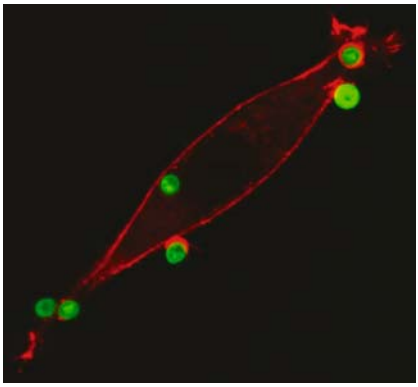


Figure 12.3. Dectin-1 can mediate the phagocytosis of zymosan and the binding and phagocytosis of yeast particles in transfected cells. Shown are Dectin-1 transfected NIH3T3 fibroblasts binding and internalising fluorescently labelled zymosan (green) via actin (red)-based phagocytic cups. Reproduced with permission from (Brown and Gordon, 2001)

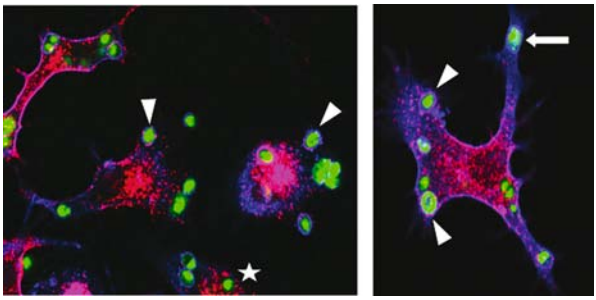


Figure 13.2. Uptake of zymosan (heat-inactivated yeast cell wall preparation derived from *Saccharomyces cerevisiae*) by immature DCs. FITC-labelled zymosan particles (green) were added to immature DCs and taken up. Cells were fixed and stained with anti-DC-SIGN- (blue) and anti-MR-antibodies (red). Most phagocytic vesicles contain both, MR and DC-SIGN (white arrow heads), while few contain exclusively MR (white arrow) or DC-SIGN (white star)

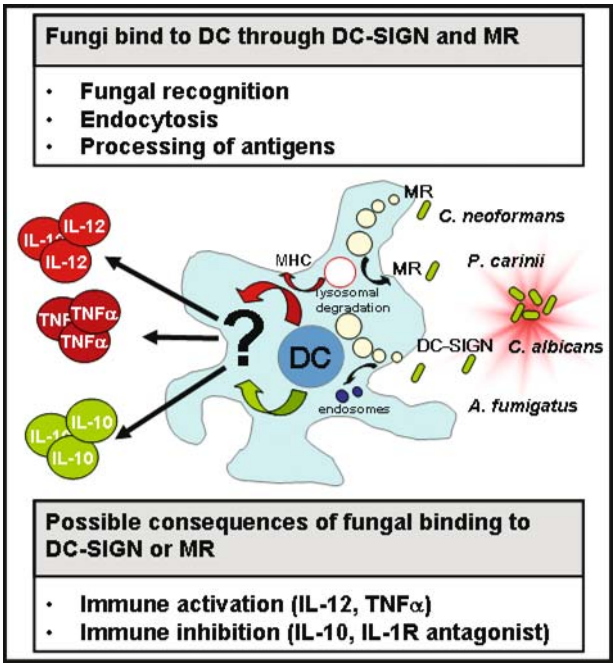
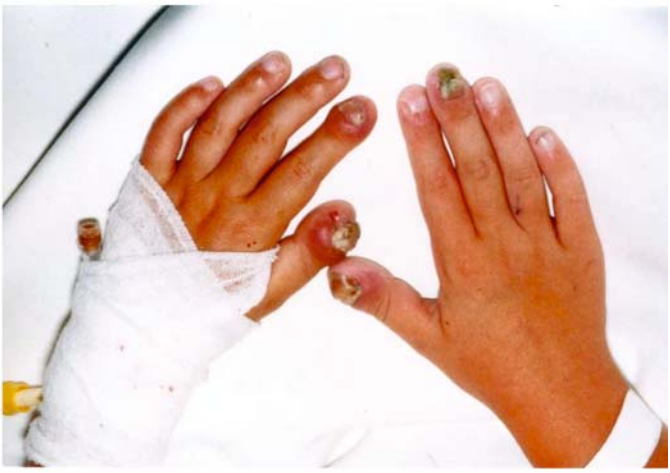


Figure 13.3. Fungi bind to DCs through DC-SIGN and MR and induce immunity or immune evasion. Binding to fungi induces DC-SIGN- and MR-mediated phagocytosis. After ligand binding MR is routed to the late endosomes while DC-SIGN ends up in early endosomes. It is intriguing that depending on the fungus, its form, and other unknown factors, fungal binding to MR or DC-SIGN may induce immune activation, while under different conditions fungi use the same receptors to evade the host immune system. The underlying molecular mechanisms are not yet known

A



B

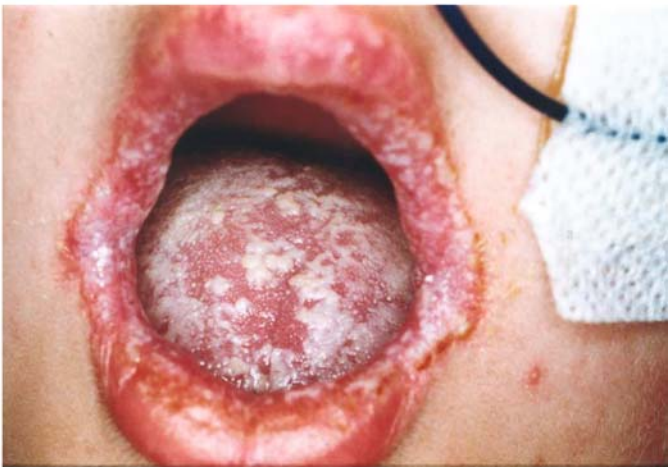


Figure 16.1. Severe, debilitating CMC of the nails (A) and mouth (B) in a six-year old girl. Reprinted with permission from Dr Mario Abinun and Professor Andrew J Cant

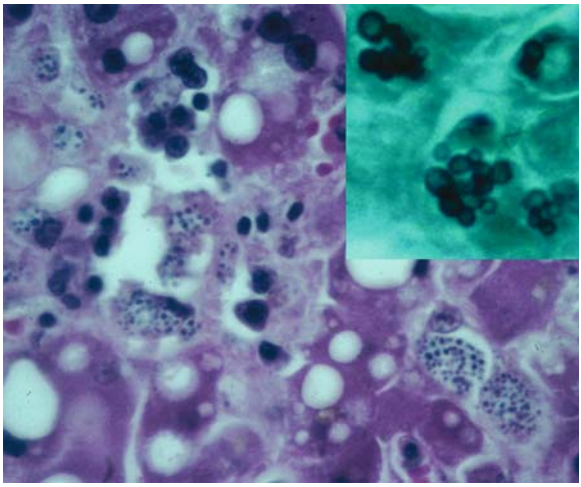


Figure 18.2. H. capsulatum in tissue. Micrographs of a liver depicting swollen and vacuolated hepatocytes and sinusoidal Kuppfer cells filled with *H. capsulatum* yeast cells. The liver section has been stained with Haematoxylin and eosin with the inset showing Gomori's methenamine silver staining. Original magnification, X400

SECTION 1

CELLS

CHAPTER 1

MACROPHAGES

SIGRID E.M. HEINSBROEK AND SIAMON GORDON
*Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford,OX1 3RE, UK**

Abstract: Macrophages are important for both tissue homeostasis and immunity. A great variety of macrophage subpopulations exist that are specialised in different functions eg, osteoclasts that remodel bone, inflammatory macrophages that orchestrate the immune response. As immune regulators macrophages recognise, internalise, degrade and present antigens. Different levels of macrophage activation can be distinguished and this influences the type of immune stimulators secreted by macrophages. Different pathogens have developed ways to evade the macrophage or influence macrophage function to their advantage. This chapter introduces the complexity of macrophage interaction with pathogens and fungal pathogens in particular

This chapter describes the function of macrophages in the immune system with emphasis on macrophage cell biology including phagocytosis of microbes and phagosome maturation. Different strategies employed by pathogens to evade macrophage killing will be discussed, including mechanisms used by some fungi.

1. DEVELOPMENT

Macrophages develop from granulocyte/monocyte precursors in the bone marrow. These precursor cells can also develop into neutrophils, dendritic cells (DC), Langerhans cells and osteoclasts depending on stimulation with growth factors. Precursor development into monocytes is mediated by macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3. Monocytes leave the bone marrow, enter the bloodstream and after approximately one day migrate into tissues where they differentiate into macrophages (Gordon, 2001). Macrophages contribute to homeostasis by clearance of apoptotic/senescent cells, tissue remodelling and repair after inflammation. Macrophages also play distinct roles in immunity; resident and recruited macrophages are highly efficient phagocytes that clear pathogens and dying cells

*e-mail: christine.holt@pathology.ox.ac.uk

G.D. Brown and M.G. Netea (eds.), Immunology of Fungal Infections, 3–25.
© 2007 *Springer.*

and can present antigen to primed T-cells. Furthermore, macrophages can secrete cytokines that affect the migration and activation of other immune cells, and reactive metabolites (reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI)) and other products that contribute to microbial killing and tissue injury (Gordon, 2001).

2. MACROPHAGE SUBPOPULATIONS

Development into tissue macrophages is mediated by factors specific to the local environment (Gordon, 1999). This renders tissue macrophages very heterogeneous, reflecting the specialisation of function adopted in different anatomical locations (Fig. 1) (Gordon and Taylor, 2005). Osteoclasts, for example, are able to remodel bone (Quinn and Gillespie, 2005). Macrophages from the lamina propria on the other hand are continuously in contact with symbiotic gut bacteria and these cells

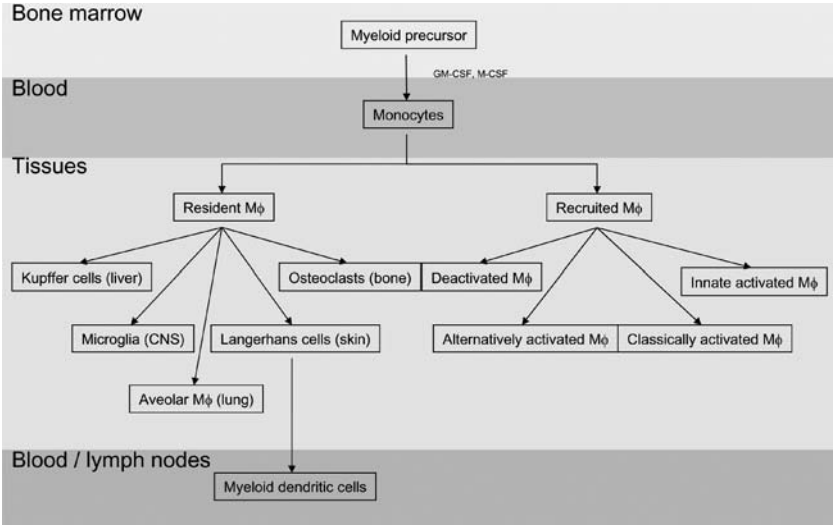


Figure 1. Macrophage heterogeneity. Myeloid precursors develop into circulating monocytes under stimulation of different factors including GM-CSF and G-CSF. Monocytes are recruited into tissues or inflammatory sites where they can differentiate into macrophages. Tissue macrophages fulfil different functions for instance Kupffer cells in the liver are involved in clearance of cells and complexes from blood, osteoclasts play an important role in bone remodelling and Langerhans cells capture epidermal antigens. The microenvironment plays an important part in the development of monocytes into these specialised cells. Macrophages recruited to inflammatory sites can be activated in different ways dependent on the environment. Upon recognition of microbes, macrophages undergo innate activation which leads to stimulation of antigen presentation and production of reactive oxygen species, nitric oxide and IFN-α/β. Macrophages become classically activated by priming with IFN-γ and a subsequent microbial trigger and alternative activation is mediated by IL-4 and IL-13. Uptake of apoptotic cells mediates deactivation of macrophages.

have a high phagocytic ability, but are less effective in producing pro-inflammatory cytokines (Smythies et al., 2005). Alveolar macrophages play a role in clearing microbes, viruses and other particles from the lung, which is an environment poor in opsonins. These cells express high levels of a range of pattern recognition receptors including scavenger receptors (McCusker and Hoidal, 1989; Palecanda et al., 1999; Taylor et al., 2002). Both local proliferation and recruitment of new precursors contribute to renewal and maintenance of the different macrophage populations (Gordon and Taylor, 2005).

3. MACROPHAGE ACTIVATION

Inflammatory monocytes are recruited and differentiate into macrophages at the site of inflammation (Van Furth, Diesselhoff-den Dulk, and Mattie, 1973). Different *in vitro* activation states have been described (Fig. 1) (Gordon, 1999). Classical activation is associated with high microbial activity, pro-inflammatory cytokine production and cellular immunity and is induced by interferon-γ (IFN-γ), and enhanced by microbial stimulation such as lipopolysaccharide (LPS). Alternative activation is stimulated by IL-4 and IL-13, which is associated with humoral immunity and tissue repair. Deactivation, which promotes an anti-inflammatory response, is induced by IL-10, transforming growth factor β (TGFβ) or ligation of inhibitory receptors (Gordon, 1999; Gordon and Taylor, 2005). It is unclear if distict activation states exist *in vivo* or whether macrophages exhibit a broad spectrum of phenotypes. It is likely that in the majority of situations the inflammatory environment will lead to exposure of macrophages to multiple stimuli with complex phenotypic consequences (Gordon and Taylor, 2005).

Inflammatory macrophages differ from resident tissue macrophages in the surface expression of different receptors. Resident macrophages in humans express LPS receptor (CD14), FcγIII receptor and high levels of chemokine receptor CX3CR1, but don't have FcγI receptor (CD64). On the other hand, inflammatory macrophages have higher CD14 expression and express CD64 besides expressing lower levels of CX3CR1 and losing CD16 expression (Gordon and Taylor, 2005). The use of human macrophages in research is limited by difficulty to access different macrophage populations in living individuals. Most research is done with the use of monocyte derived macrophages from blood which are matured into macrophages by co culture with M-CSF. Recruited macrophages can be obtained by subjecting volunteers to abrasion of a small skin area which is covered with filter paper. This is kept sterile and moist overnight after which the recruited cells can be harvested from the filter paper (Willment et al., 2005).

A frequently used model for human macrophages are macrophages from mice. These cells have very similar properties, however slight differences in receptor expression do exist; for instance resident macrophages in mice do not express MHC class II and CD14 (Gordon and Taylor, 2005). Different primary mouse macrophage populations can be studied, the most common include bone marrow derived

macrophages and peritoneal macrophages. Bone marrow derived macrophages develop by culturing bone marrow cells in M-CSF for 5–7 days. Resident peritoneal macrophages can be collected by peritoneal lavage. Elicited peritoneal macrophages are often obtained by intraperitoneal injection of small particles like bio-gel polyacrylamide beads or thioglycollate broth four days before collection. These primed elicited macrophages are terminally differentiated and proliferate partly, in the presence of M-CSF. Elicited peritoneal macrophages are different from resident macrophages, for instance Dectin-1 expression is higher on elicited macrophages while SIGN-R1, F4/80 and CD11b expression is higher on resident macrophages (Taylor et al., 2004).

Macrophages express a broad range of receptors whose expression levels vary depending on the macrophage population and activation state. The receptors are involved in a variety of macrophage functions and include responses to growth factors, cytokines, chemokines and other inflammatory mediators. Receptors are involved in migration, adhesion and antigen presentation.

4. ADHESION AND MIGRATION

Migration from blood into tissues involves a sequence of interactions between monocytes and endothelial cells, these include monocyte rolling, adhesion, polarisation and migration through the endothelium. Rolling is mediated by selectins expressed on the surface of monocytes. At this stage the monocytes are able to sense chemo-attractants such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF) or leukotriene B_4 (LTB_4), which leads to activation of β1- and β2-integrins, resulting in firm adhesion (Imhof and Aurrand-Lions, 2004). Some chemokines can be presented to the monocytes by endothelial cells. Transmembrane heparan sulphate proteoglycans expressed by endothelial cells bind chemokines in a way that leaves the binding site for monocyte chemokine receptors exposed. Initially, chemokine recognition leads to signalling cascades that activate a cytoskeletal protein called talin and a member of the RAS family of GTPases, that subsequently activate β2 integrins. Activated β2 integrins change their conformation leading to binding to ICAM1 molecules expressed by endothelial cells, resulting in strong adhesion (Kim, Carman, and Springer, 2003).

The monocytes, now adherent to the endothelium, need to migrate through the vascular barrier. This requires polarisation of the cell which is driven by integrins and the cytoskeleton. Lamellipodia which are actin-dependent flat protrusions from the cell, are formed at the anterior end. Constant vesicle transport takes place that moves lipid membrane from the golgi apparatus to the lamellipodia so that these can continue to extend; this transport is controlled by PKC-ζ. At the rear of the cell a structure forms called the uropod, which is enriched in adhesion molecules (Imhof and Aurrand-Lions, 2004). Different types of junctions have been described that maintain integrity of the endothelium and in order to migrate between adjacent

endothelial cells these junctions need to be breached. Some of the proteins involved in the formation of such junctions also play a role in monocyte migration by binding to integrins, these proteins include junctional adhesion molecules (JAMs), platelet/endothelial cell-adhesion molecule 1(PECAM1, CD31) and CD99 (Imhof and Aurrand-Lions, 2004).

5. MICROBIAL RECOGNITION BY MACROPHAGES

To eliminate pathogens macrophages, like other cells from the immune system, need to be able to distinguish self from non-self. The non-opsonic recognition of microbes is mediated by pattern recognition receptors (PRR), which recognise highly conserved molecules expressed by microbes referred to as pathogen associated molecular patterns (PAMPs) (Janeway, 1989). PAMPs are generally important for survival of the microbes and include LPS which is part of the cell wall of Gram negative bacteria, lipoteichoic acid (LTA) as part of Gram positive bacteria, mannans and β-glucans that are mainly found in the fungal cell wall and also in plants and some bacteria. Other receptors are able to sense double stranded RNA or foreign DNA of microbes (Janeway and Medzhitov, 2002).

The range of PRRs encompasses soluble serum factors, membrane bound (surface) receptors and intracellular proteins. Their functions include phagocytosis, opsonisation, activation of pro-inflammatory signalling, activation of complement and co-agglutination cascades and induction of apoptosis (Janeway and Medzhitov, 2002). Serum proteins like mannan binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein serve as opsonins for microbes. They are secreted by the liver and bind a broad range of pathogens, which leads to activation of the complement pathway and enhance phagocytosis. These soluble PRR will be discussed further in section 2 of this book. Several PRR are expressed on the surface of phagocytes (Table 1); their microbial recognition can be very specific, like Dectin-1 which binds to exposed β-glucans, or very broad like the scavenger receptors. Recognition by these receptors can lead to binding to phagocytes, phagocytosis and stimulation of a pro-inflammatory response. Toll-like receptors (TLR) form another family of membrane bound PRR. A broad array of microbes can be detected by TLRs and recognition leads to pro-inflammatory signalling. Cells also have cytosolic PRR, these include the nucleotide-binding oligomerisation domains (NODs) that recognise bacterial cell wall components, leading to apoptosis and secretion of pro-inflammatory cytokines.

Besides recognising foreign objects some PRR are also known to recognise host derived ligands. For instance, Dectin-1 has an unidentified ligand on T cells (Ariizumi et al., 2000), DC-SIGN (DC-specific ICAM-3 grabbing non-integrin) recognises intercellular adhesion molecule (ICAM)-2 and ICAM-3 (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b) and MR binds endogenous glycoproteins such as myeloperoxidase and lysosomal hydrolases (Shepherd and Hoidal, 1990).

Table 1. Pattern recognition receptors expressed at the macrophage surface

Facility	Member(s)	Selected microbial ligands
Classic C-type lectins	Mannose receptor	*C. albicans, P. carinii, M. tuberculosis, K. pneumoniae, Leishmania donovani*, HIV-1, zymosan
	DC-SIGN	HIV, Ebola virus, *Leishmania* spp.
Non-classic C-type lectins	Dectin-1	β-glucans, zymosan, *S. cerevisiae, C. albicans*
Leucine-rich repeats containing proteins	CD14	*E. coli*, LPS, LTA, peptidoglycan
	Toll-like receptors	LPS, LTA, zymosan, bacterial lipoproteins, peptidoglycan, viral proteins, flagellin, bacterial DNA
Scavenger receptors	SR-A (I and II), LOX-1, MARCO	*E. coli, S. aureus, L. monocytogenes, M. tuberculosis, Enterococcus faecalis, N. meningitidis*, LPS, LTA, bacterial DNA
Integrins	CR3, CR4	Complement coated microbes, LPS, LPG, *C. albicans, M. tuberculosis, C. neoformans*

6. TOLL LIKE RECEPTORS

The Toll receptor, previously shown to be important in development, was first found to be involved in immune defence against fungi in *Drosophila* (Lemaitre et al., 1996). Soon after mammalian homologues were characterised, and to date the TLR family is known to contain at least 11 members. Monocytes and macrophages express mRNA for most TLRs except perhaps TLR3 (Muzio et al., 2000). TLR1, TLR2 and TLR4 can be found on the surface of cells while TLR7, TLR8 and TLR9 are expressed on intracellular membrane compartments, including endosomes and endoplasmic reticulum (Ahmad-Nejad et al., 2002; Heil et al., 2003; Latz et al., 2004; Matsumoto et al., 2003). It has been proposed that intracellular compartments may be the main site for TLR recognition of microbial components (Takeda and Akira, 2005) including TLR2 which is also expressed on the surface (Underhill et al., 1999).

Microbial recognition by TLR leads to homo- or hetero- dimerisation of TLR. Upon ligation TLRs activate MyD88 -dependent and -independent signalling pathways (Akira, 2003), triggering expression of different genes that can induce many processes, including cytokine production (eg. TNFα and IL-12), nitric oxide production, actin re-organisation (West et al., 2004), phagocytosis (Doyle et al., 2004), phagosome maturation (Blander and Medzhitov, 2004; Doyle et al., 2004; Shiratsuchi et al., 2004) and induction of apoptosis. TLRs collaborate with other

pattern recognition receptors, to produce a response specific to the microbe (Underhill, 2003). The role for TLR in recognition of fungi will be discussed in chapter 11.

7. PHAGOCYTOSIS

Endocytosis is the process of taking up components of the extracellular environment, this includes pinocytosis which is the uptake of soluble molecules and small particles like viruses, a process which can be clathrin-dependent, but is independent of actin. Uptake of larger ($> 0.5\,\mu$m) particles is mediated via an actin-dependent process, called phagocytosis (Fig. 2) (Aderem and Underhill, 1999). General features of phagocytosis include receptor ligation, which results in receptor clustering, to mediate particle binding and downstream signalling. This leads to actin-based membrane motility that forms the membrane around the particle resulting in a phagosome. Actin depolymerises once the phagosome is formed, which enables the phagosome to mature by a series of fusion and fission events with endosomes and later lysosomes, forming a phagolysosome (Aderem and Underhill, 1999). During maturation of the phagosome, the pH drops and oxygen-dependent and -independent killing mechanisms are activated.

Macrophages are professional phagocytes that express a series of phagocytic receptors. The variety of receptors expressed by macrophages greatly increases the range of particles that can be phagocytosed and also provides cytosolic

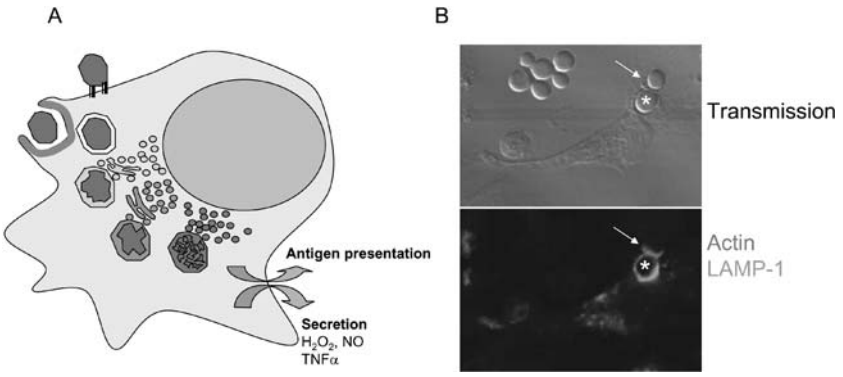


Figure 2. Phagocytosis. A) Schematic representation of phagocytosis. Engaging of phagocytic receptors leads to particle binding and signalling for cytoskeletal rearrangements. This leads to the formation of a so called phagocytic cup that results in membrane engulfment of the particle. Once a phagosome is formed it matures by fusion and fission with early endosomes, late endosomes and lysosomes, sequentially. During this process the pH of the phagosome is lowered from 6 to around 4.5. Phagocytic signalling cascades also stimulate secretion of H_2O_2, NO and TNFα. B) *Candida albicans* phagocytosis by RAW264.7 macrophages. The cells were stained for actin with TRITC phalloidin (red) and for endosomes and lysosomes with LAMP-1 (green). The first yeast is taken up in a phagosome where the lysosomes have fused (asterisk). The second yeast is in the process of being phagocytosed and there is a clear phagocytic cup present (arrow) (See Color Section.)

Table 2. Examples of macrophage phagocytic receptors (modified from (Greenberg, 1995))

Opsonin-dependent receptors	Opsonin-independent receptors
$Fc_{\gamma}R$ I, IIA, IIIA	Complement receptor 3 (CR3)
Complement receptors 1 and 3 (CR1, CR3, CR4)	Macrophage mannose receptor
IgA receptor ($Fc_{\alpha}R$)	β1 integrins
High affinity IgE receptor ($Fc_{\varepsilon}RI$)	Dectin-1
Low affinity IgE receptor (CD23, $Fc_{\varepsilon}RII$)	SIGN-R1/DC-SIGN family
Vitronectin receptor ($\alpha_{v}\beta3$)	Macrophage scavenger receptors

signalling that couples uptake to effector responses. Macrophages are able to recognise particles with more than one receptor, leading to cross-talk and synergy of the downstream signalling. This is a complex process that enables macrophages to produce a response appropriate to the ingested particle (Aderem and Underhill, 1999; Stuart and Ezekowitz, 2005). Several macrophage receptors have been suggested to mediate binding and ingestion of particles, either via opsonin-dependent or -independent recognition (Table 2), FcγR and CR3 mediated phagocytosis will be discussed below.

7.1. FcγR Mediated Phagocytosis

Fc receptors (FcRs) are receptors that recognise the Fc portion of immunoglobulins. They belong to the immunoglobulin-superfamily of proteins and consist of an α chain associated with a signaling chain, namely the β, ζ or γ (Greenberg, 1999). There are two classes of Fc receptors, one is involved in effector functions while the other transports Ig across epithelial surfaces (Ravetch, 1997). The FcγR can be divided into receptors that activate effector functions, containing immunoreceptor tyrosine-based activation motifs (ITAM) and receptors that inhibit these functions, which have an immunoreceptor tyrosine-based inhibition motif (ITIM) (Ravetch and Bolland, 2001). FcRs that mediate phagocytosis fall within the activation class and include FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIA (CD16) (Garcia-Garcia and Rosales, 2002). Macrophages express these three phagocytic FcγR and also express FcγIIB which negatively regulates phagocytosis via its ITIM motif (Hunter et al., 1998).

FcγR are some of the best understood phagocytic receptors. IgG coated particles cause clustering of FcγR which leads to phosphorylation of tyrosine within the ITAM motif by Src tyrosine kinases (Fitzer-Attas et al., 2000; Suzuki et al., 2000). The phosphorylated ITAMs recruit a range of proteins including Syk kinase (Swanson and Hoppe, 2004; Turner et al., 2000). Syk is essential for the downstream signalling of FcγR phagocytosis (Indik et al., 1995). Macrophages from Syk-deficient mice can polymerise actin into a phagocytic cup, but are unable to complete internalisation of antibody opsonised particles, however they are able to phagocytose latex beads and yeast (Crowley et al., 1997). Blocking phosphatidylinositol 3 kinase

(PI3K) blocks FcγR internalisation at the same stage (Araki, Johnson, and Swanson, 1996; Crowley et al., 1997). PI3K phosphorylation by Syk activates PI3K which leads to phosphorylation at the D-3 position of the inositol ring of phosphatidylinositides (PI). This leads to the production of several signalling molecules that influence processes like phagosome formation, phagosome maturation and NADPH oxidase assembly and activation (Swanson and Hoppe, 2004).

Cdc42 and Rac1 from the Rho family of GTPases play important roles in actin assembly for phagocytosis via FcγR. GTPases are generally in their inactive guanosine 5′-diphosphate (GDP)-bound form in the cytosol and become activated in their guanosine 5′-triphosphate (GTP)-bound form when they also become membrane bound. Guanine nucleotide exchange factors (GEF) mediate the transition from GDP to GTP and different GEF proteins are activated by PI(3,4,5)P3, a product of PI3K (Swanson and Hoppe, 2004). Activation of FcγR mediated phagocytosis is blocked by overexpression of dominant negative forms of either Cdc42 or Rac1 (Cox et al., 1997). Cdc42 and Rac1 have distinct activation patterns and contribute to phagocytosis in different ways. Cdc42 is thought to play a role in actin polymerisation for pseudopod extension (Chimini and Chavrier, 2000). Consistent with this it was shown that Cdc42 localises to the tips of advancing pseudopodia (Hoppe and Swanson, 2004). Furthermore, artificial clustering of Cdc42 near cell bound particles induced actin polymerisation, but not phagocytosis (Castellano et al., 1999). On the other hand, Rac1 is thought to play a role in phagosome closure since artificial clustering of Rac1 induces particle uptake (Castellano, Montcourrier, and Chavrier, 2000; Swanson and Hoppe, 2004). Members of the Wiskott-Aldrich Syndrome Protein (WASP) family function downstream of the Rho GTPases (Chimini and Chavrier, 2000). WASP proteins regulate the actin cytoskeleton through activation of the Arp2/3 complex (Castellano, Chavrier, and Caron, 2001). Rac1 also activates NADPH oxidase activity (Bokoch and Diebold, 2002) which is antagonised by Cdc42 (Diebold et al., 2004).

Another GTPase involved in FcγR mediated phagocytosis is Adenosine 5′-diphosphate-ribosylation factor 6 (ARF6). Macrophages expressing defective ARF6 are unable to phagocytose antibody-opsonised particles (Zhang et al., 1998). Although ARF6 plays a role in actin assembly (Radhakrishna et al., 1999), it is not required for actin polymerisation in FcγR mediated phagocytosis. However, it is important for membrane delivery to the phagosome and may also play a role in NADPH oxidase activation (Niedergang et al., 2003; Swanson and Hoppe, 2004).

Intracellular membrane sources are needed to complete phagocytosis (Greenberg and Grinstein, 2002). Membrane for the new phagosome has been proposed to come from the plasma membrane, endosomes and ER (Bajno et al., 2000; Cox et al., 2000; Garin et al., 2001; Hackam et al., 1998; Muller, Steinman, and Cohn, 1980). During invasion of trypanosomes, lysosomes have also been shown to be a source of membrane (Tardieux et al., 1992). Endosomes are most likely a primary source for this membrane as is shown by toxins that inactivate vesicle-associated membrane protein 3 (VAMP3), a SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) protein found in recycling endosomes that

plays a role in vesicle docking and fusion (Jahn, Lang, and Sudhof, 2003), and which blocks phagocytosis (Braun et al., 2004). Furthermore, fusion of VAMP3-containing vesicles precedes phagosome closure (Bajno et al., 2000). However, Allen et al. showed that antibody-opsonised bead phagocytosis was not impaired in macrophages from VAMP3-deficient mice (Allen, Yang, and Pessin, 2002). Expression of inactive Rab11, a GTPase involved in trafficking and sorting of recycling endosomes, impaired phagocytosis via FcγR (Cox et al., 2000).

Following actin polymerisation, membrane delivery and pseudopod extension the final stage of phagosome formation is phagosome closure. Myosins and PI3K amongst other signalling proteins have been shown to be involved in this last stage of phagosome formation (Swanson et al., 1999). Besides signalling for phagocytosis, FcγR can also stimulate the production of reactive oxygen intermediates and arachidonic acid metabolites and induce the secretion of TNFα, IL-1β, IL-6, chemokines and growth factors (van de Winkel and Anderson, 1991).

7.2. CR3 Mediated Phagocytosis

CR3 is also called Mac-1, Mo-1, αmβ2 or CD11b/CD18 integrin. Besides its ability to phagocytose C3bi-opsonised particles it recognises other endogenous ligands including ECM proteins, collagen, fibrinogen and ICAM-1 and -2 (Plow and Zhang, 1997). CR3 functions also as an adhesion receptor and mediates leukocyte migration. Moreover, CR3 is described to be a pattern recognition receptor able to recognise many ligands including: LPS, lipophosphoglycan (LPG), β-glucans, zymosan and *C. albicans* (Ehlers, 2000; Forsyth and Mathews, 1996; Forsyth, Plow, and Zhang, 1998; Ross, Cain, and Lachmann, 1985; Thornton et al., 1996). CR3 is a member of the β2 integrins that share the CD18 (β2) subunit. These β2 integrins are exclusively expressed by leukocytes. The CD11b subunit contains a C-terminal lectin site, a calcium binding site, an (inserted) I- domain and a small signalling domain (Ross, 2000). The lectin site and the I-domain are both involved in ligand recognition. The I-domain has overlapping, but not identical sites for binding many protein ligands, including C3bi. The lectin site has been shown to bind β-glucan, zymosan and N-acetyl-D-glucosamine (Ross, Cain, and Lachmann, 1985; Thornton et al., 1996). The interaction of *C. albicans* with CR3 is suggested to be mainly mediated by the I-domain, but this recognition is modulated by the lectin site (Forsyth, Plow, and Zhang, 1998).

Unlike FcγR, CR3 needs additional stimuli for the internalisation of particles upon recognition (Pommier et al., 1983; Wright and Silverstein, 1983). These stimuli include PKC activators such as PMA (phorbol 12-myristate 13-acetate), cytokines like TNFα or GM-CSF, microbial products like LPS, ligation of co-receptors such as FcγR, or attachment to a laminin- or fibronectin- coated substratum (Aderem and Underhill, 1999; Underhill and Ozinsky, 2002). Two mechanisms have been described to mediate activation of CR3. First, ligation of co-receptors like FcγR or selectins leads to cytoskeletal rearrangements that release CR3 from its cytoskeletal

constraints leading to CR3 clustering and activation (Jones et al., 1998; Jongstra-Bilen, Harrison, and Grinstein, 2003). The second mechanism involves ligation of G-protein-coupled receptors and is independent of actin reorganisation (Jones et al., 1998; Newton, 1998).

The phagocytic mechanisms of CR3 are different from FcγR mediated phagocytosis. Antibody-opsonised particles are engulfed by lamellipodia that project from the cell surface and tightly cover the particle interacting sequentially with IgG molecules distributed over the particle before it is drawn into the cells; this process is known as the 'zipper' mechanism (Kaplan, 1977; Silverstein, 1995). Particles phagocytosed via CR3 appear to sink into the phagocyte without apparent involvement of membrane extensions (Kaplan, 1977). The membrane is also less tightly apposed with point-like contacts with the particle, separating regions of looser membrane (Allen and Aderem, 1996). These contact areas are rich in cytoskeletal proteins like F-actin and Arp2/3 while these proteins are uniformly distributed on or near the phagosome surface in FcγR mediated phagocytosis (Allen and Aderem, 1996; May et al., 2000). Furthermore, CR3 mediated phagocytosis does not require tyrosine kinase activity, but does need intact microtubules (Allen and Aderem, 1996). Differences in phagocytic mechanisms could be explained by the differential involvement of Rho GTPases since these GTPases stimulate different actin structures (Hall, 1998). Unlike FcγR, CR3 mediated phagocytosis depends on Rho GTPase, but is independent of Cdc42 and Rac1 (Caron and Hall, 1998). However, Le Cabec et al., have also suggested that CR3 can mediate both types of phagocytosis depending on the ligand (Le Cabec et al., 2002).

A third difference between FcγR and CR3 mediated phagocytosis is that CR3 mediated phagocytosis does not automatically induce an oxidative burst and release of arachidonic acid (Aderem et al., 1985; Wright and Silverstein, 1983). This may lead to the use of CR3 as a portal of entry by pathogens. However, as suggested by Ehlers (Ehlers, 2000) the response to an infectious challenge needs to be in proportion to the threat in order to prevent unnecessary tissue damage by overactive macrophages and neutrophils. Therefore, cell activation needs additional signals besides CR3 ligation such as; receptor clustering, which indicates a high ligand density; receptor activation by costimulation by cytokines, chemokines or microbial ligands; or cooperation with other receptors, which indicates the presence of more than one foreign ligand (Ehlers, 2000).

8. PHAGOSOME MATURATION

Phagosome maturation occurs by phagosome fusion and fission with endosomes and later lysosomes, leading to the formation of a phagolysosome (Desjardins et al., 1994). The rate of phagolysosome fusion is likely to depend on the nature of the ingested particle. Both microtubules and the actin cytoskeleton are involved in phagosome maturation. Rab proteins and SNARE proteins are specific to the different organelles of the endocytic pathway and mediate the fusion of these vesicles. The endosomal compartment is a dynamic network of

tubular and vesicular membrane structures that can be divided into early and late endosomes. Early endosomes have a pH arround 6, contain small amounts of proteases and can be distinquished using specific markers like early endosomal antigen 1 (EEA1) and Rab5 while late endosomes have a slightly higher pH and contain more hydrolytic enzymes. Specific markers for late endosomes include mannose-6-phosphate receptor, Rab7 and Rab9. Lysosomes are vesicles that contain the majority of lipases and hydrolases and have a pH below 5. Lysosomal markers like lysosomal associated proteins (LAMPs) and cathepsin D can also be found in late endosomes. A method used to specifically label lysosomes includes a pulse of fluid-phase markers (e.g. fluorochrome-conjugated dextrans) followed by a long chase that localises the endocytosed marker to the lysosomes (Vieira, Botelho, and Grinstein, 2002).

As the phagosome matures it acquires markers that are specific for the organelles it is interacting with, the assembly of an ATPase complex mediates acidification and the pH lowers from neutral to around 4.5. The acidic environment affects pathogen growth, stimulates NADPH oxidase assembly and creates an optimal environment for hydrolytic enzyme activity. The phagosomal pH is also suggested to play a role in phagosome maturation (Vieira, Botelho, and Grinstein, 2002). Other regulators of phagosome maturation include calcium and different phosphoinositides. Phosphoinositides (PI) is the collective name for phosphorylated derivatives of phosphatidylinositol. PIs are membrane bound and comprise less than 10% of the total cellular phospholipids. However, these lipids are important signalling molecules in receptor-mediated signal transduction, actin remodelling and membrane trafficking (Downes, Gray, and Lucocq, 2005; Matteis, 2004). A total of eight different PIs can be produced by different combinations of phosphate groups arranged around the inositol ring. Organelle specific PI kinases and PI phosphatases mediate rapid subcellular distribution of specific PIs. This leads to recruitment, binding and activation of effector proteins that mediate downstream signalling.

The NADPH oxidase assembly at the phagosome results in the production of reactive oxygen species that are believed to mediate killing. The complex consists of six subunits that include membrane bound phagocytic oxidase subunits: gp91*phox*, p22*phox* which form flavocytochrome b upon activation, a Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2, and cytosolic subunits p40*phox*, p47*phox*, and p67*phox* that are recruited and assemble into the full complex. Upon activation, the NADPH oxidase catalyses the following reaction:

$$\mathrm{NADPH} + 2\mathrm{O}_2 \rightarrow \mathrm{NADP}^+ + 2\mathrm{O}_2{}^- + \mathrm{H}^+$$

The superoxide anions play a prominent role in oxygen dependent microbial killing; moreover O_2^- can be dismutated to hydrogen peroxide (H_2O_2), either spontaneously or by the antioxidant enzyme superoxide dismutase, and H_2O_2 may subsequently be converted into a variety of active oxygen species, including hydrogen peroxide and hydroxyl radicals. Another important enzyme involved in microbicidal activity is inducible nitric oxide synthase (iNOS) which catalyses the production of NO