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OF YEAST AND MEN: DISSECTING THE INTERACTION BETWEEN FUNGI AND IMMUNE RESPONSE

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Ai miei angeli, sempre

Ci sono mille splendidi soli aldilà delle nuvole
Proverbio indiano

To be able to listen,
we have to be very still inside,
calm as a silent witness.
Baba Harihar Ramji

Akram iti

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When it is dark enough, you can see the stars.
Persian proverb

Summary

The greatest scientific challenge of the 20th century has been the characterization of the genetic makeup of an organism, sequencing its genome and studying the function of its genes.

The first sequence of an eukaryotic genome, *Saccharomyces cerevisiae*, in 1996 paved the way to the sequence of the human genome. The functional characterization of our genes has kept scientists busy for the last 10 years. Yet the sequence of the dark side of the genome, the metagenome is now revealing the centrality for human health of an harmonious interaction between the organismal functions and the activity of the microorganisms colonizing our body.

Aim of our work is to use the best characterized microorganism *S. cerevisiae* to understand the mechanism governing a fruitful interaction between microbes and the human system.

Candida albicans and *S. cerevisiae* are ubiquitous fungal organism that often colonize the skin and the mucosal surfaces of normal individuals, without causing disease. However, when the normal host defence mechanisms are impaired (for example, in patients who are undergoing chemotherapy for malignancies, receiving immunosuppressants after an organ transplant, or patients with AIDS), they can become pathogenic.

Only in the past decade it has become clear that the innate immune system not only specifically recognizes various classes of microorganisms, but also initiates and modulates the subsequent adaptive responses that are delivered by T cells and B cells through their interactions with antigen-presenting cells, especially dendritic cells (DCs). The tasks of recognizing an invading pathogen and activating the host response are accomplished by pattern-recognition receptors (PRRs), which recognize conserved microbial components called pathogen-associated molecular patterns (PAMPs).

An association between morphogenesis and virulence has long been presumed for dimorphic fungi that are pathogenic to humans, as one morphotype exists in the environment or during commensalism, and another within the host during the disease process. For *C. albicans*, putative virulence factors include the ability to switch between saprophytic yeast and pathogenic, filamentous forms of the fungus. DCs sense either form in a specific way, resulting in distinct, T-helper (Th)-cell-dependent protective and non-protective immunities. Recent evidence suggests that the use of distinct recognition receptors contributes to the disparate patterns of reactivity observed locally in response to challenge with *C. albicans.*

The interaction between *S. cerevisiae* and human societies is so generally recognized to hypothesize domestication of strains of this species. Of the multitude of fungal species known, only a few can be considered truly pathogenic in healthy individuals. Successful resolution of pathogenic fungal disease depends on proper coordination of multiple components of the host immune response. The balance between pro- and anti-inflammatory signalling is a prerequisite for proper host-fungal interactions. Although inflammation is an essential component of the protective response to fungi, its deregulation may significantly worsen fungal diseases and limit protective antifungal immune responses.

Host/fungal interactions have so far been studied only for pathogenic fungi such as *C. albicans*, but it is not known what determines the commensalism of harmless fungi. Understanding the mechanisms of cohabitation between humans and non-pathogenic fungi is a prerequisite for controlling fungal infections. The decision on how to respond to a microorganisms, be it a pathogen or a commensal, is still primarily determined by the interaction between cell wall component and cells of the innate immune system. We investigated the rules of host-fungal cohabitation and dissected the mechanisms responsible for the differential recognition demonstrating that the skewing in the use of pathogen recognition receptors by differences in cell wall composition is a requirement for pathogenicity.

To unravel the rules of the recognition game, we used a systems biology approach to understand how different life stages of the harmless and commensal *S. cerevisiae* interact with our immune system, comparing the results to those obtained upon exposure to pathogenic microorganisms. In particular we investigated the rules of host-fungal cohabitation using human monocyte-derived DCs (moDCs) exposed to cells and spores of the yeast *S. cerevisiae* as a toolbox to dissect the role cell wall component in determining commensalism or pathogenicity traits. Combining transcriptional analysis with receptor-specific blocking and cytokine production assays, we determined that DCs respond differently to *C. albicans* and *S. cerevisiae* and in the latter case, the interplay between spores and yeasts is crucial for the commensalism of *S. cerevisiae*. We demonstrated that the differential recognition of specific mannan structures is the master regulator of the discrimination between harmful and harmless fungi. Our findings elucidate the mechanisms of commensalism, demonstrating the importance of exposing *S. cerevisiae* mannans to suppress pro-inflammatory responses.

To contrast fungal infection, augmenting the ability of the immune system to eliminate a pathogen requires a sophisticated understanding of the molecular mechanisms that are involved in pathogen recognition and in the host immune response. Widespread use of microarrays has generated large amounts of data, the interrogation of the public microarray repositories, identifying similarities between microarray experiments is now one of the major challenges. We developed a

novel method to compare microarray experiments at the pathway level, this method consists of two steps: first, generate pathway signatures, a set of descriptors recapitulating the biologically meaningful pathways related to some clinical/biological variable of interest, second, use these signatures to interrogate microarray databases. Using this approach it is possible to retrieve data from public microarray databases and perform comparisons at the pathway level using the newly structured pathways we curated in the DC-ATLAS initiative. When performing this exercise on microarray data derived from pure agonist and stimulation with the entire microorganism we were able to dissect the several components of the transcriptional response to the fungus. We also demonstrated how our approach improves the power of pathway analysis to dissect the response to different fungi. This approach proved very useful to extract from transcriptional data the regulator programs discriminating the response elicited from a pathogen and from an harmless microorganism.

The integration of a System Biology approach to functional data offers new interpretive clues to the mechanisms of fungal virulence: rather than dimorphism per se, the engagement of different recognition receptors on DCs might select the mode of fungal internalization and antigen presentation, condition the nature of the T-helper response and, ultimately, favor saprophytism or infection.

Products of the PhD activity

Beltrame, L., Rizzetto L., Paola R., Rocca-Serra P., Gambineri L., *et al.* (2009). *Using Pathway Signatures as Means of Identifying Similarities among Microarray Experiments*. PLoS ONE 4(1): e4128. doi:10.1371/journal.pone.0004128

Rizzetto, L., Kuka M., De Filippo C., Cambi A., Netea M.G., Beltrame L., Napolitani G., Torcia M.G., D'Oro U. and Cavalieri D. *Differential IL-17 production and mannan recognition explain fungal pathogenicity and commensalism.* Under revision to Journal of Immunology.

Cavalieri, D., Rivero-Guedez D., Beltrame L., Calura E., Rizzetto L., *et al. DC-ATLAS: a systems biology approach to deciphering the complexity of signal transduction pathways in dendritic cells*, in preparation

Magherini, F., Carpentieri, A., Amoresano, A., Gamberi, T., De Filippo, C., Rizzetto L., Biagini M., Pucci P., Modesti A. (2009). *Different carbon sources affect lifespan and protein redox state during Saccharomyces cerevisiae chronological ageing*. Cell Mol Life Sci. Mar; 66(5): 933-47.
This publication is subject of an experimental contribution done in parallel with the PhD activity.

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Abbreviations

µg, microgram
µl, microliter
µM, micromolar
Ab, antibody
AE, Array Express
APC, allophycocyanin
APC, antigen presenting cell
CA, *Candida albicans*
CARD9, caspase recruitment domain protein 9
cDNA, complementary deossiribonucleic acid
CFU, colony forming unit
CHO, Chinese Hamster Ovarian cell line
CLR, C-type lectin receptor
CR, complement receptor
CRD, carbohydrate recognition domain
Cy, cyanin
CWP, cell wall protein
DC, dendritic cell
DC-SIGN, Dendritic cell-specific ICAM-3-grabbing non-integrin
Dectin-1, Dendritic cell associated C-type lectin 1
Dectin-2, Dendritic cell associated C-type lectin 2
DEG, differentially expressed gene
DMSO, dimethyl sulphoxide
DPI, diphenileneiododium chloride
EDTA, ethylenediamine tetraacetic acid
EGTA, ethylene glycol tetraacetic acid
ELISA, enzyme-linked immunosorbent assay

EPN, Edinburgh Pathway Notation

FC, fold change

FcγR, Fc gamma receptor, Fc receptors for immunoglobulin G

FDR, fold discovery rate

FET, Fisher exact test

FITC, fluorescin isothiocyanate

Galectin-3, Galactose specific lectin 3

GEO, Gene Expression Omnibus

GlcNAc, N-acetylglucosamine

GM-CSF, granulocyte macrophage-colony stimulating factor

GO, Gene Ontology

GPI, glycosylphosphatidylinositol

HLA-DR, major histocompatibility complex, class II, DR

IBD, immune bowel disease

IF, Impact Factor

IFN, interferon

IgG, immunoglobulin G

IL, interleukin

IRF, interferon-regulatory factor

ITAM, immunoreceptor tyrosine-based activation-like motif

KEGG, Kyoto Encyclopedia of Gene and Genome

KOH, potassium hydroxide

LPS, lipopolysaccharide

mAb, monoclonal antibody

MAPK, mitogen-activated protein kinase

mg, milligram

MHC, major histocompatibility complex

MIM, molecular interaction map

ml, milliliter

mM, millimolar

moDC, monocyte-derived dendritic cell

MR, mannose receptor

$NaHCO_3$, sodium bicarbonate

NADPH, Nicotinamide adenine dinucleotide phosphate, reduced form

NBT, Nitroblue tetrazolium

NFkB, nuclear factor kB

ng, nanogram

nm, nanometers

N-terminal, amino terminal

nuID, nucleotide universal IDentifiers

OD, optical density

PAMPs, pathogen associated molecular pattern

PBMC, peripheral blood mononucleated cell

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PDN, Process Description Notation

PFA, paraformaldeyde

PMA, phorbol myristate acetate

PRR, pathogen recognition receptor

PS, Pathway Signature

RNA, ribonucleic acid

RMA, Robust Multichip Average procedure

ROS, reactive oxygen species

RPMI, Roswell Park Memorial Institute medium

RT-PCR, real time polymerase chain reaction

sBEF, signed binary enriched factor

SBML, System Biology markup language

SBGN. System Biology Graphical Notation

SC, *Saccharomyces cerevisiae*

SD, standard deviation

SOCS, Suppressor of cytokine signalling

SR, scavenger receptor

TF, transcription factor

TGF, transforming growth factor

TNF, tumor necrosis factor

Th, T helper cells

TLR, Toll like receptor

Treg, regulatory T cells

TRIF, TIR-domain-containing adaptor-inducing interferon

Tris, Tris(hydroxymethyl) aminomethane

YPD, yeast extract peptone dextrose

WT, wild-type

°C, degree celsius

Everything in the world exists to be end up in a book
Stephane Mallarmé

1
Introduction

The mammalian immune system consists of two different arms – innate and acquired immunity - and cooperative interaction of these two branches is required for elimination of infective pathogens with the highest possible efficiency. The innate immune system is an evolutionarily conserved system that provides the first line of protection against invading microbial pathogens and is mediated by phagocytes such as macrophages and dendritic cells (DCs; Hoffmann et al., 2003; Akira et al., 2006; Beutler et al., 2007; Medzhitov et al., 2007).

Within minutes of the invasion of the host by a pathogenic microrganism, the innate immune system is activated and coordinates the host defence during the intial hours and days of infection. The ability of the host to discriminate self from potentially harmful non-self, and act appropriately is a central feature of immune defence. Although the innate immune system is very effective in dealing with the vast majority of invading pathogens, it has been believed for many years to be non-specific and non-selective, with specificity being conferred only by the secondary activation of acquired immunity mediated by T- and B-lymphocytes.

The initial detection of pathogens is governed by the innate immune system, which is composed of cellular and humoral components with common ability to bind conserved features present on pathogenic surfaces. These pathogen associated molecular patterns (PAMPs) are the 'Achilles heel' of the microbial world as they are critical to microbial viability and therefore they are conserved from an evolutive point of view.

The dogma of the non-selective nature of the innate immune response and, in particular, the presumed non-specific recognition of microrganisms by phagocytic cells, has been challenged by the recent discovery of classes of receptors called pathogen recognition receptors (PRRs) which specifically recognize PAMPs and deliver appropriate signals to cells of the innate immune system (Janeway, 1989). The manner in which antigen presenting cells (APCs) such as macrophages and DCs recognize foreign structures and internalize them for presentation to cells of the adaptive

immune system will determine the overall quality and effectiveness of immune responses. Recognition of pathogens by PRRs leads to release of pro-inflammatory cytokines and activation of anti-bacterial mechanisms, and modulates the initiation of acquired immunity by activation of DCs.

1.1 Dendritic cells: control of immunity and tolerance

Dendritic cells (DCs), originally identified by Steinman and his colleagues (1973) represent the pacemakers of the immune response. DCs are professional APCs that are seeded throughout peripheral tissues to act as sentinels that process and present antigen to mount adequate immune responses. Depending on the type of antigen and the tissue localization, the immune response is suppressed or activated (Bancherau et al., 1998; Steinman et al., 2003).

DCs differentiate from bone marrow stem cells and migrate as precursor DCs into the blood. Immature DCs populate all body tissues where they sample either self or non-self antigens. Self antigens can be derived from their innocuous environment, such as necrotic and apoptotic cells that need to be scavenged before they disintegrate. Alternatively, non-self antigens are foreign products from invading pathogens that need to be eliminated. The main function of DCs is to capture antigen for processing and presentation as antigenic fragments on major histocompatibility complex (MHC) class I or II molecules to naïve T cells (Bancherau et al., 1998).

In a steady-state situation, prior to acute infection and inflammation, DCs are in an immature state and are not fully differentiated to carry out their known roles as inducers of immunity. It does not mean they are unrensponsive: they actively circulate through tissues and into lymphoid organs, capturing self-antigens as well as innocuous environmental proteins. In a state of alarm such as a microbial invasion, or massive cell death, immature DCs receive simultaneous activation signals through the binding of conserved molecular motifs by pattern recognition receptors, such as Toll-like receptors (TLRs) or specific TNF family members (Bancherau et al, 1998, Metzinger, 2002). This result in DC maturation and migration to secondary lymphoid organs, where DCs present the processed antigens to naïve T cells and induce antigen-specific immune responses.

Certain chemokines, adhesion molecules, and co-stimulatory molecules carefully orchestrate the maturation and migration of DCs. These factors control the differentiation stages of the DC and direct the migration of the various DC subtypes (Sallusto and Lanzavecchia, 1999). Chemokines generated within the lymph nodes attract naïve T cells toward the DC, enabling maximal exposure of MHC-peptide complexes to naïve T cells. Also, Adhesion molecules are crucial for the cellular interactions that DCs undergo during their journey from bone marrow through blood into peripheral organs and subsequently lymphoid tissues, where they enable DC–T cell interactions necessary for T cell activation.

Recently, many novel cell-surface molecules have been identified that are involved in antigen capture by DCs. In particular, DCs display a large diversity of C-type lectin receptors (CLRs) that are involved in the recognition of a wide range of carbohydrate structures on

antigens (Figdor et al., 2002, Drickamer, 1999). Especially the resident immature tissue DCs express a wide variety of C-type lectins that seem to be involved in the specific recognition of both self-antigens and pathogens.

One of the major enigmas in immunology concerns those regulatory mechanisms that maintain the unresponsiveness of self-reactive T cells in the normal repertoire to self-antigens while at the same time allowing effective immune reactions to foreign antigens to be mounted.

The ability of DCs to immune modulate a proper recognition suggests that DCs fulfill the requirements for a cell uniquely capable of discriminating between the different forms of a fungus in terms of the elicited immune response. Most pathogens express different PAMPs and trigger several classes of PRRs on a single cells simultaneously. As a result, the ultimate expression of response genes induced by a pathogen depends on the integration of these different signalling pathways. Cross talk between or even within groups of PRRs is crucial in balancing immune response through collaborative induction of positive or negative feedback mechanisms (Lee and Kim, 2007).

1.2 PRRs in antifungal immunity

Humans are continuously exposed to fungi, yet they rarely develop fungal diseases. Infections caused by fungal pathogens represent a serious health problem for immunocompromised patients (cancer, transplantation and primary immunodeficiency patients; Sternberg, 1994). Host defences against fungi are numerous and range from relatively, constitutively expressed non-specific defences to sophisticated adaptive responses that are specifically induced during infection and disease (Romani et al., 1998; Romani, 2002).

Key sensors in anti-fungal defence are fungal PRRs expressed by phagocytes and APC. Fungal PRRs can be divided in two groups that have either intrinsic microbial recognition or indirectly facilitate host-pathogen recognition (opsonic receptors). Fungal PRRs with intrinsic microbial recognition comprises members of the C-type lectin receptor (CLR) family, Toll like receptor (TLR) family, scavenger receptor (SR) family and certain integrins. Fungal PRRs can induce fungal phagocytosis, killing and/or initiate signalling leading to cytokine responses (Underhill and Ozinsky, 2002; Thoma-Uszynski et al., 2001; Weia et al., 1998).

1.2.1 Toll like receptors

TLRs are pathogen recognition receptors (Janeway and Medzhitov, 2002) that recognize a wide variety of microbial structures, including lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4), lipoteichoic acid and lipoproteins of Gram-positive bacteria (TLR2), bacterial DNA (TLR9), and viral RNA (TLR3, TLR7 and TLR8) (Figure 1.1) as well as factors secreted upon tissue damage such as heat shock proteins (Hsp 70; Akira and Hemmi, 2003;

Hemmi et al., 2000, Medzithov et al., 2001). The recognition by TLRs triggers intracellular signalling cascades that result in DC maturation and the induction of inflammatory cytokines, ultimately leading to T cell activation (Underhill and Ozinsky, 2002; Janeway and Medzhitov, 2002).

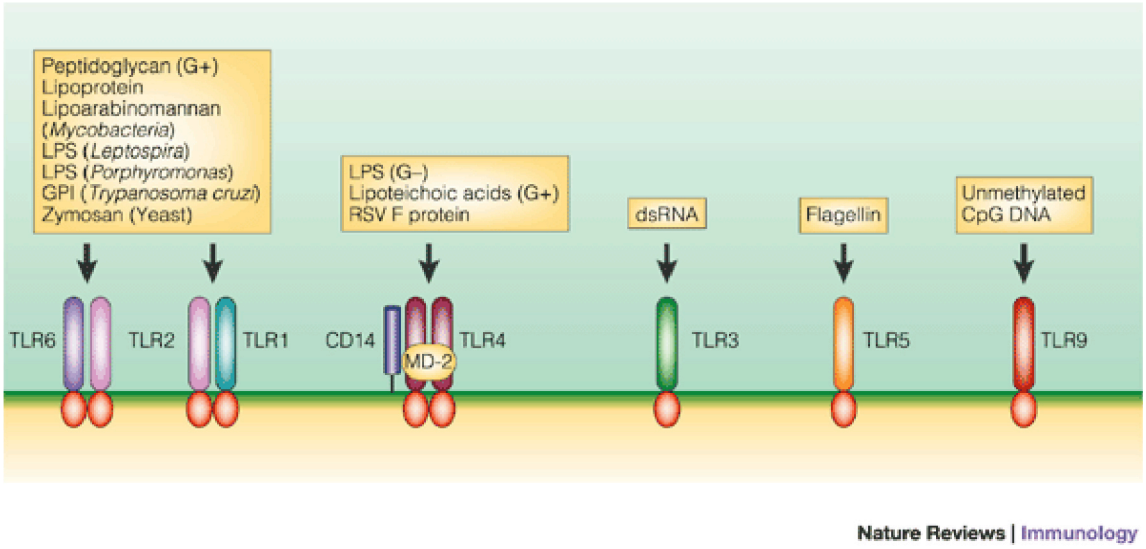


Figure 1.1. Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns (PAMPs). Recognition of lipopolysaccharide (LPS) by TLR4 is aided by two accessory proteins: CD14 and MD2. TLR2 recognizes a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. TLR3 is involved in recognition of double-stranded (dsRNA). TLR5 is specific for bacterial flagellin, whereas TLR9 is a receptor for unmethylated CpG motifs, which are abundant in bacterial DNA. G+, Gram-positive; G–, Gram negative; GPI, glycophosphoinositol; RSV, respiratory syncytial virus. *Adapted from Medzithov, 2001*

The TLR family of proteins, first described in *Drosophila*, possess extracellular leucine-rich repeat region, which are involved in microbial recognition, and an intracellular Toll/interleukin (IL)-1 receptor (R) (TIR) domain, which is necessary for signalling. Ligand recognition by TLR homo- or hetero-dimers, induces signalling cascade mediated through intracellular adaptors, including myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), which result in the activation of several transcription factors, such as NF-kB and interferon-regulatory factor 3 (IRF3). Ultimately this induces TLR-specific pattern of gene expression and the production of various cytokines and chemokines (Akira et al., 2006). Of the TLRs identified, fungal recognition appears to occur primarily through TLR2, TLR4 and TLR9 (reviewed in Netea et al., 2006). Deletion of the intracellular adaptor MyD88 renders mice highly susceptible to infections with fungi, including *Candida albicans*, *Aspergillus fumigatus* and *Criptococcus neoformans*, although the role of the individual TLRs has not been established firmly in all the diseases and is often controversial. Several research groups have

demonstrated divergent role for TLR2 and TLR4 and their importance in the control of *C. albicans* and *C. neoformans* infections, for example, is still unclear (Netea et al., 2006; Nakamura et al., 2006; Biondo et al., 2005). Thus, the TLRs undoubtedly have an important function in immunity to these pathogens, although the role of the individual receptors still remains to be completely elucidated.

1.2.2 C-type lectins

In contrast to TLRs, CLRs recognize specific carbohydrate structures on self antigens or cell wall components of pathogens. Their main function is to internalize antigens for degradation in lysosomal compartments to enhance antigen processing and presentation by DCs (Figdor et al., 2002; Engering et al., 2002). To date, several CLRs have been found to function as pathogen recognition receptors, whereas only a few have been shown to recognize self-antigens.

Within the past few years, various CLRs have been identified on DCs. The pattern of CLR expression depends on the DC subset. Whereas CLRs are abundantly expressed by immature DCs in peripheral tissues, the expression of most CLRs is rapidly down-regulated upon DC maturation. Although CLRs initially were regarded as scavenger receptors, it is now clear that they bind a variety of antigens via specific recognition of particular carbohydrate patterns with interesting and important consequences. CLR-bound antigens are processed and presented to T cells, thus enhancing antigen presentation and immune activation. Strikingly, in *vivo* stimulation of CLRs induces immune suppression, hinting to an important function of CLRs in the tolerance toward self-antigen (Steinman et al., 2003). These findings illustrate a possible function for CLRs in the recognition of a wide variety of carbohydrate structures on self-glycoproteins to allow specific homeostatic control to self antigens and to mediate cellular processes such as cell signalling, cell adhesion, and migration (Figdor et al., 2002).

Specific pathogens target CLRs to circumvent processing and presentation and to prevent immune activation. In contrast, specific pathogens benefit from their capacity to target host C-type lectins, using the function of the C-type lectins to induce non-responsiveness against their processed antigens with the aim to promote their survival by escaping immune activation.

It has been well established that T cells reactive to self-antigen are part of the normal immune repertoire of mice, non-human primates, and humans at frequencies comparable to patients with autoimmune diseases. The fact that most of us do not develop autoimmune diseases shows that the auto-reactive cells are kept at bay by an efficient and tight control mechanism. As the majority of self-antigens are glycosylated, it was postulate that tolerance is maintained by the interaction with CLRs on DCs. However, when DCs obtain stimuli that result in maturation, such as upon pathogen recognition by TLR or CD40 triggering, this signalling overrules the tolerizing effects of CLRs.

1.2.2.1 Functional characteristics of C-type lectins

CLRs form a large superfamily of transmembrane and soluble proteins that contain a carbohydrate-recognition domain (CRD) important in pathogen binding, uptake and killing. It is important to note that not all CLRs are PRRs, in fact many members of the CLR family have diverse functions (Zelensky and Gready, 2005). Members of the CLR family involved in anti-fungal immunity include Dectin-1 (DC-associated C type lectin 1, Clec7a), Dectin-2 (Clec6a), macrophage mannose receptor (MR, CD206), dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209) and Mincle (Clec4e, Clecsf9; Willment and Brown, 2008). Most CLRs on DCs are type II transmembrane proteins, with the exception of the MR and DEC-205, which are both type I transmembrane proteins (Figdor et al., 2002; Figure 1.2).

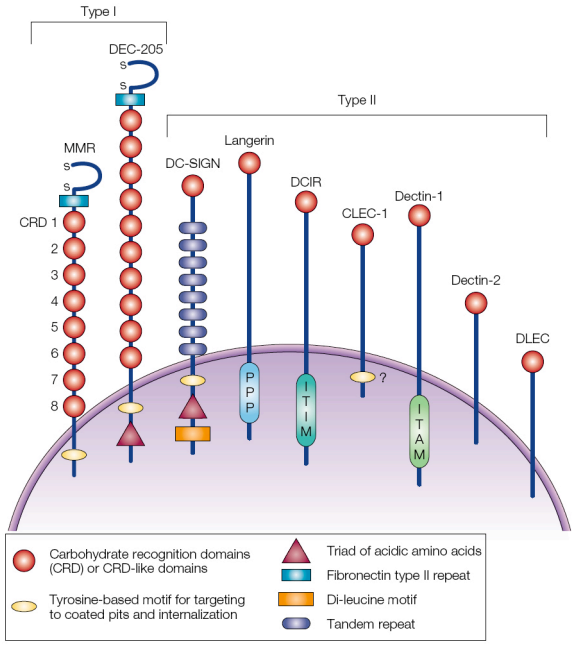


Figure 1.2. Two types of C-type lectins or lectin-like molecules are produced by dendritic cells and Langerhans cells. Type I C-type lectins (MMR and DEC-205) contain an amino-terminal cysteine-rich repeat (S–S), a fibronectin type II repeat (FN) and 8–10 carbohydrate recognition domains (CRDs), which bind ligand in a Ca2+-dependent manner. MMR binds ligand through CRD4 and CRD5. Type II C-type lectins contain only one CRD at their carboxy-terminal extracellular domain. The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs that are important for antigen uptake: a tyrosine-containing coated-pit intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Other type II C- type lectins contain other potential signalling motifs (ITIM, ITAM, proline-rich regions (P)). CLEC-1, C-type lectin receptor 1; DCIR, dendritic cell immunoreceptor; DC-SIGN, dendritic-cell specific ICAM-3 grabbing non-integrin; DLEC, dendritic cell lectin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MMR, macrophage mannose receptor. *Adapted from Figdor et al., 2002.*

All C-type lectins contain carbohydrate-binding activity based on the presence of at least one carbohydrate recognition domain (CRD; Weis et al., 1998). The CRD of the C-type lectin DC-SIGN is a globular protein that contains two Ca^{2+}-binding sites, of which one directly

coordinates the binding specificity of the carbohydrate structures (Feinburg et al., 2001). Calcium binding is essential for the function of CLRs because mutation of either Ca^{2+}-binding site in DC-SIGN leads to the loss of ligand binding (Geijtenbeek et al., 2002). Depending on the amino acid sequence, the CRD bears specificity for mannose, galactose, or fucose structures. However, binding of these carbohydrate structures to the different CLRs is also dependent on carbohydrate branching, spacing, and multivalency.

The involvement of specific C-type lectins in ligand binding is often demonstrated by the ability of specific carbohydrate components, such as mannan, or a calcium chelator to block the interaction. However, co-expression of several CLRs — with the same carbohydrate specificity and calcium-dependency — on one DC subset makes it essential to use CLR specific blocking antibodies to demonstrate specific CLR functions.

1.2.3. TLRs and CLRs cross-talk and effect on immune response

There are several indications that TLRs and CLRs communicate with each other, and it is proposed that the cross talk between TLRs and CLRs may fine-tune the balance between immune activation and tolerance (Kaufmann and Schaible, 2003; Geijtenbeek et al., 2003; Gantner et al., 2003; Brown et al., 2003). Recognition of self-antigen by CLRs alone will favor immune suppression, whereas pathogen recognition or self-recognition in a situation of danger where both TLRs and CLRs are triggered induces immune activation.

Thus, in a steady-state situation, antigens are captured by CLRs to maintain tolerance, whereas in a dangerous situation, CLR binding to the same antigen occurs in the presence of TLR triggering, and the immunostimulatory function of TLRs overrules the tolerizing function of CLRs, resulting in immune activation (Ichikawa et al., 2002).

Depending on their tissue localization and differentiation state, DCs express unique sets of TLRs and CLRs. Reflecting the large variety of CLRs on DC subsets, a large diversity of TLRs are also expressed by these DC subsets (Jarrossay et al., 2001). These distinct DC subsets carry different sets of TLRs and CLRs raises the possibility that subsets of DCs recognize distinct classes of self- and nonself-antigens to induce tolerance or activate immunity.

Intriguingly, specific pathogens target CLRs to escape immunosurveillance and to promote their survival by sequestration in DCs and by reducing their powerful antigen-presenting capacities. The intriguing question arises of whether oligosaccharides bound to specific cell structures allow DC migration to specific sites as well as DC interactions with specific T cell subsets.

Future research addressing carbohydrate-recognition profiles by C-type lectins and the regulation of glycosylation on its cellular counter-structures by post-translational modification will provide insight as to how these cell-surface receptors mediate cellular interactions and regulate DC function. Also, understanding the carbohydrate-recognition element present on self-antigens or pathogens that regulate the interaction with a specific CLR may have consequences for how the pathogen or antigen is processed by DC.

1.2.4. Pro- and anti-inflammatory signal during infection

Following activation of the innate immune system, strong pro-inflammatory signals are generated, inducing inflammation and activation of host defence. After elimination of the invading microorganisms, subsequent anti-inflammatory signals are responsible for the resolution of the inflammation. These signals are crucial, not only for the return of the immune system to homeostatic balance, but also for the protection of the host against the deleterious effects of overhelming inflammation and for subsequent tissue repair.

TLR-signals are involved in both the primary induction of inflammation and the secondary activation of anti-inflammatory mechanisms. TLRs are known to induce the release of anti-inflammatory cytokines (e.g. interleukin (IL)-10, IL-4, IL-5 and IL-13). TLR2 and TLR4-mediated signals have been shown to mediate generation of down-modulating T-regulatory cells. The human body uses regulatory T cells (Tregs) to control unwanted immune reactivity (Blustone and Abbas, 2003). Natural Tregs are localized in the thymus and inhibit T cell activation primarily via cell-cell contact, whereas inducible Tregs are distributed over the lymphoid organs and exert their suppressive activity via secretion of cytokines. In line with this, the ablation of TLR2 or TLR4 signalling results in increased mortality following overwhelming inflammation in certain experimental models.

However, whilst TLR-mediated anti-inflammatory signals are beneficial following the elimination of the pathogens, they can induce dangerous immuno-suppressive mechanisms if activated too early during a severe infection.

1.3. Fungal cell wall component recognition

Several classes of recognition receptors mediate recognition of fungal pathogens mainly by recognition of fungal cell wall components.

1.3.1 The fungal cell wall

The fungal cell wall is an essential organelle that maintains the viability of fungal cells. To be strong, yet plastic, fungal cell walls combine skeletal and matrix components in a way that resembles the engineering principles that are involved in constructing elaborate structures, such as reinforced concrete buildings, that are made of mesh and mortar.

The cells of the innate immune system recognize elements of both the skeletal and matrix components of the fungal cell wall.

The skeletal component of the cell wall of the majority of fungal pathogens, including *C. albicans*, is based on a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin (a β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc; Figure 1.3). These polymers

form hydrogen bonds between adjacent polysaccharide chains to form a tough three-dimensional network of microfibrils. Most models suggest that the skeletal components of the cell wall are found close to the cell membrane in an inner layer, although some chitin and glucan can be present throughout the thickness of the wall.

In budding yeast cells, a scar is left on the mother cell after separation of the bud, and at this site the components of the inner layers of the cell wall, such as chitin and β-(1,3)-glucan, can become exposed at the surface (Gantner et al., 2005). In addition to the glucan and chitin skeleton, the fungal cell wall contains a matrix that mainly comprises glycosylated proteins. The glycosylphosphatidylinositol (GPI)-anchor-dependent cell wall proteins (GPI-CWPs), are normally highly glycosylated with mannose-containing polysaccharides (called mannan), and carbohydrates can account for up to 90% of their molecular mass. Many CWPs have a lollipop structure with a globular domain that is presented to the outside of the cell and a Ser/Thr-rich polypeptide stem-like domain that is stabilized in the cell wall by O-linked mannan side chains. These ether-linked O-mannans are relatively short, linear polysaccharides that, in *C. albicans*, consist of one to five mannose (Man) sugars that are almost exclusively β-(1,2)-linked (Figure 1.3).

N-mannan consists of a core $Man_8GlcNAc_2$ triantennary complex to which a highly branched structure is attached, comprising up to 150 mannose sugars arranged as an β-(1,6)-linked backbone with side chains mannose and phosphomannan (Cutler, 2001; Figure 1.3).

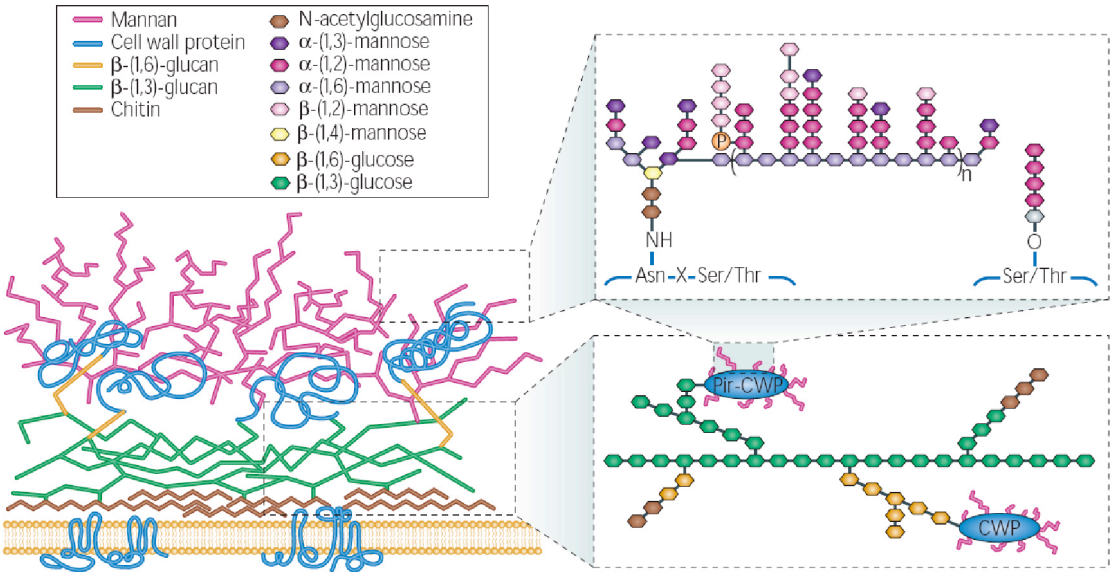


Figure 1.3. Schematic structure of *C. albicans* cell wall. *Modified from Netea et al., 2008*

1.3.2 Fungal cell wall recognition

Owing to the localization of mannoproteins and mannans in the outermost part of the cell wall, mannan detection would be expected to be one of the first steps in the recognition of *C. albicans* by the host innate immune system. However, the presence of β-glucans and chitin, especially at the level of the bud scar, is also likely to influence the recognition of *C. albicans* by leukocytes.

DCs, which are crucial for antigen processing and presentation, also express most of the PRRs that are important for the recognition of fungal pathogens (Figure 1.4).
The mosaic of PRRs that is expressed by the different immune cells ultimately determines the type of response that is initiated following recognition of *C. albicans*.

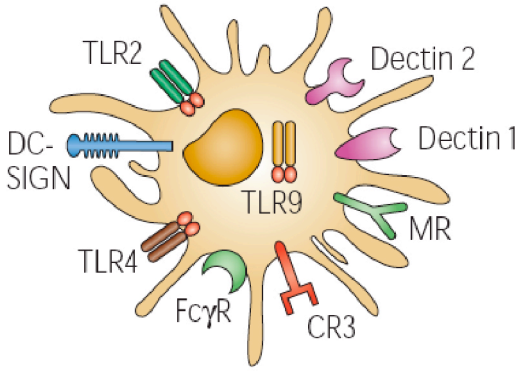


Figure 1.4. Fungal pattern recognition receptors present on DC cell surface. DC presents on cell surface several receptors specific in fungal recognition. MR; mannose receptor, CR, complement receptor, TLR, Toll like receptor. *Figure modified from Netea et al., 2008*

1.3.2.1 Mannans and mannoproteins

Both mannans and mannoproteins from the *C. albicans* cell wall have important immunostimulatory activities, ranging from stimulation of cytokine production to induction of DC maturation (Petrella et al., 2006) and T-cell immunity (Gomez et al., 1996; Gomez et al., 2000). Mannoproteins induce mainly T helper 1 (Th1)-type cytokine profiles, which have protective effects against disseminated C. albicans infection (Mencacci et al., 1994).

The first receptor on the surface of macrophages to be described as a mannan receptor was the C-type-lectin mannose receptor (MR) (Wileman et al., 1986; Stephenson and Shepherd, 1987). The MR recognizes oligosaccharides which terminate in mannose, fucose and GlcNAc (Stahl et al., 2006), and this binding is mediated by carbohydrate-recognition domains (CRDs) 4 to 8 in the extracellular region of the receptor (Linehan et al., 2000). MR preferentially recognizes β-linked oligomannoses with branched, rather than linear, structures (Kery et al., 1992). By contrast,

recognition of the shorter linear structures of O-bound mannan is performed by TLR4 (Netea et al., 2006), and results in cytokine production (Tada et al., 2002). Interestingly, TLR4 stimulation is lost during the germination of yeast into hyphae, which leads to a loss of interferon-γ (IFNγ) production capacity (van der Graaf et al., 2005).

On DCs, however, the binding of mannans is mediated through the MR and DC-SIGN. DC-SIGN is a receptor that is specifically expressed on the cell membrane of DCs; (Cambi et al., 2003) and recognizes carbohydrates, such as high-mannose structures, in a Ca^{2+}-dependent fashion with specificity being achieved through unique interactions with its ligands and tetramerization of the receptors (Koppe et al., 2005). Similar to the MR, DC-SIGN recognizes several endogenous ligands as well as several microbial pahogens. including *C. albicans*. *A. fumigatus* conidia and *Chrysosporium tropicum* (Cambi et al., 2003; Serrano-Gomez et al., 2004; Serrano-Gomez et al, 2005).

A recent study has shown that Galectin-3 on the surface of murine macrophages can discriminate between pathogenic *C. albicans* and non-pathogenic *Saccharomyces cerevisiae*, and that an association between Galectin-3 and TLR2 is involved in this process (Jouault et al., 2003).

Another lectin family member, Dectin-2, has also been described to function as a receptor for mannans of hyphal *C. albicans* (Ariizumi et al., 2000). Owing to its short intracytoplasmic tail, dectin-2 must interact with the FcγR to induce intracellular signals and, interestingly, seems to be mainly involved in the recognition of *C. albicans* hyphae (Sato et al., 2006; Robinson et al., 2009).

1.3.2.2. β-glucans

The fungal cell wall consists of approximately 60% β-glucan (Klis et al., 2001). Although initially thought to be buried beneath the mannoprotein layer, recent evidence suggests that β-glucans are exposed on the cell surface (Torosantucci et al., 2005), although possibly restricted to specific regions, such as bud scars.

The recognition of β-glucans has primarily been ascribed to two receptors, CR3 and, more recently, Dectin-1. Although other β-glucan receptors have been described, including lactosylceramide and scavenger receptors, the physiological role of these receptors is still unclear. CR3 is a widely expressed β2-integrin that recognizes several endogenous and exogenous ligands, pathogens that have been opsonized by iC3b (the inactivated form of complement component C3b) and carbohydrates, including β-glucans. Carbohydrate recognition is mediated by a lectin domain (Thornton et al., 1996; Diamond et al., 1993), which is distinct from the normal ligand-binding site (the I domain) of CR3 (Diamond et al., 1993). The lectin domain mediates recognition of both the yeast and hyphal forms of *C. albicans* (Forsyth and Mathews, 2002; Forsyth et al., 1998), as well as several other fungi.

Dectin-1 is a myeloid-expressed transmembrane receptor and possesses a single extracellular, nonclassical C-type-lectin-like domain that specifically recognizes β1,3-glucans. Dectin-1 can recognize several fungi, including *C. albicans* yeast, although it does not appear to recognize *C. albicans* hyphae (Brown and Gordon, 2003). The cytoplasmic tail of dectin-1 contains an immunoreceptor tyrosine-based activation-like motif (ITAM), which can mediate various protective responses through spleen tyrosine kinase and caspase recruitment domain protein 9 (Syk–CARD9)-dependent pathways, such as the stimulation of interleukin 2 (IL-2) and IL-10 (Rogers et al., 2005), IL-6 (Gow et al., 2008), and IL-17 production (LeibundGut-Landmann et al., 2007). Although Syk dependent signalling from Dectin-1 is sufficient for these responses, stimulation of the mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kB pathways, with subsequent production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), requires collaborative signalling with the TLR2 receptor (Brown et al., 2003; Gantner et al., 2003).

1.4 Fungal receptor and immune evasion: a host perspective of fungal virulence

Recent studies of PRR biology have shown that, while innate recognition is crucial for an efficient immune response, certain pathogens use PRR-based strategies to evade host defences.

Fungi are not merely passive participants in the infectious process, and a hypothetical set of virulence factors has been attributed to them (Cutler, 1991; Hogan et al., 1996; Calderone and Fonzi, 2001). Additionally, the interaction between fungi and the environment is profoundly affected by antigenic variability, phenotypic switching and dimorphic transition (Mitchell, 1998; Woods, 2002; Soll, 2002). It has been argued that all the traits required to establish disease should be considered fungal virulence traits (Calderone and Fonzi, 2001; Odds et al., 2001; Casadewall et al., 1999). However, as fungal virulence is only expressed in susceptible hosts, it cannot be considered an independent microbial variable (Casadewall et al., 1999; Casadewall et al., 2001; Casadewall et al., 2002).

An association between morphogenesis and virulence has long been presumed for dimorphic fungi that are pathogenic to humans, as one morphotype exists in the environment or during commensalism, and another within the host during the disease process. For *C. albicans*, putative virulence factors include the ability to switch between saprophytic yeast and pathogenic, filamentous forms of the fungus (Mitchell, 1998; Roney et al., 2002). DCs sense either form in a specific way, resulting in distinct, T-helper-cell-dependent protective and non-protective immunities. The transition to different fungal morphotypes is detected by the host immune system in a morphotype-specific fashion (Romani et al., 1998; Roney et al., 2002; Romani et al., 2002).

DCs are uniquely able to decode fungus-associated information, resulting in qualitatively different adaptive T-helper cell (Th) immune responses, both *in vitro* and *in vivo* (Fe-d'Ostiani et al., 2000; Bauman et al., 2000; Newman and Holly, 2001; Gildea et al., 2001; Richards et al., 2001; Grazziutti et al., 2001; Montagnoli et al., 2002; Bozza et al., 2002; Bacci et al., 2002). As DCs efficiently phagocytose fungi at sites of infection (Bozza et al., 2001), it follows that they are unique sensors of fungi, being able to internalize different morphotypes and either kill fungal cells or favor parasitism and/or infection. This requires DCs to be able to activate the corresponding adaptive immune response at the sites of colonization and/or infection.

1.4.1 Fungi recognition: commensalism and pathogenicity

The engagement of distinct receptors by *Candida* yeasts and hyphae translates into downstream signalling events, ultimately regulating cytokine production and co-stimulation.

TLR2 ligation can induce pro-inflammatory cytokine, but that this effect is weaker than that mediated by TLR4 (Hirschfeld et al., 2001). In contrast, TLR2 signals are strong mediators of anti-inflammatory effects. The TLR2-induced immune-suppression is either an exageration or a premature activation of the normal anti-inflammatory effects of TLRs stimulation, necessary during the recovery phase of the infection for reversing inflammation. In addition to the induction of anti-inflammatory signals through TLRs, certain fungi have developed strategies to either block or avoid their recognition by TLRs and subsequent activation of the innate defence. Thus, *Aspergillus fumigatus* evades immune recognition by germination into hyphae, with subsequent loss of TLR4 recognition, while the TLR2-mediated IL-10 pathways remain intact thus shifting the balance towards a permissive Th2-type profile (Netea et al., 2003).

A similar evasion of TLR4 recognition was documented for *C. albicans* hyphae, which stimulate mainly anti-inflammatory cytokines through TLR2 (van der Graaf et al., 2005), but which are unable to be recognized by TLR4 and thereby stimulate IL-12 or IFNγ synthesis (d'Ostiani et al., 2000; van der Graaf et al., 2005).

TLR4 is not the only PRR targeted during fungal germination. Gantner et al. (2006) demonstrated that dectin-1 recognizes the b-glucans at the level of budding scars in the yeast form, but can not recognize the β-glucans in the hyphal form, where they are shielded by a layer of mannans. In this way two major recognition system (TLR's and dectin-1) are unable to recognize *Candida* hypahe, shifting the balance towards an anti-inflammatory response.

Furthermore, the entry of yeasts signals through MR results in the production of proinflammatory cytokines, including IL-12, fungal degradation, up-regulation of co-stimulatory molecules and increased expression of MHC class II molecules. These events are all suppressed upon entry through CR3. By contrast, co-ligation of CR3 with FcγR, as in the phagocytosis of hyphae, results in upregulation of co-stimulatory molecules and the presentation of antigens by

MHC class II molecules, and the production of IL-4 and/or IL-10 (Montagnoli et al., 2002). In *vivo* studies confirmed that the opsonic dependent phagocytosis of fungi was responsible for type 2 cytokine production and Th2 cell activation, an activity counteracted by the Th1-promoting activity of non-opsonic phagocytosis through MR. That MR appears to be required for the activation of antifungal T-cell responses has also recently been demonstrated with the fungus *Cryptococcus neoformans* (Mansour et al., 2002).

There is compelling evidence that regulatory T cells (Tregs) specialized in attenuating immune responses play a crucial role in immune regulation (Roncarolo and Levings, 2000). Therefore, in addition to the Th1/Th2 balance, other mechanisms appear to be involved in regulating Th1-mediated immunity to *C. albicans* as the newly described Th17 pathway (see below).

1.5 Dendritic cell and antifungal T cell response

Immature DCs acquire antigens in peripheral tissues by a variety of mechanisms; they then mature and migrate to the T-cell areas of lymphoid organs where they 'translate' the tissue-derived information into the 'language' of Th-cells using different activating signals. In addition to initiating immunity, certain sub-populations of DCs can down-regulate immune responses (Roncarolo et al., 2001), allowing DCs to act as guardians for the induction and maintenance of peripheral T-cell tolerance and the prevention of pathological autoimmune reactions (Mellman and Steinman, 2001).

The T-cell branch of the immune system can respond to a virtually infinite variety of exogenous antigens, thus including the possibility of self-antigen recognition and dangerous autoimmune reactions. Therefore, regulatory mechanisms operate both during ontogeny within the thymus and after birth in the periphery. The control of self-reactive T cells occurs through a process of negative selection that results in apoptosis of T cells showing high affinity for self-peptides expressed at the thymic level by means of promiscuous gene expression. Self-reactive T cells escaped to negative selection are controlled in the periphery by other regulatory mechanisms, the most important being natural Foxp3 T regulatory (Treg) cells. Regulation is also required to control excessive effector T cell responses against exogenous antigens, when they become dangerous for the body. Three types of effector T cells have been recognized: (1) T helper 1 (Th1) cells, which are protective against intracellular bacteria; (2) Th2 cells, which play some role in the protection against nematodes, but are responsible for allergic reactions; (3) Th17 cells, which are probably effective in the protection against extracellular bacteria, but also play a role in the amplification of autoimmune disorders. Abnormal or excessive Th effector responses are regulated

by different mechanisms.

Redirection or immune deviation of Th1- or Th2-dominated responses is provided by cytokines (IFNγ vs. IL-4) produced by the same cell types and by the CXCR3-binding chemokines CXCL4 and CXCL10. Moreover, both Th1 and Th2 responses can be suppressed by adaptive Treg cells through contact-dependent mechanisms and/or the production of IL-10 and transforming growth factor β (TGFβ).

Finally, TGFβ1 can promote the development of both Th17 effector and adaptive Treg cells, while the contemporaneous production of IL-6 contributes to the development of Th17 cells, but inhibits Treg cells. The development of Th17 cells is also down-regulated by IL-4 produced by Th2 cells and by IFNγ produced by Th1 cells (review in Romagnani, 2006).

1.5.1 Th17 in antifungal host defence

The Th1-versus-Th2 dichotomy has dominated T cell biology for many years. However, the Th17 lineage, has now risen to prominence (Bettelli et al., 2007; Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006). Th17 cells play important roles in host defence against infection with extracellular bacterial and fungal pathogens by recruiting acute inflammatory cells into local sites of infection. In addition to host defence, ample experimental evidence supports pathological role for Th17 cells during numerous systemic and organ-specific autoimmune disease. These diseases probably result from Th17 cells directed against self-antigen. Th17 cell development occurs in the presence of transforming growth factor-β (TGF-β) and IL-6 and is opposed by Th1 cytokines. Th17 cells are maintained in the presence of the IL-12-related cytokine IL-23. Whereas IL-1β alone is capable of inducing IL-17 secretion on human central memory T cells (Acosta-Rodriguez et al., 2007a), IL-21 and TGFβ seem to be needed for driving differentiation of naive T cells (Yang et al., 2008). IL-17 induces chemokine production at sites of infection and causes recruitment of neutrophils. Because of this unique activity, Th17 cells are important in defence against extracellular pathogens (Bettelli et al., 2007; Mangan et al., 2006). Accordingly, strong Th17 responses are induced after infection with extracellular bacteria (Mangan et al., 2006).

Especially intriguing is the demonstration that while Th17 cells represent a separate and distinct T-cell lineage, pathogen-specific Th17 cells can play cooperative roles with pahogen-specific Th1 cells in host defence during some infections. Moreover, accumulating evidence demonstrates that Th17 cells can also provide protective effects during infection with more traditional pathogens. This is of paramount interest because most studies *in vitro* indicate that cytokines that promote CD4 T-cell differentiation into each lineage not only strongly reinforce other cells to differentiate into the same lineage but also potently inhibit differentiation into other T cell lineages.

T-cell subset	Protective activity	Immunopathology
Th1	Intracellular bacteria and some viruses (via macrophage activation or the increase of cytotoxic activity by NK cells and $CD8^{+}$ T cells, respectively)	Granulomatous disorders (some organ-specific autoimmune disorders, such as Hashimoto's thyroiditis; Chron's disease; sarcoidosis; others?)
Th2	Helminths (via the activity of IL-4 and and IL-13 that favour worm expulsion and of IL-5 which induces eosinophil activation)	Allergic diseases. (via IL-4 and IL-13 that induce IgE switching; IL-5 that favours eosinophil infiltration and activation, and IL-9 that induces mucus secretion in the bronchi of asthmatics)
Th17	Extracellular bacteria and some fungi (Klebsiella pneumoniae; Bacteroides fragilis; Candida albicans; Borrelia Burdorgferi; Bordetella pertussis; via the production of granulocyte-recruiting chemokines)	Chronic inflammatory and autoimmune disorders (EAE, CIA, and IBD in experimental animal models; multiple sclerosis, rheumatoid arthritis; systemic lupus erythematosus; chronic obstructive pulmonary disease)
Treg	Tolerance against self (via the suppression in the periphery of T-cell effector responses against autoantigens)	Autoimmunity (when deficient or inactive, because of Foxp3 mutations)
		Cancer (when Treg suppress tumor-specific T-cell effectors)
	Control of excessive responses against non-self (Th3, T1R, $CD4^{+}CD25^{high}Foxp3^{+}$ cells mainly acting via the release of IL-10 and TGF-b or other mechanisms)	Immunodeficiency (when hyper-active, because their suppressor mechanism is non-antigen specific)

Th, T helper; Treg, T regulatory; TGF-b, transforming growth factor-b.

Table 1.1 Overview on the possible activities of different CD4+ T cell population. *From Romagnani, 2006.*

These results suggest that additional signals with the capacity to override lineage-differentiating signals are triggered during *in vivo* infection to allow the immune system to 'fine-tune' the kinetics and balance the relative degree of T cell differentiation into multiple effector lineages during infection (see Curtis and Way, 2009).

Both Th1 (Acosta-Rodriguez et al., 2007) and Th17 (Huang et al., 2004; Milner et al. 2008; Conti et al., 2009) cells have been proposed to mediate protection against pathogenic fungi and in particular against *C. albicans*. *C. albicans* can switch from being an intracellular form, the yeast, that can be efficiently destroyed by macrophages with the help of Th1 cells, to being an extracellular form, the hyphae, that is better controlled by neutrophils (Urban et al., 2008) recruited by Th17 cells.

1.5.1.1 PRRs and Th17 priming

Which are the receptor pathways that direct the immune response toward a Th17 profile is a matter of debate, with each of the TLRs (Evans et al., 2007), Dectin-1 (LeibunGut-Landmann et al., 2007) and NOD2 (van Beelen et al., 2007) pathways individually being implicated in the induction of Th17 cells.

Dectin-1-mediated fungal recognition 'preferentially' induces IL-17–producing Th17 cells in both humans and mice. LeibundGut-Landmann *et al.* (2007) show that recognition of a fungal cell wall component by Dectin-1 is sufficient for adaptive immune activation and the induction of Th17 cell differentiation in mice. In a complementary study, Acosta-Rodriguez *et al.* (2007) show

that *C. albicans*–specific memory T cells in humans have a Th17 phenotype and a chemokine receptor expression pattern indicative of mucosal homing. It was found that the production of IL-12 and IL-23 was dissociated during fungal recognition (Acosta-Rodriguez et al., 2007). *C. albicans* is dimorphic and switches between yeast and hyphal forms, both of which are required for pathogenicity. In an *in vitro* T helper cell priming assay, yeast induced more IL-12 than IL-23 and a strong Th1 response, whereas hyphae induced only IL-23 and a strong Th17 response. Yeast stimulates both Dectins and TLRs, whereas hyphae stimulate mainly Dectin-2 (Sato et al., 2006). Thus, *C. albicans* yeast and hyphae can be distinguished by pattern recognition and induce tailored adaptive responses (Figure 1.5).

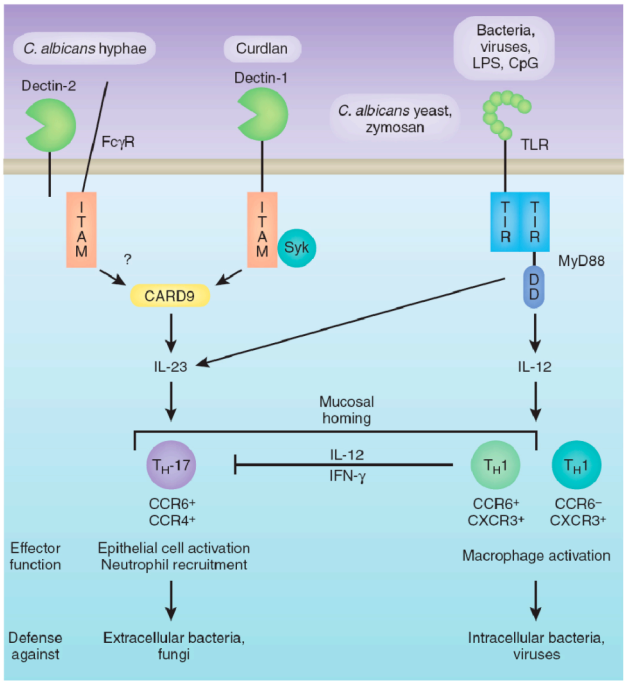


Figure 1.5. Dectins and TLRs induce distinct T helper cells in response to fungi. *Adapted from Palm and Medzithov, 2007.*

Recently, MR was described as the main pathway through which live *C. albicans* induces the Th17 response in human primary cells.

Other pathogens with multiple forms, either in response to environmental cues or as part of their life cycle, may also induce distinct T helper cell responses 'tuned' to elimination of the form that is detected.

1.6 Dendritic cell and discrimination of pathogenicity

Recent evidence suggests that the use of distinct recognition receptors contributes to the disparate patterns of reactivity observed locally in response to challenge with *C. albicans*. These findings offer new interpretive clues to the mechanisms of fungal virulence: rather than dimorphism *per se*, the engagement of different recognition receptors on dendritic cells might select the mode of fungal internalization and antigen presentation, condition the nature of the Th response and, ultimately, favor saprophytism or infection.

Incidence of fungal disease has been rising dramatically in the past several decades. Successful resolution of pathogenic fungal disease depends on proper coordination of multiple components of the host immune response. The innate immune system is crucial in forming the antifungal response, initiated with the processing and presentation of antigens by DCs. The balance between pro- and anti-inflammatory signalling is a prerequisite for successful host-fungal interactions (Richardson, 2005). Though much progress has been made in the study of the immune system under challenge from fungal pathogens, there remain numerous aspects of the tolerogenic response in fungi normally under control that are poorly understood. Understanding the mechanisms of cohabitation between humans and non-pathogenic fungi is a prerequisite for controlling fungal infections.

The best studied fungal microorganism is *Saccharomyces cerevisiae*, an ubiquitous yeast used in the production of food, wines and beer (Cavalieri et al., 2003). For the past 5 millennia human societies have coevolved with the yeast *S. cerevisiae*, a common colonizer of mucosal surfaces and part of the normal flora (Salonen et al., 2000). In addition to *C. albicans*, several strains of the *S. cerevisiae* species have been isolated from immuno-compromised individuals, such as cystic fibrosis or AIDS patients (Munoz et al., 2005; Sethi and Mandell, 1998). These findings are not in conflict with the concept that *S. cerevisiae* is 'generally recognized as safe', but are rather in agreement with the idea that pathogenicity results from an alteration in the equilibrium between microorganisms and host.

The decision on how to respond to a microorganisms, be it a pathogen or a commensal, is primarily determined by the interaction between microorganism and cells of the innate immune system. The importance of cell wall recognition in pathogenesis has been recently investigated in *C. albicans*. C-type lectins, such as Dectin-1, DC-SIGN and MR specifically mediate *C. albicans* binding and internalization by DCs (Hernanz-Falcón, et al., 2009; Newman and Holly, 2001; Cambi et al., 2008). Moreover, upon *C. albicans* stimulation, the direct recognition through the MR is sufficient for inducing a T helper (Th) 17 response in peripheral blood mononuclear cells (PBMC; van der Veerdonk et al., 2009). Mannan from this pathogen is the component capable to inducing IL-17 secretion. Interestingly, the dectin-1/TLR2 pathway synergizes with the MR-induced IL-17 secretion, revealing an interaction between C-type lectin receptors and TLRs (van de

Veerdonk et al., 2009). The IL-17 production in response to *C. albicans* corresponded with the appearance of a subset of CD4 T cells that contained cells capable of producing IL-17 or both IFNγ and IL-17.

The *S. cerevisiae* cell wall is almost exclusively composed by glycans. The interaction between the various *Saccharomyces* cell wall-associated structures with their counter-receptors, and the implications to DC function remain unknown. The only indirect evidence available indicates that *C. albicans* mannan (CA-mannan) but not *S. cerevisiae*-derived mannan (SC-mannan) is able to induce IL-17 production (van der Veerdonk et al., 2009). The differential recognition of mannans from *C. albicans* and *S. cerevisiae* is mostly likely due to the different branching of these structures: while *Candida* (CA)-derived mannan has a highly branched structure, *Saccharomyces* (SC)-derived mannan mainly contains short linear chains of mannose polymers (Cambi et al., 2008). Thus, MR is able to coordinate different responses to fungal pathogens, particularly regarding the Th17 response.

Dimorphic switch in *Candida* and germination of *Aspergillus* spores are two examples of the importance of different life stages in determining pathogenicity, possibly by exposing different cell wall components (d'Ostiani et al., 2000; Gantner et al., 2005). *S. cerevisiae* cell wall composition radically changes in different life stages and life forms, such as ascospores. Unlike *C. albicans, S. cerevisiae* life cycle alternates haploid and diploid state. Haploid cells mate and produce diploid cells. Diploid cells can divide by mitosis and grow and upon stressing conditions sporulate and undergo meiosis. Sporulation is a phenomenon that involved conformational changes in yeast cells and the final product is an *ascus* (that maintain the characteristic of the residual yeast cell wall) that encloses four spore (tetrad). The *S. cerevisiae* spores inside the *ascus* are characterized by a totally different cell wall composition with respect to cells. In particular, mature spore wall shows 4 distinct layers from innermost to outermost: a layer of mannoproteins (mannan layers), one of β-1,3 linked glucose residues (glucan layer), a chitosan layer (α–1,4 linked glucosammine residues) and a layer of spourios structure whose major constituent is cross-linked tyrosine dimers (dityrosine layer). Even if glucan content is higher than in yeast cell wall, in this one they are presumably more exposed. Mannans and glucans, the common fungal PAMPs mainly recognized by chitosan and dityrosine, undefined tyrosine dimers that represent the outermost layer of spore wall (Kreger-Van Rij, 1978; Figure 1.6).

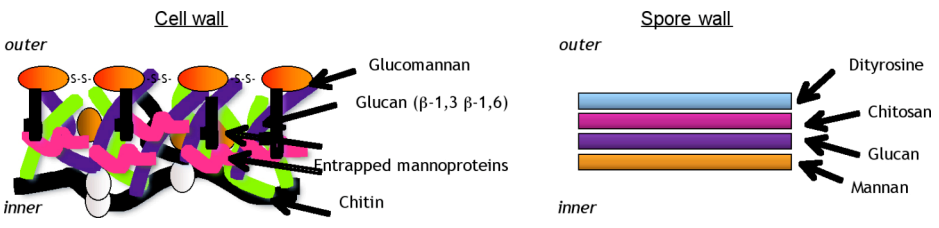


Figure 1.6. Schematic representation of components on cell and spore wall.

1.7 The complexity of immune system and System Biology approach

Interest in the innate immune response, has exploded in recent years, leading to an increased understanding of its importance in protection against and susceptibility to a range of infectious agents, in addition to the discovery of the complex communication between the innate and adaptive immune systems. This heightened focus has enabled not only an increasingly detailed dissection of many of the key signalling pathways involved (Akira, 2006; Kanneganti et al., 2007; Thompson and Locarnini, 2007) but also the realization that the innate immune response is much more complex than previously imagined (Smith and Bolouri, 2005).

Innate immunity has not to be thought as a set of discrete signalling pathways activated by a pathogen binding to a receptor; it is a complex network of interconnected pathways with activities dependent on many factors (Figure 1.7). It is now clear that single ligands can trigger multiple signal transduction pathways, although it is rare that a single stimulus would be involved in an infection.

More commonly, multiple ligands simultaneously stimulate linear signalling pathways; rather it is comprised of a complex set of integrated responses arising from a dynamic network of thousands of molecules subject to a range of receptors, which in turn activate several signalling pathways (Brikos and O'Neill, 2008). These pathways often show cross-talk, complex feedback or feed-forward loops (Hu et al., 2008) and diverse mechanisms of regulation. Considerable overlap with adaptive immunity makes many of these resources relevant to both arms of the immune response. Downstream of the pathways, specific subsets of transcription factors (TFs) are activated to initiate the appropriate gene expression response to a given stimulus (Walhout, 2006).

The immune response is also not simply a function of the host; the pathogen itself and regulated variations in its virulence along with complex interplay with the rest of the microbiome can all lead to variation in the innate immune response. Yet another layer of complexity is introduced when the cell-type and species specificity of the innate immune response are considered. Certain receptors and signalling pathways are only observed in specific cell-types, and there can be marked differences depending on the species.

The innate immune response is executed at the molecular level by a complex series of signalling pathways. In this context, pathways may be defined as a network of directional interactions between the components of a cell, which orchestrate an appropriate shift in cellular activity in response to a specific biological input or event.

A system as intricate as innate immunity necessitates the detailed level of investigation provided by systems biology approaches.

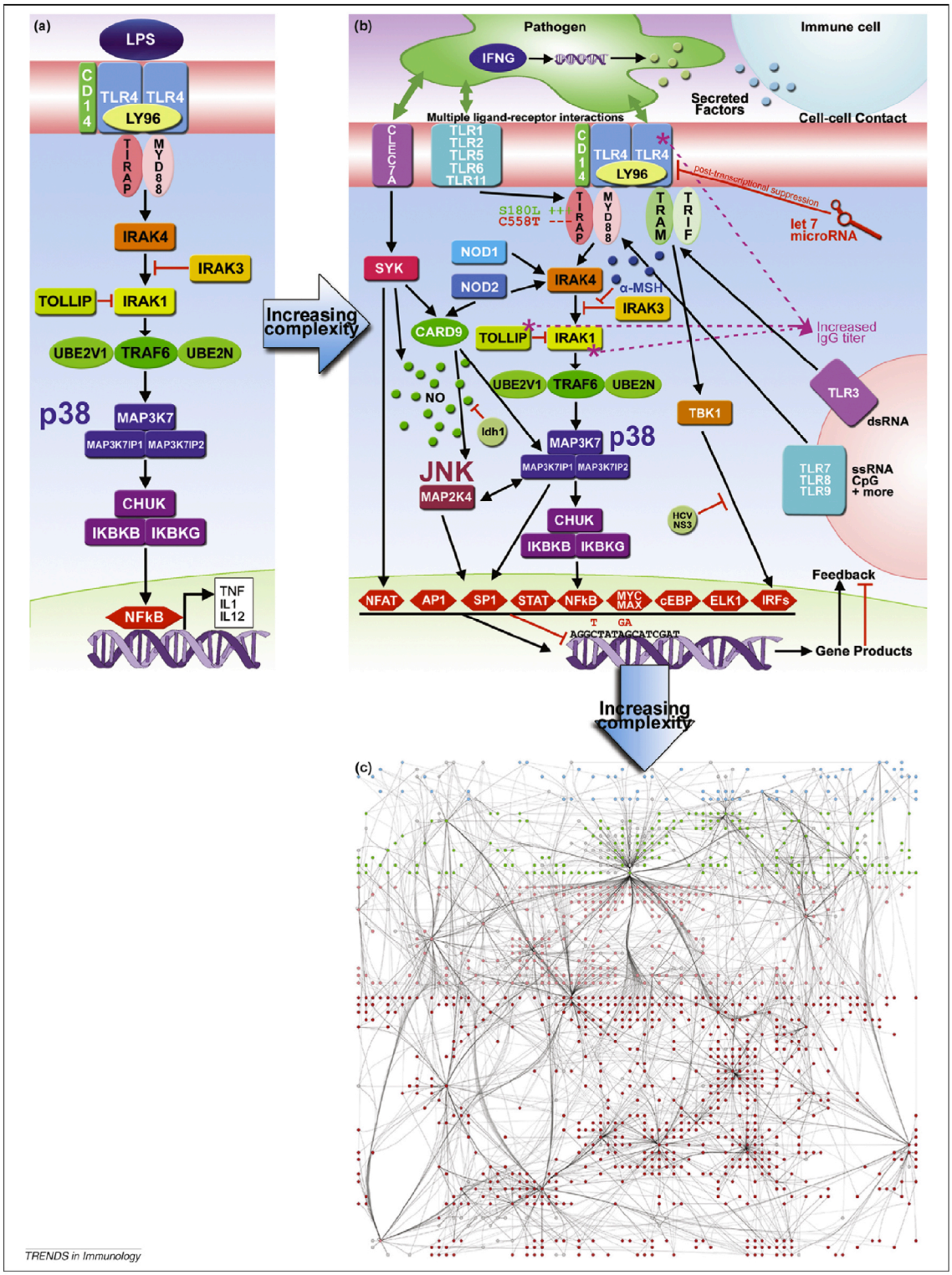


Figure 1.7. Traditional views of innate immune signalling fail to capture its full complexity. (a) Illustration of a canonical view of mammalian TLR4 signalling via NFkB, in which the process is represented as a simple linear pathway. (b) Demonstration that this simple pathway is under multiple levels of control using selected examples. (c) Illustrates a more accurate depiction of the complexity of the system. *Adapted from Gardy et al., 2009.*

1.7.1 System Biology

Systems biology represents a powerful and comprehensive paradigm for biology that stands in contrast to the reductionist approach that has tended to dominate. In the reductionist approach, researchers attempted to understand a complex entity (e.g. a cell, an organ or a disease) by breaking it down into smaller, more tractable units for study, such as genes, complexes or pathways.

The systems biology approach regards a system as more than the sum of its parts – its behavior arises not from simply the presence of its building blocks but through the complex relationships among them. Indeed, systems display what are termed emergent properties, which are behaviors made possible only through the interaction of a system's components, and which cannot be predicted by looking at a single component alone. Understanding of a biological system, its components and its emergent properties benefits from harnessing as many data types as possible, including database of genes, proteins, RNAs, small molecules and cells and their interactions, which have come out of the 'omics' era ('omics' being a catch-all phrase describing high-throughput approaches to analysing biological systems, including genomics, transcriptomics, proteomics, metabolomics and pharmacogenomics). These resources have permitted the development of new approaches, such as the use of genome-wide association studies to identify new susceptibility genes or the use of interaction data to identify more accurate biomarkers for disease progression or outcome.

1.7.2 Microarrays and transcriptional profiling

Since its first inception in 1995 (Schena et al., 1995), microarray use has increased dramatically over the years. A microarray is a made up by a solid surface (which can be a glass slide, a nylon membrane, or a silicon chip) on which thousands of DNA molecules are immobilized ('spotted') in an orderly pattern of rows and columns. Each of these molecules (named 'probes') represents a single gene. The surface with its associated probes is then hybridised with free nucleic acids ('targets', usually RNA) extracted from the sample of interest (for example a tumor tissue or a cell line) and labeled with a fluorescent dye. The target will bind with its complementary probe, and after washing under stringent conditions (to remove non specific binding) the surface is scanned using a confocal laser scanner and the fluorescent signals are quantified by specific analysis software. Every probe will have an associated expression value, making possible for a researcher to study the expression profile of thousands of genes at once.

The probes used in microarray experiments can be of different nature. A rather common approach is to use cDNA molecules, from 0.6 Kb to 2.4 Kb, containing the genes of interest and produced by PCR, which are then spotted on the array. A second approach made possible by technological advances is to use single-stranded oligonucleotides, with lengths ranging from 20 to

100 bases. Oligo-probes are designed on the basis of their thermodynamic properties and their hybridization specificity, and offer advantages over the use of cDNA probes, such as higher specificity and the fact that sequence information is the only requirement to generate them.

A number of microarray platforms exist, which differ by the way the probes are spotted on the surface. Array experiments can be designed using two different approaches, namely two-color and single-color. The two-color approach is the most common for spotted arrays, and enables the direct comparisons of two samples (e.g., two sample conditions) on a single array. RNA from the sample and its reference are labeled with two distinct fluorophores (usually cyanin-3 and cyanin-5, respectively) and then during scanning the fluorescence of both dyes is assessed separately. Then the measurements of the two fluorescent intensities are used to determine the Cy3/Cy5 ratio, often expressed in logarithmic form (log2 ratio). This is used to assess the relative expression of the sample with respect to the reference. The single-color approach is used almost exclusively on oligonucleotide arrays from Affymetrix and Illumina. After hybridization and scanning, the intensity of each probe represents the absolute expression level for its corresponding gene. In order to make comparisons between expression levels, multiple arrays with different samples need to be prepared.

A typical microarray profiling experiment usually involves either the comparison of a specific sample condition with a known reference (e.g., disease state against healthy) or how the gene expression profile changes over a series of time points (time-course experiment). As the number of genes screened is very high, careful consideration must be put into the design of experiment in order to minimize false positives and false negatives.

Due to the ability to assess expression levels of a very large number of genes simultaneously, microarrays have become a very attractive platform to study complex phenotypes, be they either physiological or pathological.

1.7.2.1 Microarray data analysis

Data analysis is a key factor for a microarray experiment. The number of genes on a microarray is painstakingly large, therefore techniques must be employed to discard data that has no relevance to the experimental design and to assist the interpretation of the results, in order to obtain biologically relevant information. As the technology improves and the resolution increases, the number of problems associated with data analysis increase as well, prompting the creation of new techniques or improvements of the existing ones.

Nevertheless, the analysis procedure in gene expression is more or less standardized and is conducted in several steps (Figure 1.8)

Analysis of a microarray experiment is a key element in obtaining reproducible data with a biological correspondence (see Verducci et al., 2006 for an overview of the challenges in data

analysis). It has been demonstrated that the analysis method can have a significant influence on the results (Allison et al., 2006), therefore a careful design of the analysis workflow is recommended. For example, signal quantification methods can influence the identification of weakly expressed (i.e., with low signal) transcripts (Irizarry et al., 2003). A poorly designed analysis method can lead to inconsistent or not reproducible results.

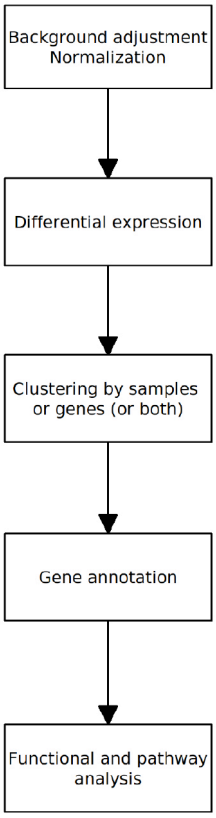


Figure 1.8: A typical analysis workflow for a microarray experiment. *Adapted from Verducci et al., 2006.*

1.7.2.2 Limitations of current approaches

Although the application of the microarray technology to the study of different biological process generated a large interest in the scientific community, most of the studies did not account adequately for the limitations of the platform (large number of genes evaluated, small number of samples) and especially of the analysis methods. Allison et al. (2006) have discussed the impact of microarray data analysis on the results. They pointed out that although some consensus has emerged in many areas, there are still some key questions that need to be answered regarding many steps of the analysis process. Among the issues still at hand, there are some related to the data validation and the need for a thorough evaluation of data analysis techniques. The choice and validation of the analysis method is especially important when published findings are compared by

independent researchers on other data sets, as a wrong choice of method may cause inconsistent results.

Usually the most common problem encountered when comparing different array data is the issue of poor overlaps, that is when lists of genes published in one study have little or no genes in common with other studies. Sample size is also a source of potential problems when evaluating predictors. When a number of candidate genes is too large when compared to the number of samples the statistical methods will suffer from overfitting, that is the prediction model will fit too tightly to the data it was derived from (training set) and therefore its accuracy will be sub-optimal when applied to independent data sets.

1.7.3 Pathway analysis and System Biology approach

Since their first inception a decade ago, microarray studies have become widely used in the research community, thanks to theirability to assess the expression of thousands of genes in a single laboratory event. The belief that such wealth of genomic information the community could not afford to lose has led to the development of microarray standards (Brazma et al., 2001; Ball et al., 2002) and databases including two major public microarray repositories, Gene Expression Omnibus (GEO; Edgar et al., 2002) and ArrayExpress (AE, Brazma et al., 2003), in the hope of enabling mining and exploration of newly acquired data space.

Identifying biologically meaningful information in relatively noisy data represents a significant tasks and so far breakthrough have been few and far between. Comparisons made on the level of gene lists obtained by different statistical methods or from different datasets hardly converge (Ein-Dor et al., 2005). As a consequence, the usefulness of the vast amounts of data stored in public repositories is subject to debate. At the same time, it is becoming important to use more than a single data set when analyzing microarray data (Xu et al., 2008) and gather hundreds or thousands of samples to develop prognostic markers (Ein-Dor et al., 2006). Reaching such a goal is difficult when information obtained from different experiments do not overlap. This is mainly because the data often has been generated with different microarray platforms, hybridization protocols, and the authors use different methods and different thresholds to calculate differentially expressed genes (DEGs; Allison et al., 2006). Finding ways to reliably compare different microarray data sets is therefore important to obtain biologically sound and reproducible information from different datasets.

In the past years collections of all the differentially expressed genes in a given condition that exclusively characterize that condition, have been proposed as ‘‘gene signatures’’ for a condition (Van’t Veer et al., 2002; Rosenwald et al., 2002; Yu et al., 2007). However, the reliability and reproducibility of such signatures has been questioned (Ranshoff, 2004; Simon et al., 2003), because of the influence of the statistical assumptions used, or errors in the methodology.

The number of inconsistencies and discrepancies when microarray data sets are compared are often reduced when approaches that take into account biologically related sets of genes, rather than single entities, are used (Manoli et al., 2006). The first methods developed with this approach aimed at identifying significantly under- or overrepresented terms in the Gene Ontology (Khatri et al., 2002). A second approach instead focuses in identifying significantly expressed gene sets (sometimes incorrectly referred as pathways or cellular networks) in a given condition using different statistical measurements: (1) Z-score (Doniger et al., 2003), (2) Gene Set Enrichment Analysis Subramanian et al., 2005), (3) signed Fisher Exact Test (Grosu et al., 2002), (4) Global test (Goeman et al., 2003) and (5) impact analysis (Draghici et al., 2007).

Recently Cavalieri's group in Florence developed a bioinformatic environment called Eu.Gene (Cavalieri et al., 2007) containing a repository of all the freely available biological pathways and different statistical methods dedicated to analyze expression datasets and assess for enrichment in biological pathways. Eu.Gene relies on 2 components, (1) a database of consistently annotated pathways collected from a number of state of the art pathway resources and (2) a module of computation implementing an array of tailored heuristics and statistical methods.
We developed a method allowing to assess similarity of samples in microarray databases. To this aim we used Eu.Gene to generate 'Pathway Signatures', recapitulating the biologically meaningful pathways related to some clinical/biological variable of interest, and used them in an analysis workflow to compare different microarray experiments.

1.7.4 Pathway and immune response

Research has made great strides towards of the goal of understanding biological processes. However, despite the recent advancements, dissecting precisely the sequence of causal and temporal effects is still a daunting task. High throughput technology such as microarrays or massive sequencing has in part exposed the problem more, as the vast amounts of data generated need to be fit in the biological landscape.

One of the ways that can be used to simplify the interpretation of said data is to associate them with a biological function or process, using the Gene Ontology (GO; Ashburner et al., 2000) or using biological pathways. For such an approach to work, the definition of what constitutes a pathway, be it a metabolic pathway or a signalling network, is essential. Unfortunately, the knowledge available on the most known pathway databases, KEGG and Reactome suffers from a series of problems that limit its usefulness.

Traditionally, representation of molecular pathways have been produced ad hoc and frequently included in reviews and original papers. Whilst they are clearly useful aids to understand biological cellular events, they are not sufficient by themselves, relying on extensive textual descriptions to explain what is shown pictorially. Recent years have seen considerable growth in

the availability of public and commercial databases offering searchable access to pathways and interaction derived from a combination of manual and automated (text mining) extraction of primary literature, reviews and large-scale molecular interaction studies. Using these tools it is possible to view a range of canonical pathway views of generate networks of interactions based on a given query.

To date, many studies have focused on either protein–protein networks or gene regulatory networks and these two types of networks are rarely combined into a single comprehensive view.

All of these efforts are let down by one or a number or key factors. The notation used in diagrams to depict one molecule's interaction with another is varied, often ambiguos and therefore limited in its ability to depict the exact nature of the relantionship between components of a pathway. There is often a lack of direct access to the experimental evidence relating to the interactions depicted or to a dataset as a whole. Similarly, labelling of the pathway components often uses non-standard nomenclature or mixes protein names form one species with that of another, such that again the reader is left uncertain as to what exactly is being shown. Finally, pathway diagrams usually focus only on a small part of biological sytem and one which often reflects the curatr's bias, such that the 'same' pathway described by different individuals may share little in common.

Whatever the source of these pathways and networks they generally suffer from graphically poor representation with ambiguity around the precise identity of what is being shown and the exact nature of their interaction. In order to address these issue different initiative started to define pathway notation and representation, from MIM (Molecular Interaction Map) notation (Kohn et al., 2006) to PDN (Process Description Notation, Kitano et al., 2005). The Edinburgh pathway notation (EPN, Moodie et al., 2006) uses a logical state-transition representation to describe biological pathways, similar to PDN. Recently, an initiative aimed to describe the signalling pathway important to macrophage activation (Raza et al., 2008) taking into account either the EPN notation either the developing international SBGN standard (System Biology Graphical Notation; Le Novére et al., 2009). The notation provides a logical context for interactions between components in the pathway; it can display the temporal order of reactions and can be mapped to the machine-readable SBML (System Biology Markup Language; Hucka et al., 2003). This scheme and the SBGN desire to develop pathways map 'readable' by a biologist. While the attempt to represent events in a detailed, accurate and logical fashion, the interpretation of the available literature useful for the reconstraction of the individual events is a unavoidably flawed process. Determining what constitutes good evidence for an interaction and what does not, is often difficult to judge for those who is not an expertise in the field.

A possible solution lies in the expert curation of pathways specific for the biological system that is being studied. As part of this effort, we recently proposed the DC-ATLAS initiative (Cavalieri et al., submitted) as a curated, highly specific pathway database for the DCs.

The thousand pink flowers of peach blossom bloom in spring. But its color is One.
Keizan Zenji

Aim of the study

The sequence of the dark side of the genome, the metagenome is now revealing the centrality for human health of an harmonious interaction between the organismal functions and the activity of the microorganisms colonizing our body.

We propose that the best characterized bakers yeast *Saccharomyces cerevisiae* can play a fundamental role in unraveling the rules of the interaction between the genome and the meta genome.

This work focused on the use of *S. cerevisiae* to understand the mechanism governing a fruitful interaction between microbes and the human system by dissecting the dendritic cell immune response to foodborne yeast and pathogenic fungi.

We pursued different strategies to understand the mechanisms of fungal recognition:

- By using the existing expression profiling, to allow integration between '–omics' data, we developed an algorithm to assign 'pathway signatures' to different dataset making them comparable.
- To understand the temporal signalling and the single pattern recognition receptor contribution to fungi recognition we contributed to the reconstruction of DC signalling pathways in the initiative DC-ATLAS.
- In order to dissect the signalling due to the single fungal cell wall recognition with respect to the complexity represented by the entire yeast we performed a traditional expression profiling analysis to identify the differentially expressed genes, their function and the pathway signatures associated to the single signal.
- By integrating microarray data with functional data we investigated the immune response of DCs to harmless and opportunistic pathogenic fungi, comparing *S. cerevisiae* cells, spores and *C. albicans* hyphae.

This work had two main goals: first, the implementation of an analytical approach that would facilitate the interpretation of the '-omics' results and increase the comparability between different data sets, lessening the problems associated with the use of different types of data and array platforms; and the development of new pathway structure to allow temporal dissection of the immune response associated to the pattern recognition receptor sensing; secondly, by integration of different results, to investigate the immune response that discriminates between friends or foes.

The important thing is not to stop questioning
Albert Einstein

2
Experimental Procedures

2.1 Yeast culture and preparation

2.1.1 *Saccharomyces cerevisiae* cell stimuli

Saccharomyces cerevisiae strain SK1 (*MAT*a/α *HO gal2 cup*^{S} *can1*^{R} *BIO*, Kane SM and Roth J. 1974 Bacteriol. 118: 8-14) was cultured in complete medium (YPD, 2% yeast extract, 1% peptone, 2% glucose) for 18 hours, then collected, washed twice with sterile water and resuspended at 10^8 cells/ml.

S. cerevisiae strains BY4741 (genotype, *Mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* and BY4741 *och1* (*Mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OCH1*::kanMX4) were cultured in complete medium till exponentially phase and treated as before.

2.1.2 Spore stimuli

In order to test homogeneous yeast populations, pure spore cultures were obtained by using this strain whose sporulation efficiency is 100%. To prepare spores, cells were grown on YPD plates, replicated on SPOIV medium (2% potassium acetate, 0.25% yeast extract, 0,1% glucose) and sporulation was assessed by optical microscopy. Zymolyase (2 mg/ml) was used to digest ascum and liberate spores. We initially performed experiments to evaluate zymoliase sensitivity of *asci* for this specific strain and we assessed that 15 minutes incubation was the minimum time needed to damage the *ascus*, without touching the spore cell wall. Zymoliase was inactivated by heat (65°C for 2 minutes) and washed away carefully together with the remainings of the empty *ascus*, by resuspending twice the spore culture in 500 µl of distilled water centrifuging and discarding the supernatant; the spores were then resuspended to a concentration of 10^8 cells/ml. In order to exclude damage on the spore cell wall or effects on spore viability 100 µl of a culture of 1000 spores per ml was plated on YPD in triplicate, the spores showed 100% viability. The

procedure used allows exposing DCs to the pure spore culture and to analyze the response to the spores alone, eliminating confounding effects of the presence of the *ascus*. Although from a mycological point of view the term ascospores might seem more appropriate than spores, we will use the term spores to specifically indicate that the effects we are observing are only due to the spores and not to the *ascus*.

We observed that after 24 hours of culture in RPMI 1640 (GIBCO-BRL), spores were germinating and the exponentially growing cells were starting to divide. Therefore any analyses performed at later time points would be affected by this altered state, while no interference should be present until 24 hours.

2.1.3 *Candida albicans* hyphae stimuli

Serotype A *C. albicans* strain SC5314 was cultured overnight in Saboraud medium at 28°C and then shifted to RPMI medium for 18 hours at 37°C to allow hyphal growth. The culture was treated as for yeast cells. After microscopical inspection of the purity of hyphal culture, this was treated as for yeast cells. It should be noted that in all the experiments performed in this study live microrganisms were used.

2.2 Microrganism survival following uptake by DCs

After 6 hour of stimulation, DCs were collected, washed 3 times with PBS, treated with zymolyase, washed twice and cells, lysated with a hypotonic solution (KCl 0.05%), were plated on YPD. Survival of yeast cells, spores or hyphae after uptake was reported as percentage of colony forming units after 3 days relative to the total number of cells growing in the absence of DCs exposure. To assess the importance of ROS production in DC killing ability, DPI (10 μM) was added 30 minutes before stimulation and survival of microorganisms was assessed using the same method. When evaluating survival after exposure, the possible effect of DMSO and DPI on the stimuli was taken into account.

2.3 Dendritic cell preparation and stimulation

2.3.1 Monocyte isolation

Peripheral Blood Mononucleated Cells (PBMCs) were isolated from buffy coat blood sample from healthy volunteers by Ficoll-Hypaque density gradient centrifugation (Biochrom AG). Monocytes were isolated from low density PBMCs by magnetic enrichment with anti-CD14 beads

(Miltenyi Biotec). MACS® Technology took place within MACS Columns. When a MACS Column is placed in a MACS Separator, a strong permanent magnet, a high-gradient magnetic field is induced on the column matrix. Unlabeled cells passed through and they were collected; labeled cells were released after removal of the column from the magnet. For isolation of monocytes, a positive selection was used. Positive selection means that the desired target cells are magnetically labeled and isolated as the magnetically retained cell fraction. Both fractions, labeled and unlabeled, were recovered and used.

2.3.2 Dendritic cell differentiation

After isolation, monocytes were cultured in RPMI 1640 (GIBCO-BRL) supplemented with glutamine (Sigma), penicillin and streptomycin (GIBCO-BRL), and 10% heat-inactivated fetal calf serum (Hyclone) in the presence of GM-CSF (800 U/ml, Gentaur) and recombinant IL-4 (1000 U/ml, Gentaur) for 6 days to allow DC differentiation (Sallusto e Lanzavecchia, 1994).

2.3.3 Dendritic cell stimulation

DC activation was induced by yeast in the different life stages. The control stimuli used were lipopolysaccharide (LPS 1 μg/ml), Curdlan (100 μg/ml), Zymosan (100 μg/ml, Sigma-Aldrich). Depending on the experiments, moDCs were added at different concentrations. Serial dilution of yeast preparations was added to the moDCs at different stimuli:DC ratios.

2.4 Isolation and priming of T cells

2.4.1 $CD4^+$ purification

Total $CD4^+$ T cells were obtained from PBMC by negative isolation with a combination of magnetic sorting (Miltenyi Biotec). Untouched isolation was performed by depletion of undesired cells. Non-target cells were magnetically labeled and eliminated from the cell mixture. The non-magnetic, untouched cell fraction contained the target cells.

2.4.2 $CD4^+$ T cell priming

moDCs were stimulated for 12 hours with spores/yeast, washed and plated with autologous $CD4^+$ T cells at a ratio of 1:20. For RT-PCR cells were collected after 24, 48 and 72 hours or 5 days of co-culture. Supernatants were collected after 5 days of co-culture for cytokine detection.

2.5 Cytokine production

After the times indicated, supernatants were collected and cytokine detection was performed. MesoScale Assay 7-spot (Meso Scale Discovery) or Luminex® Assay (Invitrogen) were used for detection of IL-1β, IL-8, IL-6, TNFα, IL-10, IL-12p70, IFNγ and IL-17A according to the manufacturer's instructions.

These procedures allowed simultaneously measurement of different cytokines in the same supernatants. The multiplex assay format differs from conventional ELISA in one significant way: the multiplex capture antibody is attached to a polystyrene bead whereas the ELISA capture antibody is attached to the microplate well. The use of the suspension bead-based technology enables multiplexing. Microspheres are internally dyed with red and infrared fluorophores of differing intensities. Each bead is given a unique number, or bead region, allowing differentiation of one bead from another. Beads covalently bound to different antibodies can be mixed in the same assay, utilizing a 96-well microplate format.

At the completion of the sandwich immunoassay, beads were read, using the Bioplex® 200™ detection system, in single-file by dual lasers for classification and quantification of each analyte.

Alternatively, ELISA assay was performed according to the manufacturer's instructions. ELISA kits were from Biosource rather than ELISA for IL-12p70 that was from R&D System and IL-17 from e-Bioscience.

In blocking experiment, after 2 hors of exposure with the inhibitor, supernatants were collected after 8 or 24 hours or for T cell cytokine detection, 5 days of incubation.

2.6 Quantitative real time polimerase chain reaction (PCR)

Total RNA was extracted with the Rneasy Mini Kit (Qiagen). Random hexamer and reverse transcriptase kit (SuperScript II, Invitrogen) were used for cDNA synthesis. Quantitative real-time PCR for *FOXP3* gene expression was performed using primer pairs and SYBR Green PCR Master Mix (Applied Biosystem). Transcripts for *IL-6*, *IL-17A,* and *IFNγ* were quantified with Applied Biosystems predesigned TaqMan Gene Expression Assays and reagents according to the manufacturer's instructions. Quantification of the PCR signals was performed by comparing the cycle threshold (Ct) value of the gene of interest with the Ct value of the reference gene *GAPDH*. Values are expressed as fold increase of mRNA relative to that in unstimulated cells.

2.7 IL-12p70 blocking assay

2.7.1 IL-12p70 inhibition by cytochalasin D

DCs were exposed to cytochalasin D (10 μg/ml, TebuBio) for 30 minutes at 4°C. After washing with cold PBS, DCs were stimulated with *S. cerevisiae* yeast cells or spores at a DC:stimuli ratio of 4:1 for 24 hours. IL-12p70 production was assessed by ELISA.

2.7.2 IL-12p70 blocking assay

In order to assess the importance of IL-12p70 in balancing Th1/Th17 response two different experiments were performed.

In one case, DCs were stimulated for 8 hours with live spores of *S. cerevisiae* and *C. albicans* hyphae at a stimuli:DC ratio of 4:1 in the presence of different concentration of human recombinant IL12p70 (0, 1, 10 and 100 ng/ml).

In the second experiment, DCs were pre-incubated with different concentrations (0, 0.1, 1, 10, 100 μg/ml) of a monoclonal anti-human IL-12 antibody for 2 hours, then stimulated for 8 hours with live S. *cerevisiae* cells in a stimuli:DC ratio of 4:1.

The ability of these stimulated DCs to drive $CD4^+$ T cells polarization towards a Th1 response was assessed by measuring of IFNγ and IL-17 in T cells 5-day culture supernatants.

2.8 ROS production

To determine ROS production we used the modified version by Choi et al. (2006) of the microscopic Nitroblue tetrazolium (NBT, Sigma-Aldrich) assay. Briefly, DCs were stimulated with spores or yeasts, washed, supplemented with 0.1 mg/ml of NBT, which allows the precipitation of formazan particles in presence of ROS. Blue formazan particles were dissolved using 2M KOH and DMSO and its absorbance was measured using a microplate reader at 620 nm. The absorbance of dissolved NBT increased in proportion to cell number, incubation time, and stimulus concentration.

2.9 Internalization assay

2.9.1 Phagocytosis assay

Yeasts/spores were biotinylated using 10 mg/ml sulfo-NHS-LC-biotin (Sigma-Aldrich) in 50 mM $NaHCO_3$ pH 8.5 for 2 hours at 4°C. The remaining reactive biotin molecules were inactivated by incubation in 100 mM Tris-HCl pH 8.0 for 40 minutes at 4°C. DCs were then

treated with biotinylated spores/yeasts. After 1 hour, cells were permeabilized and labeled with aHLA-DR-FITC. After zymolyase treatment, intracellular yeasts/spores were detected using APC-labeled streptavidin and analyzed by flow cytometry.

2.9.2 Receptor mediated-internalization assay

Immature DCs were allowed to adhere onto fibronectin and subsequently incubated with FITC-labeled yeast or spores for 5, 15, 30 minutes at 37°C. At the end of the incubation period, the samples were fixed in 4% PFA, permeabilized in Methanol, and labeled for DC-SIGN and/or MR using specific mAb and isotype-specific fluorescent secondary Abs. Samples were analyzed using a Zeiss LSM 510 confocal microscope. Alexa647-conjugated goat-anti-mouse IgG, and Alexa568 goat-anti-mouse IgG2b were from Molecular Probes.

2.10 Receptor involvement assay

2.10.1 Competition assay

DCs were exposed to laminarin (500 μg/ml, Sigma-Aldrich), mannan (500 μg/ml, Sigma-Aldrich) and chitin (500 μg/ml, Sigma-Aldrich) for 30 minutes at 37°C. After washing with PBS, DCs were exposed to yeast cells or spores at a concentration of 4:1 for 6 hours. Inhibition ability of TNFα production was assessed by ELISA.

2.10.2 Binding assay

Labeling of *S. cerevisiae* cells or spores was performed as previoulsy described (Cambi et al., 2003). The binding of DCs or CHO-DC-SIGN to *S. cerevisiae* yeast cells or spores was measured by flow cytometry using the FACS Calibur (BD Biosciences) and performed as already described. To test the effects of various reagents on ligand binding the following concentrations were used: different carbohydrates 150 μg/ml, mannose (100 μM), Isotype control, AZN-D1, AZN-D2, AZN-D3 anti-DC-SIGN, anti-MMR, and anti-β2-integrin (30 μg/ml), EGTA and EDTA 2 mM. Incubation was performed in 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% bovine serum albumin. FITC-labeled yeast cells or spores were added in a stimuli:DC ratio of 10:1. After 30 minutes of incubation at 37°C, cell-*S. cerevisiae* conjugates were analyzed by flow cytometry., DCs were labeled with anti-CD45-APC and CHO-DC-SIGN with PKH26 (Sigma-Aldrich) to discriminate cells binding FITC-labeled yeast/spores particles from yeast/spores aggregates.

Mannan derived from *C. albicans* (CA-mannan) and glucan were a kind gift of Prof. David Williams and were previously isolated (Chorvatovicová et al., 1999; Lowman et al., 2003). The

following Abs were used: mAb AZN-D1, AZN-D2 and AZN-D3 anti-DC-SIGN (Geijtenbeek et al., 2000), NKI-L19, anti-b_2-integrins. mAb anti-MMR and mAb DCN46 anti-DC-SIGN from BD Pharmingen, APC-conjugated mAb anti-CD45RO from Becton Dickinson. Alexa647-conjugated goat-anti-mouse IgG, and Alexa568 goat-anti-mouse IgG2b were from Molecular Probes.

2.11 *In vivo* survival assay

To evaluate spores survival during gastrointestinal transit, one-month old rats were fed for 4 weeks on a daily basis with water containing $1X10^7$ yeast SK1 spores (n=3) or cells (n=3). A group of rats (n=3) was fed with water containing any stimuli as control. From 1 to 4 weeks, faecal samples were collected and after serial dilution were plated on YPD medium with chloramphenicol (100 ng/ml, Sigma-Aldrich) to allow growth of CFU. After three days, CFU were counted. Survival of spores was referred to the number of CFU grown after 3 days.

2.12 DC transcriptional analysis

2.12.1 Hybridization protocol

To obtain the experiments useful for the development of the algorithm, $3X10^6$ DCs were either not stimulated or stimulated with yeast grown in exponential phase. Cells were harvested after 4 hours of stimulation. RNA extraction was done with TRIzol reagent (Invitrogen). Sample pre-processing and biotin labeling were performed using the Affymetrix GeneChipH cDNA Synthesis Kit and IVT Labeling Kit (Affymetrix) according to the manufacturer's protocols. Microarray were then hybridized on Affymetrix GeneChipH HG-U133A 2.0 microarrays, and scanned according to the manufacturer's instructions on a GeneChipH Scanner 3000 (Affymetrix). Extraction, hybridization and scanning were performed by the Genopolis consortium (University of Milano-Bicocca, Italy). Microarray data have been submitted to GEO (accession number GSE13901).

To obtain a detailed picture of DC maturation process after yeast and spores internalization, $2x10^6$ DCs were cultivated with cells of *S. cerevisiae* cells, spores, hyphae of *C. albicans* or without any stimuli in a ratio of 4:1. After 4 hours, cells were collected. RNA preparation, labeling, hybridization on a HT12 array (Illumina), and scanning were performed according to Illumina instructions by Genomics Lab, Wellcome Trust Centre for Human Genetics (Univeristy of Oxford). Microarray data have been submitted to Array Express (accession number E-MTAB-135). To unravel the specific contribution of cell wall components on fungi recognition, $2x10^6$ DCs were cultivated with *S. cerevisiae* cells, spores in a ratio of 4:1 or mannan, curdlan and

zymosan (100 μg/ml) or without any stimuli. After 4 hours, cells were collected. RNA preparation, labeling, hybridization on a HT12 array (Illumina), and scanning were performed according to the Illumina reference protocols by Genomics Lab, Wellcome Trust Centre for Human Genetics (Univeristy of Oxford). The analysis was performed on four different donors.

2.12.2 Array pre-processing

Affymetrix array data files (CEL files) were pre-processed and normalized using the Robust Multichip Average procedure (RMA). Annotations were updated following a procedure devised by Dai and co-workers (Dai et al., 2005). Computation was performed with the RMAExpress program (http://rmaexpress.bmbolstad.com)

Bead-summary data saved from Illumina BeadStudio was pre-processed in several steps. Firstly, the background signal was assessed and corrected using the intensity signal from the control probes present on the array, then quantile normalization was performed. In addition to background correction, Illumina probe identifiers were converted to nucleotide universal IDentifiers (nuIDs) (Du et al., 2007) specific for the nucleotide sequence of each probe. The computation was performed using the lumi package (Du et al., 2008), written in the R programming language.

2.12.3 Differential expression and annotation

Differential expression analysis was carried out using the Rank Product algorithm (Hong et al., 2006), taking into account the differences between donors. p-values estimating differential expression were corrected for multiple testing (FDR) and genes with a corrected p-value <= 0.05 were selected.

Differentially expressed gene lists from the various data sets were uploaded in the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov)) to perform functional annotation. The up-regulated and down-regulated gene lists were annotated separately.

2.12.4 Pathway analysis

Pathway analysis was performed with Eu.Gene (Cavalieri et al., 2005), over a set of 80 pathways selected by the DC-THERA consortium or the new pathway set DC-ATLAS, developed during this period and collecting several manullay curated DC-specific pathway (see dedicated section).

Eu.Gene identifies biological pathways, which are enriched in up-regulated or down-regulated genes (therefore determining if the pathways themselves are up or down-regulated) using

a statistical method, the Fisher's Exact Test (FET; Grosu et al., 2002). Also, it provides two measures to assess if a pathway is significantly deregulated:

- a p-value (result of the statistical analysis)
- the 'Coverage' parameter. The latter is defined as the number of genes in the data set present in the pathway versus the total genes of the pathway.

Pathways with a low coverage may be false positives, because despite being significant, the number of genes represented in the pathway itself is little.

2.12.4.1 Fisher Exact Test

Prior to analysis, the transcriptional data was converted into $\log_2$ ratios using the following procedure. For each data set, treated and untreated sample data were separated, and the expression level of each gene in each treated sample was divided by the expression level of the same gene in the corresponding paired untreated sample, and the resulting ratio was $\log_2$ transformed, obtaining a list of log2 ratios for each gene in each treated sample. The transformed expression data were then used for Fisher's Exact Test (FET). Then, in an effort to reduce inter donor variability, the mean of the ratios for all replicates in a specific condition was calculated. The resulting ratios were used to perform pathway analysis using the FET.

The upper and lower cut-offs for FET were determined from the 2s interval of the binomial distribution of expression values (Segota et al., 2008). The algorithm was then run using the hypergeometric distribution without approximation. Each pathway was associated to a signed p-value and a matrix of signed p-values for all samples was obtained for each data set. In order to provide a correction for multiple testing, p-values were adjusted following the procedure by Benjamini et al. (2001). Resulting p-values from the FET analysis were then transformed prior to clustering (see below).

2.12.4.2 p-value transformation (FET)

The FET p-value tables from Eu.Gene were transformed in order to be used for hierarchical clustering. Two approaches were used:

- *Pathway Enrichment Factor (PEF)*: p-values were transformed into PEF as follows:

  $$\pm\log_2(1/\text{pvalue})$$

  where the sign is determined by the Sign of the p-value obtained from the FET analysis in Eu.Gene.

– *signed Binary Enrichment Factors (sBEFs):* p-value were transformed in sBEF as:

± 1 if p-value ≤ 0.05 depending on Sign

0 if p-value < 0 or Sign is not defined

In the case of sBEFs, after transformation they were filtered to exclude non-significant pathways in all the samples (i.e., rows containing only zeroes in all samples).

2.12.4.3 Clustering

Hierarchical clustering was performed with the TIGR Multiexperiment Viewer (TMeV), version 4.1.1 (Saeed et al., 2003). Specifically, we used standard hierarchical clustering and clustering with support trees: in the latter case pathways and samples were bootstrapped over 100 and 1000 iterations. Euclidean distance was used as distance metrics, with average linkage clustering.

2.13 Statistics

Student's t-test was used to evaluate the statistical significance of the results, by comparison between spores and yeast or between control and blocking condition. Results were classified as non significant ($p > 0.05$), significant ($p < 0.05$) and highly significant ($p < 0.01$).

Not all who wander are lost
J.R.R. Tolkien

3
Pathway signatures generation

3.1 Background

Since their first inception a decade ago, microarray studies have become widely used in the research community, thanks to their ability to assess the expression of thousands of genes in a single laboratory event.

The number of inconsistencies and discrepancies when microarray data sets are compared are often reduced when approaches that take into account biologically related sets of genes, rather than single entities, are used (Manoli et al., 2006).

We developed a method allowing to assess similarity of samples in microarray databases. To this aim we used Eu.Gene to generate 'Pathway Signatures', recapitulating the biologically meaningful pathways related to some clinical/biological variable of interest, and used them in an analysis workflow to compare different microarray experiments.

3.2 Data set selection

In particular, we decided to use as reference experiments a data sets composed of 106 experiments (grouped in 7 sets) focused on the exposure of *Homo sapiens* dendritic cells (DCs) to different stimuli (Table 3.1).

Following up the data set collection, data were pre-processed and subject to pathway analysis (see Methods). Then, we formulated a prediction basing on biological evidence against which the method would be tested. We performed a wet lab experiments stimulating DCs with cells of the yeast *Saccharomyces cerevisiae* in exponential growth phase (analyzed in the same manner as the public data) and we predicted that this experiment would show similarity with the samples from GEO data set GSE6965 (gene expression profile of monocyte-derived DCs stimulated for 6 hours with germinating germ tubes of *Aspergillus fumigatus*).

Isolation of human monocytes and differentiation on dendritic cells (DC) were performed

as described in the Experimental procedures section.

Table 3.1. Data set summary. Dendritic cells data sets obtained from GEO

GEO ID	Description	Type	Sample no.	Reference	Year
GSE8658	PPARγ regulated gene expression in human DCs	Ligand response	63	Szatmari et al., 2007	2007
GSE7247	DCs Compare the Similarity of Endogenous and Exogenous Antigens	Loading methodology comparison	10	Decker et al., 2006	2006
GSE4984	moDCs Maturation	Dendritic cell maturation	12	Fulcher et al., 2006	2006
GSE6090	DC-SIGN initiates an immature DCs phenotype triggering Rho activation that is used by HIV-1	Receptor signalling	6	Hodges et al., 2007	2007
GSE6965	Gene expression profiling of human DCs after infection with A. fumigatus	Dendritic cell activation	4	Mezger et al., 2008	2008
GSE5679	Comparative gene expression profile of PPARγ and RARa ligand treated human dendritic cells	Ligand response	11	Szatmari et al., 2006	2008

3.3 Selection of the pathway set

To identify biologically meaningful changes in our data sets, we assembled a dedicated pathway set. The pathway database present in Eu.Gene was searched for pathways involved in immune response and signal transduction by key transcription factors involved in immune response. All pathways were defined as gene lists (containing genes associated with the pathway). The set was prepared excluding pathways containing less than three elements. Out of the remaining pathways, we built a selection where pathways were classified by their level of immunological relevance. Out of the 1038 human pathways available, 80 were selected to be part of the set.

3.4 Preprocessing and pathway analysis

Prior to the analysis, microarray raw data were transformed into absolute-scale values and processed following the procedure outlined by Beltrame et al. (2009): firstly, ratios between each treated condition and the unstimulated controls were calculated.. The resulting ratios were used to perform pathway analysis using the Fisher's Exact Test (FET).

With the aim of selecting the most representative FET threshold, FET thresholds were selected from the 2s interval of the binomial distribution of the expression values.

When using FET, we addressed the question of how to use the collection of p-values for a defined set of pathways to generate a pathway signature with the aim of comparing different microarray experiments.

Firstly, we corrected the p-values for multiple testing as described in the Experimental procedures section then our first attempt was to transform the p-values into a measure whose magnitude would express the degree of significance, and to that purpose, we transformed p-values into Pathway Enrichment Factors (PEFs).

Secondly, we transformed p-values in signed binary enrichment factors (sBEFs) (see Experimental procedures section): these factors categorized p-values into three classes:

1. significantly up-regulated,
2. significantly down-regulated, and
3. not significantly affected.

We then evaluated the performance of these metric, as expressed in the ability of assessing similarity according to the expectation that biologically similar experiments should show identical or similar pathway profiles (defined as collections of PEFs or sBEFs used for testing).

3.5 Validation of the method

The clustering of signed pathway enrichment factors (sPEFs) gave results that were agreeing with our prediction, as wet lab experiments (analyzed in the same manner as the public data) show similarity with the samples from GEO data set GSE6965. The Pathway Signatures of ad-hoc sample produced stimulating DCs with cells of the yeast *S. cerevisiae* in exponential growth phase, clustered together with profile of moDCs stimulated for 6 hours with germinating germ tubes of *A. fumigatus* (Figure 3.1).

In detail, the two samples belonged to the same sub-cluster and were the most closely associated. Pathways which shared a similar profile among the two samples included the Toll-like receptor signalling pathway, JAK-STAT signalling, and cytokine-cytokine receptor interaction (Figure 3.2).

Since we expect the two stimuli to elicit signalling from the same pathogen associated molecular patterns (PAMPs) these experiments were in principle the nearest stimulations to the yeast one, among those present in the selected dataset. The results therefore show that pathway-level signatures identify samples with similar biological characteristics and totally confirmed the expectation. These findings show that FET pathway based metrics are a powerful tool to identify similarity between experiments.

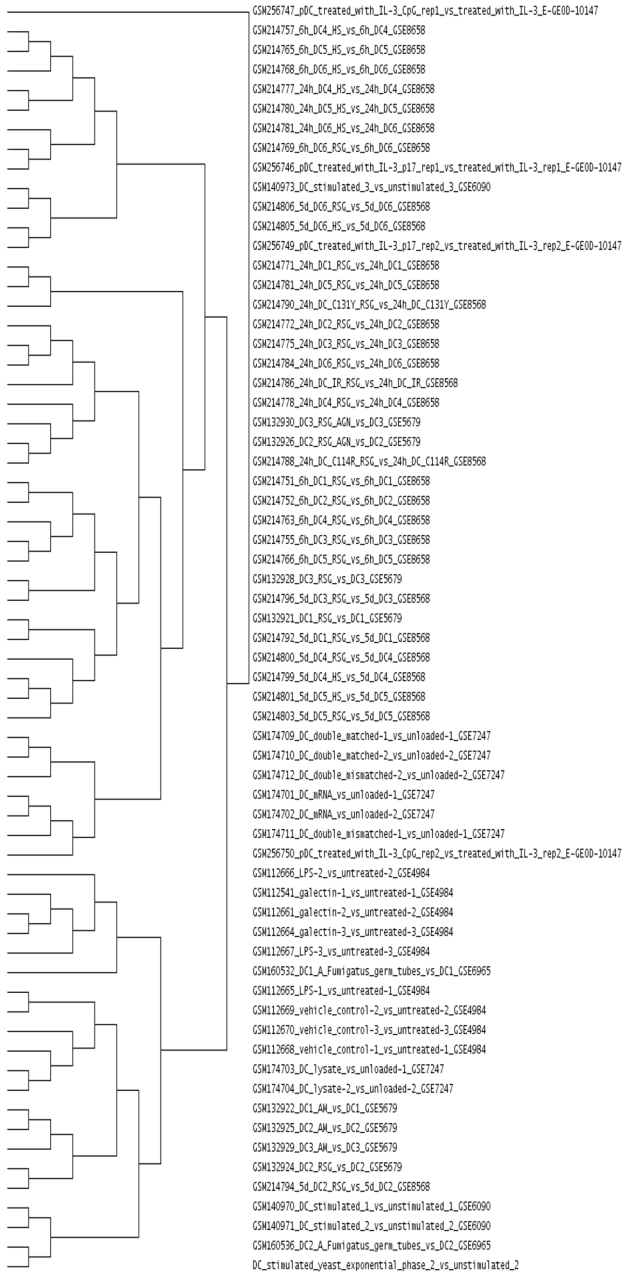


Figure 3.1. Sample clustering of Fisher's Exact Test samples on dendritic cell data. Sample tree originated from the clustering of FET signed Binary Enrichment Factors (sBEFs) values with Euclidean distance.

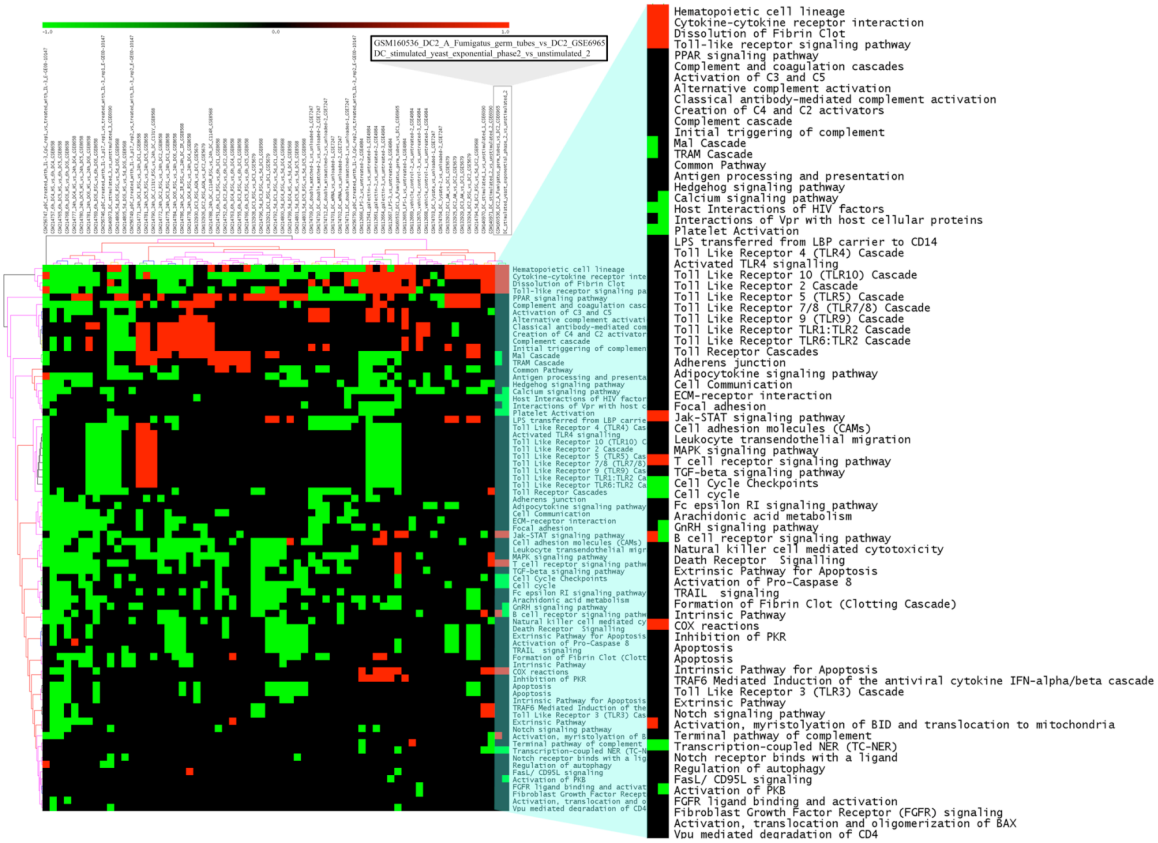


Figure 3.2. Clustering of signed Binary Enrichment Factors (sBEFs) obtained from the FET analysis on dendritic cell samples. Colored spots indicate significant (p#0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black). The inset shows the clustering of sample GSM160356 from data set GSE6965 with the in-house dendritic cell experiment

3.6 Discussion

With the aim to propose a novel method to compare microarray experiments at the pathway level, our work addressed three major tasks:

1. defined a metric measuring the probability of a set of pathways to be related to some clinical/biological variable of interest and the relative importance of that set in the context of the biological problem;
2. proposed a method using these pathway signatures to assess relative similarity of the experiments;
3. proved the validity of this method applying it to two different well defined biological problems on two independent collections of microarray experiments.

As a proof of concept we decided to use data sets from *Homo sapiens* because the majority of the experiments stored in GEO and ArrayExpress are from human samples. We chose transcriptomic datasets from DCs as they have some clear advantages respect to other fields. Firstly, the cell type is well-defined, which enables the study of the alterations in gene expression following stimulation. Secondly, there is the possibility to perform prediction and hypothesis-driven functional genomics studies aiming at the reconstruction of the networks of molecular interactions characterizing specific DC differentiation programs.

The aim of the work was to propose a procedure to assess similarity between microarray experiments at the pathway level generating 'Pathway signatures' (PS) for a set of experiments and use these signatures to interrogate microarray databases.

The need for better methods to identify similarities in microarray data sets arises from the fact that although the analysis techniques have constantly improved over the past years, one of the biggest hurdles remains the comparability among distinct data sets produced by different researchers and laboratories, resulting in lists of genes which do not overlap, or overlap in a very limited fashion.

The improvements observed when comparing different experiments at the pathway level (Manoli et al., 2006) is coherent with the assumption that genes never act alone in a biological system, but participate in a cascade of networks, an approach overlooked by gene-based analyses.

The selection of the statistical method used to measure enrichment is central to our approach. Different metrics have been proposed to integrate the probability of alteration of a sector of the cellular network (pathway) and the relative importance of that pathway in the context of the biological problem, such as the probability vector (Grosu et al., 2002), the impact factor (IF; Draghici et al., 2007). Other methods have been devised for the identification of regulatory modules and their regulation program by integrating genome-wide location and expression data (Tavazoie et al., 1999; Segal et al., 2003; Segal et al., 2004; Segal et al., 2005).

However, to our knowledge, these methods have not been employed to compare a large number of experiments assembled from different microarray data sets. To make more immediate FET p-values had to be transformed to improve interpretation: p-values express a probability, and the smaller they are the more significant the result is; while from a point of view it is better to express pathway enrichment as a number that it is either categorical or the more significant the greater it is. That is the reason we first used the logarithm of reciprocal of the p-value (the Pathway Enrichment Factor), to express the measure in a scale that would avoid interpretation problems, the PEF was finally obtained multiplying the value for a sign representing the 'direction' of a pathway, with the same approach described in (Grosu et al., 2002). Our results showed that clustering PEFs grouped samples according to their biological similarity.

Biclustering of PEFs on the DC data sets gave concordant and biologically relevant results, the responses elicited by stimulation with *S. cerevisiae* follow the same downstream signalling as

the ones in response to the fungus *A. fumigatus* (Buentke e Scheynius et al., 2003; van de Veerdonk et al., 2008; Roeder et al., 2004; Tada et al., 2002).

The observation that sBEFs, PEFs and PSs, identify similarity between samples that are biologically meaningful, indicates that the categorical transformation of the p-values does not affect the observed result, concluding that the observed similarity has a biological meaning rather than resulting from a manipulation of the data.

We can conclude that the ability to investigate at the single sample makes the Fisher's Exact Test conceptually appropriate to search for similarities among experiments in a microarray database, that contains a number of hybridizations that should be interrogated without necessarily specifying the membership to a data set or another.

Our results show that using pathway signatures in conjunction with hierarchical clustering with support trees is a powerful and useful technique to compare experiments produced by different people and laboratories with greater power than with the traditional analysis techniques. The results are also easier to interpret and discover biologically meaningful implications, making this approach an ideal candidate to analyze data from different sources. PS generated using sBEFs can be useful as ''barcodes'' to classify experiments in microarray databases and clustering of sBEFs can be a useful way to query experiments in databases according to their similarity at a pathway level. Thus, we propose to store PSs as an additional experimental annotation in the microarray databases and implement methods using pathway signatures to query experiments in public databases and concurrent analyses of subsets of experiments.

If people never did silly things nothing intelligent would ever get done
Ludwig Wittgenstein

4

DC ATLAS: towards the reconstruction of the pathway of the dendritic cells

4.1 Background

Research has made great strides towards of the goal of understanding biological processes. However, despite the recent advancements, dissecting precisely the sequence of causal and temporal effects is still a daunting task. The description of the causal inferences between a set of proteins or genes is usually described as a pathway. Pathways are currently generic in terms of cell type and topography, and a possible solution lies in the expert curation of pathways specific for the biological system that is being studied. The results presented in this chapter are part of a larger effort, in the context of the consortium DC-THERA, the DC-ATLAS initiative (Cavalieri et al., submitted).

Exposure of DCs to various stimuli leads to profound changes in their phenotypic and functional properties, a process commonly referred to as activation or maturation. Depending on the nature of the stimuli, and their interactions with specific cell-surface or intracellular receptors, the signals are translated by the fundamental machinery of the cell into appropriate cellular responses (Huang et al., 2001).

DC-ATLAS provides an integrated description of the interactions operating in DCs.
This initiative aims at establishing a complete 'road-map' of intracellular signalling pathways and regulatory networks by defining the methodological bases, and proposing a computational infrastructure, suitable for the investigation of the networks of molecular interactions characterizing activation programs of DCs.

4.2 Goals and organization of the DC-ATLAS initiative

The overarching goals of DC-ATLAS are to define the structure, interconnection, and regulation of signalling pathways in DCs, to create a consensus description of these pathways using a language amenable to modelling and statistical analysis, and to foster a systems biology approach for understanding the biology of this immunologically central cell type. The DC-ATLAS initiative aimed to establish both the methodological basis and computational infrastructure required for investigating the networks characterizing the activation programs of DCs. The methodological aims were to develop a model describing the molecular interactions occurring in DCs, and to define the appropriate ontological terms describing these interactions. The interactions were annotated precisely with information on the cell type and organism in which supportive evidence has been obtained, and the reasons for which the process as a whole can be considered to be operative in DCs. The pathways were curated manually and constructed using controlled vocabularies and hierarchies.

Strategically, the project relied on close collaboration between members of an interdisciplinary research group, the DC-ATLAS curation team, which integrated the biological, bioinformatic and statistical expertise required to ensure the development of a systemic approach to the analysis of a complex biological system.

4.3 Pathway structure in DC-ATLAS

Interactions were organized in functional coherent modules: a set of modules, describing the transfer of the signal from the receptor to the outcome, defines a pathway. The selected pathways have been used to populate the DC-PATH pathway repository, created by using a SBGN compliant data model and dedicated controlled vocabularies.

Pathways were constructed according to a DC-ATLAS specific data model.

The data model was built to unambiguously describe pathways, their genes and proteins, and their interactions. Pathways are shown as sets of nodes (genes, small molecules, etc.) connected by lines that represent interactions. Genes have been identified using the official symbol provided by HUGO Gene Nomenclature Committee (HGNC, www.genenames.org, Bruford *et al.* 2008) symbol and their Entrez Gene ID (www.ncbi.nlm.nih.gov/Entrez; Maglott et al., 2005; 2007).

We reached a consensus on the structure, interconnection, and regulation of signalling pathways using an ontological language amenable to modeling and statistical analysis. Specifically, controlled vocabularies were built to clearly annotate the components, their interactions and their location. These vocabularies were created by integrating with DC-specific terms the Open Biomedical Ontologies, and allowed to reduce to a widely accepted representation the complexity

of molecular interactions, such as complex formation, nuclear translocation and post-translational modifications.

The graphical representations of the pathways were performed with the tool DC-PathEDIT, allowing the design of the pathways in which proteins (Nodes) react (Edges) with each other. DC-PathEDIT permits the selective visualization of reactions that have been proven to occur in dendritic cells, those that have only been documented in other cell types, and hypothetical reactions that remain to be formally established.
The data model divided pathways into 3 different modules:

1- the portion of the pathway implicated in sensing the stimulus (receptor/sensing module);
2- the principal signal transduction modules situated downstream of the receptor (transduction modules);
3- the biological outcome, such as cytokine or chemokine production, apoptosis, migration and differentiation (outcome modules).

The pathways were curated by creating a gene list, a graphical representation and a text file, containing a controlled vocabulary compliant detailed description of the pathway itself. Pathways have been curated on the basis of information available in existing databases and literature, as well as of experimental evidence generated in the laboratories of the researchers involved in the DC-ATLAS project. The curation process led to the definition of pathways not yet present in any other databases, which exclusively describe interactions proven in DCs

In the case of pathways already described in public databases, disproven interactions were eliminated and significant newly established connections were added. In particular, the Outcome modules included a major amount of novel information derived from functional genomics data combined with physiological information.

4.4 Curation *ex novo* of Dectin-1

Many pathways, as Dectin-1 or DC-SIGN, are novel in that they are currently not available in other systematic pathway collections (Kanehisa, M. *et al. 2008;* Vastrik, I. *et al 2007)*. The intracellular signalling pathways induced by these C-type lectins modulate the responses of other PRRs such as TLRs, but also exert functions independent from other PRRs (vonVliet et al., 2007)

Dectin-1 was originally cloned as a dendritic cell surface molecule capable of delivering co-stimulatory signals to T cells (Ariizumi et al, 2000). It was subsequently shown to be expressed more widely on myeloid cells including macrophages, dendritic cells and neutrophils (Robinson et al, 2006; Taylor et al, 2002) and identified as a receptor for β-glucans (Brown and Gordon 2001).

Dectin-1 specifically recognizes soluble or particulate β-1,3- and/or β-1,6-glucans, which are found primarily in the cells walls of fungi but also plants and some bacteria (fungi and micobacteria, Yadav and Schorey, 2006).

Dectin-1-deficiency is associated with impaired recruitment of inflammatory leukocytes and inflammatory mediator production at the site of infection. Myeloid cell activation by dectin-1 is controlled by inherent cellular programming, with distinct macrophage and dendritic cell populations responding differentially to the engagement of this receptor. The inflammatory response is further modulated by the progression of the phagocytosis, with 'frustrated phagocytosis' resulting in dramatically augmented inflammatory responses. Dectin-1 in isolation is sufficient to drive a potent inflammatory response in a context-dependent manner. This has implications for the mechanism by which myeloid cells are activated during fungal infections and the processes involved in the therapeutic manipulation of the immune system via exogenous dectin-1 stimulation or blockade.

Trough this novel pathway, Dectin-1 also collaborates with the TLRs to induce pro-inflammatory responses, such as TNFα (Brown et al, 2006). The first evidence for collaboration between TLR2 and Dectin-1 in coordinating inflammatory gene induction came from studies with zymosan (Gantner et al, 2003; Brown et al, 2003). Dectin-1 and TLR2 signals have also been reported to contribute to cytokine induction by *A. fumigatus* conidia (Hohl et al, 2005; Steele et al, 2005; Gersuk et al, 2006). How this collaboration takes place is unknown, as the read out is largely cytokine production as Dectin-1 and the TLR synergise/collaborate but it seems that it is not at signalling level.

Despite the information collected in years to understand the molecular mechanisms that underlie Dectin-1 function, a complete picture of the intracellular signalling lacks.

In the DC-ATLAS initiative, we used the literature information available to reconstruct the intracellular signalling leading from initial recognition to DC maturation, describing the pathway as an assembly of modules, sensing, signal transduction and outcome (Figure 4.1, for legend, Figure 4.2) and as a description of the reaction (Appendix A and B) and a list of genes (Appendix C).

4.5 Discussion

DC-ATLAS represents a major advance relative to other existing databases with respect to eight key aspects:

1- it is focused on DCs signalling pathways being thus cell type specific;
2- it is the first immunological and bioinformatics integrated project that will allow the analysis of high throughput data in a DC-specific manner;
3- it contains many pathways that are not currently available in other pathway collections;

4- it contains pathways that have been curated manually by prominent experts in the field, and have been in certain cases validated with laboratory experiments;
5- it contains pathways that have been constructed using controlled vocabularies and hierarchies, allowing automatic processing of the data for various applications, including pathway analysis;
6- it adopted a data model composed of interconnected modules that provide a structural overview of the mechanisms, from receptors via signal mediators to effectors. The modules allow to appreciate the time in which the signal take place overcoming the issues of the current generic pathway structure that masks specific events in a plethora of generic interactions;
7- DC-ATLAS is one of the first pathways databases SBGN compliant and
8- it represents a consensus established by a large number of scientists, comprising essentially the entire European scientific community with expertise in DCs.

The division of pathways into different modules will be critical for the statistics of pathway analysis. Furthermore, the modules can be used to define or reconstruct general signal transduction pathways in immunology.

The possibility of verifying key biological information relevant to the DC-specific modules, including data from patients, will permit an improved description of the hallmarks of DC maturation. The latter can not be achieved with the current generic pathway structure, which masks specific events in a plethora of generic interactions.

Our data model allows the dissection of signal transduction pathways involved in DC activation by means of a Systems Biology approach. Expert curated DC-ATLAS pathways represents a valuable tool to emphasize established facts as well as highlight the limits of our knowledge with respect to the hierarchy of events leading to effective immune responses. The appropriate application of computational methods will contribute to fill current gaps in the bioinformatics analysis of genomic data, in which the integration of different types of information remains a major, and partially unresolved, computational issue. Furthermore, the ability of DC-ATLAS to identify gaps in our current knowledge will help to focus future research efforts, and lead to the design of specific experiments aimed at reconciling interactions and findings documented in human and mouse DCs, and at establishing relationships between pathways operating in DCs and other cell types.

Finally, DC-ATLAS will provide a knowledge base on DC biology that will prove to be valuable for rationalizing information present in the literature. The ability of DC-ATLAS to identify gaps in our current knowledge will help to focus future research efforts, and will lead to the design of specific experiments aimed at reconciling interactions and findings documented in

human and mouse DCs, and at establishing relationships between pathways operating in DCs and other cell types.

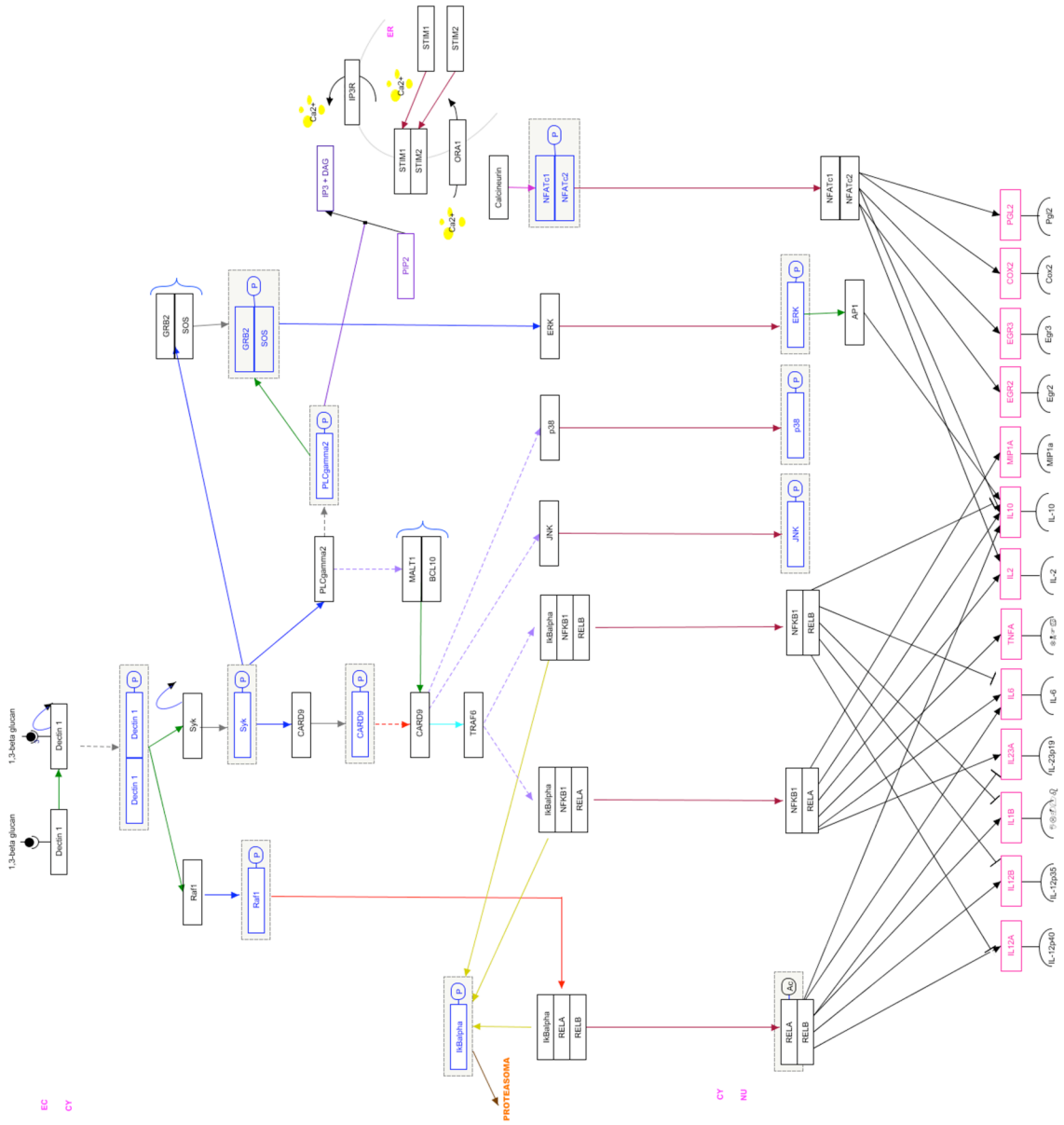


Figure 4.1. Graphical representation of Dectin-1 with DC-PathEDIT.

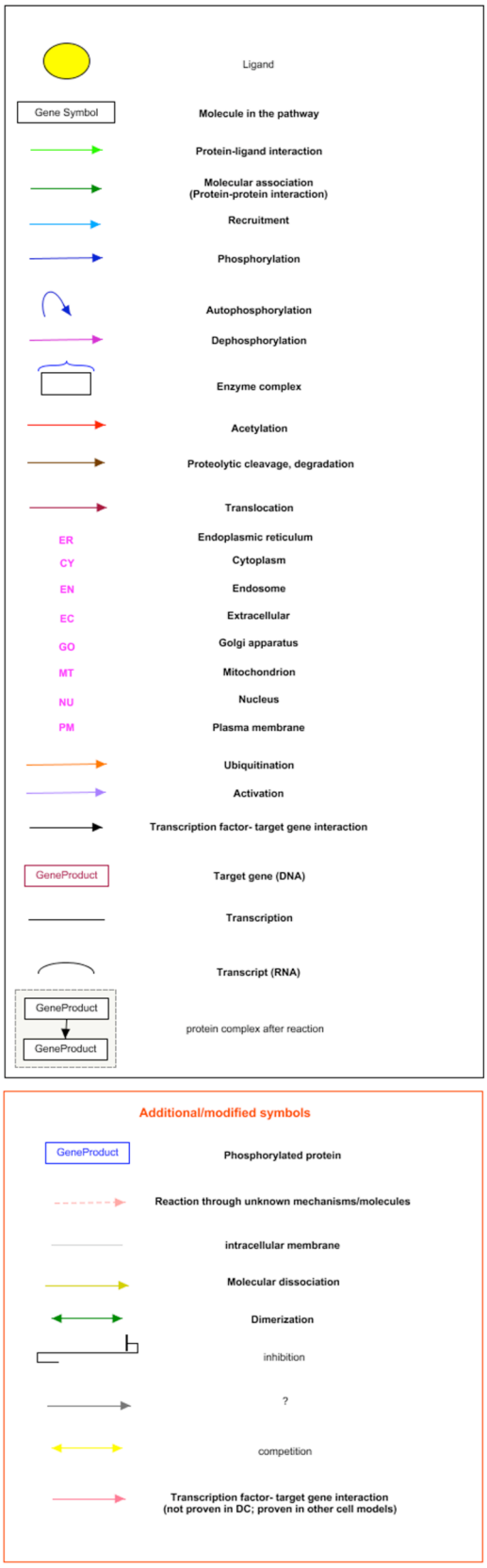


Figure 4.2. Legend of symbols used to represent the different reaction/interaction in the pathway using DC-PathEDIT.

Embrace the variety of differences, here is the richness
Zhuangzi

5

Differential immune response to pathogenic and harmless fungi

5.1 Background

The decision on how to respond to a microorganisms, be it a pathogen or a commensal, is primarily determined by the interaction between microorganism and cells of the innate immune system.

Host/fungal interactions have so far been studied only for pathogenic fungi such as *C. albicans*, but it is not known what determines the commensalism of harmless fungi. In particular, the rules governing the interaction of *S. cerevisiae* and our immune system are almost completely unknown. Following this observation, we attempted to understand the mechanism by which the host immune system discriminates friends from foes in the interplay with microorganisms.

5.2 Pathway analysis allows distinguishing different DC response to fungi

In order to dissect the response of DCs to fungi at the pathway level, we performed a pathway analysis on 7 microarray data sets, which included dendritic cells stimulated with the supposed harmless *S. cerevisiae*, in form of cells and spores, and the two opportunistic pathogen, *C. albicans* and *A. fumigatus* (Table 5.1), using the DC-ATLAS pathway set (see Chapter 4). The analysis results were then converted to pathway signatures (see Experimental Procedures) and clustered (Figure 5.1 and 5.2). We observed that the *Saccharomyces* yeast cell samples formed a separate cluster, indicating a peculiar DCs response to distinguish between pathogenic and non-pathogenic fungi. Interestingly, the two yeast spores data sets grouped closely to the *C. albicans* samples (see separate section for discussion).

Table 5.1. Selection of microarray experiments challenged with fungi

dataset	origin	stimulation	platform
hmoDC – *S. cerevisiae* yeast cells	Our lab	4 hours	Affymetrix
hmoDC – *S. cerevisiae* spores	Our lab	4 hours	Affymetrix
hmoDC – *S. cerevisiae* yeast cells	Our lab	4 hours	Illumina
hmoDC – *S. cerevisiae* yeast cells	Our lab	4 hours	Illumina
hmoDC – live *C. albicans* hyphae	Our lab	4 hours	Illumina
hmoDC – heat killed *C. albicans* hyphae	G. Shuler lab	3 hours	Illumina
hmoDC – *A. fumigatus* conidiae	GSE6965	6 hours	Affymetrix

In general terms, we observed a strong inter-donor variability, exhibited by the fact that single replicates of the same experiment did not show a consistent grouping when clustered. Also, we noticed a very marked 'platform effect' in the clusters, that is samples from the same microarray platform had the tendency of grouping together, regardless of the stimulus.

In an effort to improve the specificity of the analysis and to reduce the platform and donor variability effects, we ran the same analysis on the mean expression ratios for each data set (Figur 5.2). The cluster showed a clear reduction of the platform effect, as the various stimuli grouped together. In particular, yeast spores formed a close cluster along with *A. fumigatus*, with *C. albicans* and *Saccharomyces* yeast samples being more distant in the cluster tree. Interestingly, heat-killed *Candida* samples formed a separate cluster as compared with the stimulation with live *C. albicans*. This is probably due to the several changes in exposure of cell wall components consequent to heat killing.

The activation states of the various pathway modules showed a number of consistent features across all data sets. These consistent features are in general describing the common response to fungi, irrespectively of the fact if these are pathogenic or not. Globally the expression dataset shows significant enrichment in genes of the pathways specifically involved in fungi recognition such as the C-type lectins, DC-SIGN and Dectin-1, signalling or Toll like receptor (TLR) 2/6 signalling.

Figure 5.1. Clustering of signed Binary Enrichment Factors (sBEFs) obtained from the FET analysis on dendritic cell samples. Colored spots indicate significant (p#0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black). The inset shows the clustering of *S. cerevisiae* cells stimulated DCs.

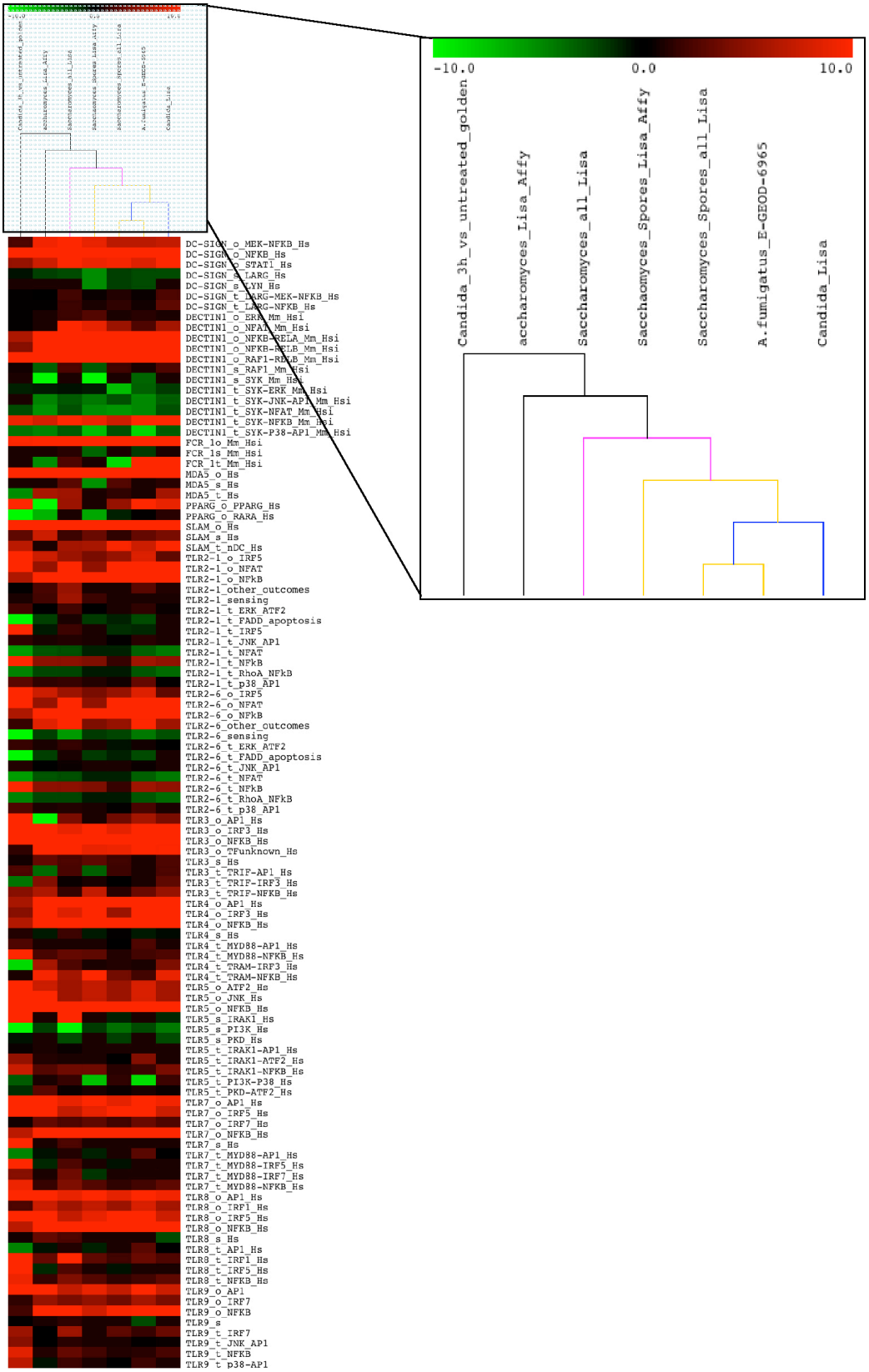


Figure 5.2. Clustering of Pathway Enrichment Factor (PEF) obtained from the FET analysis on dendritic cell samples. Colored spots indicate significant (p#0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black). The inset shows the cluster tree.

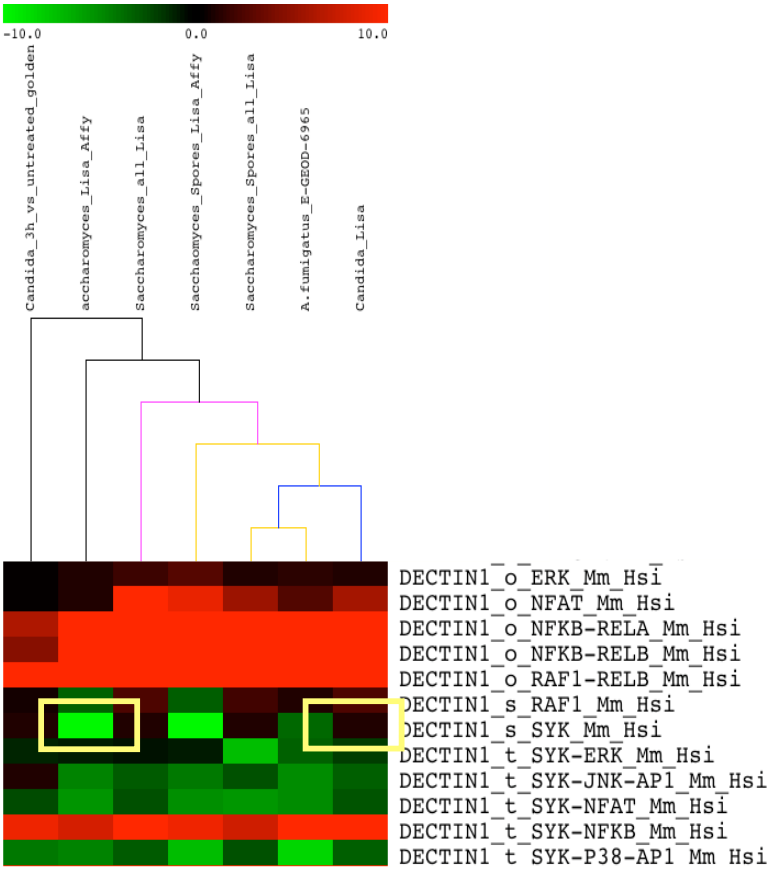
Upon stimulation with non-pathogenic yeasts or yeast forms the receptor/sensing modules of Dectin-1, or TLR 2/6 pathways were mostly down-regulated, and part of the transduction modules for these pathways was either down-regulated as well, or only weakly activated. DC-SIGN receptor/sensing module was down-regulated for all the data sets. This underlines the importance of C-type lectins, such as Dectin-1, DC-SIGN and the macrophage mannose receptor (MR) to specifically mediate fungal recognition (Hernanz-Falcón et al., 2009; Newman and Holly, 2001; Cambi et al., 2008). This is a very important finding; so far it was unclear what happened to gene expression of the genes producing the mRNAs of the proteins responsible of the first initial interaction between the dendritic cell and the fungi.

We clearly observed that these genes were switched off at 3-4 hours after the exposure to the microorganism. This result is therefore consistent with the fact that the experiments in the dataset analyzed were performed at 3-4 hours. It is reasonable to think that this down-regulation means that the cell is priming its response towards to fungi. The observation we made can be compared to a 'snapshot', a view of the state of DCs in a specific moment of their maturation process, a period where DCs are already matured and ready to prime differential Th responses. In this scenario the cell's fate has been decided it does not make sense in the cell economy to continue maintaining the molecular repertoire dedicated to sensing. The significant downregulation we report is in agreement with the observation that the expression of C-type lectins is very sensitive to maturation stimuli, leading to down-regulation as DCs mature (van Kooyk, 2008).

5.2.1 Differential involvement of Dectin-1 in fungi recognition

The Dectin-1 receptor/sensing module exhibited different regulation between *C. albicans* and the rest of the samples: the former was not significantly active, while for the other samples it showed clear down-regulation. The datasets we compared derived from DCs stimulated with *C. albicans* hyphae and are consistent with previous observations that Dectin-1 recognized yeast form and not filaments of *C. albicans* **(**Gantner et al., 2005).

The genes present in the Dectin-1 receptor/sensing module were expressed consistently with the global direction of expression of the module (Figure 5.3) confirming the validity of our enrichment method.



Sample	Gene	Fold change (log)
Saccharomyces cells stimulation	*SYK*	-1.3
Candida hyphae stimulation	*CLEC7A*	0.38

Figure 5.3. Dectin-1 modular structures. In yellow, the differences at the sensing level, between *C. albicans* hyphae and *S. cerevisiae* stimulation were underlined and the differential gene expressions were reported in the table.

As we hypothesize that the down-regulation of the receptor/sensing module observed at 3-4 hours is a time dependent phenomenon, we investigated the behavior of these genes with a time course experiment spanning several time points, using real-time PCR. We stimulated DCs from 3 donors with *S. cerevisiae* cells and hyphae of *C. albicans* and we performed a RT-PCR after 1, 2, 4, 8 and 12 hours (Figure 5.4). As predicted, the mRNA levels of Dectin-1 (*CLEC7A*) and of its adaptor *SYK* markedly increased at early time points with respect to the immature DCs (up to two hours following stimulation with *S. cerevisiae*). The expression then decreased over time indicating a consequent lack of persistent stimulation.

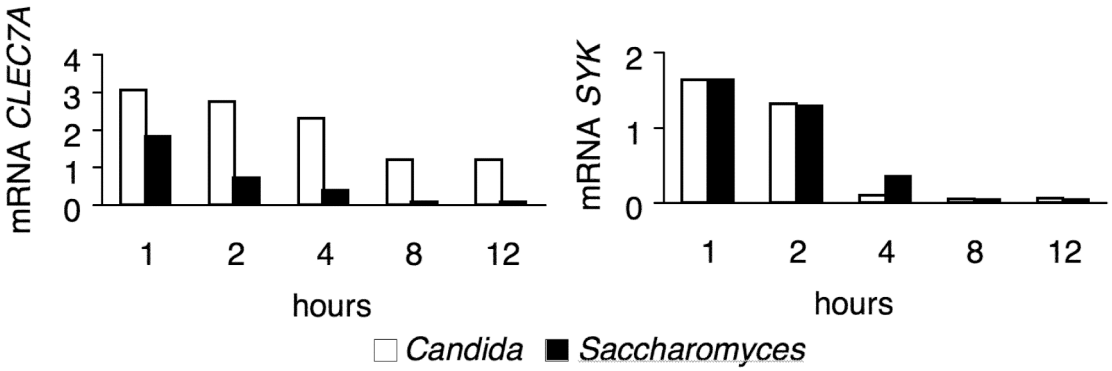


Figure 5.4. *DECTIN-1* and *SYK* gene expression on DCs upon challenge with *C. albicans* and *S. cerevisiae*. Gene expression was assessed in real-time PCR on DCs stimulated with *S. cerevisiae* cells and hyphae of *C. albicans* for 1, 2, 4, 8 and 12 hours (N=3).

RT-PCR spanning several time points also confirmed the down-regulation of *CARD9* (Figure 5.5) that we had observed with microarray analysis. Interestingly, the *C. albicans*-stimulated samples showed a sustained expression of *CLEC7A* and *SYK* even after 4 hours.

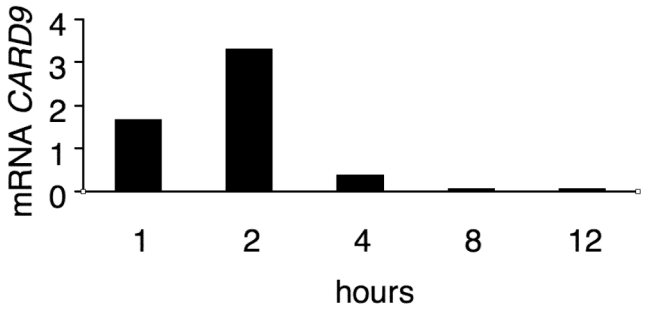


Figure 5.5. *CARD9* gene expression on DCs upon challenge with *S. cerevisiae*. Gene expression was assessed in real-time PCR on DCs stimulated with *S. cerevisiae* cells 1, 2, 4, 8 and 12 hours. (N=3)

With regards to the transduction modules of Dectin-1, they were down-regulated in all samples, with the exception of the transduction module leading to NFkB, which was up-regulated. This suggests a role for NFkB signalling in the regulation of the temporal events related to fungal immune responses.

Interestingly, considering the transduction that lead to Calcium dependent activation of the transcription factor *NFAT*, genes coding for the calcium channel (*ORA1*) and its adaptors *STIM1* and *STIM2* were found downregulated upon *A. fumigatus* and *S. cerevisiae* stimulation, and the gene *NFATC1* result differentially upregulated with respect to *Candida*. The upregulation of *STIM2* is essential if Ca^{2+} signalling is present (Lioudyno et al., 2008).

The outcome modules are significantly up-regulated, indicating that a large part of the transduction signal has already reached the later part of the pathways. In addition to the results we found regarding Dectin-1, our findings indicate that the genes present in the outcome modules of DC-SIGN exert an important role in the recognition of fungi, as previously reported at the protein level (Serrano-Gomez et al., 2005, Cambi et al., 2005).

In general terms, our results show that the signal travels through the network starting very early following stimulation, prompting a rapid commitment of the affected DCs.

5.3 Dissection of the pathway response for cell wall components

Fungal recognition is not the result of one microbial component interaction with a single recognition receptor, but a complex network of interacting receptors and ligands. Depending on the type of receptors involved and also the organization of the receptors at the cell surface, the outcome can be completely different. It can result in simple resolution of pathogen but also lead to a vigorous adaptive immune response or lead to tolerance induction.

To determine the role of the single components in the fungal cell wall as opposed to the multiple receptor stimulation by whole organisms, we carried out additional experiments, stimulating DCs with components of the fungal cell wall. In particular, we focused on the various components of the *S.cerevisiae* cell wall, a structure whose main components include β-glucans and mannan (Figure 5.6).

We performed a transcriptionl analysis on moDCs stimulated with zymosan A, a cell wall extract from *S. cerevisiae* (containing both β-glucans and mannan and other glycan recognized by several receptors, including C-type lectins such as Dectin-1, MR and DC-SIGN and TLRs, such as TLR2 and 4), curdlan, a β-glucan preparation, mannan derived from *S. cerevisiae* and the whole fungus, represented by *S. cerevisiae* cells. Cells were collected after 4 hours of stimulation. Pathway analysis was carried out using Eu.Gene (Cavalieri et al., 2007), and PEFs were clustered against the whole organisms (Figure 5.7).

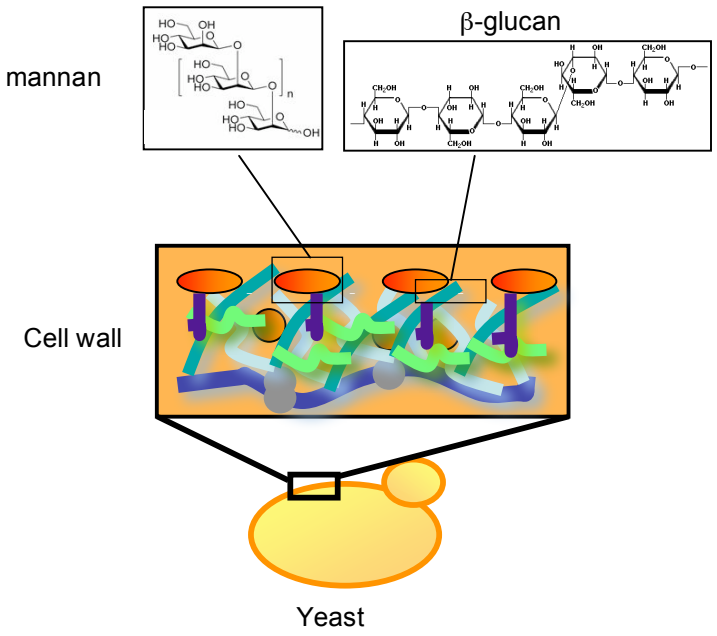


Figure 5.6. *S. cerevisiae* cell wall and components. Yeast cell wall is a dinamic structure which main components are mannan and β-glucan.

DCs stimulated with mannan (thus without β-glucan) showed a completely different pathway profile than the rest of the samples, which were groped together. Curdlan stimulated DCs were closely associated with *Saccharomyces* spores, while zymosan, curdlan, *S. cerevisiae*, and *C. albicans* formed separate, distinct groups.

As represented in Figure 5.7, all the outcome modules, which overlap to some degree, were activated. Mannan signalling resulted in repression of the genes for the receptor module of DC-SIGN, curdlan specifically down regulated Dectin-1 sensing module, Zymosan downegulated DC-SIGN and Dectin-1 sensing modules while activating TLR2 and TLR4 pathway.

A number of other receptor pathways resulted regulated in different way across the stimuli. Several of the TLR pathways show overlap in the elements that contribute to the signalling but this does not necessarily means that they are specifically involved in the signalling. Moreover, we identified 'key players' in the recognition game: the differential regulation of the signalling which leads to AP-1 transcription factor activation is likely a factor which differentiates the response to β-glucan containing stimulation with respect to mannan. Lastly, the endosomal pathways were differentially regulated among RNA presenting stimuli with respect to single cell wall component. These observations need further investigation.

We did not obtain the same results when using publicly available pathways, as the differences between stimuli were not discernible (Figure 5.8). We could assess differences at the cluster level but we could not infer any indication about the signalling itself.

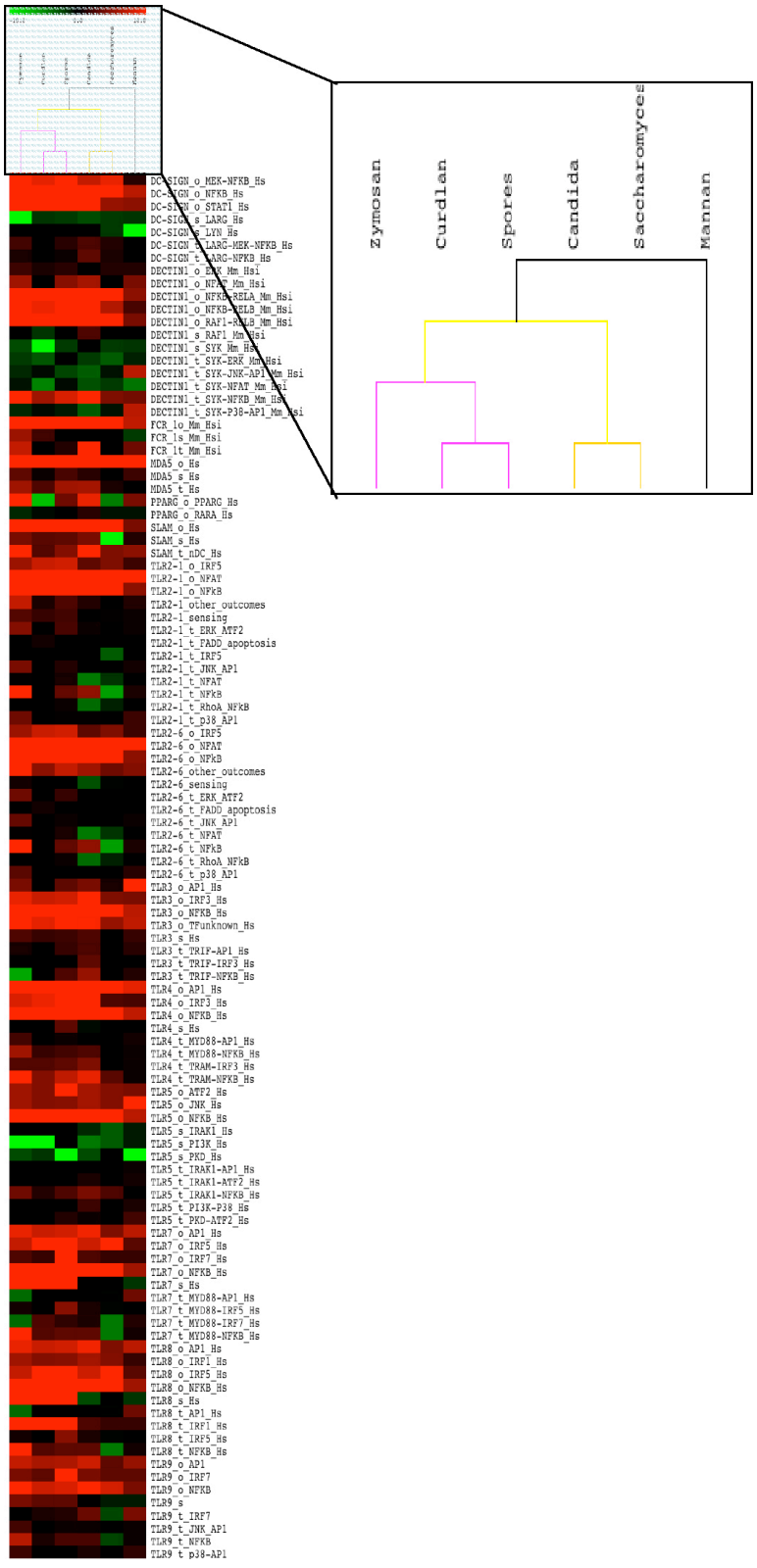


Figure 5.7. Clustering of Pathwat Enrichment Factors (PEFs) obtained from the FET analysis on dendritic cell samples challenged with cell wall components. Colored spots indicate significant (p#0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black). The inset shows the cluster tree.

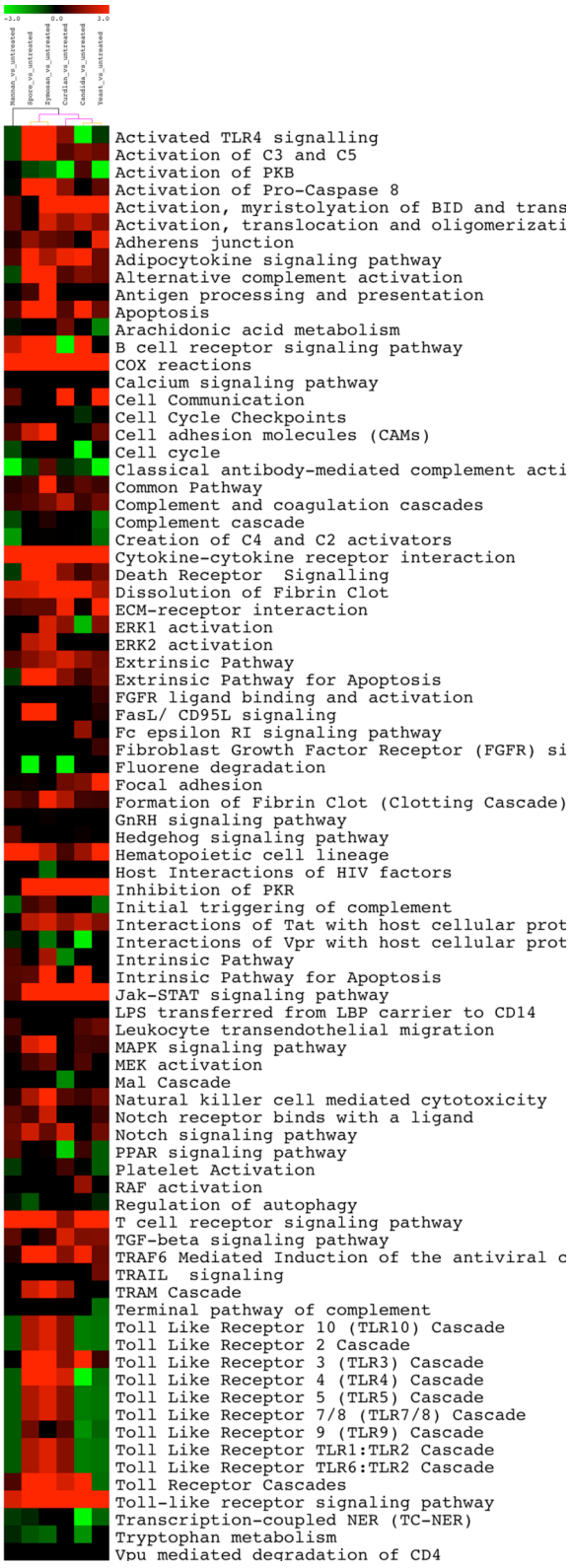


Figure 5.8. Clustering of Pathway Enrichment Factors (PEFs) obtained from the FET analysis on dendritic cell samples challenged with cell wall components using public pathways. Colored spots indicate significant (p#0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black). The inset shows the cluster tree.

The affected pathways represent all the genes involved in the complete process, without any indication of the temporal recruitment of the molecules: we observed a general activation of the immune response pathway in all conditions. In particular we observed the activation of pathways such as Toll Like Receptor signalling pathway, JAK-STAT signalling, COX reaction and cytokine-cytokine receptor interaction. T cell receptor signalling pathway and apoptotic pathway were also significantly activated.

In contrast with the DC-ATLAS data, it was not possible to identify the state of the signal in a specific part of the pathway. The Toll-like signalling pathway, from KEGG, shows a general up-regulation in all the conditions but it includes information derived from signalling of all TLRs, without distinction. On the other hand, the Reactome-curated pathways attempt to describe the contribution of each TLR: nevertheless, it was still difficult to determine which pathway discriminated the response to the various stimuli. Furthermore, the traditional, publicly available pathways were not able to show the integration of signals derived from the interaction between DCs and a complex stimulus. For example, mannan showed a TLR activation pattern similar to the one of the entire fungus.

5.3.1 From whole genome picture to genes: the harmless *S. cerevisiae*

In order to obtain a detailed description of immune response activated by the different PAMPs we performed a comparative analyis on differential expressed genes (DEG).
The analysis between the single cell wall components indicated clearly that DCs stimulated with curdlan and mannan shared less responsive elements and as expected zymosan, that contains both curdlan and mannan, induced a complex level of higher gene activation (Figure 5.9). The genes activated in response to the three stimulation conditions showed a common proinflammatory response indicated by upregulation of genes coding for cytokines (*IL1A, ILB, IL6, IL8, TNFA*), chemokines (*CXCL1, CXCL2, CCL20*). In addition prostaglandin related gene (*PTGS*), cell adhesion molecules and matrix-metallo proteinases (*MMP10*) were found differently regulated (Figure 5.9C).

The comparative analysis of zymosan-stimulated DCs and curdlan-stimulated DCs showed that zymosan but not curdlan is able to activate the expression of some genes involved in Th1 response such as *IL-12A* and *IL-12B* genes, prompted the parallel stimulation of TLR2. This difference in *IL12* genes expression was not found in mannan stimulation comparison. Zymosan-stimulated DCs showed expression of *CCR7* gene known to be involved in the maturation process and in the migration process to lymphnodes and of *RND1* gene that regulates the organization of active cytoskeleton in response to extracellular facto (Nobes et al 2008).

However, although the strong activation of Th1 polarizing factor (Figure 5.10) balancing the Th17 factor, zymosan-stimulated DCs activated several genes, such as *IL-10* and its receptor

IL-10R, that mediate immunosuppressive signals of IL-10 inhibiting proinflammatory signals and *SOCS2*, a member of a family genes associated with the suppression of cytokine production (Figure 5.10). We also found upregulated *TRAF3* gene, responsible for stress activated kinase response and DUSP genes (*DUSP10* and *DUSP2*), a family genes involved in counter-mechanism to limit innate immune response and TLR signalling acting on different MAPKs (Konstantin and Tamas, 2007). These observations configure a self-regulated activation process by activation of negative-feedback mechanisms, which can restrain and terminate the innate immune response.

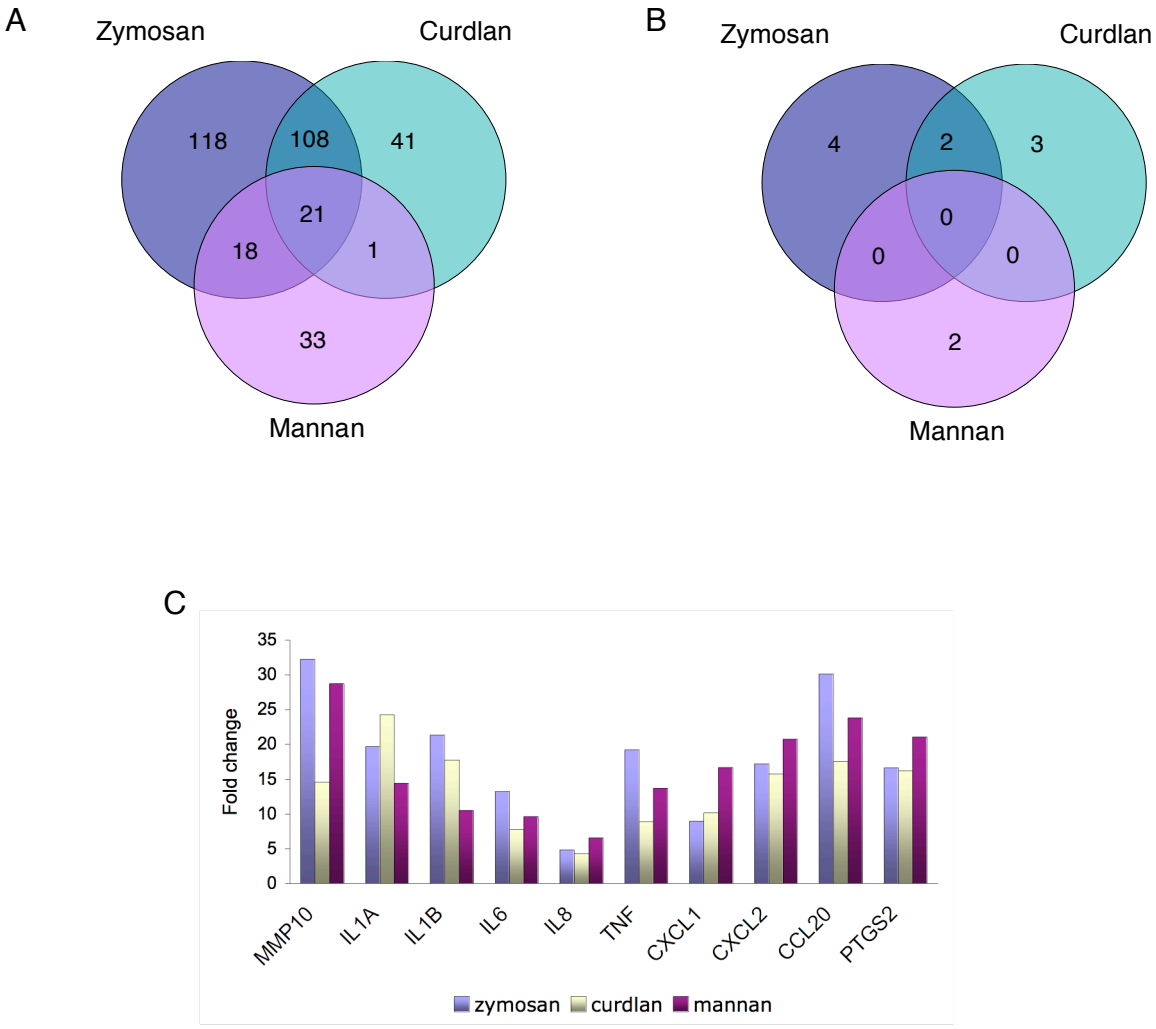


Figure 5.9. Immune response (IR) genes activated different cell wall components in moDCs. Transcriptional analysis was performed on moDCs after 4 hours of stimulation with curdlan, zymosan, mannan or without any stimuli. (A) and (B) Venn diagram of upregulated and downregulated DEGs, respectively. (C) IR differentially regulated genes ($p <= 0.05$) commonly expressed among the three stimulation condition.

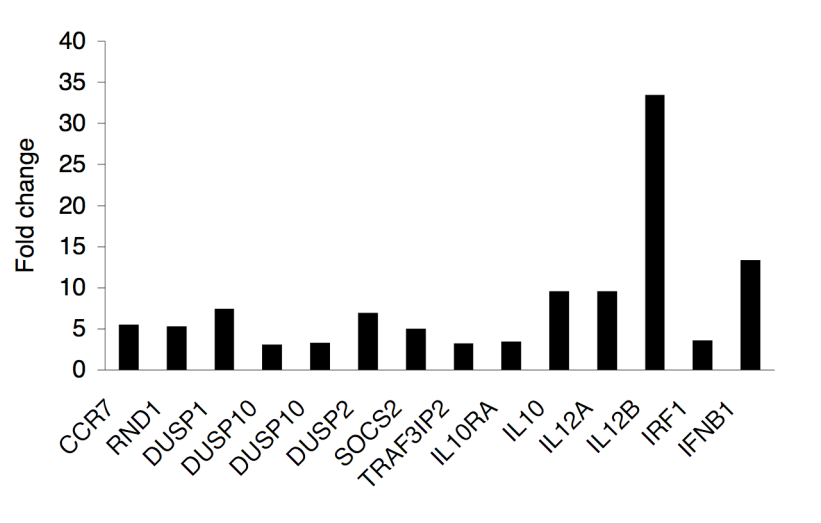


Figure 5.10. Immune response (IR) genes activated by Zymosan in moDCs. IR differentially regulated genes (p <= 0.05) uniquely expressed in Zymosan-stimulated DCs compared to Curdlan-stimulated DCs.

The comparative analysis among the different stimulation condition showed, in the four stimulation conditions, 21 genes commonly activated (Figure 5.11). Among these, genes coding for cytokines, chemokines and immune receptors, were up-regulated compared to the unstimulated control.

The Gene Ontology analysis of differentially expressed genes showing for all the stimulation condition a marked representation of biological process involved in immune response and recognition of external stimuli (Figure 5.12).

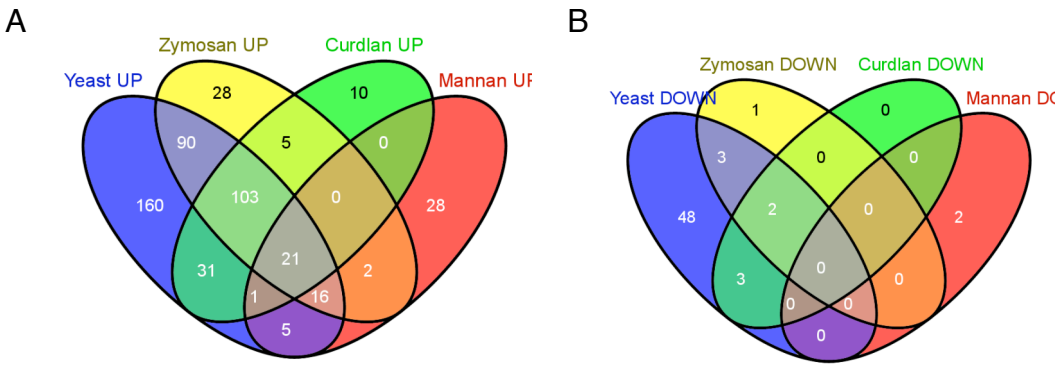


Figure 5.11. Immune response (IR) genes activated by different cell wall components and *Saccharomyces cerevisiae* in moDCs. Transcriptional analysis was performed on moDCs after 4 hours of stimulation with curdlan, zymosan, mannan and *S. cerevisiae* or without any stimuli. (A) and (B) Venn diagram of upregulated and downregulated DEGs, respectively.

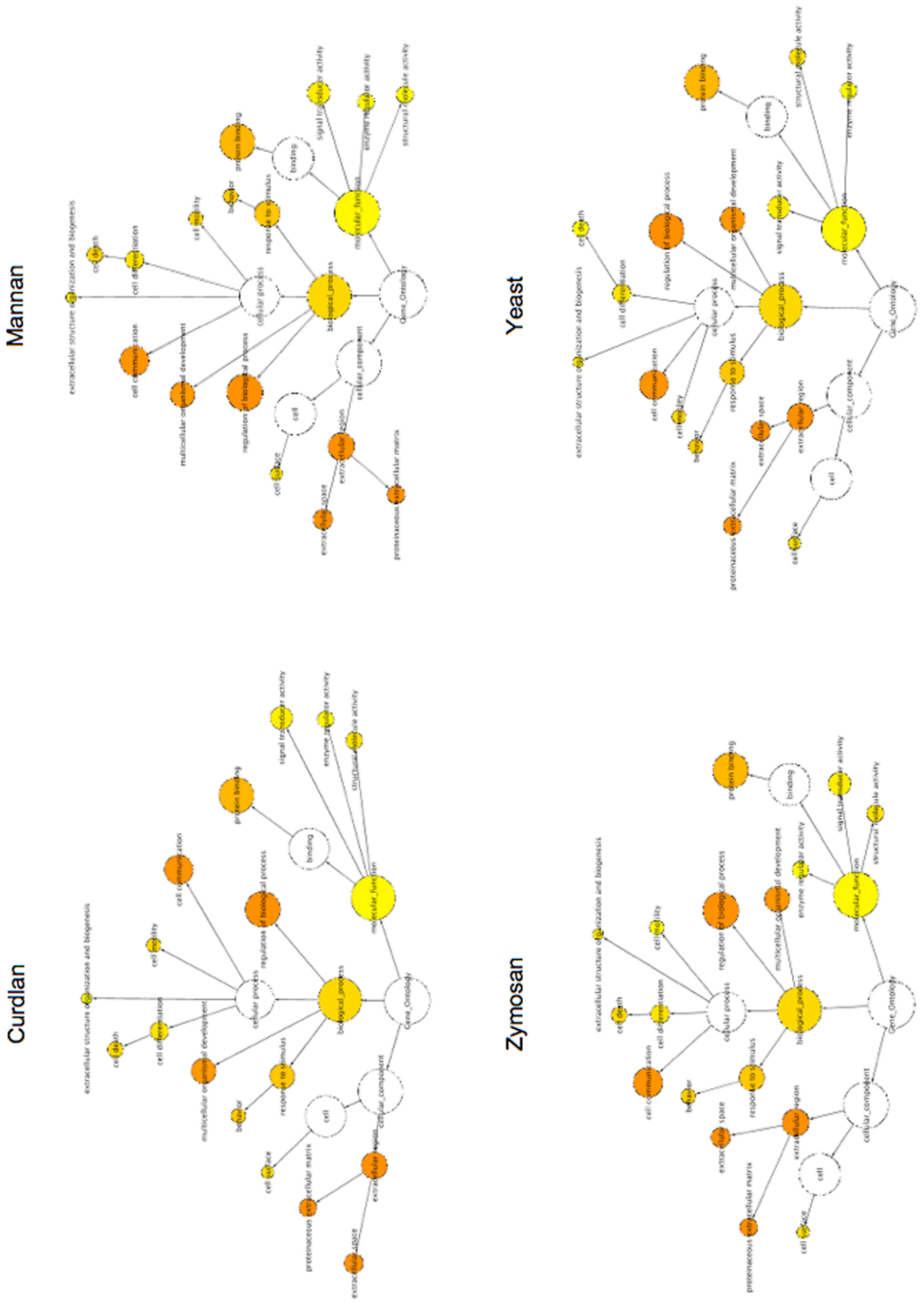


Figure 5.12. GO_Slim enrichment of DC stimulated with curdlan, mannan, zymosan and *S. cerevisiae.* GO_SLIM was calculated by using the Bingo tools on Cytoscape.

The comparison between yeast-stimulated DCs and zymosan-stimulated DCs, showed a common marked expression of several pro-inflammatory mediators and factors involved in the maturation process of DCs, including a remarkable activation of *IL12A* and *IL12B* genes coding for the Th1 polarizing factor IL-12p70 (Figure 5.13). They also showed an increased expression of the *CCR7* and *CCL20* genes, known to be involved in the maturation and migration to lymph nodes (Hansonn et al, 2006), of the *PTX3* gene known to be involved in phagocytosis (Diniz et al., 2004). However, the strong induction of pro-inflammatory and Th1 polarizing cytokine gene activation induced by yeast was counter-balanced by an increased expression of factors involved in immune modulation which limit DC activation as well as T cell effector function such as *IL-10*, *INDO* and *IL2RA* (Romani, 2008; Steinman et al., 2003; Romani and Puccetti, 2007). Genes for the Th17 polarizing factors IL-1β and IL-6 were also found to be expressed showing that the balancing among the different Th polarizing cytokine is peculiar to the proper maturation program of stimulated DCs.

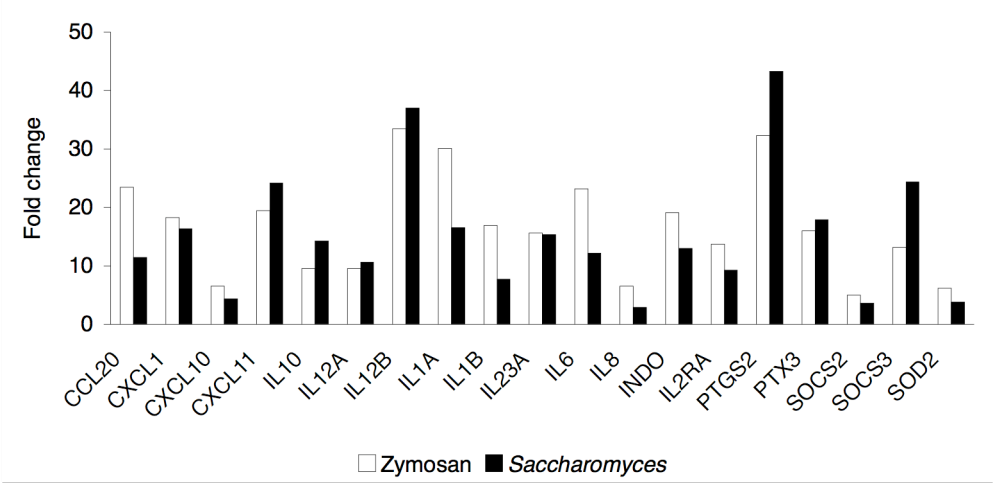


Figure 5.13. Compared dendritic cell immune response (IR) to zymosan and *S. cerevisiae*. IR differentially regulated genes ($p <= 0.05$) commonly expressed among the two stimulation condition,

Overall, these core responses suggest that TLR and C-type lectins-cell surface activation, set the stage for the more intense TLR-dependent and -independent responses generated by phagocytosed yeast cells.

The key difference between pure stimuli and entire cells could be represented as a precise program, involving signalling from cell surface receptors within the first two hours, a proper response that requires switching off the response from surface sensors, and a final activation of the response prompted by stimuli deriving from the phagosome. Live yeast cells also more intensely or exclusively regulated several genes associated with cell damage and repair than zymosan (Figure

5.14). This finding could be related to the capacity to initiate programmed cell death responses. Gene transcripts within this group included *PARP10* (Poly ADP-ribose polymerase10). The catalytic activity of PARPs has been shown to be stimulated by DNA strand breaks, which occur during programmed cell death. *PARP10* has not been previously associated with apoptosis (Kleine et al., 2008), suggesting that phagocytosis of *S. cerevisiae* may initiate novel mechanism of cell damage and repair.

Yeast cells compared to zymosan stimulation are able to induce a differential regulation of type I interferons and type I interferon associated genes (*IFIH1, IFIT1, IFIT2, IFIT3*). These responses are of particular relevance given that lipoprotein mediated TLR2-derived signals do not induce type I interferons (Toshchakov et al., 2002; Kawai and Akira, 2007). Several other TLRs, including TLR7, TLR8 and TLR9, are able to launch distinct signalling pathways from within phagosomal vacuoles that differentially regulate type I interferons (Stockinger et al., 2000).

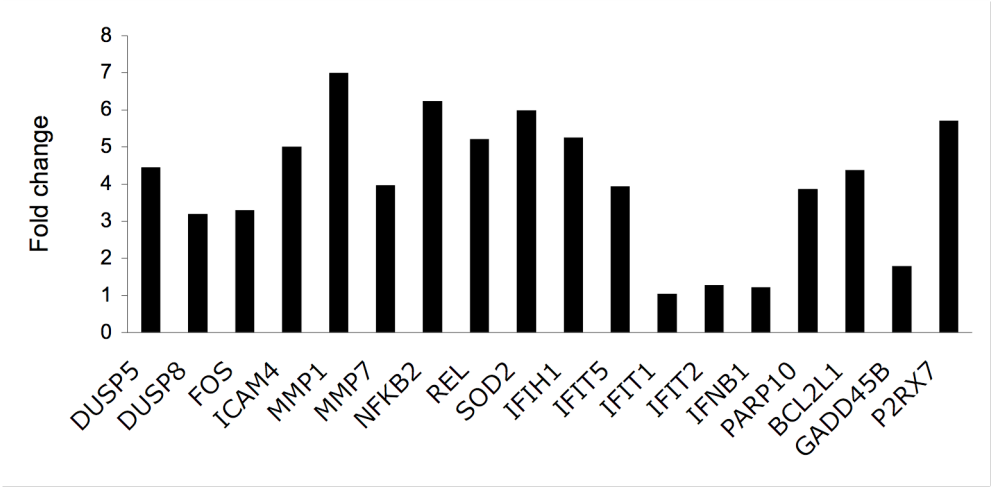


Figure 5.14. Dendritic cell immune response (IR) to *S. cerevisiae*. IR differentially regulated genes (p <= 0.05 more expressed upon *S. cerevisiae* cell stimulation than Zymosan stimulation.

Particularly important for the development of phagosomal signalling was the markedly enhanced secretion of IL-1β in response to yeast. Unlike other pro-inflammatory cytokines (i.e. TNF-α), which are linearly induced by TLR activation, the production of biologically active IL-1β requires the integration of NF-κB-mediated transcription of pro-IL-1β followed by activated caspase-1 cleavage of the inactive cytokine (Ogura et al., 2006). It requires processing into its active form by activated caspase-1. Caspase-1 is activated within a multiprotein complex called the inflammasome (Weiss et al., 2007) in response to diverse stimuli. Stimulation of cell surface purinergic receptors by exogenous ATP, following human monocyte activation, is a known mechanism by which bacterial pathogens can lead to cleavage of caspase-1 (Piccini et al., 2008). Released ATP engages the cell's purinergic ion channel receptors (P2XR7) inciting release of

intracellular K^+ which in turn generates signals that lead to assembly of the inflammasome and activation of caspase-1.

By transcriptional analysis we observed the overexpression of *P2XR7* gene and by Elisa measurement, presence of active IL-1β only after yeast cells stimulation (Figure 5.15).

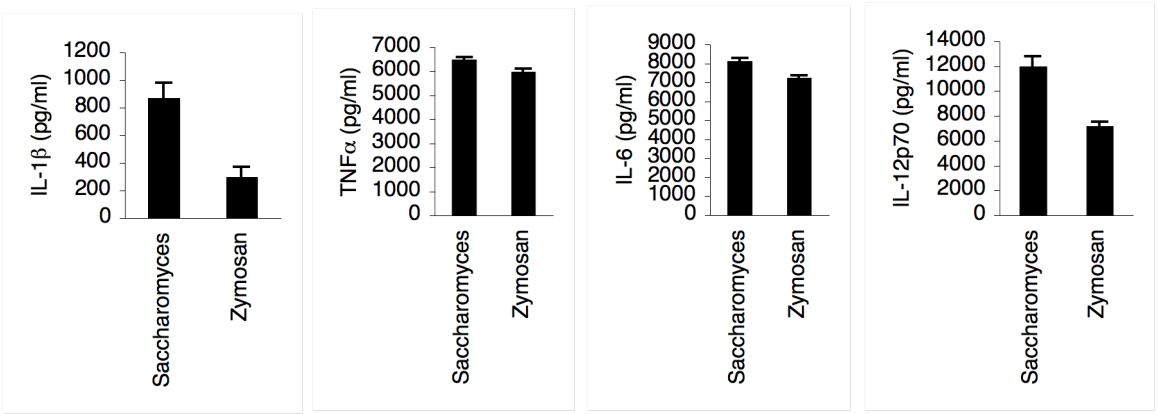


Figure 5.15. Cytokine production after *S. cerevisiae* and zymosan stimulation. Elisa were performed for IL-1β, TNFα, IL-6 and IL-p70 measurement on supernatants of DCs stimulated for 24 hours with *S. cerevisiae* or zymosan.

Collectively our results highlight the ability of phagocytosed *S. cerevisiae* to induce diverse and more intense innate immune signals which are mechanistically distinct from those generated when different glycan engage cell surface PRRs.

5.3.2 From whole genome picture to genes: the opportunistic pathogen *C. albicans*

The ability of phagocytosed yeast to induce a stronger innate immune response with respect to single cell component, is evidenced in DC response upon *C. albicans* stimulation.

The comparative analysis showed a marked inflammatory response induced by *C. albicans*, represented by the upregulation of mediators and factors involved in the maturation process of DCs (Figure 5.16) such as genes coding for co-stimulatory molecules (A), genes coding for cytokines and chemokines (B), genes involved in inflammation and apoaptosis (C) and several transcription factors (D). The upregulation of genes coding for cytokines is different to what observed after *S. cerevisiae* cells stimulation suggesting a different Th polarizing cytokines production leading to a Th17 response instead to a Th1.

Upon live *C. albicans* hyphae internalization, the inflammosome is activated as suggested by the trascriptional analysis (Figure 5.17) and the clearly upregulation of *NLRP3* gene, important for inflammosome complex formation and production of the active form of IL-1β (Gross et al., 2009).

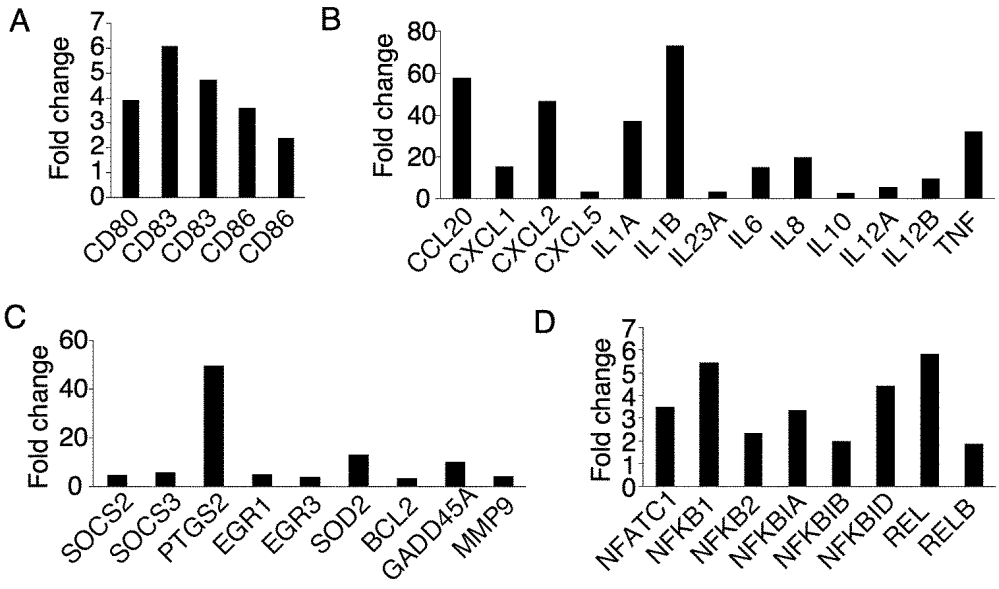


Figure 5.16. Immune response (IR) genes activated by *C. albicans* hyphae in moDCs. (A) IR genes coding for co-stimulatory molecules (A), genes coding for cytokines and chemokines (B), genes involved in inflammation and apoaptosis (C) and several transcription factors (D) differentially regulated genes ($p <= 0.05$) in *Candida*-stimulated DCs.

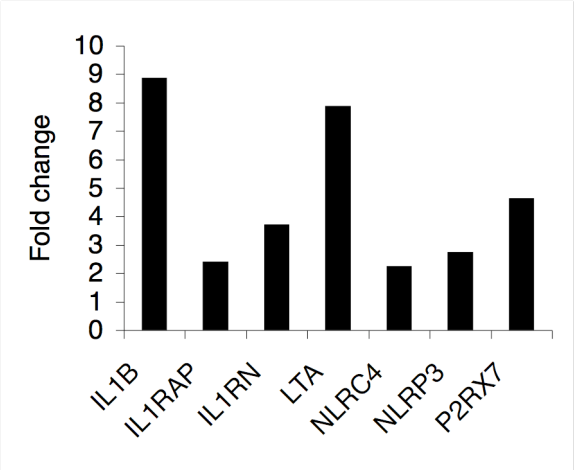


Figure 5.17. Inflammasome-related genes activates in DCs stimulated with live *C. albicans* hyphae. Inflammasome activation related genes differentially regulated ($p <= 0.05$) in *Candida*-stimulated DCs

It's worthwile to note that after 4 hours of stimulation, *CLEC7A* gene, coding for Dectin-1, is still upregulated as a lack of involvement, while *CLEC4A* gene, coding for Dectin-2 is downregulated as recently reported in the involvement of *C. albicans* hyphae recognition (Robinson et al., 2009, Figure 5.18)

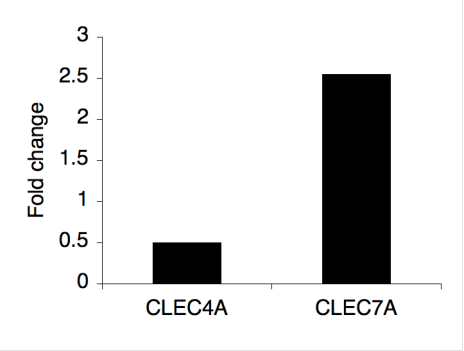


Figure 5.18. Different involvement of Dectin-1 and Dectin-2 recognition of hyphal form of *C. albicans*. *CLEC7A* and *CLEC4A* were differently regulated by DCs in response to *C. albicans* hyphae.

5.3.3 Pathogenic versus non pathogenic fungi

The importance of cell wall recognition in pathogenesis has been recently investigated in *C. albicans*. Dimorphic switch in *Candida* and germination of *Aspergillus* spores are two examples of the importance of different life stages in determining pathogenicity, possibly by exposing different cell wall components (d'Ostiani et al., 2000; Gartner et al., 2005). *S. cerevisiae* cell wall composition radically changes in investigated the immune response to *S. cerevisiae,* in cell form and spores, and hyphae of *C. albicans.*

Through transcriptional analysis on moDCs exposed to these fungi we obtained lists of differentially expressed genes (DEGs) for each stimulation compared to unstimulated controls. 1179 genes were commonly activated in the three stimulation conditions as indicated by the Venn Diagram (Figure 5.19). Differentially expressed gene (DEG) analysis showed an up-regulation of a wide range of DEGs involved in immune responses and inflammation, such as genes coding for cytokines, chemokines and immune receptors. The regulation of genes coding for the receptors, Toll like receptor (TLR) 2 and TLR1 and Dectin-1, the adaptor proteins Syk and Card9, and co-stimulatory molecules and metalloproteinases. outlined a common maturation process between *S. cerevisiae* and *C. albicans* stimulation. The Gene Ontology analysis of DEGs showed for all the stimulation condition a marked representation of biological process involved in immune response and recognition of external stimuli, as represented for spore stimulation in Figure 5.20.

In addition to the commonly activated genes a set of 174 genes was differentially expressed only after *S. cerevisiae* cells stimulation, while 444 and 507 genes were uniquely differentially expressed after *C. albicans* and *S. cerevisiae* spores stimulation, respectively.

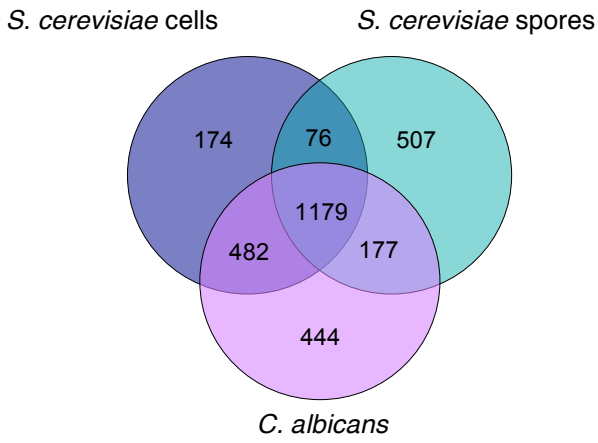


Figure 5.19. Venn diagram of differentially expressed genes (DEGs). Transcriptional analysis was performed on moDCs after 4 hours of stimulation with *S. cerevisiae* spores, exponentially growing yeast cells, *C. albicans* hyphae or without any stimuli.

Figure 5.20. GO_Slim enrichment of DC stimulated with spores. GO_SLIM was calculated by using the Bingo tools on Cytoscape

The comparative analysis of *Saccharomyces* cells-stimulated moDCs and *Candida* hyphae-stimulated moDCs, showed a marked expression of several pro-inflammatory mediators and factors involved in the DC maturation process, including the activation of *IL12A* (Figure 5.21A). The strong induction of pro-inflammatory and Th1 polarizing cytokine genes induced by *S. cerevisiae* cells was counter-balanced by an increased expression of factors involved in immune modulation which limit DC activation as well as T cell effector function as underlined before.

Compared to *S. cerevisiae* stimulation, *Candida*-stimulated moDCs showed an increased expression of the *CCR7* and *CCL20* genes, known to be involved in the maturation and migration to lymph nodes (Hansoon et al., 2007). Genes for the Th17 polarizing factors IL-1β and IL-6 were also found to be more expressed in *Candida*-stimulated DCs (Figure 5.21B), showing that different fungi induce peculiar maturation program of stimulated DCs.

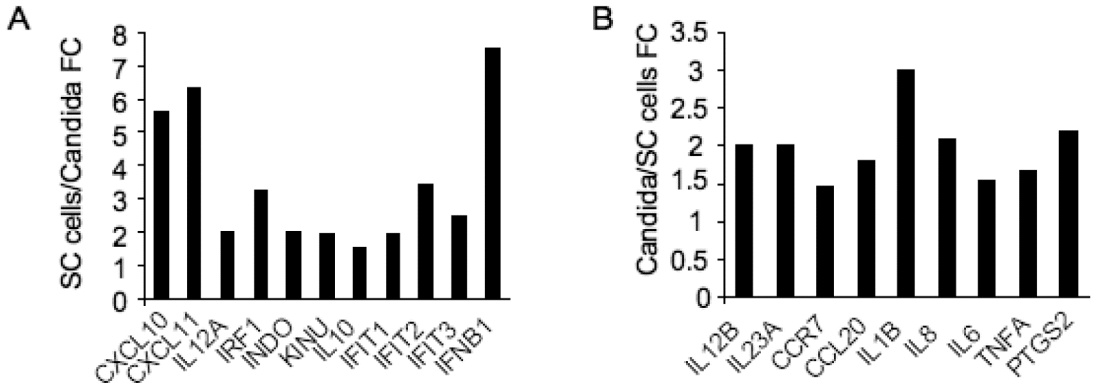


Figure 5.21. Immune response (IR) genes activated by *Saccharomyces cerevisiae* cells or spores and *C. albicans* hyphae in moDCs. (A) IR differentially regulated genes ($p <= 0.05$) more expressed in *Saccharomyces* (SC) cells-stimulated DCs compared to *Candida*-stimulated DCs. (B) IR differentially regulated genes ($p <= 0.05$) more expressed in *Candida*-stimulated DCs compared to *Saccharomyces* cells-stimulated DCs.

The comparison of *S. cerevisiae* cells and spores stimulation showed a strong induction of pro-inflammatory and Th1 polarizing cytokine gene activation, counter-balanced by an increased expression of factors involved in immune modulation (Figure 5.22A). Conversely, genes for the Th17 polarizing factors IL-1β and IL-6 were found more expressed in spore-stimulated DCs (Figure 5.22B), in a *Candida* like manner, suggesting that different life stages of *S. cerevisiae* can induce a different maturation program on DCs.

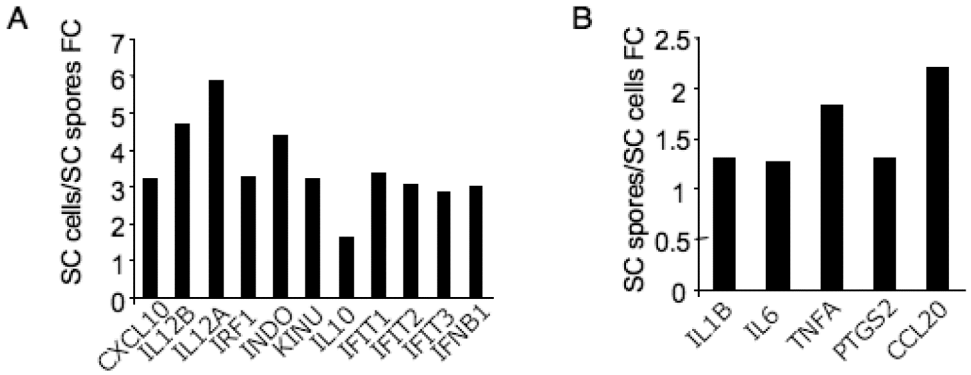


Figure 5.22. Immune response (IR) genes activated by *Saccharomyces cerevisiae* cells or spores and *C. albicans* hyphae in moDCs. (A) IR differentially regulated genes (p <= 0.05) more expressed in *Saccharomyces* cells-stimulated DCs compared to *Saccharomyces* spores-stimulated DCs. (B) IR differentially regulated genes (p <= 0.05) more expressed in *Saccharomyces* spores-stimulated DCs compared to *Saccharomyces* cells-stimulated DCs

To investigate the regulation of pathways and cellular networks in our samples, we performed a pathway analysis and clustered the results as signed Binary Enrichment Factors (sBEF; Figure 5.23; Beltrame et al., 2009). We observed a general activation of the immune response pathway in all conditions. In particular we observed the activation of pathways such as Toll Like Receptor signalling pathway, JAK-STAT signalling, COX reaction and cytokine-cytokine receptor interaction. T cell receptor signalling pathway and apoptotic pathway were also significantly activated in all the three stimulation conditions. Furthermore, the clustering analysis separated *C. albicans* and *S. cerevisiae* in two distinct groups. This finding showed the different DCs response to pathogenic and non-pathogenic fungi. Futhermore, the clustering outlined different pathway signatures between *S. cerevisiae* cells and spores.

These findings indicate that *S. cerevisiae* spores are 'teleologically' similar to *Candida albicans* hyphae in terms of ability to induce a DC response, possibly related to the different cell wall component disposition among *S. cerevisiae* cells and spores.

5.4 Discussion

Host/fungal interactions have so far been studied only for pathogenic fungi such as *C. albicans*, but it is not known what determines the commensalism of harmless fungi.

We attempted to address the mechanism by which the host immune system discriminates friends from foes in the interplay with microorganisms, by using a pathway analysis approach. This approach allows comparison of different microarray experiments in order to obtain a detailed photograph of the genomic response at the time of the experiment. During dendritic cell sensing of

the microrganism and the maturation program that lead to the proper Th polarization to cope better the invading pathogen, several changes either at transcriptional level either at protein level occur.

Through the comparison of different whole genome transcriptional analysis at the pathway level, we confirm the presence of a peculiar transcriptional program governing the recognition of fungi, be it a pathogenic or a commensal fungi.

DC-ATLAS, the collection of pathway for PRR signalling we are curating, allows to dissect the temporal events characterizing the signalling cascades leading to initial recognition to maturation, describing the pathway as an assembly of modules, sensing, signal transduction and outcome. The results of statistical analysis based on DC-ATLAS are much improved respect to the results one can obtain from existing pathway databases using the same statistical test. DC-ATLAS reduces overlap between pathways and as a consequence improves the capture of specificity of a signalling program respect to another. Despite this substantial improvement in many cases pathways that are not specifically meant to respond to a stimulus are called as significantly altered. The reason for this lack of consistency is that the two selected pathways share a substantial element in common that fools the statistical test used, or the approach used for multiple test correction. This consideration implies that substantial work needs to be invested in tailoring the multiple test correction to the section where the correction wants to be applied. This will require further interaction with experts in statistics to improve the statistical applications to specific section of the module analysis.

The overall upregulation of the otucome modules in DC stimulated with various stimuli was in principle due to the time the experiments were performed. That is, at that point in time DCs are matured and ready to prime differential Th response. Thanks to the division in modules, we could assess the temporal signalling of the events, since the sensing and transduction modules were downregulated. In particular we can observed differential involvement of receptors due to differences in cell wall components disposition (i.e *C. albicans* hyphae) or distinguish the single cell components contribution the response to a whole fungus by discrimination of the preferential signal flow of different cell wall components.

Most studies so far have analyzed DC activation induced by single microbial compounds. However, pathogens express several TLR and C-type lectins agonists that may engage different receptors at different times and in distinct cellular compartments. By pathway analysis and differential transcriptional analysis we observed that cell wall components such as mannan and curdlan integrate their stimulation as observed by using zymosan as stimuli.

Further, after internalization yeast presents other PAMPs to endosomal receptors with respect to the single cell wall components. Immune cell activation by yeast has generally been ascribed to outer membrane lipoprotein-TLR1/2 and Dectin-1 (Clec7a) mediated inflammatory responses. Our study results also make obvious that a far more intense and diversified innate immune response coincides transcriptionally of live yeast and maturation of the phagosome. Most

prominently, the innate immune signals generated by phagocytosed live *S. cerevisiae* led to an enhanced TLR-mediated pro- and anti-inflammatory cytokine output. TLRs continuously sample the extracellular environment and inform the cell to react to PRRs by facilitating cellular responses via inflammatory pathways which culminate in cytokine production and cell activation (Lien et al., 1999; Kaway and Akira, 2007).

Human DCs, which originate as monocytes in the peripheral blood, express a substantial complement of both cell surface as well as endosomal TLRs (Chuang and Ulevitch, 2000). The transcriptional responses elicited by both live *S. cerevisiae* and zymosan, for the most part were representative of cell surface TLR1/2 and dectin-1 mediated activation. These responses were perhaps best exemplified by the differential regulation of cytokines as well as several other chemokines. Surface signals were also capable of up-regulating several genes associated with molecules that regulate TLR responses, including *SOCS3* (Yasukawa et al., 2003).
The enhanced TLR-mediated cytokine production could be the result of several nonexclusive mechanisms broadly divided into three categories;

1. a more efficient activation of recruited cell surface and endosomal TLR1/2 receptors by yeast glycans,
2. engagement of additional endosomal TLRs by internalized and degraded yeast cells, and
3. cooperation between multiple TLR receptors from within the phagosomal vacuole.

The transcriptional activation of genes conding for TNF-α, IL-1β, IL-6 and IL-10 in DCs, suggests that TLR2 is available for signalling,
TLR7, TLR8 and TLR9, all of which are known to be expressed in endosomal membranes (Kawai and Akira, 2006) may also play an important role in generating the enhanced cytokine responses to internalized yeast cells.

The initial recognition of the cell surface create a temporal window for stimulation of other receptors that intensify, complement and sustains the DC activation process.
The integration of multiple stimuli over a defined temporal window might allow a more effective response to invading pathogens than to soluble microbial products. This mechanism substains the ability of DCs of integrating signals from pathogens, cytokines and T cells, leading to the generation of an adaptive immune response of the appropriate class (Reis e Sousa, 2004)
Infact, DCs represent a critical source of interleukin 12 (IL-12), a cytokine that is key in innate responses and drives T helper type 1 (Th1) polarization (Trinchieri, 2003), but also other cues driving T cell differentiation have been described. One such factor is IL-23, which shares the p40 chain with IL-12 but pairs this with a unique p19 chain. IL-23 drives the differentiation of inflammatory T cells capable of secreting large amounts of tumor necrosis factor (TNF) and IL-17, which mediate tissue damage in autoimmune diseases (Cuang et al., 2003; Langrish et al., 2004).

The transcriptional analysis indicated that, far from being inactive microbes, *S. cerevisiae* yeast cells activate a defined pathway of immune responses in moDCs, balancing each other in order to avoid potentially harmful inflammation processes. The DC transcriptional response to pathogenic fungi has been previously in part addressed. The transcriptional report of DCs stimulated with a *C. albicans* strain defecting in filamentous development in experimental conditions largely superimposable to that used in our paper was reported by Huang et al. (2001). The overall response, taking into account the different technology used in that experiment, is more closely related to *S. cerevisiae* cells than to *C. albicans* hyphae. Infact some of the genes (e.g *CD83*, *IL12A*, *TNFA*, *IL6*, *IL1B* and more) found differentially regulated by Huang et al., were found also differentially regulated in a similar manner in our experiment by using DCs stimulated with *S. cerevisiae* cells.

Furthermore, the immune response to spores, a peculiar S. cerevisiae life stage, is much closer to the response to the well known pathogen *C. albicans*, indicating that the changes in *S. cerevisiae* cell wall composition during sporulation could influence DC recognition.

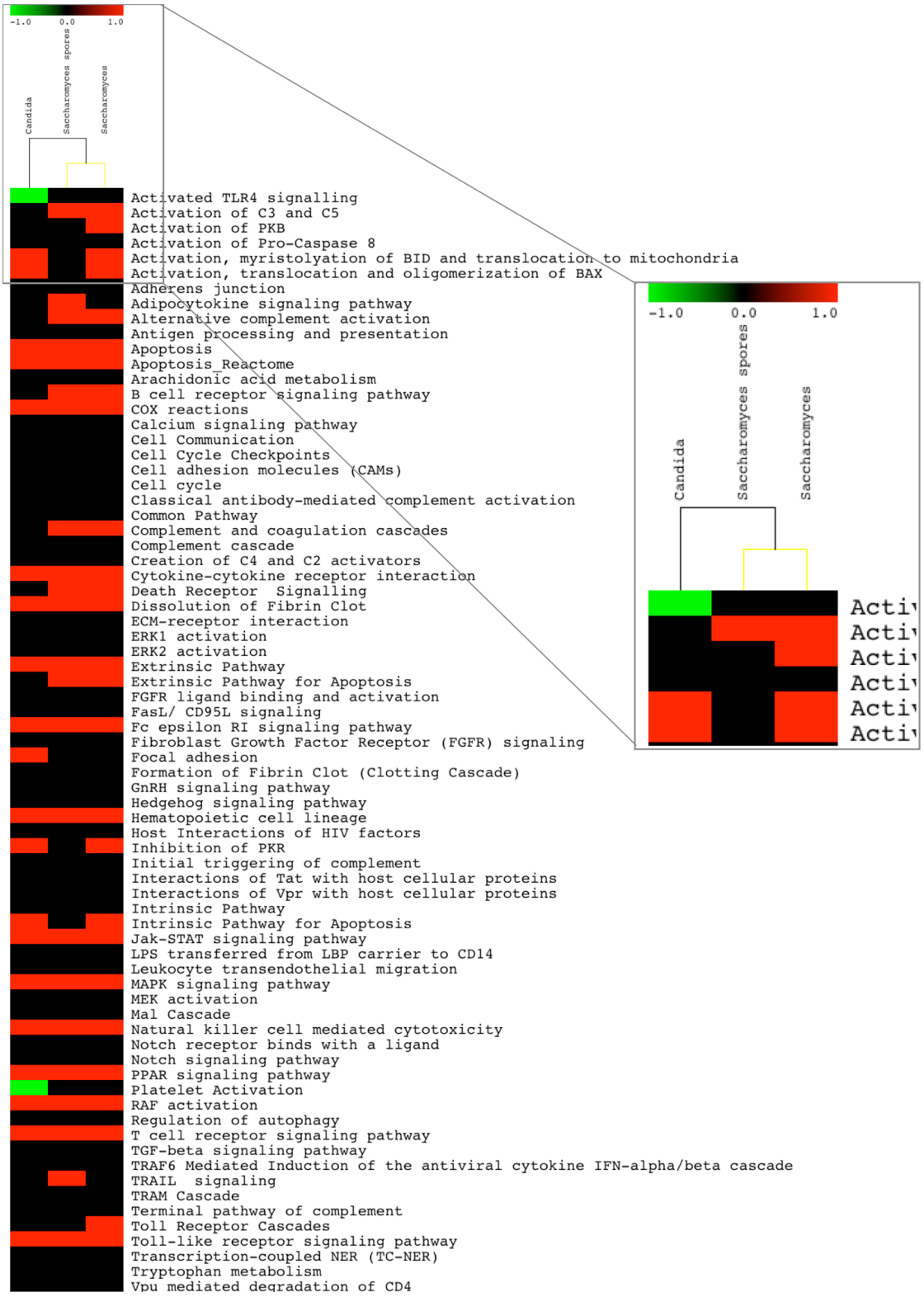


Figure 5.23. Spores induce a response closed to *C. albicans*. Clustering of signed Binary Enrichment Factors using Euclidean distance using support trees on DC stimulated with *S. cerevisiae* cells, spores and *C. albicans*. Colored spots indicate significant (p = 0.05) up- (red) or down- (green) regulation. The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black).

Nothing in life is to be feared, it is only to be understood
Marie Curie

6

Differential IL-17 production and mannan recognition explain fungal commensalism and the insurance of pathogenicity

6.1 Background

Unlike *C. albicans, S. cerevisiae* life cycle alternates haploid and diploid state. Haploid cells mate and produce diploid cells. Diploid cells can divide by mitosis and grow and upon nitrogen starvation sporulate and undergo meiosis. Sporulation is a phenomenon that involved conformational changes in yeast cells and the final product is an *ascus* (that maintain the characteristic of the residual yeast cell wall) that encloses four spore (tetrad), which wall is arranged differently from cell one. In particular, mature spore wall shows 4 distinct layers from innermost to outermost: a layer of mannoproteins (mannan layers), one of β-1,3 linked glucose residues (glucan layer), a chitosan layer (α-1,4 linked glucosammine residues) and a layer of indeterminant structure whose major consistuent is cross-linked tyrosine dimers (dityrosine layer). Even if glucan content is higher than in yeast cell wall, in this one they are presumably more exposed Mannans and glucans are also found in the vegetative cell wall. Chitosan and dityrosine are unique to the spore. (Coluccio et al., 2004).

The transcriptional results showed that that *S. cerevisiae* spores are 'teleologically' similar to *Candida albicans* hyphae in terms of ability to induce a DC response, possibly related to the different cell wall component disposition among *S. cerevisiae* cells and spores.

6.2 Spores circumvent the DCs activation process

To assess the cytokine response of moDCs to *S. cerevisiae* treatment, we exposed moDCs to yeast or spores for 24 hours in a concentration (expressed as stimuli:DC ratio) of 10:1. Cytokine production in supernatants was evaluated by Mesoscale or Elisa assay. In agreement with the microarray data presented in the previous section, yeast cells promoted an increased production of

IL-12p70 and IL-10 compared to spores (Figure 6.1). In order to understand if this behaviour could be related to pathogenic fungi, DC stimulation with C. *albicans* hyphae was also performed in parallel.

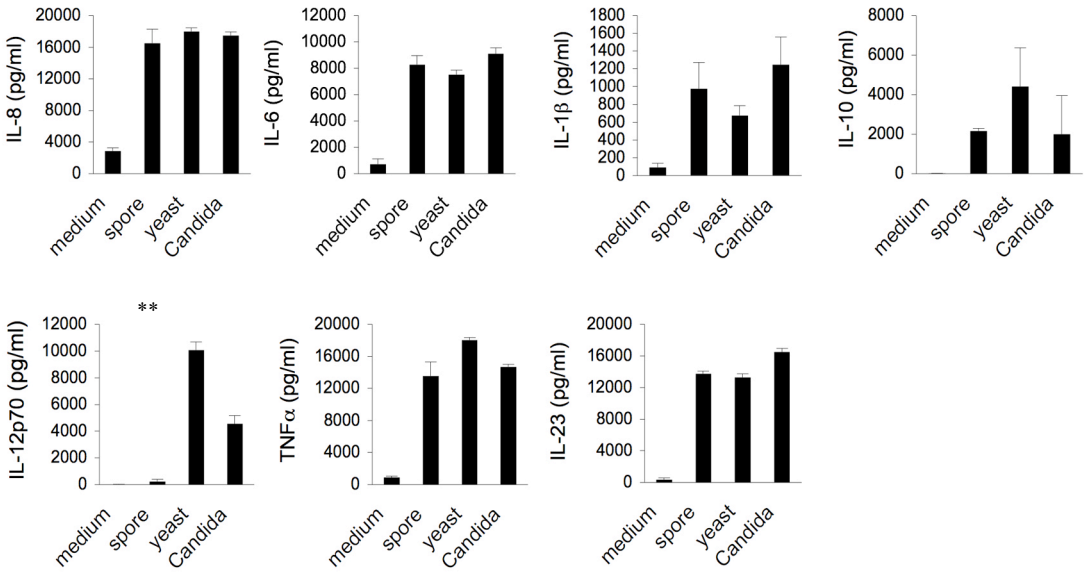


Figure 6.1 Cytokine profile of spore-stimulated and yeast-stimulated DCs. DCs were stimulated for 24 hours with spore or yeast of S. cerevisiae and surnatants were analyzed for presence of released cytokines. Data are representative of mean±s.d. (N=3), **p<0.01.

The absence of IL-12p70 induction upon spore stimulation was observed even after massive or prolonged stimulation (Figure 6.2A and B). In particular DCs were exposed at different concentrations of yeast cells (black squares) or spores (open circles) and IL-12p70 was evaluated after 24 hours in culture supernatants. Otherwise, DCs were exposed to spores and yeast cells at 10:1 ratio and IL12p70 was evaluated after 2 (gray bars), 8 (white bars) and 30 hr (black bars) of stimulation.

To rule out that zymoliase treatment could interfere in DCs recognition of spores, crucial experiments such us the IL-12p70 production by DCs were performed in parallel by DC stimulation with liberated spores, liberated spores treated with zymoliase for 5 minutes more, and untreated *asci*. Upon *asci* stimulation, the level of IL-12p70 was comparable to that induced upon spores challenge (Figure 6.3). This demonstrated that the 15 minutes zymoliase treatment does not affect the spore wall structure in a way to interfere with the immune response.

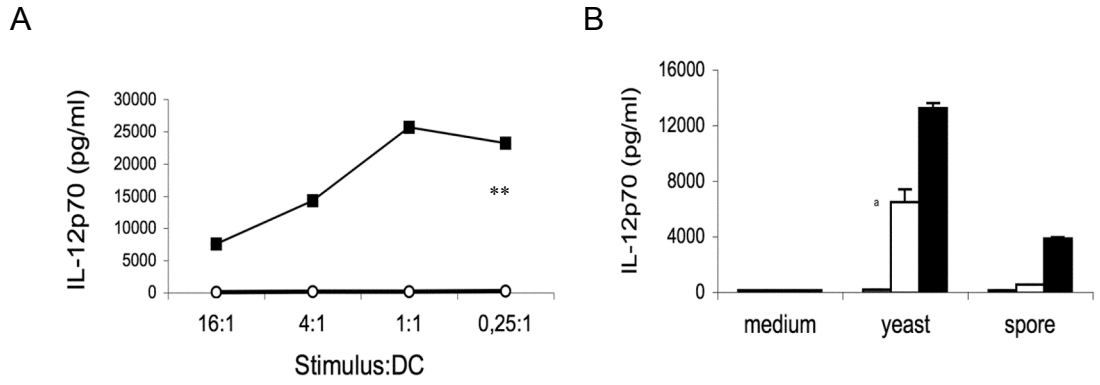


Figure 6.2 IL-12 production by spore-stimulated and yeast-stimulated DCs. (A) IL-12p70 was evaluated after exposure to different concentration of stimuli (spores, white circles; yeast cells, black squares); (B)) IL-12p70 was measured after 2 (grey bars), 8 (white bars) and 30 hours (black bars). Data are representative of mean±s.d. (A, N=3, B, N=4), **p<0.01.

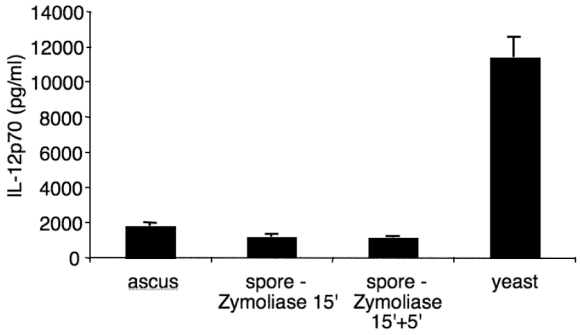


Figure 6.3. Zymoliase treatment does not influence IL-12p70 production by spores. To rule out that zymoliase treatment could interfere in DCs recognition of spores, IL-12p70 production by DCs were performed by stimulating DCs with liberated spores (*asci* treated for 15 minutes), liberated spores treated for 5 minutes more and *asci* (without zymoliase treatment) at stimuli: DC ratio of 10:1 for 24 hr, IL-12p70 production was assessed by ELISA.

IL-12p70 production in the presence of *C. albicans* depends on internalization of the pathogen by DCs (Chiani et al., 2000). By blocking internalization of *S. cerevisiae* yeasts and spores using cytochalasin D (10 μg/ml), an inhibitor of actin polymerization, we showed that IL-12p70 synthesis induced by the yeast form was strongly suppressed (Figure 6.4A), suggesting that cytokine production was dependent upon *S. cerevisiae* internalization.

The lack of IL-12p70 production by spore-stimulated DCs could be related to a lower phagocytosis rate of spores, thus we evaluated the ability of moDCs to internalize spores. We performed confocal fluorescent microscopy and flow cytometry analysis of DCs exposed to yeast and spores at stimuli:DC ratio of 4:1. DCs were treated with zymolyase to detect intracellular yeasts/spores only, labeled with aHLA-DR-FITC and biotinylated yeasts/spores were labeled with streptavidin-APC (SA-APC) (Figure 6.4B and C).

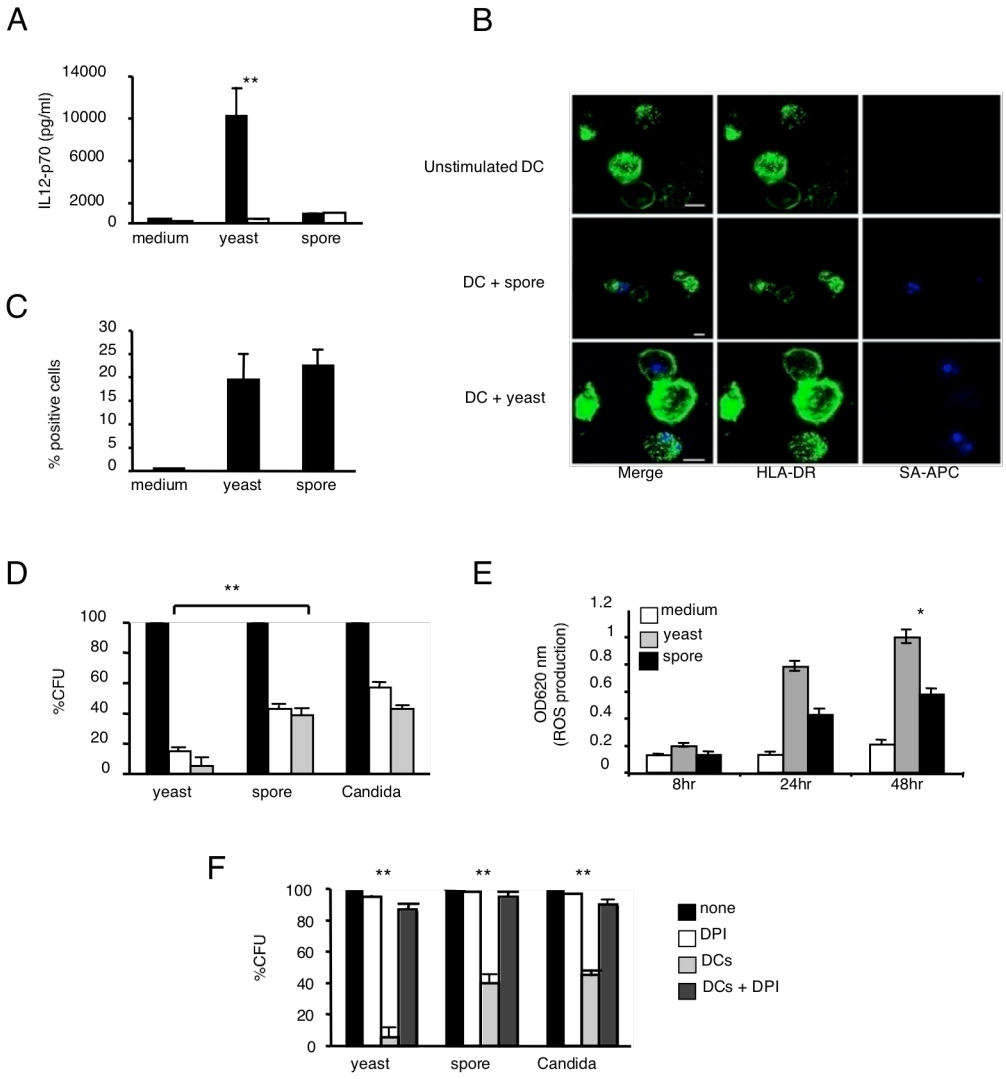


Figure 6.4. Internalization, survival rate of yeast or spore forms of *S. cerevisiae* and induction of ROS production by DCs. (A) DCs pre-treated or not with cytochalasin D (10 □g/ml) were stimulated for 24 hr with yeast cells or spores. IL-12p70 was measured by ELISA. (B) Confocal fluorescent microscopy and (C) flow cytometry of DCs exposed to yeast and spores at stimuli:DC ratio of 4:1. DCs were treated with zymolyase to detect intracellular yeasts/spores only, labeled with aHLA-DR-FITC and biotinylated yeasts/spores were labeled with streptavidin-APC (SA-APC). (D) *S. cerevisiae* spores, yeast cells and *C. albicans* hyphae survival after culturing with DCs. Stimuli were cultured alone (black bars) or with DCs (4:1 ratio). After 6 hr of exposure, DCs were treated (gray bars) or not (white bars) with zymolyase and lysated. Data are expressed as percentage of CFU of yeast growing. (E) ROS production by DCs in response to stimuli. DCs were stimulated with yeast cells (gray bars), spore (black bars) or without any stimuli (white bars) at a 4:1 ratio. NBT assay was performed after the time indicated. (E) NADPH oxidase-induced ROS account for the killing of *S. cerevisiae* cells. Stimuli were cultured alone or with DCs (4:1 ratio) in presence of absence of DPI (10 μM). After 6 hr of exposure, DCs were treated with zymolyase (white bars) and lysated. DPI did not influence viability of stimuli. Data are expressed as percentage of CFU of yeast growing. Data are representative of three separate experiments (mean±s.d, A, B, C, D, E, F). Statistical comparisons between samples are represented as horizontal bars, *p<0.05, **p<0.01.

We found that spores, as well as yeast cells, similarly interacted with DCs and that spores were equally internalized as observed after elimination of surface-bound spores by zymolyase treatment (Figure 6.4B and C).

This indicates that spores and yeast cells are equally taken up by DCs.

6.2.1 Spores could evade degradation process by DCs

We next investigated the degradation process of *S. cerevisiae* yeast cells, spores and *C. albicans* hyphae in the phagosome by evaluating the survival following their uptake by moDCs. We cultured moDCs in the presence of *S. cerevisiae* yeast cells or spores and *C. albicans*, then lysated the DCs and plated on yeast growth medium. As shown in Figure 6.4D, significantly more colonies were obtained from internalized spores or *C. albicans* compared to *S. cerevisiae* yeasts after 6 hours of exposure.

We therefore measured ROS production by DCs cultured with yeast or spores for 8, 24 and 48 hours (Figure 6.4E) by measures of formazan accumulation after ROS oxidation (see Experimental Procedures).

The reduced production of ROS in the presence of spores together with the intrinsic resistance of spore wall structure to acidic pH might account for the increased intracellular survival ability of spores compared to the yeast cell.

ROS production is one of the parameters, that most likely reflects the differences in intracellular killing: previous studies demonstrated the important role on NADPH oxidase-induced ROS in DC killing of *C. albicans*. In order to link production of ROS and survival of spores upon internalization, we performed survival experiments in the presence of DPI, an inhibitor of flavoproteins including the NADPH oxidase. DPI-treated DCs lack ROS production in response to PMA (Vulcano et al., 2004), therefore DPI is a useful reagent to investigate NADPH oxidase activity in DC. DPI treatment (10 μM) abrogated the ability of DCs to kill *S. cerevisiae* cells without affecting spore survival (Figure 6.4F). These results indicate that ROS produced by NADPH oxidase play an important role in DC fungicidal activity and that the observed major survival of spore could suggest an escape mechanisms to oxidative damage as reported for *C. albicans* (Donini et al., 2007). We can speculate that the lack of IL-12p70 production and the reduced production of ROS might all be mechanisms by which spores circumvent the DC activation process.

To obtain indications on the possible relevance of differential survival *in vivo,* we measured survival of *S. cerevisiae* cells and spores fed to rats by plating stool samples after 1 to 4 weeks of treatment. Furthermore, in faecal sample of rats treated with spores we assessed the presence of spores. The results indicated that *S. cerevisiae* spores can survive the passage through

the gut, while yeast cells cannot (Table 6.1), but does not allow to conclude anything on the mechanism. Further in depth investigations of the molecular mechanisms and on the role of different cell types, including the response to *Candida* hyphae, will be fundamental to clarify the role of DCs in determining differential survival.

Table 6.1. Survival of *S. cerevisiae* through rat intestinal tract.

TREATMENT	1 week*	2 weeks*	3 weeks*	4 weeks*
Control	0 CFU/ml	0 CFU/ml	0 CFU/ml	1.2 CFU/ml
Yeast cells	2±0.5 CFU/ml	3±1 CFU/ml	4±1 CFU/ml	3.5±1 CFU/ml
Spores	2000±100 CFU/ml	2500±100 CFU/ml	2500±100 CFU/ml	4000±250 CFU/ml

*data expressed as colony forming unit (CFU) grown after plating 1 ml of faecal sample

6.3 DCs stimulated by diverse forms of *S. cerevisiae* prime a different T helper response

Whether Th1 or Th17 responses arise depends on the balance between the Th1 polarizing factor IL-12p70 and the Th17 polarizing factors (IL-1, IL-6 and IL-23 in humans) with IL-12p70 being dominant over Th17 polarizing factors (Bettelli et al., 2008). The evidence that moDCs released IL-12p70 in the presence of yeast cell and almost no IL-12p70 in the presence of spores prompted us to ask whether moDCs, which were matured in the presence of these two forms of *S. cerevisiae,* differed in their capacity to promote Th1 and Th17 responses. $CD4^+$ cells were co-cultured in a ratio of 20:1 with autologous moDCs, which matured for 12 hours with *S. cerevisiae* cells or spores. Quantitative real-time PCR assays were performed to assess the expression over time of *IL-6* and *IL-17A* genes, encoding for cytokines involved in Th17 polarization, and the *IFNG* gene, encoding for the main cytokine produced by Th1 cells on $CD4^+$ T cells stimulated with spore or yeast-stimulated DCs (Figure 6.5A). The analysis showed an increase of *IL-17A* mRNA and of IL-6 mRNA in T cells exposed to spores- and *C. albicans*-stimulated DCs compared to unstimulated DCs just after 24 hours of co-culture. By contrast yeast-stimulated DCs did not induce *IL-17A* gene activation and only slightly increased *IL-6* gene activation (Figure 6.5A). The *INFG* gene was activated by all the stimuli (Figure 6.5B). The expression of the *FOXP3* gene was found to be increased in T cells after 5 days of co-culture with yeast-stimulated DCs only (Figure

6.5C) suggesting that yeast cells might induce the differentiation of a Treg population, as also supported by microarray data (Figure 5.22A).

In co-culture experiments with autologous $CD4^+$ T lymphocytes, we used *C. albicans* hyphae stimulation as control stimulus for IL-17 induction. As expected, IL-12 producing yeast-matured DCs promoted a pure Th1 response, inducing the release of IFNγ only (Figure 6.6A). In contrast, spore-stimulated DCs, such as the pathogenic fungus *C. albicans*, induced the release of a substantial amount of IL-17 together with IFNγ, consistent with the presence of a Th17 response (Figure 6.6). Thus, the low levels of IL-12p70 induced by spore stimulation were sufficient to induce a Th1 response but they could also allow the effect of Th17 polarizing factors on IL-17 production by $CD4^+$ T cells.

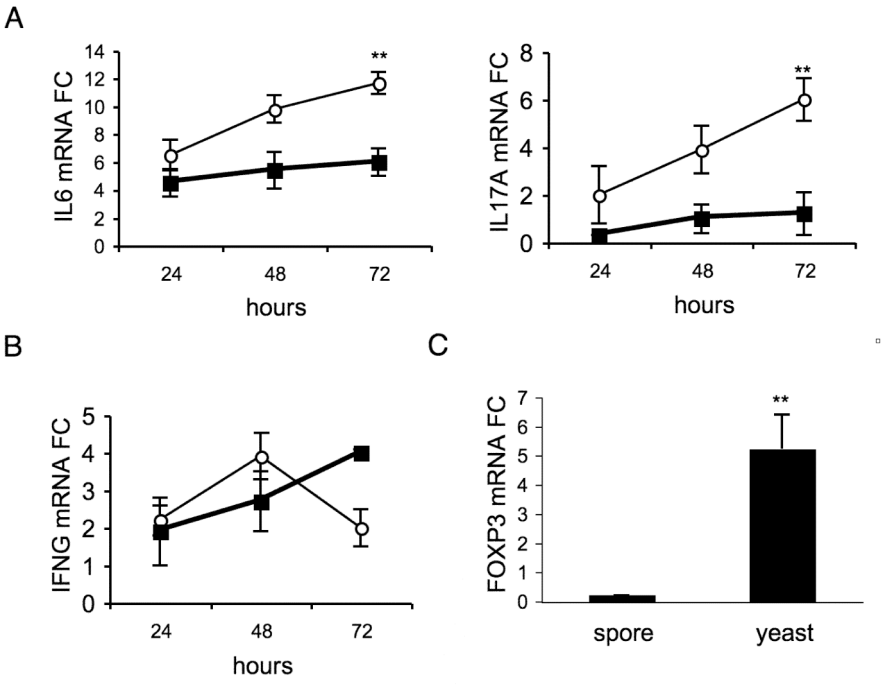


Figure 6.5. Effect of spore-stimulated DCs or yeast-stimulated DCs on $CD4^+$ effector T cell differentiation. (A) *IL6, IL17A* and (B) *IFNG* gene expression in $CD4^+$ T-cells exposed to spores (white circles) or yeast cell (black square) for 24, 48 and 72 hours; (C) *FOXP3* gene expression in $CD4^+$ T-cells exposed to spores or yeast cell for 5 days. All data are presented as mean±s.d (A, B, N=3).

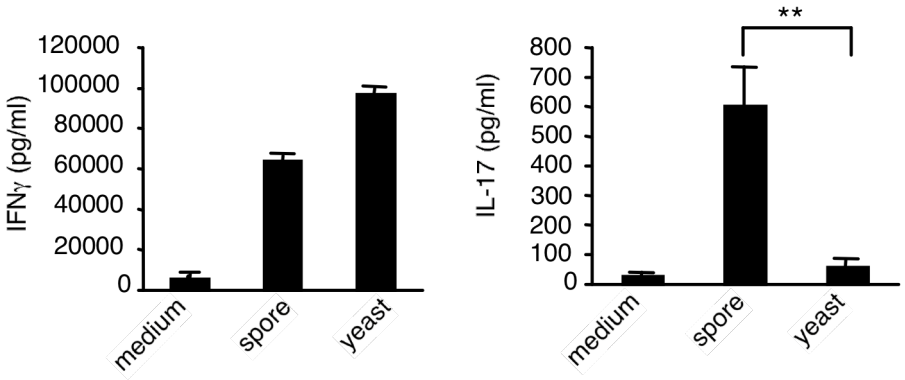


Figure 6.6. Effect of spore-stimulated DCs or yeast-stimulated DCs on cytokine production by $CD4^+$ effector T cell. IL-17 and IFNγ measurement in 5-day culture supernatants of $CD4^+$ T cells produced in response to yeast- and spore-stimulated DCs

6.3.1 IL-12p70 antagonizes Th17 priming

To assess the importance of IL-12p70 in the balance of Th1/Th17 responses we cultured DCs with spores or *C. albicans* hyphae in the presence of human recombinant IL-12p70. The added IL-12p70 during DC stimulation by spores rescued the ability of DCs to polarize a pure Th1 responses, in a dose dependent fashion as well as antagonized the IL-17 induction by *C. albicans* (Figure 6.7A), indicating that high amount of IL-12 can indeed counteract the effect of the Th17 polarizing factor. To address this observation in yeast stimulated DCs, we used an antibody against IL-12 to block the activity of the cytokine. The block of IL-12 activity favoured Th17 cell activation (Figure 6.7B). This demonstrates the important role exerted by IL-12 in balancing the fungal immune response.

6.4. *S. cerevisiae* recognition by DCs: differential involvement of MR and DC-SIGN

As previously reported in the Introduction, different receptors are responsible of recognition and internalization of conserved structure of yeast cell wall. C-type lectins, such as Dectin-1, DC-SIGN and the macrophage mannose receptor (MR) specifically mediate *C. albicans* binding and internalization by DCs (Hernanz-Falcón et al., 2009; Newman and Holly, 2001; Cambi et al., 2003; Cambi et al., 2008).

While β-glucan is specifically recognized by Dectin-1 (Reid et al., 2009), N-linked mannan is the fungal carbohydrate structure specifically recognized by MR and DC-SIGN directly influencing the production of pro-inflammatoy cytokines (Cambi et al., 2008).

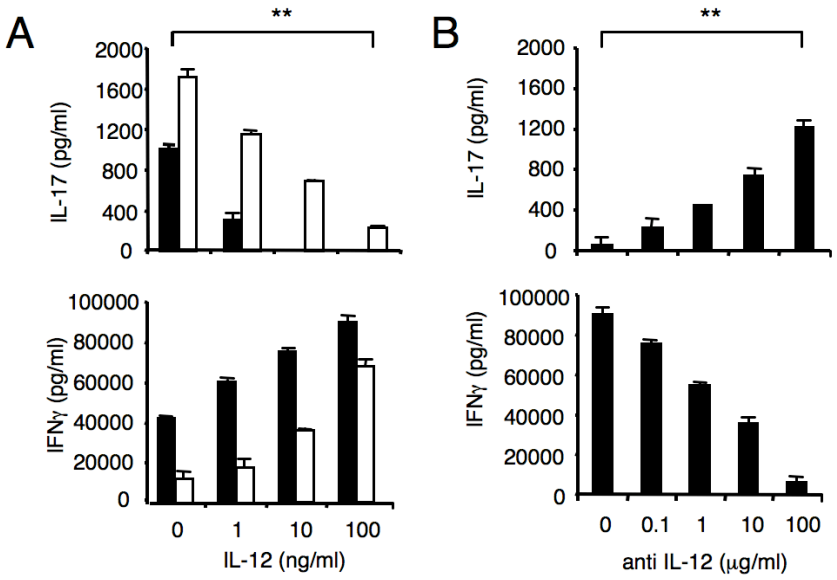


Figure 6.7. IL-12p70 drives the balancing between Th1 and Th17 response. (A) Human recombinant IL-12p70 rescue Th1 polarization upon *S. cerevisiae* spore and *C. albicans* hypahe stimulation in a dose dependent fashion. DCs were treated with spores or C. albicans for 8 hr in presence of hrIL12-p70 and ability of DCs to polarize a Th1 or a Th17 response were assessed by IFNγ and IL-17 measurement, respectively, in 5-day culture supernatants of $CD4^+$ T cells. (B) Blocking IL-12p70 activity by using an antibody specific for IL-12 results in a loss of IFNγ production and promotes IL-17 production in 5-day culture supernatants of $CD4^+$ T cells in response to yeast- stimulated DCs. Statistical comparisons between samples are represented as horizontal bars, *p<0.05, **p<0.01.

To identify which receptors are involved in the differential recognition of *S. cerevisiae* spores, we performed experiments with competitive ligands of the structures most commonly involved in fungal recognition: laminarin for dectin-1, mannan for mannose receptor and chitin for chitin receptors (Netea et al, 2006). Untreated DCs or DCs pre-exposed for 2 hours to the receptor agonists, laminarin, mannan and chitin (100 μg/ml. respectively) were incubated with yeasts and spores for 6 hours. Blocking each of these receptors resulted in an inhibition of TNFα production in DCs stimulated by yeast cells (Figure 6.8A).

On the contrary, none of the three competitor ligands was able to inhibit TNFα release in spore-stimulated DCs, suggesting that the molecular pathways involved in spore recognition could be different from those commonly implicated in yeast-DC interaction.

Human DCs are able to bind *C. albicans* through MR (Newman and Holly, 2001) and DC-SIGN (Cambi et al., 2003). In order to assess if these receptors could contribute to the different recognition of yeast cells or spores by DCs, we used the transfectant (CHO) cell line stably expressing DC-SIGN to investigate the ability of *S. cerevisiae* cells or spores to bind DC-SIGN in the absence of any other known fungal receptors.

PKH206-labeled transfectant (CHO) cells stably expressing DC-SIGN were exposed to FITC-labeled yeast (black bars) or spores (white bars). mAbs AZN-D1/D2/D3 and L19 were used to block DC-SIGN and β2-integrin, respectively. By flow cytometry we assessed that DC-SIGN clearly mediates adhesion to both yeast cells and spores (Figure 6.8B). Blocking-antibodies against DC-SIGN significantly inhibited binding of *S. cerevisiae* by CHO-DC-SIGN. Furthermore, the calcium chelator EGTA abrogated binding. This Ca^{2+} dependence confirms that the C-type lectin domain of DC-SIGN mediates binding to yeast cells and spores.

To get further insight into the specificity of DC-SIGN recognition, we analyzed several purified fungal cell wall components for their capacity to inhibit the interaction between DC-SIGN and *S. cerevisiae*. In particular we used SC-mannan, CA-mannan, β-glucan or mannose.
CA-mannan was the only carbohydrate that inhibited DC-SIGN binding to *S. cerevisiae*. SC-mannan was not able to inhibit binding of spores as previously observed for *C. albicans* (Cambi et al., 2003).

To determine the receptor repertoire involved in the interactions between DCs and *S. cerevisiae*, we performed similar experiments using moDCs and blocking MR and DC-SIGN either with purified fungal cell wall glycans (Figure 6.8C) or with antibodies (Figure 6.8D). Blocking of β_2-integrin receptors was used as negative control. In particular, CD45-APC-labeled DCs were incubated with FITC-labeled yeast (black bars) or spores (white bars) in the presence or absence of anti-DC-SIGN mAb, a mixture of anti-DC-SIGN Abs, anti-MR mAb, EDTA and anti-β2-integrin antibody as control or SC-mannan, CA-mannan, β-glucan or mannose. As before, basal binding (TSA) is set as 100%.

Again, blocking-antibodies against DC-SIGN significantly inhibited binding of spores and yeast cells by DCs, suggesting that DC-SIGN plays an important role in *S. cerevisiae* recognition. Interestingly blocking of MR by using antibody or mannose only affected the DC ability to bind yeast cells, whereas had no effect in the binding to spores (Figure 6.8).

To assess if the different recognition of spores and yeast cells by DCs influences the internalization we performed a phagocytosis assay, labeling specifically MR and DC-SIGN. By confocal microscopy the double labeling or MR and DC-SIGN showed that, after yeast cell stimulation, the two receptors co-localized in the same phagocytic vesicle (Figure 6.9).

This indicates that yeast cells were internalized by DCs in vesicles containing both MR and DC-SIGN as well as mutually exclusive vesicles, as previously reported for *C. albicans* internalization (Cambi et al., 2005). In contrast, spores were internalized by DCs in vesicles containing DC-SIGN only as shown by the lack of co-localization of the two receptors (Figure 6.9).

These results clearly demonstrate that DCs use a different PRR repertoire to recognize different life stages of the same microorganism.

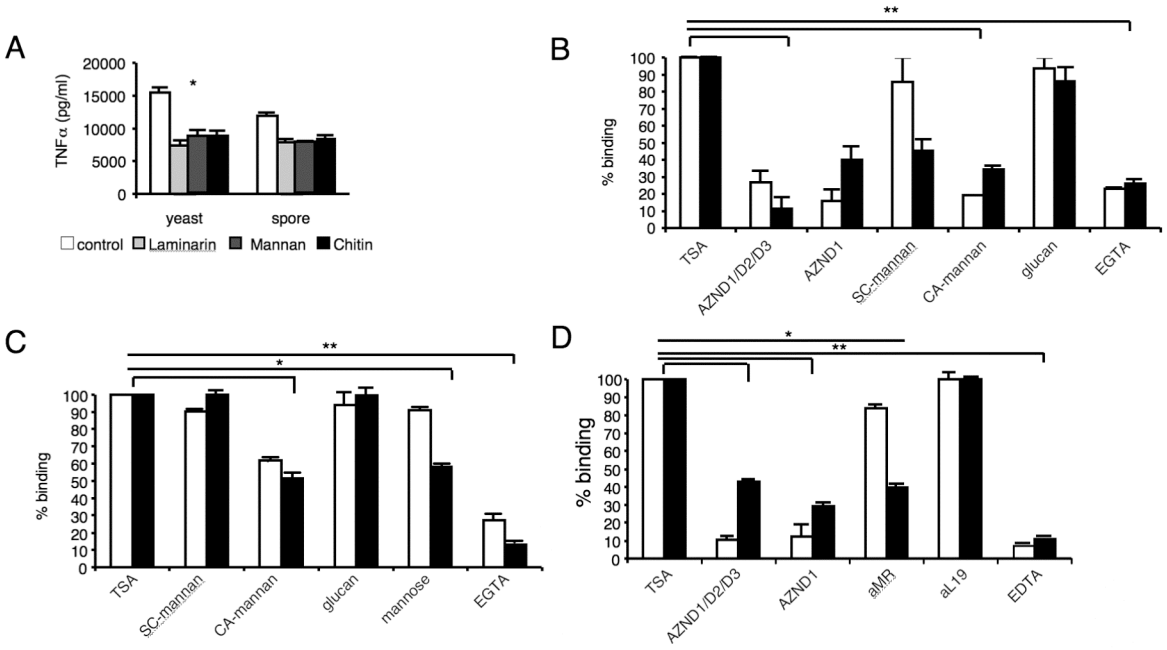
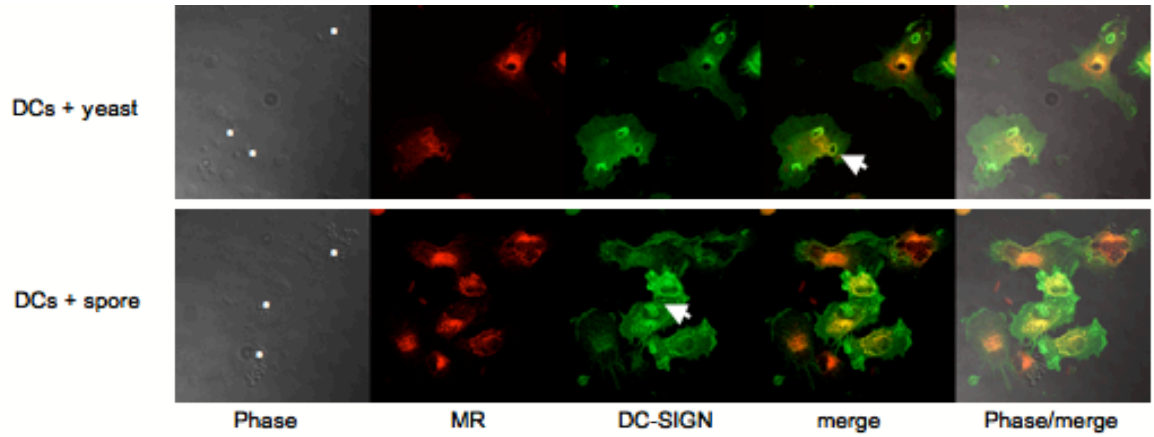


Figure 6.8. MR and DC-SIGN involvement in *S. cerevisiae* recognition. (A) Untreated DCs or DCs pre-exposed to receptor agonists, laminarin, mannan and chitin were incubated with yeasts and spores for 6 hr. TNFα production was inhibited by receptor agonist treatment only in yeast-stimulated DCs. (B) DC-SIGN specifically binds to both *S. cerevisiae* cells and spores. PKH206-labeled transfectant (CHO) cells stably expressing DC-SIGN were exposed to FITC-labeled yeast (black bars) or spores (white bars). mAbs AZN-D1/D2/D3 and L19 were used to block DC-SIGN and β2-integrin, respectively. Addition of EGTA showed that DC-SIGN-mediated binding is Ca^{2+} dependent. (C) and (D) Immature DCs binds *S. cerevisiae* trough C-type lectins. CD45-APC-labeled DCs were incubated with FITC-labeled yeast (black bars) or spores (white bars) in the presence or absence of anti-DC-SIGN mAb, a mixture of anti-DC-SIGN Abs, anti-MR mAb, EDTA and anti-β2-integrin Ab as control or SC-mannan, CA-mannan, β-glucan or mannose. Basal binding (TSA) is set as 100%. All data are presented as mean±s.d (N=3). Statistical comparisons between samples are represented as horizontal bars, *$p<0.05$, **$p<0.01$.



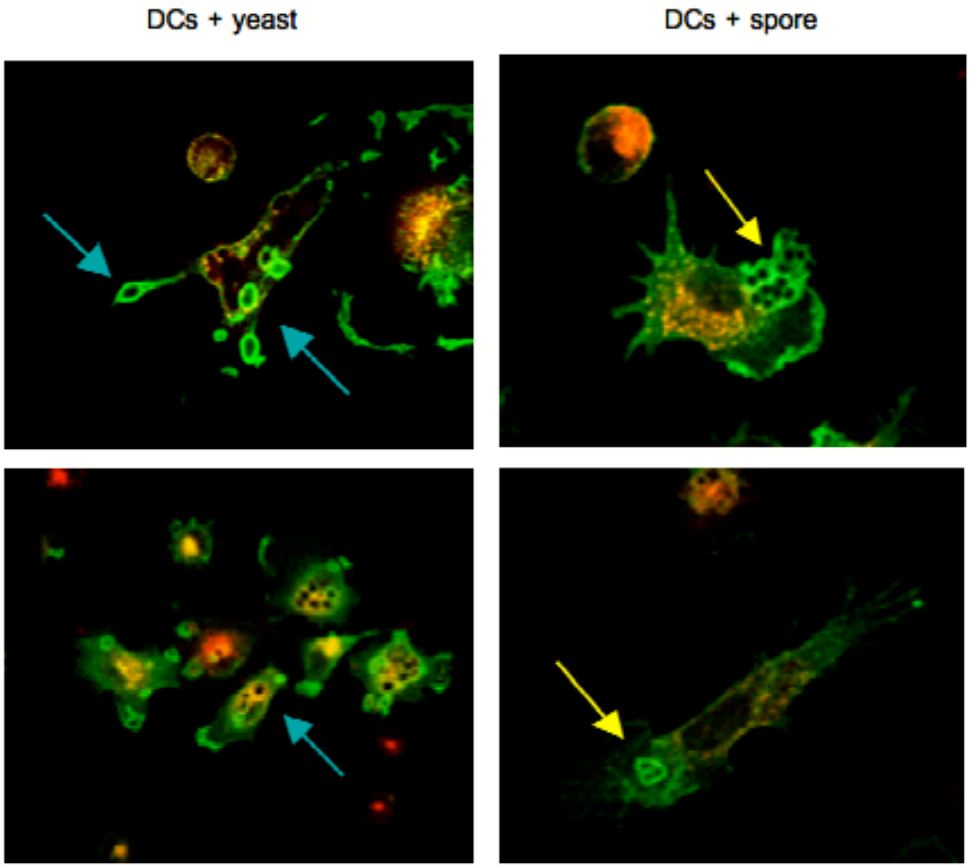


Figure 6.9. **Differential involvement of MR and DC-SIGN in S. cerevisiae spore and cell recognition.** Labeling of MR (in red) and DC-SIGN (in green) show considerable colocalization with yeast cells (asterisk). Spores are internalized by DC-SIGN-containing vesiscle only. All data are presented as mean±s.d (N=3).

6.5 Role of exposed mannan in defining immune responses to *S. cerevisiae*

It was recently demonstrated that the MR is the main pathway trough which *C. albicans* induces a Th17 response. The interaction between the various *Saccharomyces* cell wall-associated structures with their counter-receptors, and the implications to DC function remain unknown. The only indirect evidence available indicates that *C. albicans* mannan (CA-mannan) but not *S. cerevisiae*-derived mannan (SC-mannan) is able to induce IL-17 production upon PBMC stimulation (van der Veerdonk, 2009), implying that the different branches of the two structures profoundly influence the recognition of fungi and the subsequent Th response.

Since SC-mannan itself cannot induce IL-17 production despite interacting with MR (Cambi et al., 2008) and spores lack exposed mannan, we further investigated the specific role of this branched carbohydrate in stimulating the innate *S. cerevisiae* immune response. We assessed by stimulation of immature DCs with SC-mannan that mannan itself stimulated IL-12p70 production by DCs (Figure 6.10) indicating that the exposure of SC-mannan could influence the Th response priming. Thus, DCs were exposed or not to an excess of SC-mannan (100 mM) for 2 hours and stimulated with spores for 8 hours. Then the co-colture with $CD4^+$ T cells was performed. IL-12p70, IFNγ and IL-17 were measured. The amount of IL-12p70 produced by SC-mannan was sufficient to inhibit the IL-17 production by T cells induced by spores (Figure 6.10). This suggests that the lack of SC-mannan in spore may be responsible for the low IL-12 production.

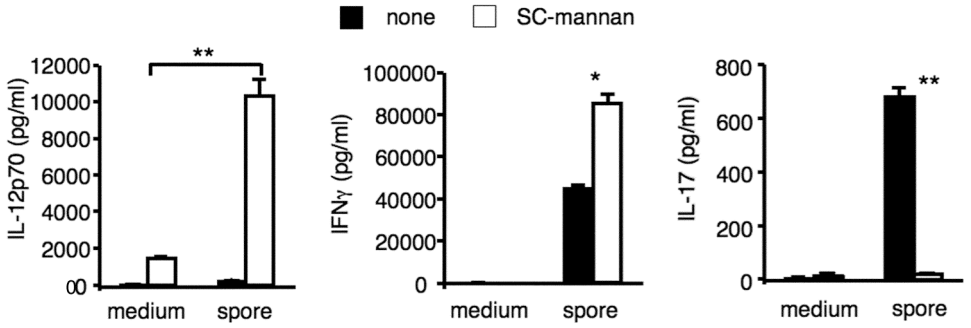


Figure 6.10. *S. cerevisiae* derived-mannan inhibits Th17 response induced by spores. DCs were exposed (white bars) or not (black bars) to an excess of SC-mannan (100 mM) for 2 hours and stimulated with spores for 8 hours. Thus the co-colture with $CD4^+$ T cells was performed. IL-12p70, IFNγ and IL-17 were measured. All data are presented as mean±s.d (N=3).

From previous reports it is known that *Candida och1* mutant, defective in mannosylation process, failed to interact with DCs supporting the important role played by N-linked mannan in the binding of *C. albicans* to DCs (Cambi et al., 2008). In contrast, in *S. cerevisiae*, *OCH1* gene is not only required for a proper mannosylation but also for masking the β-glucan and the immune

recognition by the Dectin-1 pathway (Wheeler and Fink, 2007) involved in Th17 priming (Acosta-Rodriguez et al., 2007).

This prompted us to further investigate the effects of absence of exposed mannan in Th17 induction by *S. cerevisiae* spores. DCs were exposed to cells of different *S. cerevisiae* strains (SK1, BY4741 wild type, BY4741 *och1* mutant), to *C. albicans* hyphae and to SK1 spores for 12 hours and their capacity to prime Th17 or Th1 polarization of T cells was assessed by IL-12, IL-17 and IFNγ measurement by ELISA. Stimulation with *S. cerevisiae och1* mutant yeast cells polarized a Th1/Th17 population towards the type of responses induced by spores (Figure 6.11).

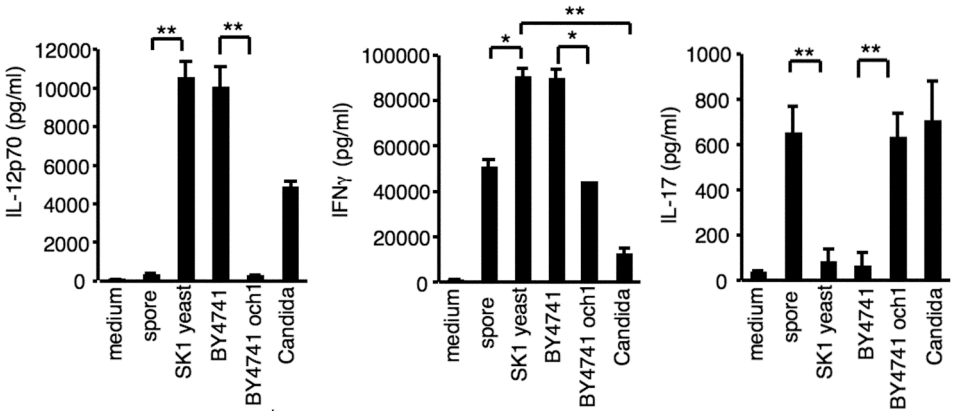


Figure 6.11. Absence of exposed mannan induces Th17 polarization. DCs were exposed to cells of different *S. cerevisiae* strains (SK1, BY4741 wild type, BY4741 *och1* mutant), to *C. albicans* and to SK1 spores for 12 hr and their capacity to prime Th17 or Th1 polarization of T cells was assessed by IL-12, IL-17 and IFNγ measurement by ELISA. All data are presented as mean±s.d (N=3).

Stimulation with *C. albicans* hyphae polarized a Th17 response as previously demonstrated (van der Veerdonk et al., 2009). These data demonstrate that the presence of exposed mannan on the cell wall influences the immune response mounted by the DCs to properly respond and tolerate *S. cerevisiae* yeast cells, differently to what occur with *C. albicans* hyphae.

We demonstrated that spores are recognized by DC-SIGN and not by MR and we know that SC-mannan is a ligand for DC-SIGN as well as MR. To understand the relative contribution of these two receptors in *S. cerevisiae* Th response, we exposedDCs to blocking antibodies before exposure to spores and yeast cells. The blocking of MR induced the decrease in IL-12p70 production and an increase of IL-17 induction upon yeast cells stimulation, while blocking of DC-SIGN showed a slightly effect on IL-17 production (Figure 6.12).

Thus, we show that in contrast to *C. albicans* hyphae stimulation, *S. cerevisiae* cells interaction with DCs, exerted by mannan, favours the IL-12/IFNγ axis, while the absence of exposed mannan in spores induces a Th17 response.

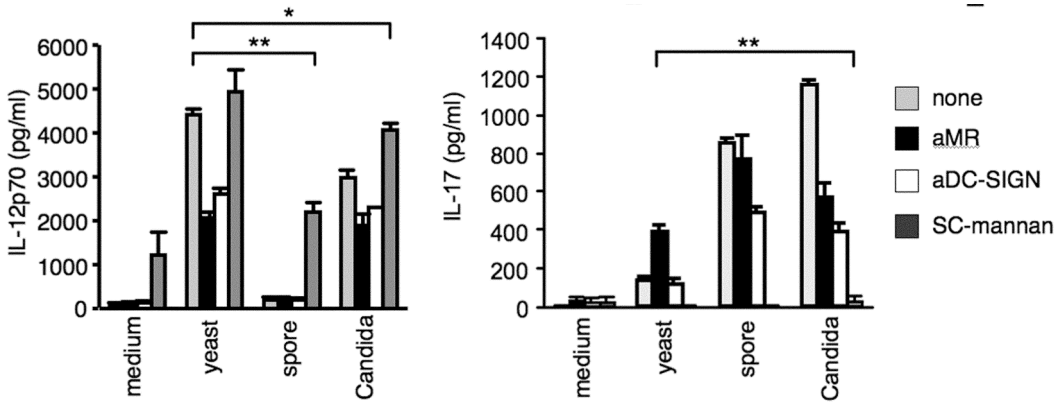


Figure 6.12. Specific role of mannan structures for interaction of host cells with *S. cerevisiae.* DCs were exposed to *S. cerevisiae* cells, spores and *C. albicans* (purple bars). mAbs were used to block MR (black bars) or DC-SIGN (white bars) or exposure to SC-mannan (light blue bars) were used. After 12 hours, IL-12-p70 was measured and after 5 days of co-colture IL-17 was assessed on T cells supernatants. All data are presented as mean±s.d (N=3).

6.6 Discussion

Our results demonstrate the usefulness of *S. cerevisiae* as a tool to learn the rules of cohabitation between yeast and its host. The relationship between humans and *S. cerevisiae* is so strong that some strains of this species are often referred to as domesticated organisms (Liti et al., 2009). Our results demonstrate that, in spite of the lack of IL-12p70 production by DCs, spores, a peculiar form of *S. cerevisiae*, induce a Th1/Th17 polarization of CD4 T cell precursors. In contrast the activation of suppressive mechanisms, such as the increased expression of the *IDO* gene in DCs and the induction of a $CD4^+$ $FOXP3^+$ Treg population, was observed upon yeast cell stimulation.

Furthermore, in contrast to what happened with yeast cells, spores seem to interact with DC with a reduced activation of the NADPH oxidase and inducing a poor fungicidal activity of these cells as previously reported for *C. albicans* (Donini et al., 2007). Further investigation on the nature of this possible oxidative damage escape mechanisms will be carried out in follow-up studies.

We observe that similarly to what reported for *C. albicans* (Cambi et al., 2008), interaction between *S. cerevisiae* yeast and DCs is mediated by mannan rather than other fungal cell wall components. While recognition of mannan and mannose by DC-SIGN and MR plays an important role in binding and phagocytosis of *S. cerevisiae* cells by human DCs, DC-SIGN alone seems to have the major role in spore recognition. Our data suggest that the outermost layer of dityrosine present on spore wall could hide to DCs important carbohydrates, influencing immune detection by eluding important PAMPs.

We demonstrate that the interplay between spores and yeasts is crucial for the commensalism of *S. cerevisiae*. The differential induction of a Th1 response versus a Th17 response is determined by the structure of the cell wall of these two forms of the fungus. Our results indicate that SC-mannan could favor a specific individual recognition pathway through mannan-recognizing receptors such as MR, DC-SIGN or TLR4 that lead to different cytokine release and the polarization of a Th1 response. By contrast, the absence of exposed mannan contributes to the Th17 induction by the spores.

We show that *S. cerevisiae och1* mutant, defective in N-mannosylation process, induces a Th1/Th17 response comparable to that induced by spores, highlighting the importance of the presence of the mannan layer to address the T cell response to better cope with the invading pathogen.

The differential recognition of mannan from *C. albicans* and *S. cerevisiae* is most likely due to the different branching of these structures. In contrast to SC-mannan, CA-mannan induces potent Th17 responses (van der Vendoonk et al., 2009), which suggests that MR is differently used by *C. albicans* and *S. cerevisiae* to induce an immune response. The relative contribution of DC-SIGN in Th17 priming is under investigation.

Our results show that in moDCs *S. cerevisiae* spores are able to activate different molecular pathways with respect to yeast cells.

The evidence that *S. cerevisiae* spores do not induce IL-12p70 and are resistant to phagocytosis suggests that sporulation may be a mechanism for circumventing adaptive immune responses as well as the Th1 responses that protect against intracellular pathogens. Both Th1 (Acosta-Rodriguez et al., 2007) and Th17 (Huang et al., 2004; Milner et al., 2008; Conti et al., 2009) cells have been proposed to mediate protection against pathogenic fungi and in particular against *C. albicans*. *C. albicans* can switch from being an intracellular form, the yeast, that can be efficiently destroyed by macrophages with the help of Th1 cells, to being an extracellular form, the hyphae, that is better controlled by neutrophils (Urban et al., 2008) recruited by Th17 cells. This is consistent with the differential chemokine profile we observed in cells challenged with the yeast or the hyphae. Indeed Th1 cells express CXCR3 and migrate towards IFN dependent chemokines, such as CXCL10 and CXCL11, which are preferentially induced in response of the yeast. In turn, Th17 cells express CCR6 and are recruited in response of CCL20, which is preferentially expressed in the presence of the hyphae. The pattern of chemokine expression induced by the spore is similar to that induced by the hyphae. This might represent a mechanism to escape the Th1 responses mediated by the CXCR3-CXCL10 axis and to favour Th17 responses which might be beneficial for the pathogen. Indeed here we show that the enhancement of Th17 responses in presence of *S. cerevisiae* spores, via increased IL-1β and IL-6 release, favours spore expulsion. This finding may have relevance for autoimmune diseases such as inflammatory bowel disease (IBD) where *S. cerevisiae* spores could play a role in the inflammation process mediated by a mixed Th1/Th17

response. Interestingly, anti-*S. cerevisiae* antibodies can be detected in a subset of patients with Crohn's disease and are considered to be markers of active disease (Standaert-Vitse et al., 2006). The capacity of spores to promote IL-17 release may be a possible link between *S. cerevisiae* and the pathogenesis of this disease and therefore sporulation could be a potential target for therapy.

It has been recently shown that survival of *S. cerevisiae* spores in *Drosophila melanoglaster* gut is increased compared to yeast cells (Coluccio et al., 2008) and that spores are thus more resistant to innate mechanisms of protection. In this work, we find that spores but not cells of *S. cerevisiae* can be isolated from faecal samples of rats treated with spores or yeasts. This result indicates the functional implications of differential immune recognition of spores with respect to yeast cells. Reports of spores in mammalian intestine are rare probably due to the habit of culturing faecal samples rather than observing them by microscopy. This result opens new avenues for further investigation on the *in vivo* nature of the immune response in animal models and IBD's patients in future studies

Tolerance requires the ability of the microbe to circumvent inflammatory signals evolved as response to potentially pathogenic microorganisms. We demonstrate that the differential recognition of specific mannan structures is the master regulator of the discrimination between harmful and harmless fungi, outlining the importance to deeper insight on the specific contribution of each receptor in fungal recognition.

The higher capacity of SC-mannan to induce IL-12/Th1 protective response is likely to play a major role for the non-pathogenicity of *S. cerevisiae* and its commensalism in humans. Our findings are ecologically important as insects and mammalians that feed on fruits and fermented beverages are continuously contaminated with yeasts that can potentially colonize the gut. Sporulation may have evolved in yeast to allow host clearance while surviving the immune response. This trait may be advantageous and explain the maintenance of the ability to sporulate, as indicated by the high sporulation rates of many of the naturally existing strains (Mortimer, 2000). The yeast ecological cycle could therefore possibly include mammals as hosts and vectors.

Creativity requires the courage to let get go of certainties.
Erich Fromm

7
Discussion

Despite life-threatening systemic fungal infections has risen steadily over the past three decades, although this increase might now be reaching a plateau. The increased prevalence of fungi as agents of disseminated infection has been restricted to patients in whom surgical or chemotherapeutic interventions and/or underlying immunological deficiencies have allowed fungi to overwhelm the protective host defence mechanisms. In healthy and immunologically normal individuals, the innate immune system is an efficient sentinel that provides protection from the thousands of fungal species that humans regularly encounter.

Candida albicans and *Saccharomyces cerevisiae* are ubiquitous fungal organism that often colonizes the skin and the mucosal surfaces of normal individuals, without causing disease. However, when the normal host defence mechanisms are impaired they can become a pathogen.
Until recently, little was known about the ways in which antigen presenting cells, the major players in innate immunity, recognized *C. albicans* as a pathogenic and *S. cerevisiae* as a harmless microorganism, or how the fungal–leukocyte interaction triggers an inflammatory response. The dogma that had been blindly accepted over the past 50 years was that although effective, innate immunity was non-specific and 'rather primitive and dumb'. However, this simplistic model, in which innate immunity performs only simple 'ingest and destroy' tasks, could not explain how innate immune cells recognize microbial pathogens as 'non-self', or why different responses are triggered by different classes of microorganisms, be they pathogenic or commensal. Only in the past decade has it become clear that the innate immune system not only specifically recognizes various classes of microorganisms, it also initiates and modulates the subsequent adaptive responses that are delivered by T and B cells through their interactions with antigen- presenting cells, especially dendritic cells (DCs). The tasks of recognizing an invading pathogen and activating the host response are accomplished by pattern-recognition receptors (PRRs), which recognize conserved microbial chemical signatures called pathogen-associated molecular patterns

(PAMPs).

An association between morphogenesis and virulence has long been presumed for dimorphic fungi that are pathogenic to humans, as one morphotype exists in the environment or during commensalism, and another within the host during the disease process. For *C. albicans,* putative virulence factors include the ability to switch between harmless yeast and pathogenic, filamentous forms of the fungus. DCs sense either form in a specific way, resulting in distinct, T helper cell-dependent protective and non-protective immunities. Recent evidence suggests that the use of distinct recognition receptors contributes to the disparate patterns of reactivity observed locally in response to challenge with *C. albicans*. The interaction between *S. cerevisiae* and human societies is so generally recognized to hypothesize domestication of strains of this species.

Successful resolution of pathogenic fungal disease depends on proper coordination of multiple components of the host immune response. The balance between pro- and anti-inflammatory signalling is a prerequisite for safe host-fungal interactions. Although inflammation is an essential component of the protective response to fungi, its deregulation may significantly worsen fungal diseases and limit protective antifungal immune responses.

Host/fungal interactions have so far been studied only for pathogenic fungi such as *C. albicans*, but it is not known what determines the commensalism of harmless fungi. Understanding the mechanisms of cohabitation between humans and non-pathogenic fungi is a prerequisite for controlling fungal infections. We investigated the rules of host-fungal cohabitation and dissected the mechanisms responsible for the differential recognition demonstrating that the skewing in the use of pathogen recognition receptors by differences in cell wall composition is a requirement for pathogenicity.

The immune response is a complex entity with many possible inputs, influences and outcomes, and systems biology holds the promise of allowing us to both better understand its nature, and generate predictions and hypotheses about its behaviour under particular conditions. Immunity is not simply the product of a series of discrete linear signalling pathways; rather it is comprised of a complex set of integrated responses arising from a dynamic network of thousands of molecules subject to multiple influences. Its behavior often cannot be explained or predicted solely by examining its components.

The comparison of the immune response to pure agonists and entire cells clearly illustrated how without addressing the complexity of the response to entire fungi it is impossible to model the immune response. In particular the exposure to entire living cells highlighted the importance of signalling from different cellular compartments dissecting the interplay between outer membrane and phagosomal receptors. Our result indicate the importance of the temporal window between the

initial recognition of the fungus at the cell surface and the stimulation of the other receptors that intensify, complement and sustains the DC activation process.

The integration of multiple stimuli over a defined temporal window is a requirement for an effective response that can be understood only when studying the challenge with pathogenic and non-pathogenic microorganisms in addition to soluble microbial products. This mechanism is of paramount importance for the generation of an adaptive immune response of the appropriate class. Our transcriptional analyses indicated that one of the major differences between stimulation of DCs with *S. cerevisiae* yeast cells and *Candida* hyphae activate a common defined pathway of immune responses, that differs importantly for the differential regulation of sustained transcription of C-type lectins in *Candida* hyphae, contrary to repression of sensing proteins genes in DCs treated with *S. cerevisiae*. This evidence supports the observation that the activation of the phagosomal pathways is partially or totally missing from the stimulation with *Candida* hyphae (similarly to *S. cerevisiae* spores) and could be associated with a lack of switch repressing signalling from cell membrane an activating signalling from the phagosome, visible in yeast but not in hyphae. This temporally restricted switch could be crucial in moDCs, balancing tolerogenic and inflammatory responses, in order to guarantee microbial recognition and avoid potentially harmful sustained inflammation processes.

The transcriptional analysis indicated that, far from being inactive microbes, *S. cerevisiae* yeast cells activate a defined pathway of immune responses in moDCs, balancing each other in order to avoid potentially harmful inflammation processes.

The type of immune response mounted is primarily determined by the initial interaction between the pathogen and the cells of innate immune system. The ability to eradicate fungal infection has been associated to Th1 response. Conversely, a Th17 polarization has been described in several pathological conditions and is activated in response to pathogenic fungi.
It was previously shown that the important differences in the structure of *Saccharomyces*-derived mannan and *Candida*-mannan determine differential responses. In contrast to *Saccharomyces* mannan, *Candida*-mannan induces potent Th17 response, which suggests that MR is differently used by *C. albicans* and *S. cerevisiae* to induce an immune response. We used human monocyte-derived dendritic cells exposed to cells and spores of the yeast *S. cerevisiae* as well as *C. albicans* hyphae, as a toolbox to dissect the role of surface mannans which are central for pattern recognition.

Combining transcriptional analysis with receptor-specific blocking and cytokine production assays, we determined that DCs respond differently to *C. albicans* and *S. cerevisiae* and in the latter case, the interplay between spores and yeasts is crucial for the commensalism of *S. cerevisiae.* In contrast with the yeast cell wall, mannan are present only in the innermost layer of

the spore wall preventing mannan recognition. We identified that, while yeast cells are recognized either by MR and DC-SIGN, spores are recognized by DC-SIGN only, suggesting that not only MR acts differently in response to pathogenic and non-pathogenic fungi but also in response to different form of the same microorganism.

Our results indicate that *S. cerevisiae* mannan favor a specific individual recognition pathway through mannan-recognizing receptors, leading to different cytokine release and the polarization of a Th1 response. In contrast, the absence of exposed mannan on the spore surface contributes to the Th17 induction by the spores. We demonstrated that the differential recognition of specific mannan structures is the master regulator of the discrimination between harmful and harmless fungi. The higher capacity of *Saccharomyces*-mannan to induce IL-12/Th1 protective response is likely to play a major role for the non-pathogenicity of *Saccharomyces* and its commensalism in humans, in contrast with *Candida* mannan-Th17 induced response.

We demonstrate that the spores are able to elicit a Th17 response, hypothesizing this as a mechanism of escaping host clearance that ultimately may favour yeast dissemination. Thus, we investigated the potential of yeast spores to survive passage through the human host and be disseminated in new environments.

Our results bear importantly on the ecological significance of sporulation and on the central role of fungal cell wall recognition in discrimination between friends and foes.

Conclusion

Science may set limits to knowledge, but should not set limits to imagination
Bertrand Russel

The research presented here aimed at using a Systems Biology approach to dissect the response of dendritic cells to fungi. Systems Biology requires the acquisition of information on the different levels of regulation of a biological system and its integration in the development of models, that could predict the outcome of stimuli and changes in variables controlling the dynamic nature of the system. This work combined traditional wet-lab work and genome wide analyses of transcription and gene regulation, with computational and bioinformatic methods to dissect the response of dendritic cells to fungi, in particular to the harmless *Saccharomyces cerevisiae*. Dissecting the poorly characterized immune response to this non-pathogenic yeast is of paramount importance to identify the thin line that render a saprophytic fungus an opportunistic pathogen one.

Extending our studies to different forms of clinical isolates of *S. cerevisiae* will be crucial to fully understand the rules of proper recognition of fungi and might lead to development of new drugs to treat fungal infections or Immune Bowel Disease.

From an ecological perspective the observation that spores evolved strategies to survive better than yeast cells in mammalian viscera, and spore-mediated Th17 response could suggest spores as a mean to circumvent host clearance, that ultimately may favor the use of mammals as a vectors for yeast dissemination. The evidence that *S. cerevisiae* cells induce a tolerogenic response explains its description as non pathogenic microorganism. The finding that only spores are capable of inducing IL-17-mediated inflammatory response might provide the missing link between the presence of anti-*S. cerevisiae* antibody and inflammatory bowel diseases, and sporulation, a potential target for therapy.

Understanding the molecular mechanisms governing immune response discrimination of pathogenic and non pathogenic microorganisms is the only way to the understanding of aberrant immune phenomena such as autoimmunity and chronic inflammatory diseases as well as informing the design of vaccine, immune-modulatory drugs and novel antifungal drugs.

Bibliography

Acosta-Rodriguez, E.V., Napolitani, G., Lanzavecchia, A., and Sallusto, F. (2007). Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol*. 8(6): 639-46.

Akira, S., and Hemmi, H. (2003). Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett*. 85: 85–95.

Akira, S. (2006). TLR signalling. *Curr. Top. Microbiol. Immunol*. 311: 1–16.

Allison, D.B., Cui, X., Page, G.P., Sabripour, M. (2006). Microarray data analysis: from disarray to consolidation and consensus. *Nat Rev Genet* 7: 55–65.

Ariizumi, K. et al. (2000). Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem*. 275: 11957–11963.

Arp, R. and Smith, B. (2008). Ontologies of cellular networks. *Science signalling* 1, mr2.

Ashburner, M. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* 25(1),: 25-29.

Bacci, A. *et al.* (2002). Dendritic cells pulsed with fungal RNA induce protective immunity to Candida albicans in hematopoietic transplantation. *J. Immunol.* 168: 2904–2913.

Ball, C.A., *et al*. (2002). Standards for microarray data. *Science* 298: 539. 17.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature* 392,: 245–252.

Bauman, S.K. *et al*. (2000). Dendritic cells in the induction of protective and nonprotective anticryptococcal cell-mediated immune responses. *J. Immunol.* 165: 158–167

Beltrame, L., Rizzetto, L., Paola, R., Rocca-Serra, P., Gambineri, L., Battaglia, C., Cavalieri, D. (2009). Using pathway signatures as means of identifying similarities among microarray experiments. *PLoS One*. 4(1): e4128.

Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N., Golani, I. (2001). Controlling the false discovery rate in behavior genetics research. *Behav. Brain. Res.* 125: 279–284.

Bettelli, E. *et al.* (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.

Bettelli, E., Korn, T., Oukka, M., and Kuchroo, VK. (2008). Induction and effector functions of T(H)17 cells. *Nature* 19: 453(7198), 1051-1057.

Bettelli, E., Oukka, M. and Kuchroo, V.K. (2007). T(H)-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol*. 8: 345–350

Beutler, B. *et al.* (2007). Genetic analysis of resistance to viral infection. *Nat. Rev. Immunol*. 7(10): 753-66

Biondo, C. et al. (2005). MyD88 and TLR2, but not, TLR4, are required for host defence against Cryptococcus neoformans. *Eur. J. Immunol.* 35: 870-878.

Bluestone, J.A., and Abbas, A.K. (2003). Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol*. 3: 253–57

Bozza, S. *et al.* (2002) Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J. Immunol.* 168: 1362–1371

Brazma, A., *et al.* (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat, Genet,* 29: 365–371.

Brazma, A., *et al.* (2003). ArrayExpress–a public repository for microarray gene expression data at the EBI. *Nucleic Acids Res.* 31: 68–71.

Brikos, C. and O'Neill, L.A. (2008). Signalling of toll-like receptors. *Handb. Exp. Pharmacol*. 21–50

Brown, G.D., and Gordon, S. (2001). Immune recognition. A new receptor for beta-glucans. *Nature* 413: 36-37.

Brown, G.D., *et al.* (2003). Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med*. 197: 1119–24

Brown, G.D., and Gordon, S. (2003). Fungal-glucans and mammalian immunity. *Immunity* 19: 311–315

Brown, G.D., and Gordon, S. (2005). Immune recognition of fungal -glucans. *Cell. Microbiol*. 7: 471–479

Bruford, E.A., *et al.* (2008).The HGNC Database in 2008: a resource for the human genome. *Nucleic acids research* 36: D445-448

Buentke, E., Scheynius, A. (S.d.) Dendritic cells and fungi. *APMIS* 111: 789–96.

Calderone, R.A. and Fonzi, W.A. (2001). Virulence factors of Candida albicans. *Trends Microbiol.* 9: 327–334

Cambi, A. *et al.* (2003). The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. *Eur. J. Immunol.* 33: 532–538

Cambi, A., Koopman, M., Figdor, C.G. (2005). How C-type lectins detect pathogens. *Cell. Microbiol*. 7(4): 481-488.

Cambi, A., *et al.* (2008). Dendritic cell interaction with Candida albicans critically depends on N-linked mannan. *J. Biol. Chem*. 283: 20590-20599.

Casadevall, A. *et al.* (1999). Host–pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* 67: 3703–3713

Casadevall, A. *et al.* (2001). Host–pathogen interactions: the attributes of virulence. *J. Infect. Dis.* 184,:337–344

Casadevall, A. *et al.* (2002). What is a pathogen? *Ann. Med*. 34: 2–4

Cavalieri, D., *et al.* (2007). Eu.Gene Analyzer a tool for integrating gene expression data with pathway databases.*Bioinformatics* 1;23(19): 2631-2632

Cavalieri, D., McGovern, P.E., Hartl, D.L., Mortimer, R., and Polsinelli, M. (2003). Evidence for S. cerevisiae fermentation in ancient wine. *Journal of molecular evolution* 57 Suppl 1: S226-232.

Chiani, P., Bromuro, C., and Torosantucci, A. (2000). Defective induction of interleukin-12 in human monocytes by germ-tube forms of Candida albicans. *Infection and immunity* 68: 5628-5634.

Choi, H.S., Kim, J.W., Cha, Y.N., and Kim, C. (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of immunoassay & immunochemistry* 27: 31-44.

Chorvatovicová, D., Machová, E., Sandula, J., Kogan W. (1999). Protective effect of the yeast glucomannan against cyclophosphamide-induced mutagenicity. *G. Mutat Res.* 21: 117-122.

Chuang, T.H., Ulevitch, R.J. (2000) Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur. Cytokine Netw*. 11: 372–378.

Coluccio, A.E., Rodriguez, R.K., Kernan, M.J., and Neiman, A.M. (2008). The yeast spore wall enables spores to survive passage through the digestive tract of Drosophila. *PLoS ONE* 6; 3(8), e2873

Coluccio, A., Bogengruber E., Conrad M.R. Dresser, P. Briza M. E., and Neiman AM. (2004). Morphogenetic Pathway of Spore Wall Assembly in *Saccharomyces cerevisiae. Eukariotic Cell* 3 (6); 1464-1475.

Conti, H.R., *et al*. (2009). Th17 cells and IL-17 receptor signalling are essential for mucosal host defence against oral candidiasis. *J. Exp. Med*. 16: 299-311.

Cua, D.J. *et al.* (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744–748.

Curtis, M.M., and Way, S.S. (2009). Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology* 126(2): 177-185.

Cutler, J. E. (2001). N-glycosylation of yeast, with emphasis on Candida albicans. *Med. Mycol*. 39: 75–86.

Cutler, J.E. (1991). Putative virulence factors of Candida albicans. *Annu. Rev. Microbiol*. 45: 187–218.

Decker, W.K., *et al.* (2006). Double loading of dendritic cell MHC class I and MHC class II with an AML antigen repertoire enhances correlates of T-cell immunity in vitro via amplification of T-cell help. *Vaccine.* 24(16): 3203―3216.

Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L. and Springer, T. A. (1993). The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. *Cell Biol.* 120: 1031–1043.

Doniger, S.W., *et al.* (2003). MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol*. 4: R7.

Donini, M., Zenaro E., Tamassia N., and Dusi S. (2007). NADPH oxidase of human dendritic cells: role in Candida albicans killing and regulation by interferons, dectin-1 and CD206. *Eur. J. Immunol.*. 37(5): 1194-1203.

d'Ostiani, C.F. *et al.* (2000). Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. *The Journal of experimental medicine* 191: 1661-1674.

Draghici, S., *et al.* (2007) A systems biology approach for pathway level analysis. *Genome Res.* 17: 1537–1545.

Drickamer, K. (1999). C-type lectin-like domains. *Curr. Opin. Struct. Biol*. 9: 585–90.

Du, P., Kibbe, W.A., Lin, S.M. (2007). nuID: a universal naming scheme of oligonucleotides for illumina, affymetrix, and other microarrays. *Biol. Direct.* 31: 2-16.

Du, P., Kibbe, W.A., Lin, S.M. (2008). lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 1(24): 1547-1548.

Edgar, R., Domrachev, M., Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 30: 207–210.

Ein-Dor, L., Kela, I., Getz, G., Givol, D., and Domany, E. (2005). Outcome signature genes in breast cancer: is there a unique set? *Bioinformatics* 21: 171–178.

Ein-Dor, L., Zuk, O., and Domany, E. (2006). Thousands of samples are needed to generate a robust gene list for predicting outcome in cancer. *Proc. Natl. Acad. Sci.* USA 103: 5923-5928.

Engering, A., *et al.* (2002). The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J. Immunol*. 168: 2118–2126.

Fè-d'Ostiani, C. *et al*. (2000). Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans: implications for initiation of T helper cell immunity in vitro and in vivo. *J. Exp. Med.* 19: 1661–1674.

Feinberg, H., Mitchell, D.A., Drickamer, K., and Weis, W.I. (2001). Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science* 294: 2163–2166.

Figdor, C.G., van Kooyk, Y., and Adema, G.J. (2002). C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Immunol.* 2: 77–84.

Forsyth, C.B., and Mathews, H. L. (2002). Lymphocyte adhesion to Candida albicans. *Infect. Immun*. 70: 517–527.

Forsyth, C B., Plow, E. F., and Zhang, L. Interaction of the fungal pathogen Candida albicans with integrin CD11b/CD18: recognition by the I domain is modulated by the lectin-like domain and the CD18 subunit. J. *Immunol.* 161: 6198–6205.

Fulcher, J.A., *et al.* (2006). Galectin-1-matured human monocyte-derived dendritic cells have enhanced migration through extracellular matrix. *J. Immunol.* 177(1): 216―226.

Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., and Underhill, D.M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197: 1107–1117.

Gantner, B.N., Simmons, R.M. and Underhill, D.M. (2005). Dectin-1 mediates macrophage recognition of Candida albicans yeasts but not filaments. *EMBO J.* 24: 1277–1286.

Gardy, J.L., Lynn, D.J., Brinkman, F.S.L., and Hancock, R.E.W. (2009). A systems biology approach to immunology: focus on innate immunity. *Trends Immunol*. 20: 6.

Geijtenbeek, T.B.H., *et al.* (2002). Identification of different binding sites in the dendritic cell-specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1. *J. Biol. Chem.* 277: 11314–11320.

Geijtenbeek, T.B.H., *et al.* (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197: 7–17.

Geijtenbeek, T.B.H, *et al.* (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell.* 3; 100(5): 575-585.

Gildea, L.A. *et al.* (2001). Histoplasma capsulatum yeasts are phagocytosed via very late antigen-5, killed, and processed for antigen presentation by human dendritic cells. J. *Immunol.* 166: 1049–1056.

Goeman, J.J., Geer, S.A.V.D., Kort, F.D., Houwelingen, H.C.V. (2003). A global test for groups of genes: testing association with a clinical outcome. *Bioinformatics* 20: 93–99.

Gomez, M.J. *et al.* (2000). Biochemical and immunological characterization of MP65, a major mannoprotein antigen of the opportunistic human pathogen Candida albicans. *Infect. Immun*. 68: 694–701.

Gomez, M.J. *et al.* (1996). Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-Candida cell-mediated immune responses in humans. *Infect. Immun*. 64: 2577–2584.

Gow, N.A.R. *et al.* Recognition of Candida albicans-glucan by dectin-1 induces cytokines and has non-redundant effects on the activation of innate immunity. *J. Infect. Dis*.in press.

Grazziutti, M. *et al.* (2001). Dendritic cell-mediated stimulation of the in vitro lymphocyte response to Aspergillus. *Bone Marrow Transplant*. 27: 647–652.

Gross, O.,*et al.* (2006). Card9 controls non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442: 651-656.

Gross, O., *et al.* (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature. 21; 459(7245): 433-436.

Grosu, P., Townsend, J.P., Hartl, D.L., Cavalieri, D. (2002). Pathway Processor: a tool for integrating whole-genome expression results into metabolic networks. *Genome Res.* 12: 1121–1126.

Hansson, M., *et al.* (2006). Dendritic cells express CCR7 and migrate in response to CCL19 (MIP-3beta) after exposure to Helicobacter pylori. *Microbes Infect.* 8(3): 841-850.

Hemmi, H., *et al.* (2000). A Toll-like receptor recognizes bacterialDNA. *Nature* 408: 740–745.

Hernanz-Falcón, P., Joffre, O., Williams, D.L., Reis e Sousa, C. (2009). Internalization of Dectin-1 terminates induction of inflammatory responses. *Eur. J. Immunol*. 39(2): 507-513.

Hirschfeld, M., *et al.* (2001) Signalling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun. 69: 1477–1482.

Hodges A, Sharrocks K, Edelmann M, Baban D, Moris A, Schwartz O, et al. (2007). Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. Nat Immunol. Giu ;8(6):569—577.

Hoffmann, J.A. (2003). The immune response of Drosophila. *Nature* 426(6962): 33-38.

Hogan, L.H. *et al.* (1996). Virulence factors of medically important fungi. *Clin. Microbiol. Rev.* 9: 469–488.

Hong, F. *et al*. (2006). RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* 22: 2825-2827.

Hu, X. *et al.* (2008). Regulation of interferon and Toll-like receptor signalling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol. Rev.* 226: 41–56.

Huang, Q. *et al*. (2001). The plasticity of dendritic cell responses to pathogens and their components. *Science* 294: 870-875.

Huang, W., Na, L., Fidel, P.L., and Schwarzenberger, P. (2004). Requirement of interleukin-17A for systemic anti-Candida albicans host defence in mice. *J. Infect. Dis.* 190: 624-631.

Hucka, M., et al. (2003). The systems biology markup language (SBML): a medium for representation and exchange of bio- chemical network models. *Bioinformatics* 19(4): 524-531.

Ichikawa, H.T., Williams, L.P., and Segal, B.M. (2002). Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J. Immunol*. 169: 2781–2787.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4(2): 249–264.

Janeway, C.A. Jr., and Medzhitov, R. (2002). Innate immune recognition. *Annu. Rev. Immunol.* 20: 197–216.

Janeway, C.A Jr., (1989). Autoimmune disease: immunotherapy by peptides? Nature 341(6242): 482-483.

Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F., and Lanzavecchia, A. (2001). Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur. J. Immunol.* 31: 3388–3393.

Jouault, T. *et al.* (2009). Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by Candida albicans. *Cell .Microbiol.* 11: 1007-1015.

Jouault, T. *et al.* (2003). Candida albicans phospholipomannan is sensed through Toll-like receptors. *J. Infect. Dis.* 188: 165–172.

Jouault, T. *et al.* (2006). Specific recognition of Candida albicans by macrophages requires galectin-3 to discriminate Saccharomyces cerevisiae and needs association with TLR2 for signalling. *J. Immunol.* 177: 4679–4687.

Kanehisa, M. *et al.* (2008). KEGG for linking genomes to life and the environment. *Nucleic acids research* 36: D480-484.

Kanneganti, T.D. *et al.* (2007). Intracellular NOD-like receptors in host defence and disease. *Immunity* 27: 549–559.

Kaufmann, S.H., and Schaible, U.E. (2003). A dangerous liaison between two major killers: Mycobacterium tuberculosis and HIV target dendritic cells through DC-SIGN. *J. Exp. Med.* 197(1): 1-5.

Kawai, T., and Akira, S. (2006). TLR signalling. *Cell Death Differ*. 13: 816–825.

Kawai, T., and Akira, S. (2007). TLR signalling. *Semin. Immunol.* 19: 24–32.

Kery, V., Krepinsky, J.J., Warren, C.D., Capek, P. and Stahl, P.D. (1992). Ligand recognition by purified human mannose receptor. *Arch. Biochem. Biophys.* 298: 49–55.

Khatri, P., Draghici, S., Ostermeier, G.C., and Krawetz, S.A. (2002). Profiling gene expression using onto-express. *Genomics* 79: 266–270.

Kitano, H., Funahashi, A., Matsuoka, Y., Oda, K. (2005). Using process diagrams for the graphical representation of biological networks. *Nat. Biotechnol.* 23(8): 961-966.

Kleine, H., *et al.* (2008). Substrate-Assisted Catalysis by PARP10 Limits Its Activity to Mono-ADP-Ribosylation. *Molecular Cell.* 32: 57–69.

Klis, F.M., de Groot, P. and Hellingwerf, K. (2001). Molecular organization of the cell wall of Candida albicans. *Med. Mycol.* 39: 1–8.

Kohn, K.W., Aladjem, M.I., Weinstein, J.N., and Pommier, Y. (2006). Molecular interaction maps of bioregulatory networks: a general rubric for systems biology. Mol Biol Cell. 17(1): 1-13.

Koppel, E.A., van Gisbergen, K.P., Geijtenbeek, T.B., and van Kooyk, Y. (2005). Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell Microbiol.* 7(2): 157-165.

Kreger-Van Rij, N. J. (1978). Electron microscopy of germinating ascospores of Saccharomyces cerevisiae. *Arch. Microbiol.* 117: 73-77.

Langrish, C.L. *et al.* (2004). IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol. Rev.* 202: 96–105.

Le Novère, N. *et al.* (2009). The Sytem Biology Graphical Notation. *Nat Biotechnol.* 27(8) : 735-741.

Leibundgut-Landmann, S. *et al.* (2007). SYK- and CARD9- dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nature Immunol*. 8: 630–638.

Lien, E., *et al.* (1999). Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem*. 274: 33419–33425.

Lioudyno, M.I., *et al.* (2008). Orai1 and STIM1 move to the immunological synapse and are up-regulated during T cell activation. *Proc. Natl. Acad. Sci. USA*. 12; 105(6): 2011-2016.

Liti, G., *et al.* (2009). Population genomics of domestic and wild yeasts. *Nature* 19; 458(7236): 337-341.

Lowman, D.W., Ferguson, D.A., Williams, D.L. (2003). Structural characterization of (1-->3)-beta-D-glucans isolated from blastospore and hyphal forms of Candida albicans. *Carbohydr. Res.* 4, 338(14), 1491-1496.

Maglott, D., Ostell, J., Pruitt, K.D. and Tatusova, T. (2005).Entrez Gene: gene-centered information at NCBI. *Nucleic acids research* 33: D54-58.

Maglott, D., Ostell, J., Pruitt, K.D. and Tatusova, T. 31 (2007).Entrez Gene: gene-centered information at NCBI. *Nucleic acids research* 35: D26.

Mangan, P.R. *et al.* (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231–234.

Manoli, T., *et al.* (2006). Group testing for pathway analysis improves comparability of different microarray datasets. *Bioinformatics* 22: 2500–2506.

Mansour, M.K., *et al.* (2002). Optimal T cell responses to Cryptococcus neoformans mannoprotein are dependent on recognition of conjugated carbohydrates by mannose receptors. *J. Immunol.* 168: 2872–2879.

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science* 296: 301–305.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature* 449(7164): 819-826.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews Immunology* 1, 135-145.

Mellman, I. and Steinman, R.M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.

Mencacci, A. *et al*. (1994). A mannoprotein constituent of Candida albicans that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. *Infect. Immun*. 62: 5353–5360.

Mezger, M., *et al.* (2008). Proinflammatory response of immature human dendritic cells is mediated by dectin-1 after exposure to Aspergillus fumigatus germ tubes. *J .Infect. Dis*. 97(6): 924-931.

Milner, J.D., *et al.* (2008). Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 10, 452(7188): 773-776.

Mitchell, A.P. (1998). Dimorphism and virulence in Candida albicans. *Curr. Opin. Microbiol.* 1: 687–692.

Montagnoli, C. *et al.* (2002). The interaction of fungi with dendritic cells: implications for Th immunity and vaccination. *Curr. Mol. Med.* 2: 485–505.

Moodie, S.L., S.A. Goryanin, I., Ghazal, P. (2006). A Graphical Notation to Describe the Logical Interactions of Biological Pathways. *Journal of Integrative Bioinformatics* 3(2): 11.

Mortimer, R.K., (2000). Evolution and variation of the yeast (Saccharomyces) genome. *Genome Res*. 10(4): 403-409. Erratum in: Genome Res Jun;10(6):891.

Muñoz, P., *et al.* (2005). Saccharomyces cerevisiae fungemia: an emerging infectious disease. *Clin. Infect. Dis*. 40: 1625-1634.

Nakamura, K., *et al*. (2006). Limited contribution of Toll-like receptor 2 and 4 to the host response to fungal infections pathogen, Cryptococcus neoformans. *FEMS Immunol. Med. Microbiol.* 47: 148-154.

Netea, M.G., *et al.* (2003). Aspergillus fumigatus evades immune recognition during germination through loss of TLR4-mediated signal transduction. *J. Infect. Dis.* 188: 320–326.

Netea, M.G., *et al.* (2006). Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* 116: 1642–1650.

Netea, M.G. *et al.* (2006). Recognition of fungal pathogens by Toll-like receptors. *Curr, Pharm. Des*. 12: 4195-4201.

Netea, M.G., Brown, G.D., Kullberg, B.J., and Gow, N.A. (2008). An integrated model of the recognition of Candida albicans by the innate immune system. *Nature reviews* 6: 67-78.

Newman, S.L., and Holly, A. (2001). Candida albicans is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect. Immun.* 69: 6813–6822.

Odds, F.C. *et al.* (2001). Fungal virulence studies come of age. *Genome Biol.* 2: 1009.

Ogura, Y., Sutterwala, F.S., and Flavell, R.A. (2006). The inflammasome: first line of the immune response to cell stress. *Cell.* 126: 659–662.

Piccini, A., *et al.* (2008). ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proc. Natl. Acad. Sci. USA.* 105: 8067–8072.

Pietrella, D., Bistoni, G., Corbucci, C., Perito, S. and Vecchiarelli, A. (2006). Candida albicans mannoprotein influences the biological function of dendritic cells. *Cell. Microbiol.* 8: 602–612.

Pulendran, B. *et al.* (2001). Sensing pathogens and tuning immune responses. *Science* 293: 253–256.

Ransohoff, D.F. (2004). Rules of evidence for cancer molecular-marker discovery and validation. *Nature Reviews Cancer* 4: 309–314.

Raza, S., *et al.* (2008). A logic-based diagram of signalling pathways central to macrophage activation. *BMC Syst. Biol.* 23: 2: 36.

Reid, D.M., Gow, N.A., Brown, G.D. (2009). Pattern recognition: recent insights from Dectin-1. *Curr. Opin. Immunol.* 21(1): 30-37.

Reis e Sousa, C. (2001). Dendritic cells as sensors of infection. *Cell* 14: 495–498.

Reis e Sousa, C. (2004). Activation of dendritic cells: translating innate into adaptive immunity. *Curr. Opin. Immunol.* 16: 21–25.

Richards, J.O. *et al.* (2001). Dendritic cells pulsed with Coccidioides immitis lysate induce antigen-specific naive T cell activation. J. Infect. Dis. 184(9): 1220-1224.

Richardson, M.D. (2005). Changing patterns and trends in systemic fungal infections. *J. Antimicrob. Chemother.* 56(Suppl 1): 5–11.

Robinson, M.J., et al. (2009). Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med*..206(9): 2037-2051.

Robinson, M.J., Sancho, D., Slack, E., LeibundGut-Landmann, S., and Reis e Sousa, C. (2006). Myeloid C-type lectins in innate immunity. *Nat. Immunology* 7(12): 1258-1265.

Roeder, A., *et al.* (2004) Toll- like receptors as key mediators in innate antifungal immunity. *Med. Mycol.* 42: 485–98.

Rogers, N.C. *et al.* (2005). SYK-dependent cytokine induction by dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22: 507–517.

Romagnani, S. (2006). Regulation of the T cell response Clinical and Experimental *Allergy* 36: 1357–1366

Romani, L. and Puccetti, P. (2007). Controlling pathogenic inflammation to fungi. *Expert review of anti-infective therapy* 5: 1007-1017.

Romani, L. (2002). Innate immunity against fungal pathogens. In Fungal Pathogenesis: Principles and Clinical Applications (Calderone, R.A. and Cihlar, R.L., eds), pp. 401–432, Marcel Dekker.

Romani, L. (2002). Overview of the fungal pathogens. In Immunology of Infectious Diseases (Kaufmann, S.H.E. et al., eds), pp. 25–37, ASMPress.

Romani, L. (2008). Cell mediated immunity to fungi: a reassessment. *Med. Mycol*. 46: 515-529.

Romani, L. *et al*. (1998). Immunity to fungi. *Res. Immunol.* 149: 277–281.

Roncarolo, M.G. and Levings, M.K. (2000). The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol.* 12: 676–683.

Roncarolo, M.G. *et al.* (2001). Differentiation of T regulatory cells by immature dendritic cells. *J. Exp. Med.* 193, F5–F9.

Rosenwald, A., *et al.* (2002). The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N. Engl. J. Med.* 346: 1937–1947.

Saeed, A.I., *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34: 374–378.

Sallusto, F., and Lanzavecchia, A. (1999). Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J. Exp. Med.* 189: 611–614.

Sallusto, F. and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of experimental medicine* 179: 1109-1118.

Salojin, K., and Oravecz, T. (2007). Regulation of innate immunity by MAPK dual-specificity phosphatases: knockout models reveal new tricks of old genes. *J. Leukoc. Biol.* 81(4): 860-869.

Salonen, J.H., *et al.* (2000). Fungal colonization of haematological patients receiving cytotoxic chemotherapy: emergence of azole-resistant Saccharomyces cerevisiae. *The Journal of hospital infection* 45: 293-301.

Sato, K., *et al.* (2006). Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor chain to induce innate immune responses. *J. Biol. Chem.* 281: 38854–38866.

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary dna microarray. *Science* 270(5235): 467–470.

Segal, E., Friedman, N., Kaminski, N., Regev, A., and Koller, D. (2005). From signatures to models: understanding cancer using microarrays. *Nat. Genet.* 37 Suppl: S38–S45.

Segal, E., Friedman, N., Koller, D., and Regev, A. (2004). A module map showing conditional activity of expression modules in cancer. *Nat. Genet.* 36: 1090–1098.

Segal, E., *et al.* (2003). Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. *Nat. Genet.* 34: 166–176.

Segota, I., Bartonicek, N., and Vlahovicek, K. (2008). MADNet: microarray database network web server. *Nucleic Acids Res.*; doi:10.1093/nar/gkn289.

Serrano-Gómez, D., Domínguez-Soto, A., Ancochea, J., Jimenez-Heffernan, J.A., Leal, J.A., and Corbí, A.L. (2004). Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin mediates binding and internalization of Aspergillus fumigatus conidia by dendritic cells and macrophages. *J. Immunol.* 173(9): 5635-5643.

Serrano-Gómez, D., Leal, J.A., and Corbí, A.L. (2005). DC-SIGN mediates the binding of Aspergillus fumigatus and keratinophylic fungi by human dendritic cells. *Immunobiology* 210(2-4): 175-83.

Sethi, N. and Mandell, W.. (1988). Saccharomyces fungemia in a patient with AIDS. *New York state journal of medicine* 88: 278-279.

Simon, R., Radmacher, M.D., Dobbin, K., McShane, L.M. (2003). Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J. Natl. Cancer. Inst.* 95: 14–18.

Smith, K.D. and Bolouri, H. (2005). Dissecting innate immune responses with the tools of systems biology. Curr. Opin. Immunol. 17, 49–54.

Soll, D.R. (2002). Molecular biology of switching in Candida. In Fungal Pathogenesis: Principles and Clinical Applications (Calderone, R.A. and Cihlar, R.L., eds), pp. 161–182, Marcel Dekker.

Stahl, P.D., Rodman, J.S., Miller, M.J. and Schlesinger, P. H. (1978). Evidence for receptor-mediated binding of. glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc. Natl Acad. Sci. USA* 75, 1399–1403.

Standaert-Vitse, A., *et al.* (2006). Candida albicans is an immunogen for anti-Saccharomyces cerevisiae antibody markers of Crohn's disease. *Gastroenterology* 130(6): 1764-1775.

Steinman, R.M., Hawiger, D., Nussenzweig, M.C. (2003). Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21: 685–711.

Steinman, R., and Cohn, Z. Identification of a novel cell type in peripheral lymphoid organs of mice. (1973). *J. Exp. Med.* 137: 1142-1162.

Stephenson, J.D. and Shepherd, V.L. (1987). Purification of the human alveolar macrophage mannose receptor. *Biochem. Biophys. Res. Commun.* 148: 883–889.

Sternberg, S. (1994). The emerging fungal threat. *Science* 266: 1632-1634.

Stockinger, S., and Decker, T. (2008). Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. *Immunobiol.* 213: 889–897.

Subramanian, A., *et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U S A* 102: 15545–15550.

Szatmari, I., *et al.* (2006). PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *J. Exp. Med*. 203(10) :2351—2362.

Szatmari, I., *et al*. (2007). PPARgamma regulates the function of human dendritic cells primarily by altering lipid metabolism. *Blood.* 110(9): 3271—3280.

Tada, H., *et al.* (2002). Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiol. Immunol.* 46: 503–512.

Tarca, A.L. *et al.* (2009).A novel signalling pathway impact analysis. *Bioinformatics* 25: 75-82.

Tavazoie, S., Hughes, J.D., Campbell, M.J., Cho, R.J., and Church G.M. (1999). Systematic determination of genetic network architecture. *Nat. Genet.* 22: 281–285.

Taylor, P.R., *et al.* (2002). The beta glucan receptor Dectin-1, is predomimantly expressed on the surface od cells of the monocyte/macrophage and neutrophil lineages. *J. Immunol.* 23: 901-944.

Thoma-Uszynski, S., *et al.* (2001). Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* 291: 1544–1547.

Thompson, A.J., and Locarnini, S.A. (2007) Toll-like receptors, RIG-Ilike RNA helicases and the antiviral innate immune response. *Immunol. Cell Biol.* 85: 435–445.

Thornton, B.P., Vetvicka, V., Pitman, M., Goldman, R.C., and Ross, G.D. (1996). Analysis of the sugar specificity and molecular location of the -glucan- binding lectin site of complement receptor type 3 (CD11b/CD18). *J. Immunol.* 156: 1235–1246.

Torosantucci, A., *et al.* (2005). A novel glyco-conjugate vaccine against fungal pathogens. *J. Exp. Med.* 202: 597–606.

Toshchakov, V., *et al.* (2002). TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat. Immunol.* 3: 392–398.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.

Underhill, D.M., and Ozinsky, A. (2002). Tolllikereceptors: key mediators of microbe detection. *Curr. Opin. Immunol.* 14: 103–110.

Urban, C.F., Reichard, U., Brinkmann, V., and Zychlinsky, A. (2008). Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol.* 8(4): 668-768.

Van't Veer, L.J., *et al.* (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415: 30–536.

van der Graaf, C.A., Netea, M.G., Verschueren, I., van der Meer, J.W.M., and Kullberg, B.J. (2005). Differential cytokine production and Toll-like receptor signalling pathways by Candida albicans blastoconidia and hyphae. *Infect. Immun.* 73(11): 7458-7464.

van der Veerdonk, F.L., Kullberg, B.J., van der Meer, J.W.M., Gow, N.A.R, and Netea, M.G. (2008). Host-microbe interactions: innate pattern recogni ti on of fungal pathogens. *Curr. Opin. Microbiol.* 11: 305–312.

van der Veerdonk, F.L., *et al.* (2009). The macrophage mannose receptor induces IL-17 response to Candida albicans. *Cell Host Microbe* 5: 1-12.

van Kooyk, Y. (2008). C-type lectins on dendritic cells: key modulators for the induction of immune responses. *Biochem. Soc. Trans.* 36(Pt 6): 1478-1481.

Vastrik, I., *et al.* (2007).Reactome: a knowledge base of biologic pathways and processes. *Genome biology* 8: R39.

Veldhoen, M. *et al.* (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.

Verducci, J.S., *et al.* (2006). Microarray analysis of gene expression: considerations in data mining and statistical treatment. *Physiol. Genomics* 25(3): 355–363.

Vulcano, M., *et al.* (2004). Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *Eur. J. Immunol.*. 1; 173(9): 5749-5756

Walhout, A.J. (2006) Unraveling transcription regulatory networks by protein-DNA and protein-protein interaction mapping. *Genome Res*. 16: 1445–1454.

Weis, W.I., Taylor, M.E., and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. *Immunol. Rev.* 163: 19–34.

Weiss, D.S., Henry, T., and Monack, D.M. (2007). Francisella tularensis: activation of the inflammasome. *Ann N Y Acad Sci*. 1105: 219–237.

Wheeler, R.T., and Fink, G.R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog.* 2: e35.

Wileman, T.E., Lennartz, M.R., and Stahl, P D. (1986). Identification of the macrophage mannose receptor as a 175-kDa membrane protein. *Proc. Natl Acad. Sci. USA* 83: 2501–2505.

Willment, J.A., and Brown, G.D. (2008). C-type lectin receptors in antifunga immunity. *Trends Microbiol*. 16: 27-32.

Woods, J.P. (2002). Dimorphism in human pathogens. In Fungal Pathogenesis: Principles and Clinical Applications (Calderone, R.A. and Cihlar, R.L., eds), pp. 99–113, Marcel Dekker.

Xu, L., Tan, A.C., Winslow, R.L., and Geman, D. (2008). Merging microarray data from separate breast cancer studies provides a robust prognostic test. *BMC Bioinformatics* 9: 125.

Xu, S., Huo, J., Lee, K.G., Kurosaki, T., and Lam, K.P. (2009). Phospholipase Cgamma2 is critical for Dectin-1-mediated Ca2+ flux and cytokine production in dendritic cells. *J. Biol. Chem.* 13; 284(11): 7038-7046.

Yadav, M., and Schorey, J.S. (2006) The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood.* 108(9): 3168-3175.

Yasukawa, H., *et al.* (2003). IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* 4: 551–556.

Yu, J., *et al.* (2007). Pathway analysis of gene signatures predicting metastasis of node-negative primary breast cancer. *BMC Cancer* 7: 182.

Zeeberg, B.R., *et al.* (2003). GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol.* 4: R28.

Zelensky, A.N., and Gready, J.E. (2005). The C-type lectin-like domain superfamily. *FEBS J.* 272: 6179-6217.

Appendix

Appendix A

The following is the text of the short description of the reaction involved in Dectin-1 signalling.

DECTIN-1 SIGNALLING

SENSING MODULE – SYK

- The 1,3-β-glucan particles binds to the C-type lectin Dectin-1 in glucan containing phagosome.
- The adaptor Dectin-1 dimerizes and becomes tyrosine phosphorilated.
- Syk binds to dectin-1 dimer and autophosphorilates.

TRANSDUCTION MODULE – NFkB - RELA

- Syk autophosphorilates and phosphorilates the transmembrane adaptor CARD9
- The effector molecule BLC10 binds to MALT1 by PLCG2 activity.
- After a conformational change CARD9 binds to the complex BLC10:MALT1.
- The complex binds to TRAF6.
- The protein TRAF6 binds to the serine/threonine kinases TAK1, TAB1, TAB2 (TRAF6:TAK1:TAB1:TAB2).
- The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) translocates from the cytoplasmic membrane to the cytosol.
- The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) binds the E2 ubiquitin ligases Uev1A and Ubc13 and the protein TRAF6 is ubiquitinated.
- The protein TRAF6 ubiquitinated controls the unknown process that leads to the activation of the serine/threonine kinaseTAK1 phosphorylated.
- The serine/threonine kinase TAK1-phosporilated phosphorilates the serine/threonine kinases IKK-alpha and IKK-beta of the complex IKK (IKK-alpha-IKK-beta-IKK-gamma)
- The phosphorylation of the transcriptional regulator IkB-alpha by the IKK complex phosphorylated leads the translocation of the general transcription factor NFkB p65 complexed with c-Rel into the nucleus.
- The transcriptional regulator IkB-alpha is ubiquitinated and degraded in the proteosome.

OUTCOME NFkB - RELA

- The transcription factor NFkB translocates in the nucleus.
- The transcription factor NFkB binds DNA and activates the transcription of the genes: IL-23A, IL-6, IL-2, IL-10, TNF-alpha, MIP2.

TRANSDUCTION MODULE – NFkB - RELB

- Syk autophosphorilates and phosphorilates the transmembrane adaptor CARD9
- The effector molecule BLC10 binds to MALT1 by PLCG2 activity..
- After a conformational change CARD9 binds to the complex BLC10:MALT1.
- The complex binds to TRAF6.
- The protein TRAF6 binds to the serine/threonine kinases TAK1, TAB1, TAB2 (TRAF6:TAK1:TAB1:TAB2).
- The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) translocates from the cytoplasmic membrane to the cytosol.
- The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) binds the E2 ubiquitin ligases Uev1A and Ubc13 and the protein TRAF6 is ubiquitinated.
- The protein TRAF6 ubiquitinated controls the unknown process that leads to the activation of the serine/threonine kinaseTAK1 phosphorylated.
- The serine/threonine kinase TAK1-phosporilated phosphorilates the serine/threonine kinases IKKa and IKKb of the complex IKK (IKK-alpha:IKK-beta:IKK-gamma)
- The phosphorylation of the transcriptional regulator IkB-alpha by the IKK complex phosphorylated leads the translocation of the general transcription factor NFkB complexed with RelB into the nucleus.
- The transcriptional regulator IkB-alpha is ubiquitinated and degraded in the proteosome.

OUTCOME NFkB-RELB

- The transcription factor NFkB translocates in the nucleus.
- The transcription factor NFkB complexed with RelB binds DNA and activates the transcription of the genes: CCL17, CCL22.
- The transcription factor NFkB complexed with RelB binds DNA and repress the transcription of the genes IL10, IL12A, IL12B, IL6, IL1B

TRANSDUCTION MODULE -ERK

- Syk autophosphorylates and phosphorylates the complex Grb2/Sos and PLCG
- The phosphorylated Grb2/Sos:PLCG2 complex phosphorylates the transcription factor ERK.
- The transcription factor ERK translocates into the nucleus.

OUTCOME ERK

- The transcription factor ERK translocates in the nucleus.
- The transcription factor ERK binds to AP1.
- AP1 binds to DNA and activates the transcription of the genes of the cytokines: IL-10.

TRANSDUCTION MODULE NFAT

- Syk autophosphorylates and phosphorylates PLC-g2.
- PLCG2 hydrolyzes the membrane phospholipid PIP_2 to IP_3 and DAG.
- IP_3 opens IP_3R which permits Ca^{2+} efflux from ER Ca^{2+} stores.
- The ER Ca^{2+} sensor STIM1 and STIM2 sense the resulting reduction of ER Ca^{2+} stores via their paired N-terminal EF hands located in the ER lumen.
- After Ca^{2+} dissociation from the EF, STIM proteins aggregate into small cluster in the ER membrane and trigger store-operated Ca^{2+} entry via the CRAC channel, ORAI1.
- Ca^{2+} influx elevates intracellular Ca^{2+} concentration and activates the calcineurin-NFAT pathway by dephosphorylation of NFAT.
- The dephosphorylated transcription factors NFATC1 and NFATC2 translocate into the nucleus.

OUTCOME NFAT

- The dephosphorylated transcription factor NFAT translocates into the nucleus.
- The transcription factor NFAT binds DNA and activates the transcription of the genes of the cytokines: EGR2, EGR3, COX2, IL2, IL10, PGE2, IL12.

TRANSDUCTION MODULE JNK

- Syk phosphorylates CARD9
- CARD9 binds BLC10 and MALT1
- With an unknown process, JNK phosporilates.

TRANSDUCTION MODULE p38

- Syk phosphorylates CARD9
- CARD9 binds BLC10 and MALT1
- With an unknown process, p38 phosporilates

SENSING MODULE – RAF1

- The 1,3-b-glucan particles binds to the C-type lectin Dectin-1 in glucan containing phagosome.
- The C-type lectin Dectin-1 dimerizes and becomes tyrosine phosphorilated.
- The C-Type lectin Dectin-1 dimer autophosphorilates.
- The C-type lectin Dectin-1 phosphorilates the MAP kinase Raf1 through Pac and Src kinases.

TRANSDUCTION MODULE - RAF1

The subunit RelB of the transcription factor NFkB complexes with the subunit RelA of the transcription factor NFkB.

The MAP kinase Raf1 acetylates NFkB RelA by CPB and p300

The inactive transcription factor NFkB translocates into the nucleus.

OUTCOME MODULE – RAF1

The transcription factor NFkB translocates into the nucleus.

The transcription factor NFkB induces the transcription of the genes IL12A, IL12B, IL1B, IL10, IL6 and represses the transcription of the gene IL23A.

Appendix B

The following is the text of the long description of the reaction involved in Dectin-1 signalling.

DECTIN-1

Dectin-1 is the archetypal signalling, non-Toll like pattern recognition receptor that play a protective role in immune defence to fungi. Dectin-1 was originally cloned as a dendritic cell surface molecule capable of delivering co-stimulatory signals to T cells (Ariizumi et al, 2000). It was subsequently shown to be expressed more widely on myeloid cells including macrophages, dendritic cells and neutrophils (Robinson et al, 2006; Taylor et al, 2002) and identified as a receptor for β-glucans (Brown and Gordon 2001). Dectin-1 specifically recognizes soluble or particulate β-1,3- and/or β-1,6-glucans, which are found primarily in the cells walls of fungi but also plants and some bacteria (fungi and micobacteria, Yadav and Schorey, 2006).

Dectin-1-deficiency is associated with impaired recruitment of inflammatory leukocytes and inflammatory mediator production at the site of infection. Myeloid cell activation by dectin-1 is controlled by inherent cellular programming, with distinct macrophage and dendritic cell populations responding differentially to the engagement of this receptor. The inflammatory response is further modulated by the progression of the phagocytosis, with 'frustrated phagocytosis' resulting in dramatically augmented inflammatory responses. Dectin-1 in isolation is sufficient to drive a potent inflammatory response in a context-dependent manner. This has implications for the mechanism by which myeloid cells are activated during fungal infections and the processes involved in the therapeutic manipulation of the immune system via exogenous dectin-1 stimulation or blockade.

Trough this novel pathway, dectin-1 also collaborates with the TLRs to induce pro-inflammatory responses, such as TNFα (Brown et al, 2006). The first evidence for collaboration between TLR2 and Dectin-1 in coordinating inflammatory gene induction came from studies with zymosan (Gantner et al, 2003; Brown et al, 2003). Dectin-1 and TLR2 signals have also been reported to contribute to cytokine induction by *A. fumigatus* conidia (Hohl et al, 2005; Steele et al, 2005; Gersuk et al, 2006).

Analysis of the transcriptional responses of macrophages and dendritic cells to fungi has revealed collaboration between TLR2 and Dectin-1 signalling to amplify the TLR2 response and promote the induction of proinflammatory cytokines.

How this collaboration takes place is unknown, as the read out is largely cytokine production as Dectin-1 and the TLR synergise/collaborate but it seems that it is not at signalling level.

The major work on understanding Dectin-1 recognition was done in mouse myeloid cells, bone marrow derived macrophage or bone marrow derived dendritic cells.

There are some evidences in human DCs. The involvement in this DCs was specified during the text. By contrast, if the interaction is proven only in BMDCs, this was not specificated.

Dectin-1 signalling

The 1,3-β-glucan particles binds to the C-type lectin Dectin-1. On ligand binding Dectin-1 dimerizes and becomes tyrosine phosphorylated, presumably by Src kinases and induces an intracellular signalling cascade that results in various cell-specific responses.

Dimerization is not known to be the specific trigger for Dectin-1 activation. In fact crosslinking with antibodies and non-particulate β-glucans do not stimulate the receptor (eg laminarin is a soluble b-glucan that is used as an antagonist). In short only particulate ligands have been shown to trigger Dectin-1. This mechanisms is known as 'frustrated phagocytosis' (Rosas et al, 2008). They propose that β-glucan particles are too big to be phagocytosed and act as Dectin-1 agonists. This was also shown by Caetano Reis e Sousa' s group (Hernanz-Falcon et al.,2009). In summary clustering of Dectin-1 by particulate β-glucans induces the intracellular signalling cascades. A robust cellular activation leading to gene transcription may only be achieved if the particle is too large to be phagocytosed.

Despite requiring only the membrane-proximal tyrosine for signalling, the cytoplasmic tail of dectin-1 , the ITAM domain can interact with spleen tyrosine kinase, Syk. The nature of this interaction is unknown but it is proposed to occur to the bridging of two dectin-1 molecules (Rogers et al., 2005). Whereas interactions with Syk can directly induce cellular responses, such as respiratory burst and IL-10 IL-2, TNFα and IL-6 production (Leibundgut-Landmann S 2007). Syk contributes to phagocytosis of zymosan particles in DCs.

Trough this novel pathway, dectin-1 also collaborates with the TLRs to induce pro-inflammatory responses, such as TNFα (Brown et al, 2006).

Besides Syk, Dectin-1 induces a second signalling pathway trough the kinase Raf1 that was independent of the Syk pathway but converges at the level of NFkB activationto control adaptive immunity to fungi. Moreover, Dectin-1 cross-talk with various TLRs was dependent on Raf-1 signalling.

Dectin-1 can induce a robust TNF response in DCs without TLR enagaement (Leibundgut-Landmann et al, 2007). This underlines a major feature of Dectin-1 signalling in that it differs greatly between cell types. Therefore although DCs and macrophages are closely related the outcomes of Dectin-1 triggering on these two cell types differs greatly. This is best studied by Rosas et al (2008). So one cannot make assumptions from one cell type to another. Goodridge et al (2009) have recently expanded on this showing the coupling of Card9 to NFkB is cell type dependent. Here they show the split is not Macrophage Vs DC but also between sub-population of macrophages and DCs.

In summary, the ability of Dectin-1 to engage signals that lead to gene transcription (eg TNF) varies greatly between different sub-populations of macrophages and DCs. This may be governed by the ability of Card9 to induced NFkB. Curdlan stimulation induced also rapid Syk-dependent phosphorylation of the mitogen-activated protein kinases p38, Erk and Jnk.

In cells where Dectin-1 does not trigger a robust gene induction, it is still able to potently syngerise with TLRs.

SENSING MODULE - SYK

The 1,3-β-glucan particles binds to the C-type lectins Dectin-1 in glucan containing phagosome (see frustrated phagocytosis comment above). On ligand binding Dectin-1 dimerizes (see dimerisation comment above) and becomes tyrosine phosphoryilated. Syk is recruited by a tyrosine phosphorylation in the ITAM portion of receptor (Rogers et al 2005; Gantner et al, 2006).

Usually, dually phosphorylated ITAM domains recruit tandem SH2 containing kinase of the Syk family (Underhill et al, 2005, Rothfuchs et al, 2007). Although Dectin-1 has a dual tyrosine motif, only tyrosine 15 is required. This differs from all other ITAMs (Rogers et al 2005). This binding triggers autophosphorylation (Turner et al., 2000). The kinase Syk was recruited.

Ref: Gantner et al, 2006; Rogers et al, 2005; Underhill et al, 2005, Rothfuchs et al, 2007; Futterer et al., 1998

TRANSDUCTION MODULE SYK – NFkB-RELA

Following ligation of Dectin-1, Syk kinase I activated and phosphorylates a number of substrates. This leads to recruitment of phospholipase Cγ-2 (PLCγ-2, Xu et, 2009) contributing to its activation and production of inositol bisphosphate and diacylglycerol (DAG, Schulze-Luehrmann et al, 2006). Syk also mediates activation of the PI-3K pathway as measured by phosphorylation of protein kinase B, PKB, a downstream substrate of PI-3K (unpublished data, Dennehy and Brown, 2007). DAG or PI-3K products contribute to the activation of PKC isoforms. Card9 is shown to be important for Dectin-1 signalling and it was further proposed that during Dectin-1 signalling, a Card9-Bcl10-Malt1 complex was formed instead to activate NF-κB, (Gross et al, 2006; Xu et al, 2009).

PLCγ-2 signalling might be important to induce the association of Malt1 with Bcl10 but not Card9 with Bcl10. Although it's not known the hierarchy of the interactions of these three proteins, there are indications that PLCγ2 signalling is required for the proper stoichiometry and stable assembly of Card9-Bcl10-Malt1 complex in Dectin-1 signalling. Thus PLCγ2 plays an important role in coupling Dectin-1 recognition of fungal PAMPs to the activation of NF-κB in BMDCs via inducing the assembly of the Card9-Bcl10-Malt1 complex and the subsequent activation of IKKα/β and degradation of IκB proteins.

In particular, Syk phosphorylates the transmembrane adaptor caspase recruitment domain 9 CARD9 (Gross et al., 2006). The effector molecule BLC10 binds to the mucosa-associated lymphoid tissue 1 (MALT1) through PLCγ-2 activity. After a conformational change, CARD9 binds to the complex BCL10:MALT1 (Xu et al 2009). The complex binds the TNF receptor-associated factor 6 (TRAF6) (Rawlings et al 2006).

The TRAF6 complex binds to the IkB kinases leading to phosphorylation and degradation of IkB and NFkB activation by formation of the complex RelA-cRel-p52.

There are no evidences of TRAF6 engagement downstream of Dectin-1. If this is an extension from other cell types it can't necessarily be applied here. Therefore the gaps could fill to NFkB via TRAF6 and the downstream effectors listed below. It's not known at the moment if there is a direct link between TRAF6 and NFkB activation.

Maybe it's happen as TRAF6 signalling in TLR cascade, where
the protein TRAF6 binds to the serine/threonine kinases TAK1, TAB1, TAB2 (TRAF6:TAK1:TAB1:TAB2).
The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) translocates from the cytoplasmic membrane to the cytosol.
The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) binds the E2 ubiquitin ligases Uev1A and Ubc13 and the protein TRAF6 is ubiquitinated.
The protein TRAF6 ubiquitinated controls the unknown process that leads to the activation of the serine/threonine kinaseTAK1 phosphorylated.
The serine/threonine kinase TAK1-phosporylated phosphorylates the serine/threonine kinases IKKα and IKKβ of the complex IKK (IKKα-IKKβ-IKKγ)
The phosphorylation of the transcriptional regulator IκBα by the IKK complex phosphorylated leads the translocation of the transcription factor NFκB into the nucleus.
The transcriptional regulator IκBα is ubiquitinated and degraded in the proteosome.
The TRAF6 complex binds to the IkB kinases leading to phosphorylation and degradation of IkB and NFkB activation
The phosphorylation of the transcriptional regulator IκBα by the TRAF6 complex leads the translocation of the general transcription factor NFκB into the nucleus.
The transcriptional regulator IκBα is ubiquitinated and degraded in the proteosome.

Ref: Gross et al., 2006; Rawlings et al 2006; Dennehy and Brown 2007, Xu et al, 2009, Gringhuis et al., 2009.

OUTCOME NFκB - RELA

The transcription factor NFκB translocates in the nucleus.
The transcription factor NFκB binds DNA and activates the transcription of the genes: IL23A, IL6, IL2, IL10, TNα, MIP2 .

The IL-23 production is proven also in moDCs (Gerosa et al, 2008). moDCs in response to β-glucan produce also IL-10, TNFα, and IL-1β (Carmona et al, 2006)but it is not known the involvement of the transcription factor NF-kB.

Ref: Leibundgut-Landmann S et al, 2007; Gantner et al, 2003; Rogers et al, 2005; Ariizumi et al, 2000, Goodridge et al 2009, Gerosa et al, 2008, Carmona et al, 2006.

TRANSDUCTION MODULE SYK – NFkB- RELB

Besides the activation of canonical NFkB subunits p65 (RelA) and c-Rel, dectin-1 induced Syk signalling activates the non canonical NFkB subunit RelB, making dectin-1 the only know pathogen recognition receptor to induce the non canonical NFkB pathway by mediating the association between RelB and p52.

The mechanism under this activation in unknown. Problably the mechanism is similar to the classical NFkB activation, where:
In particular, Syk phosphorylates the transmembrane adaptor caspase recruitment domain 9 CARD9 (Gross et al., 2006). The effector molecule BLC10 binds to the mucosa-associated lymphoid tissue 1 (MALT1) through PLCγ-2 activity. After a conformational change, CARD9 binds to the complex BCL10:MALT1 (Xu et al 2009). The complex binds the TNF receptor-associated factor 6 (TRAF6) (Rawlings et al 2006).
The TRAF6 complex binds to the IkB kinases leading to phosphorylation and degradation of IkB and NFkB activation by formation of the complex RelB-p52.

There are no evidences of TRAF6 engagement downstream of Dectin-1. If this is an extension from other cell types it can't necessarily be applied here. Therefore the gaps could fill to NFkB via TRAF6 and the downstream effectors listed below. It's not known at the moment if there is a direct link between TRAF6 and NFkB activation.

Maybe it's happen as TRAF6 signalling in TLR cascade, where
the protein TRAF6 binds to the serine/threonine kinases TAK1, TAB1, TAB2 (TRAF6:TAK1:TAB1:TAB2).
The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) translocates from the cytoplasmic membrane to the cytosol.
The complex of the serine/threonine kinases TAB1-TAB2 phosphorylated-TAK1 phosphorylated and the protein TRAF6 (TAB1:TAB2-p:TAK1-p:TRAF6) binds the E2 ubiquitin ligases Uev1A and Ubc13 and the protein TRAF6 is ubiquitinated.
The protein TRAF6 ubiquitinated controls the unknown process that leads to the activation of the serine/threonine kinaseTAK1 phosphorylated.
The serine/threonine kinase TAK1-phosporylated phosphorylates the serine/threonine kinases IKKα and IKKβ of the complex IKK (IKKα-IKKβ-IKKγ).
The phosphorylation of the transcriptional regulator IκBα by the IKK complex phosphorylated leads the translocation of the transcription factor NFκB into the nucleus.
The transcriptional regulator IκBα is ubiquitinated and degraded in the proteosome.
The TRAF6 complex binds to the IkB kinases leading to phosphorylation and degradation of IkB and NFkB activation
The phosphorylation of the transcriptional regulator IκBα by the TRAF6 complex leads the translocation of the general transcription factor NFκB into the nucleus.
The transcriptional regulator IκBα is ubiquitinated and degraded in the proteosome.

Ref: Hayden et al., 2004, Gringhuis et al., 2009

OUTCOME SYK - NFkB - RELB

The non canonical NFkB transcription factor acts as a negative regulator of transcription of the immune genes, balancing the response and the Th1/Th17 polarizing factor production.

The subunits of the transcription factor NFkB, RelB-p52 translocate into the nucleus.
The subunits of the transcription factor NFkB, Rel B-p52 induce the transcription of the genes CCL17 and CCL22 and represses the transcription of the genes IL12A, IL12B, IL1B, IL10, IL6.

TRANSDUCTION MODULE ERK

Besides inducing NF-κB, Dectin-1 also activates MAPKs, which are important for signalling the production of inflammatory cytokines in BMDCs.
In TLR signalling, the activation of ERK and JNK can lead to the induction of the transcription factor AP-1 that contributes substantially to the production of inflammatory cytokines (Vanden Bush et al, 2008: Agrawal et al, 2003). Consistent with this, the engagement of Dectin-1 by curdlan could lead to the induction of AP-1 as demonstrated in BMDCs. The signal transduced by PLCγ2 is required to activate AP-1 during Dectin-1 signalling (Xu et al, 2009).
It has been clearly showed that zymosan activation of ERK in DCs is Syk dependent (Slack EC et al 2007). The inference of this is that this will also be true of Dectin-1. It was clearly shown that this mechanisms is Myd88 and TPL2 independent.

Syk autophosphorylates and phosphorylates the complex Grb2/Sos and PLCγ–2
The phosphorylation of the Grb2/Sos:PLCγ–2 complex leads to ERK phosphorilation (Xu et al, 2009) and the translocation of the MAPK ERK into the nucleus.

Ref. Vanden Bush et al, 2008; Agrawal et al, 2003; Slack et al 2007; Xu et al., 2009

OUTCOME ERK

The transcription factor ERK translocates in the nucleus.
The transcription factor ERK binds to AP1 (Xu et al, 2009) .
AP1 binds to DNA and activates the transcription of the genes of the cytokines: IL-10.
ERK is also required to stabilize IL-2 mRNA (Slack et al, 2007).

Ref. Slack EC et al 2007, Xu et al, 2009.

TRANSDUCTION MODULE JNK

Besides inducing NF-κB, Dectin-1 also activates MAPKs, which are important for signalling the production of inflammatory cytokines in BMDCs.
In TLR signalling, the activation of ERK and JNK can lead to the induction of the transcription factor AP-1 that contributes substantially to the production of inflammatory cytokines (34, 35).

Consistent with this, the engagement of Dectin-1 by curdlan could also lead to the induction of AP- 1 as demonstrated by the robust binding of consensus DNA sequences by nuclear extract prepared from wildtype BMDCs. In contrast, the activation of AP-1 was minimal in curdlan treated plcγ2-/- BMDCs, suggesting that signal transduced by PLCγ2 is required to activate AP-1 during Dectin-1 signalling (Xu et al, 2009).

Syk phosphorylates the transmembrane adaptor caspase recruitment domain 9 CARD9 (Gross et al., 2006). After a conformational change, CARD9 binds the downstream effector molecule BLC10 and the mucosa-associated lymphoid tissue 1 (MALT1) (Ruland, 2008).
The process leading to JNK phosphorylation is unknown.

Ref. Leibundgut-Landmann S et al, 2007, Xu et al, 2009.

TRANSDUCTION MODULE p38

CARD9 in macrophages is recruited to phagosomes where it coordinates signalling to p38 MAPK, even in the absence of a connection to the NF-kB pathway. It is the same in DCs.

Syk phosphorylates the transmembrane adaptor caspase recruitment domain 9 CARD9 (Gross et al., 2006). After a conformational change, CARD9 binds the downstream effector molecule BLC10 and the mucosa-associated lymphoid tissue 1 (MALT1) (Ruland, 2008).
The process leading to p38 phosphorylation is unknown.

Ref. Leibundgut-Landmann et al, 2007 Goodridge et al, 2009

TRANSDUCTION MODULE NFAT

Zymosan and live C. albicans triggers NFAT activation trough calcineurin pathway such as for NFAT activation in lymphocytes (Goodridge et al, 2007). This activation is PLCγ-2 dependent (Xu et al, 2009).

Syk autophosphorylates and phosphorylates PLCγ-2 (Xu et al 2009). PLCγ-2 hydrolyzes the membrane phospholipids PIP_2 to IP_3 and DAG.
IP_3 opens IP_3R which permits Ca^{2+} efflux from ER Ca^{2+} stores (first evidence of this flux, Xu et al, 2009)
The ER Ca^{2+} sensor STIM1 and STIM2 sense the resulting reduction of ER Ca^{2+} stores via their paired N-terminal EF hands located in the ER lumen.
After Ca^{2+} dissociation from the EF, STIM proteins aggregate into small cluster in the ER membrane and trigger store-operated Ca^{2+} entry via the CRAC channel, ORAI1.
Ca^{2+} influx elevates intracellular Ca^{2+} concentration and activates the calcineurin-NFAT pathway by dephosphorylation of NFAT.
The dephosphorylated transcription factors NFATc1 and NFATc3 translocate into the nucleus.

Ref. *Goodridge et al, 2007,* Xu et al 2009

OUTCOME NFAT

The dephosphorylated transcription factor NFATc1 and NFATc3 translocate into the nucleus.
The transcription factor NFATc1 and NFATc3 binds DNA and activates the transcription of the genes: EGR2, EGR3, COX2, IL2, IL10, PGE2, IL12.

Ref. Goodridge et al 2007; Xu et al 2009

SENSING MODULE RAF1

Besides Syk, Dectin-1 induces a second signalling pathway trough the kinase Raf1 that was independent of the Syk pathway but integrated with it at the level of NFkB activation. The dectin-1 induced Raf-1 signalling. repressed the Syk-induced RelB activity and increased p65 transcription activityto induce Th1- and Th17-polarizing cytokines in response to both curdlan and C. albicans. Moreover, Dectin-1 cross-talk with various TLRs was dependent on Raf-1 signalling.

The 1,3-β-glucan particles binds to the C-type lectins Dectin-1 in glucan containing phagosome (see frustrated phagocytosis comment above). On ligand binding Dectin-1 dimerizes (see dimerisation comment above) and becomes tyrosine phosphoryilated. Syk is recruited by a tyrosine phosphorylation in the ITAM portion of receptor (Rogers et al 2005; Gantner et al, 2006).
Usually, dually phosphorylated ITAM domains recruit tandem SH2 containing kinase of the Syk family (Underhill et al, 2005, Rothfuchs et al, 2007). Although Dectin-1 has a dual tyrosine motif, only tyrosine 15 is required. This differs from all other ITAMs (Rogers et al 2005). This binding triggers autophosphorylation (Turner et al., 2000). The kinase Raf1 is recruited.

Ref: Gantner et al, 2006; Rogers et al, 2005; Underhill et al, 2005, Rothfuchs et al, 2007; Futterer et al., 1998; Gruighuis et al., 2009-06-10

TRANSDUCTION MODULE Raf1

Dectin-1 induced Raf1-signalling repressed Syk-induced RelB activity and increased p65 transactivation activity to induce cytokine expression. Raf1 signalling phosphorylates RelA/p65 at Ser276, which regulates cytokine expression at two distinct levels: RelB inactivaition and p65 acetylation by the HAT CBP and p300.
The formed p65-RelB complex of the transcription factor NKkB translocates into the nucleus.

OUTCOME MODULE RAF1

The formed p65-RelB complex of the transcription factor NKkB translocates into the nucleus.
The formed p65-RelB complex of the transcription factor NKkB activates the transcription of the genes IL12A, IL12B, IL1B, !L10, IL6 and represses the transcription of the gene IL23A.

REFERENCES:

Agrawal, S., Agrawal, A., Doughty, B., Gerwitz, A., Blenis, J., Van, D. T., and Pulendran, B. (2003) *J. Immunol.* 171, 4984-4989

Ariizumi K, Shen GL, Shikano S, Xu S, Ritter R, Kumamoto T, Edelbaum D, Morita A, Bergstresser Pr, Takashima A, 2000. *Critical role of myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase in high burden systemic infection of mice with* Candida albicans. J Infect Dis 185: 1833-1837.

Brown GD and Gordon S, 2001. *Immune recognition. A new receptor for beta-glucans*. Nature 413:36-37.

Carmona EM, Vassallo R, Vuk-Pavlovic Z, Standing JE, Kottom TJ, Limper AH. *Pneumocystis cell wall beta-glucans induce dendritic cell costimulatory molecule expression and inflammatory activation through a Fas-Fas ligand mechanism.* J Immunol. 2006 Jul 1;177(1):459-67

Dennehy KM, Brown GD, 2007. *The role of beta glucan receptor Dectin-1 in control of fungal infection*. J Leukocyte Biology 82:253-258

Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, Kasprowicz DJ, Kellar K, Pare J, van Dyke T, Ziegler S, Ununtmaz D, Polendran B, 2006. *Yeast zymosan, a stimulus for TLR2 and Dectin-1, induces regulatory antigen presenting cells and immunological tolerance*. J Clin Invest 116:916-928

Fütterer K, Wong J, Grucza RA, Chan AC, Waksman G. *Structural basis for Syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide*.J Mol Biol. 1998 Aug 21;281(3):523-37

Gantner BN, Simmons RM, Underhill DM, 2006. *Dectin-1 mediaes macrophage recognition of Candida albicans yeast but not filaments*. EMBO J 24:1277-1286.

Gantner BN, Simmons, Canavera SJ, Akira S, Underhill DM, 2003. *Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2*. J. Exp Med 197: 1107-1117

Gerosa F, Baldani-Guerra B, Lyakh LA, Batoni G, Esin S, Winkler-Pickett RT, Consolaro MR, De Marchi M, Giachino D, Robbiano A, Astegiano M, Sambataro A, Kastelein RA, Carra G, Trinchieri G. *Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells.* J Exp Med. 2008 Jun 9;205(6):1447-61

Goodridge H, Simmons R, Underhill DM, 2007 *Dectin-1 stimulation by Candida albicans triggers NFAT activation in macrophages and dendritic cells.* J immunol

Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, Ruland J, 2006. *Card9 controls non-TLR signalling pathway for innate anti-fungal immunity*. Nature 442:651-656.

Hara H, Ishihara C, Takeuchi A, Imanishi T, Xue L, Morris SW, Inui M, Takai T, Shibuya A, Saijo S, Iwakura Y, Ohno N, koseki H, Yoshida H, Penninger JM, Saito T, 2007. *The adaptor protein CARD9 is essential for the activation of myeloid cells trough ITAM-associated and Toll-like receptors*. Nature Immunology,

Leibundgut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown Gd, Ruland J, Reis e Sousa G, 2007. *Syk and CARD9- dependent coupling of innate immunity to the induction ot T helper cells that produce interleukin 17*. Nat Immunol

Oh-hora M, Rao A, 2008. *Calcium signalling in lymphocytes*. C Opinion Immunol 20:250-258

Rawlings DJ, Sommer K, Moreno-García ME. *The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes*. Nat Rev Immunol. 2006 Nov;6(11):799-812.

Robinson MJ, Sancho D, Slack E, LeibundGut-Landmann S, Reis e Sousa C, 2006. *Myeloid C-type lectins in innate immunity*. Nature immunology 7

Rogers NC, Slack EC, Edwards AD, Nolte MA, Shulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD, Reis e Sousa C, 2005. *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins*. Immunity 22:507-517.

Rothfuchs AG, Bafica A, Feng CG, Egen JG, Williams DL, Brown GD, Sher A. *Dectin-1 interaction with Mycobacterium tuberculosis leads to enhanced IL-12p40 production by splenic dendritic cells*. J Immunol. 2007 Sep 15;179(6):3463-71.

Ruland J. *CARD9 signalling in the innate immune response.* Ann N Y Acad Sci. 2008 Nov;1143:35-44

Shin DM, Yang CS, Yuk JM, Lee JY, Kim KH, Shin SJ, Takahara K, Lee SJ, Jo EK, 2008. *Mycobacterium abscessus activates macrophage immune response via a physical and functional interaction between TLR2 and dectin-1*. Cell Microbiol 10:1608-1621.

Slack EC, Robinson MJ, Hernanz-Falcon p, Brown G, Williams DL, Schweighoffer E, Tybulewicz, Reis e Sousa C, 2007. *Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulaed with zymosan*. Eur J Immunol 37:1600-1612

Suram S, Brown GD, Ghosh M, Gordon S, loper R, Taylor PR, Akira S, Uematsu S, Williams DL, Leslie CC, 2006. *Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor*. J Biol Chem 281:5506-5514.

Taylor PR, Brown Gd, Reid DM, Willment JA, Martinez-Pomares L, Gordon S, Wong SY, 2002. *The beta glucan receptor Dectin-1, is predomimantly expressed on the surface od cells of the monocyte/macrophage and neutrophil lineages*. J Immunol 23:901-944.

Underhill DM, Rossnagle E, Lowell CA, Simmons RM. *Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production*. Blood. 2005 Oct 1;106(7):2543-50. Epub 2005 Jun 14.

Yadav M, Schorey JS. *The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria*. Blood. 2006 Nov 1;108(9):3168-75. Epub 2006 Jul 6.

Vanden Bush, T. J. and Bishop, G. A. (2008) *Eur. J. Immunol.* 38**,** 400-409

Appendix C

The following is the gene list of the components involved in Dectin-1 signalling, dissected in modules.

Sensing/Receptor Module - SYK

Gene Symbol	Official Name	GeneID
DECTIN-1	CLEC7A	64581
SYK	SYK	6850

Sensing/Receptor Module - RAF

Gene Symbol	Official Name	GeneID
DECTIN-1	CLEC7A	64581
RAF1	RAF1	5894

Transduction module - NFkB

Gene Symbol	Official Name	GeneID
SYK	SYK	6850
CARD9	CARD9	64170
BCL10	BCL10	8915
MALT1	MALT1	10892
TRAF6	TRAF6	7189
TAB1	MAP3K7IP1	10454
TAB2	MAP3K7IP2	23118
TAK1	MAP3K7	6885
IKKg	IKBKG	8517
IKKa	CHUK	1147
IKKb	IKBKB	3551
IkBa	NFKBIA	4792
NF-kB	NFKB1	4790
NF-kB	RELA	5970
cREL	REL	5966
RELB	RELB	5971

Transduction module - ERK

Gene Symbol	Official Name	GeneID
SYK	SYK	6850
GRB2	GRB2	2885
SOS2	SOS2	6655
PLCγ	PLCG1	5335
ERK	MAPK1	5594

Outcome module - ERK

Gene Symbol	Official Name	GeneID
ERK	MAPK1	5594
IL2	IL2	3558
IL10	IL10	3586

Outcome module - NFkB RELA

Gene Symbol	Official Name	GeneID
NF-κB	NFKB1	4790
NF-κB	RELA	5972
IL2	IL2	3558
IL10	IL10	3586
IL6	IL6	3569
IL23	IL23A	51561
TNF	TNF	7124
MIP2	CXCL2	2920

Transduction module - NFAT

Gene Symbol	Official Name	GeneID
SYK	SYK	6850
PLCγ	PLCG1	5335
STIM1	STIM1	6786
STIM2	STIM2	57620
ORAI1	ORAI1	84876
NFATC1	NFATC1	4273
NFATC2	NFATC2	4772
IP3R	ITFR1	3708
calcineurin	PP3CB	5532
calcineurin	PP3CA	3330

Outcome module - NFkB RELB

Gene Symbol	Official Name	GeneID
NF-κB p50	NFKB1	4790
NF-κB p65	RELB	5971
IL1B	IL1B	3553
CCL17	CCL17	6361
CCL22	CCL22	6367
IL12A	IL12A	3592
IL12B	IL12B	3593
IL1B	IL1B	3553
IL10	IL10	3586
IL6	IL6	3569

Outcome module - NFAT

Gene Symbol	Official Name	GeneID
NFAT1	Nfatc2	18019
EGR2	EGR2	1959
EGR3	EGR3	1960
COX2	PTGS2	5743
IL2	IL2	3558
IL10	IL10	3586

Outcome module - RAF

Gene Symbol	Official Name	GeneID
NF-κB p50	NFKB1	4790
NF-κB p65	RELA	5970
RELB	RELB	5971
IL12A	IL12A	3592
IL12B	IL12B	3593
IL1B	IL1B	3553
IL10	IL10	3586
IL6	IL6	3569
IL23	IL23A	51561

Transduction module - JNK

Gene Symbol	Official Name	GeneID
SYK1	SYK	6850
CARD9	CARD9	64170
BCL10	BCL10	8915
MALT1	MALT1	10892
JNK	MAPK8	5599

Transduction module - RAF

Gene Symbol	Official Name	GeneID
NF-κB p50	NFKB1	4790
NF-κB p65	RELA	5970
CBP	CREBBP	1387
p300	EP300	2033
RELB	RELB	5971

Transduction module - p38

Gene Symbol	Official Name	GeneID
SYK1	SYK	6850
CARD9	CARD9	64170
BCL10	BCL10	8915
MALT1	MALT1	10892
p38	MAPK14	1432

When your heart speaks, take good notes.
Judith Campbell

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