Differential IL-17 Production and Mannan Recognition Contribute to Fungal Pathogenicity and Commensalism

Lisa Rizzetto, Mirela Kuka, Carlotta De Filippo, Alessandra Cambi, Mihai G. Netea, Luca Beltrame, Giorgio Napolitani, Maria Gabriella Torcia, Ugo D'Oro and Duccio Cavalieri

J Immunol 2010;184;4258-4268; Prepublished online 12 March 2010;
doi:10.4049/jimmunol.0902972
http://www.jimmunol.org/content/184/8/4258

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Differential IL-17 Production and Mannan Recognition Contribute to Fungal Pathogenicity and Commensalism

Lisa Rizzetto,*[,1] Mirela Kuka,[†,1] Carlotta De Filippo,* Alessandra Cambi,[‡] Mihai G. Netea,[§] Luca Beltrame,* Giorgio Napolitani,[¶] Maria Gabriella Torcia,[‖] Ugo D'Oro,[†] and Duccio Cavalieri*

In this study, we present evidence of differential Th17 responses in human monocyte-derived dendritic cells exposed to the pathogenic *Candida albicans* or the nonpathogenic *Saccharomyces cerevisiae.* We use different forms of the microorganisms, cells, hyphae, and spores, as a toolbox to dissect the role of surface mannan in the fungal immune response. In contrast to the *S. cerevisiae* yeast cell-induced Th1 response, dendritic cells stimulated with spores or *C. albicans* hyphae induce cellular responses shifted toward Th17 differentiation. The differential recognition of specific mannan structures is the master regulator of the discrimination between harmful and harmless fungi. The switch between spores and yeast is crucial for the commensalism of *S. cerevisiae* and depends on the use of a different receptor repertoire. Understanding the role of cell wall recognition during infection might lead to understanding the boundaries between safety and pathogenicity. ***The Journal of Immunology*****, 2010, 184: 4258–4268.**

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 (IR). The innate immune system is crucial in forming the antifungal response, initiated with the processing and presentation of Ags by dendritic cells (DCs). The induction and modulation of DCs depend largely on the sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Depending on which PRR is engaged, DCs can release different polarizing factors capable of instructing the differentiation of the T cell type to better cope with the invading pathogen (1). The balance between pro- and anti-inflammatory signaling is a prerequisite for successful host–fungal interactions (2).

DC maturation can be induced by stimulation with yeast extract, suggesting that exposure to yeasts/fungi has been one of the major forces driving the evolution of the DC-mediated IR.

Although 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 nonpathogenic fungi is a prerequisite for controlling fungal infections. Of the multitude of fungal species known, only a few can be considered truly pathogenic in healthy individuals.

The best-studied fungal microorganism is *Saccharomyces cerevisiae*, a ubiquitous yeast used by the food industry in the production of food, wines, and beer (3).

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 (4). In addition to *Candida albicans*, several strains of the *S. cerevisiae* species have been isolated from immunocompromised individuals, such as cystic fibrosis or AIDS patients (5, 6). 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 microorganism, be it a pathogen or a commensal, is still primarily determined by the interaction between the microorganism and the 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 the macrophage mannose receptor (MR), specifically mediate *C. albicans* binding and internalization by DCs (7–9). Moreover, upon *C. albicans* stimulation, the direct recognition through the MR is sufficient for inducing a Th17 response in PBMCs (10).

The *S. cerevisiae* cell wall is almost exclusively composed of 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 mannan derived from *C. albicans* (CA-mannan), but not mannan derived from *S. cerevisiae* (SC-

*Department of Preclinic and Clinic Pharmacology and [‖]Department of Clinic Physiopathology, University of Firenze, Firenze; [†]Novartis Vaccines, Siena, Italy; [‡]Department of Tumor Immunology, Nijmegen Center for Molecular Life Science, Radboud University Nijmegen Medical Centre; [§]Department of Medicine and Nijmegen Institute for Infection, Inflammation and Immunity, Radboud University, Nijmegen, The Netherlands; and [¶]Institute of Research in Biomedicine, Bellinzona, Switzerland

[1]L.R. and M.K. contributed equally to this work.

Received for publication September 9, 2009. Accepted for publication February 12, 2010.

This work was supported by funding from the European Community's Project FP6 DC-THERA EU LSHB-CT-2004-512074 and Project FP7 SYBARIS (Grant Agreement 242220) and by Grant MIUR PRIN 519MIUR060. A.C. was supported by Veni Grant 916.66.028 from The Netherlands Organization of Scientific Research. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Address correspondence and reprint requests to Dr. Duccio Cavalieri, University of Firenze, Viale Pieraccini 6, Firenze 50139, Italy. E-mail address: duccio.cavalieri@unifi.it

The online version of this article contains supplemental material.

Abbreviations used in this paper: CA-mannan, mannan derived from *Candida albicans*; DC, dendritic cell; DEG, differentially expressed gene; DPI, diphenyleneiodium chloride; FC, fold change; IBD, inflammatory bowel disease; IR, immune response; moDC, monocyte-derived dendritic cell; MR, mannose receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; ROS, reactive oxygen species; sBEF, signed binary enrichment factor; SC, *Saccharomyces* cell; SC-mannan, mannan derived from *Saccharomyces cerevisiae*; YPD, yeast peptone dextrose

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902972

mannan), is able to induce IL-17 production (10), implying that the different branches of the two structures profoundly influence the recognition of fungi and the Th response subsequently elicited.

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 (11, 12). *S. cerevisiae* cell wall composition radically changes in different life stages and life forms, such as ascospores. Ascospores are the outcome of the yeast meiotic cycle and produce haploid progeny. The ecological significance of sporulation, besides the generation of variability and interaction with the immune system, are still largely unknown. Sporulation is a phenomenon that involves conformational changes in yeast cells, and the final product is an ascus (which maintains the characteristic of the residual yeast cell wall) that encloses four spores (a tetrad). The *S. cerevisiae* spores inside the ascus are characterized by a totally different cell wall composition with respect to cells, whereas the common fungal PAMPs mainly recognized by DCs, such as β-glucan and mannan, are covered by chitosan (α−1,4-linked glucosamine residues) and by a layer of indeterminant structure whose major constituent is cross-linked tyrosine dimers (13). These outermost layers are unique features of the spore (14).

We used a systems biology approach to understand how different life stages of the harmless and commensal *S. cerevisiae* interact with our immune system, and to unravel the rules of the recognition game between pathogenic and nonpathogenic 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 of cell wall components in determining commensalism or pathogenicity traits.

In addition, we discussed the potential of yeast spores to survive passage through a 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 discriminating between friends and foes.

Materials and Methods

Reagents

The culture medium used was RPMI 1640 (Life Technologies BRL, Grand Island, NY) supplemented with glutamine (Sigma-Aldrich, St. Louis, MO), penicillin and streptomycin (Life Technologies BRL), and 10% heat-inactivated FCS (Thermo Scientific HyClone Products, Waltham, MA). GM-CSF and human recombinant IL-4 were from Gentaur (Brussels, Belgium). The control stimuli used—LPS (1 μg/ml), Curdlan (100 μg/ml), Zymosan (100 μg/ml), and the receptor agonists laminarin, mannan, chitin, Sulfo-NHS-LC-biotin, and NBT—were purchased from Sigma-Aldrich. ELISA kits were obtained from BioSource (Camarillo, CA), except for ELISA for IL-12p70, which was from R&D Systems (Minneapolis, MN), and IL-17, from eBioscience (San Diego, CA). Cytochalasin D was from TebuBio (Milan, Italy). FITC was from Fluka (Castle Hill, New South Wales, Australia). Mannan and D-mannose were from Sigma-Aldrich. CA-mannan and glucan were a kind gift from Prof. David Williams and were previously isolated (15, 16). The following Abs were used: mAb AZN-D1, AZN-D2 and AZN-D3 anti-DC-SIGN (17), NKI-L19, and anti-β_2-integrins. mAb anti-MMR and mAb DCN46 anti-DC-SIGN were obtained from BD Pharmingen (San Diego, CA); APC-conjugated mAb anti-CD45RO from BD Biosciences (San Jose, CA); and Alexa647-conjugated goat-anti-mouse IgG, and Alexa568 goat-anti-mouse IgG2b from Molecular Probes (Eugene, OR). PKH26 dye was purchased from Sigma-Aldrich. Human recombinant IL-12p70 was from obtained from Tema Ricerca (Bologna, Italy), and monoclonal anti-human IL-12 Ab was purchased from R&D Systems.

Yeast culture and preparation

S. cerevisiae diploid strain SK1 (MATa/α HO gal2 cupS can1R BIO) was cultured in complete medium (2% yeast extract, 1% peptone, 2% glucose, 0.1% glucose [YPD]) for 18 h, then collected, washed twice with sterile water, and resuspended at 10^8 cells/ml. 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 and replicated on SPOIV medium (2% potassium acetate, 0.25% yeast extract); a condition of nitrogen starvation, conducive to sporulation, was assessed by optical microscopy. Zymolyase (2 mg/ml; Sigma-Aldrich, St. Louis, MO) was used to digest asci and liberate spores (18). Experiments performed to evaluate zymolyase sensitivity of asci for this specific strain showed that 15 min of incubation was the minimum time needed to dissolve the ascus, without touching the spore cell wall. Zymolyase was inactivated by heat (65˚C for 2 min) and washed away carefully, together with the remainder of the empty ascus; the spores were then resuspended to a concentration of 10^8 cells/ml. The zymolyase treatment did not affect spore cell wall composition, and spore viability was assessed by plating 100 μl culture (1000 spores/ml) on YPD in triplicate and obtaining 100% viability. We will use the term spores and not the more appropriate term ascospores to specifically indicate that the effects we are observing are due only to the spores, not to the ascus. Germination of the spores was observed after 24 h of culture in RPMI, and the exponentially growing cells were starting to divide. Therefore, any analyses performed at later time points would be affected by this altered state, whereas no interference should be present until after 24 h. *S. cerevisiae* strains BY4741 and BY4741 *och1* (kindly provided by Neil Gow, University of Aberdeen, Aberdeen, Scotland) were cultured in complete medium until the exponential growth phase and treated as before. *C. albicans* SC5314 strain was cultured overnight in Saboraud medium at 28˚C and then shifted to RPMI medium for 18 h at 37˚C to allow hyphal growth. After microscopic inspection for purity, the hyphal culture was treated as for yeast cells. It should be noted that in all the experiments performed in this study live microorganisms were used.

Microorganism survival following uptake by DCs

After 6 h of stimulation, DCs were collected, washed three times with PBS, treated with zymolyase, and washed twice; 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 the percentage of CFUs after 3 d relative to the total number of cells growing in the absence of DC exposure. In selected experiments, diphenyleneiodium chloride (DPI; 10 μM) was added 30 min 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.

DC preparation and stimulation

PBMCs were isolated from buffy coat blood sample from healthy donors from the Transfusion Unit of the Careggi Hospital (Florence, Italy) by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany). The experimental plan was approved by the local ethical committee, and informed consent was obtained from all donors. Monocytes were isolated from low-density PBMCs by magnetic enrichment with anti-CD14 beads (Miltenyi Biotec, Bergish Gladbach, Germany). Cells were cultured in the presence of GM-CSF (800 U/ml) and recombinant IL-4 (1000 U/ml) for 6 d to allow DC differentiation (19). DC activation was induced by yeast in the different life stages. Depending on the experiments, moDCs were added at different concentrations. Serial dilution of live yeast preparations was added to the moDCs at different stimuli/DC ratios.

Isolation and priming of T cells

Total $CD4^+$ T cells were obtained from PBMC by negative isolation with a combination of magnetic sorting (Miltenyi Biotec) and incubated at a T/DC ratio of 20:1 with autologous moDCs exposed for 12 h with live spores/yeast. For real-time PCR, cells were collected after 24, 48, and 72 h or 5 d of coculture. Supernatants were collected after 5 d of coculture for cytokine detection.

Cytokine production

After the times indicated, supernatants were collected, and cytokine detection was performed. MesoScale Assay 7-spot (Meso Scale Discovery, Gaithersburg, MD) was used for detection of IL-1β, IL-8, IL-6, TNF-α, IL-10, and IL-12p70, according to the manufacturer's instructions. Alternatively, ELISA assay was performed according to the manufacturer's instructions. In the blocking experiment, after 2 h of exposure with the inhibitor, supernatants were collected following 8 h or 24 h or 5 d of incubation.

Quantitative real time PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). Random hexamer and reverse transcriptase kit (SuperScript II; Invitrogen,

Carlsbad, CA) were used for cDNA synthesis. Quantitative real-time PCR for *FOXP3* gene expression was performed using primer pairs and SYBR Green PCR Mater Mix (Applied Biosystems, Foster City, CA). Transcripts for *IL-6*, *IL-17A,* and *IFN-γ* 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 value of the gene of interest with the cycle threshold value of the reference gene *GAPDH*. Values are expressed as fold increase (fold change [FC]) of mRNA relative to that in unstimulated cells.

IL-12p70 inhibition by cytochalasin D

DCs were exposed to cytochalasin D (10 μg/ml) for 30 min at 4˚C. After washing with cold PBS, DCs were stimulated with live yeast or spores at a stimuli/DC ratio of 4:1 for 24 h. IL-12p70 production was assessed by ELISA.

IL-12p70 blocking assay

DCs were stimulated for 8 h with live spores of *S. cerevisiae* and *C. albicans* hyphae at a stimuli/DC ratio of 4:1 in the presence of different concentrations of human rIL-12p70 (0, 1, 10, and 100 ng/ml). Alternatively, DCs were preincubated with different concentrations (0, 0.1, 1, 10, and 100 μg/ml) of a monoclonal anti-human IL-12 Ab for 2 h, then stimulated for 8 h with live *S. cerevisiae* cells in a stimuli/DC ratio of 4:1. IFN-γ and IL-17 productions were assessed in T cells 5-d culture supernatants.

Phagocytosis assay

Live yeasts/spores were biotinylated using 10 mg/ml sulfo-NHS-LC-biotin in 50 mM $NaHCO_3$, pH 8.5, for 2 h at 4˚C. The remaining reactive biotin molecules were inactivated by incubation in 100 mM Tris-HCl, pH 8.0, for 40 min at 4˚C. DCs were then treated with biotinylated spores/yeasts. After 1 h, 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.

NBT assay

To determine reactive oxygen species (ROS) production, we used the modified version by Choi et al. (20) of the microscopic NBT assay.

Competition assay

DCs were exposed to laminarin (500 μg/ml), mannan (500 μg/ml), and chitin (500 μg/ml) for 30 min at 37˚C. After washing with PBS, DCs were exposed to live yeast cells or spores at a concentration of 4:1 for 6 h. Inhibition ability of TNF-α production was assessed by ELISA.

S. cerevisiae binding assay

Labeling of live *S. cerevisiae* cells or spores was performed as previously described (21). 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-β_2-integrin (30 μg/ml); and 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% BSA. FITC-labeled yeast cells or spores were added in a stimuli:DC ratio of 10:1. After 30 min 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 to discriminate cells binding FITC-labeled yeast/spore particles from yeast/spore aggregates.

Internalization assay

Immature DCs were allowed to adhere onto fibronectin and subsequently incubated with FITC-labeled live yeast or spores for 5, 15, and 30 min at 37˚C. At the end of the incubation period, the samples were fixed in 4% paraformaldehyde, permeabilized in methanol, and labeled for DC-SIGN and/or MMR, using specific mAb and isotype-specific fluorescent secondary Abs. Samples were analyzed using a Zeiss (Oberkochen, Germany) LSM 510 confocal microscope.

In vivo survival assay

The experimental protocol was designed in conformity with the recommendations of the European Economic Community (86/609/CEE) for the care and the use of laboratory animals, was in agreement with the Good Laboratory Practices, and was approved by the Animal Care Committee of the University of Florence (Florence, Italy). One-month-old rats were fed for 4 wk on a daily basis with water containing live 1×10^7 SK1 yeast cell or 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 wk, fecal samples were collected and after serial dilution were plated on YPD medium with chloramphenicol (100 ng/ml) to allow growth of CFU. After 3 d, CFU were counted. Survival of spores referred to the number of CFU grown after 3 d.

DC transcriptional analysis

A total of 2×10^6 DCs were cultivated with live cells of *S. cerevisiae* cells, spores, hyphae of *C. albicans,* or without any stimuli in a ratio of 4:1. After 4 h, the time after which the spores are not germinating, cells were collected. RNA preparation, labeling, hybridization on a HT12 array (Illumina, San Diego, CA), and scanning were performed according to the Illumina reference protocols.

Array preprocessing

Bead-summary data saved from Illumina BeadStudio was preprocessed in several steps. First, 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 (22) specific for the nucleotide sequence of each probe. The computation was performed using the lumi package (23), written in the R programming language.

Data have been submitted to the Array Express repository (experiment ID E-MTAB-135, www.ebi.ac.uk/microarray-as/ae/).

Differential expression and annotation

Differential expression analysis was carried out using the Rank Product algorithm (24), taking into account the differences between donors. The p values estimating differential expression were corrected for multiple testing (false discovery rate), and genes with a corrected p value ≤ 0.05 were selected. Gene differential expressions of the stimulated cells with respect to the unstimulated control were expressed as FC in natural scale. A FC < 0.5 means downregulation. A FC > 2 means upregulation. To compare the different stimulation conditions, a ratio between the FC of the interested gene was done.

Pathway analysis

Pathway analysis was performed with Eu.Gene (25), over a set of 80 pathways selected by the DC-THERA consortium. Prior to the analysis, microarray raw data were transformed into absolute-scale values and processed following the procedure outlined by Beltrame et al. (26): First, ratios between each treated condition and the unstimulated controls were calculated. Then, in an effort to reduce interdonor 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 Fisher's exact test, and the resulting signed p values were transformed into signed binary enrichment factors (sBEFs). sBEFs were clustered, using Euclidean distance as metric, bootstrapping the data over 1000 iterations. This computation was carried out with the TIGR Multiexperiment Viewer 4.4.

Statistics

A Student t test was used to evaluate the statistical significance of the results, by comparison between spores and yeast or between control and blocking conditions. Results were classified as nonsignificant ($p > 0.05$), significant ($p < 0.05$), and highly significant ($p < 0.01$).

Results

Differential IR to pathogenic and harmless fungi

To understand the rule of proper recognition, we investigated the IR to different life stages of *S. cerevisiae,* a 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 with unstimulated controls. A total of 1179 genes were commonly activated in the three stimulation conditions (Fig. 1*A*, Supplemental Table I). Gene clustering showed an upregulation of a wide range of DEGs involved in IRs and inflammation, such as genes coding for cytokines, chemokines, and immune receptors (Supplemental Fig. 1). The upregulation of genes coding for the receptors, TLR-2 and TLR-1

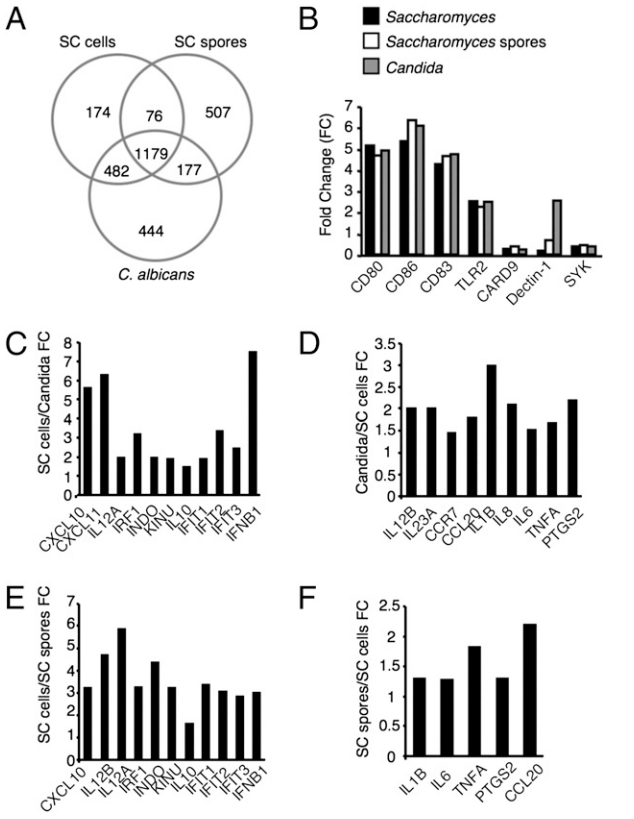

FIGURE 1. IR genes activated by *Saccharomyces cerevisiae* cells or spores and *C. albicans* hyphae in moDCs. Transcriptional analysis was performed on moDCs after 4 h of stimulation with *S. cerevisiae* cells and spores, with *C. albicans* hyphae, or without any stimuli. *A*, Venn diagram of DEGs. The full cluster is available as Supplemental Fig. 1. *B*, Selection of IR differentially regulated genes ($p \leq 0.05$) expressed upon *S. cerevisiae* or *C. albicans*. The differential expression is expressed as FC. *C*, IR differentially regulated genes ($p \leq 0.05$) more expressed in SC cell-stimulated DCs compared with *Candida*-stimulated DCs. *D*, IR differentially regulated genes ($p \leq 0.05$) more expressed in *Candida*-stimulated DCs compared with *SC*-stimulated DCs. *E*, IR differentially regulated genes ($p \leq 0.05$) more expressed in SC-stimulated DCs compared with *Saccharomyces* spore-stimulated DCs. *F*, IR differentially regulated genes ($p \leq 0.05$) more expressed in *Saccharomyces* spore-stimulated DCs compared with SC-stimulated DCs. SC, *Saccharomyces* cell.

and Dectin-1, the adaptor proteins Syk and Card9, and costimulatory molecules outlined a common maturation process between *S. cerevisiae* and *C. albicans* stimulation (Fig. 1*B*). Despite the upregulation of TLR, the C-type lectin Dectin-1 downregulated upon engagement. In contrast to what occurred upon *S. cerevisiae* yeast cell/spore challenge, *C. albicans* hyphae did not induce downregulation of Dectin-1 (*CLEC7A*), presumably indicating lack of recognition by Dectin-1. The downregulation of the gene coding for the adaptor Syk suggests a possible engagement of Dectin-2 in hyphal response (27).

In addition to the commonly activated genes, a set of 174 genes was differentially expressed only after *S. cerevisiae* cell stimulation, whereas 444 and 507 genes were uniquely differentially expressed after *C. albicans* and *S. cerevisiae* spore stimulation, respectively. The comparative analysis of *Saccharomyces* cell-stimulated moDCs and *Candida* hyphae-stimulated moDCs showed a marked expression of several proinflammatory mediators and factors involved in the DC maturation process, including the activation of *IL12A* (Fig. 1*C*). The strong induction of proinflammatory and Th1 polarizing cytokine genes induced by *S. cerevisiae* cells was counterbalanced by an increased expression of factors involved in immune modulation that limit DC activation as well as T cell effector function, such as IL-10, KINU, and IDO (28–30). Genes for the Th17 polarizing factors IL-1β and IL-6 were also found to be more expressed in *Candida*-stimulated DCs (Fig. 1*D*), showing that different fungi induce a peculiar maturation program of stimulated DCs. Furthermore, *S. cerevisiae* cells activated a stronger induction of *IFNB1* [recently associated with an internalized TLR2 recognition (31)] and of IFN-dependent genes and chemokines involved in Th1 responses (32) and in the mechanism of intracellular protection from pathogen invasion, whereas *Candida* promotes a preferential expression of *CCL20*, which has been associated with Th17 responses (33–35) that are protective against extracellular pathogens.

The comparison of *S. cerevisiae* cell and spore stimulation showed a strong induction of proinflammatory and Th1 polarizing cytokine gene activation, counterbalanced by an increased expression of factors involved in immune modulation (Fig. 1*E*). Conversely, genes for the Th17 polarizing factors IL-1β and IL-6 were found more expressed in spore-stimulated DCs (Fig. 1*F*), in a *Candida*-like manner, suggesting that different life stages of *S. cerevisiae* can induce a different maturation program of DCs.

To investigate the regulation of pathways and cellular networks in our samples, we performed a pathway analysis and clustered the results as sBEFs (26) (Fig. 2). We observed a general activation of the IR pathway in all conditions. In particular, we observed the activation of pathways, such as the TLR signaling pathway, JAK-STAT signaling, COX reaction, and cytokine–cytokine receptor interaction. The TCR signaling pathway and apoptotic pathway were also significantly activated in all three stimulation conditions. Furthermore, the clustering analysis separated *C. albicans* and *S. cerevisiae* into two distinct groups. This finding suggested a different DC response to pathogenic and nonpathogenic fungi. The comparison among *S. cerevisiae* cells, spores, *C. albicans* hyphae, and *A. fumigatus* (data obtained from a public repository) strengthened this observation (Supplemental Fig. 2). These findings suggested that *S. cerevisiae* spores are teleologically similar to *C. 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.

Spores circumvent the DC activation process

To assess the cytokine response of moDCs to *S. cerevisiae* treatment, we exposed moDCs to yeast or spores for 24 h. In agreement with the microarray data, yeast cells, compared with spores, promoted an increased production of IL-12p70 and IL-10 (Fig. 3*A*). To understand if this behavior could be related to pathogenic fungi, DC stimulation with C. *albicans* hyphae was also performed in parallel.

The absence of IL-12p70 induction upon spore stimulation was observed even after massive or prolonged stimulation (Fig. 3*B*, 3*C*).

To rule out that zymolyase treatment could interfere in DCs recognition of spores, crucial experiments, such as IL-12p70 production by DCs, were performed in parallel by DC stimulation with liberated spores, liberated spores treated with zymolyase for 5 min more, and untreated asci. Upon asci stimulation, the level of IL-12p70 was comparable to that induced upon spore challenge (Supplemental Fig. 3). This demonstrated that the 15-min zymolyase treatment does not affect the spore wall structure in a way that will interfere with the IR.

IL-12p70 production in the presence of *C. albicans* depends on internalization of the pathogen by DCs (36). By blocking internalization of *S. cerevisiae* yeasts and spores, using cytochalasin D, an inhibitor of actin polymerization, we showed that IL-12p70 synthesis induced by the yeast form was strongly suppressed (Fig. 4*A*), suggesting that cytokine production was dependent upon *S.*

FIGURE 2. Spores induce a response closed to *C. albicans*. Clustering of sBEFs using Euclidean distance that uses support trees on DC stimulated with *S. cerevisiae* cells and spores and with *C. albicans*. Colored spots indicate significant ($p = 0.05$) upregulation (red) or downregulation (green). The colors of the dendrogram indicate the percentages of the tree support (significance), from 50% (pink) to 100% (black).

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 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 stimuli by zymolyase treatment (Fig. 4*B*, 4*C*).

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

We next investigated the degradation process of *S. cerevisiae* yeast cells and spores and *C. albicans* hyphae in the phagosome by evaluating 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 them on yeast growth medium. As shown in Fig. 4*D*, significantly more colonies were obtained from internalized spores or *C. albicans,* compared with *S. cerevisiae* yeasts, after 6 h of exposure. We therefore measured ROS production by DCs cultured with yeast or spores for 8, 24, and 48 h (Fig. 4*E*). 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 with yeast cells.

ROS production is one of the parameters that most likely reflect differences in intracellular killing: Previous studies demonstrated the important role of NADPH oxidase-induced ROS in DC killing of *C. albicans*.

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 NADPH oxidase, which is known to abrogate ROS production by DCs in response to PMA (37). DPI treatment (10 μM) abrogated the ability of DCs to kill *S. cerevisiae* cells without affecting spore survival (Fig. 4*F*). These results indicate that ROS produced by NADPH oxidase play an important role in DC fungicidal activity and suggest that the increased survival of spores could be related to an escape mechanism from oxidative damage, as reported for *C. albicans* (38).

We can speculate that the lack of IL-12p70 production and the reduced production of ROS might be mechanisms by which spores circumvent the DC activation process.

DCs stimulated by diverse forms of S. cerevisiae *prime a different Th 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 (39). The evidence that moDCs released IL-12p70 in the presence of yeast cells 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. 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 (Fig. 5). The analysis showed an increase of *IL-17A* mRNA and of IL-6 mRNA in T cells exposed to spore-stimulated and *C. albicans*-stimulated DCs, compared with unstimulated DCs just after 24 h of coculture. By contrast, yeast-stimulated DCs did not induce *IL-17A* gene activation and only slightly increased *IL-6* gene activation (Fig. 5*A*). The *INFG* gene was activated by all the stimuli. The expression of the *FOXP3* gene was found to be increased in T cells after 5 d of coculture with yeast-stimulated DCs only (Fig. 5*B*), suggesting that yeast cells might induce the differentiation of a regulatory T cell population, as also supported by microarray data (Fig. 1*C,* Supplemental Table I). In coculture experiments with autologous $CD4^+$ T lymphocytes, we used *C. albicans* hyphae stimulation as the control stimulus for IL-17 induction. As expected, IL-12–producing, yeast-matured DCs promoted a pure Th1 response, inducing the release of IFN-γ only (Fig. 5*C*). In contrast, spore-stimulated DCs, such as the pathogenic fungus *C. albicans*, induced the release of a substantial amount of IL-17 together with IFN-γ, consistent with the presence of a Th17 response (Fig. 5*C*). 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. 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 rIL-12p70. The added IL-12p70 during DC stimulation by spores rescued the ability of DCs to polarize a pure Th1 response in a dose dependent fashion, as well as antagonized IL-17 induction by *C. albicans* (Fig. 6*A*), indicating that high amounts of IL-12 can indeed counteract the effect of the Th17

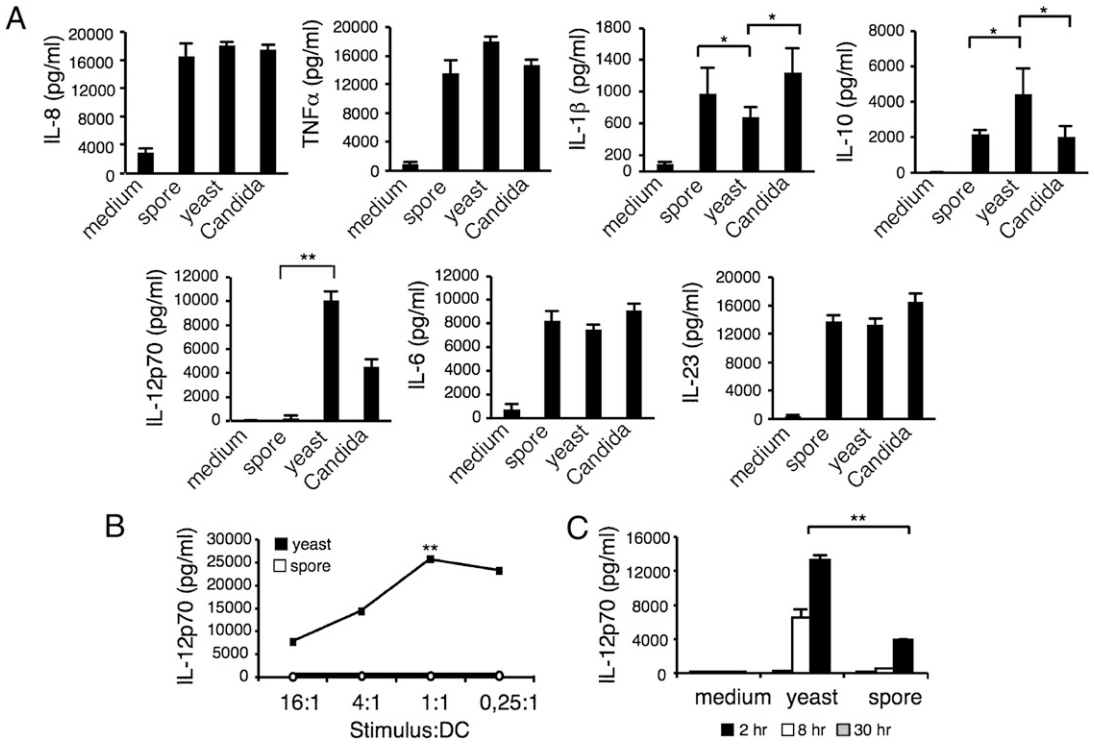

FIGURE 3. Cytokine profile of spore-stimulated and yeast-stimulated DCs. *A*, DCs were exposed for 24 h to *S. cerevisiae* spores or yeast cells, to *C. albicans,* or without any stimuli in a concentration (expressed as stimuli/DC ratio) of 10:1. Cytokine production in supernatants was evaluated by Meso Scale or ELISA assay. Spore-matured DCs induced more production of IL-6 and IL-1β and failed to induce IL-12p70 production, compared with yeast-stimulated DCs, which induced IL-10 production. *B*, DCs were exposed at different concentrations of yeast cells (■) or spores (○), and IL-12p70 was evaluated after 24 h in culture supernatants. *C*, DCs were exposed to spores and yeast cells at 10:1 ratio, and IL-12p70 was evaluated after 2 (gray bars), 8 (white bars), and 30 h (black bars) of stimulation. Data are representative of mean ± SD (*A*, $n = 3$; *B*, $n = 3$; *C*, $n = 4$). Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

polarizing factor. To address this observation in yeast-stimulated DCs, we used an Ab against IL-12 to block the activity of the cytokine. The block of IL-12 activity favored Th17 cell activation (Fig. 6*B*). This finding demonstrates the important role exercised by IL-12 in balancing the fungal IR.

MR and DC-SIGN are differently involved in S. cerevisiae recognition by DCs

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 MR, and chitin for chitin receptors (40). Blocking each of these receptors resulted in an inhibition of TNF-α production in DCs stimulated by yeast cells (Fig. 7*A*). On the contrary, none of the three competitor ligands was able to inhibit TNF-α 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 (8) and DC-SIGN (9). To assess whether 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.

Binding studies demonstrated that DC-SIGN clearly mediates adhesion to both yeast cells and spores (Fig. 7*B*). Blocking-Abs 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 gain 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*. 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* (9). 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 (Fig. 7*C*) or with Abs (Fig. 7*D*). Blocking of β_2-integrin receptors was used as negative control. Again, blocking-Abs 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, using Ab or mannose, affected only the DC ability to bind yeast cells, whereas it had no effect on the binding to spores. To assess whether the different recognition of spores and yeast cells by DCs influences the internalization, we performed a phagocytosis assay, specifically labeling MR and DC-SIGN. By confocal microscopy, the double labeling showed that, after yeast cell stimulation, MR and DC-SIGN colocalized in the same phagocytic vesicle (Fig. 7*E*, Supplemental Fig. 4). This observation 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 (41). In contrast, spores were internalized by DCs in vesicles containing DC-SIGN only as shown by the lack of colocalization of the two receptors (Fig. 7*E*, Supplemental Fig. 5). These results clearly demonstrate that DCs use a different PRR repertoire to recognize different life stages of the same microorganism.

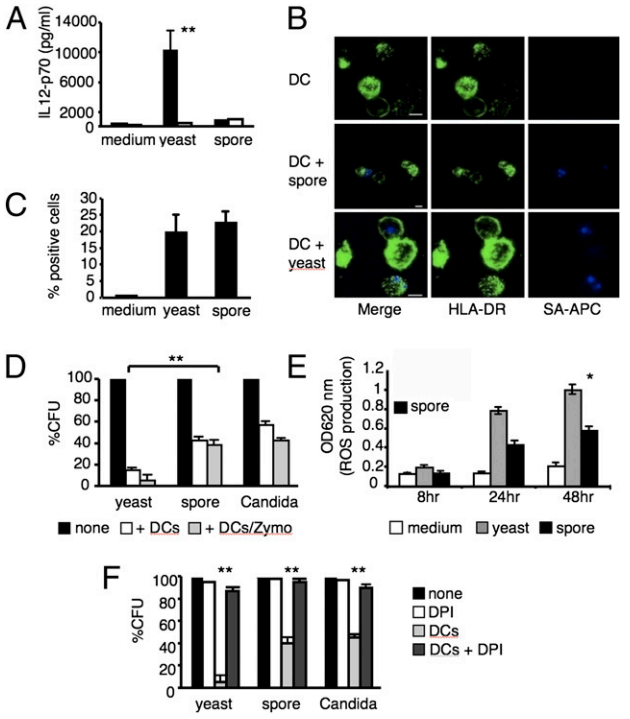

FIGURE 4. Internalization, survival rate of yeast or spore forms of *S. cerevisiae* and induction of ROS production by DCs. *A*, DCs pretreated or not with cytochalasin D (10 μg/ml) were stimulated for 24 h with yeast cells or spores. IL-12p70 was measured by ELISA. Confocal fluorescent microscopy (*B*) and flow cytometry (*C*) 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*, Survival of *S. cerevisiae* spores and yeast cells and *C. albicans* hyphae after culturing with DCs. Stimuli were cultured alone (black bars) or with DCs (4:1 ratio). After 6 h 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), spores (black bars), or without any stimuli (white bars) at a 4:1 ratio. NBT assay was performed after the time indicated. *F*, NADPH oxidase-induced ROS account for the killing of *S. cerevisiae* cells. Stimuli were cultured alone or with DCs (4:1 ratio) in the presence of absence of DPI (10 μM). After 6 h 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 (*A–F*, mean ± AD). Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

Specific role of mannan structures for interaction of host cells with S. cerevisiae

It was recently demonstrated that the MR is the main pathway through which *C. albicans* induces a Th17 response upon PBMC stimulation. Because SC-mannan itself cannot induce IL-17 production, despite interacting with MR (10), and spores lack exposed mannan, we further investigated the specific role of this branched carbohydrate in stimulating the innate *S. cerevisiae* IR. SC-mannan itself stimulated IL-12p70 production by DCs; furthermore, the amount of cytokine produced was sufficient to inhibit IL-17 production by T cells induced by spores (Fig. 8*A*). This finding suggests that the lack of SC-mannan in spores may be responsible for low IL-12 production.

From previous reports, it is known that *Candida och1* mutant, defective in the mannosylation process, failed to interact with DCs, supporting the important role played by *N*-linked mannan in the

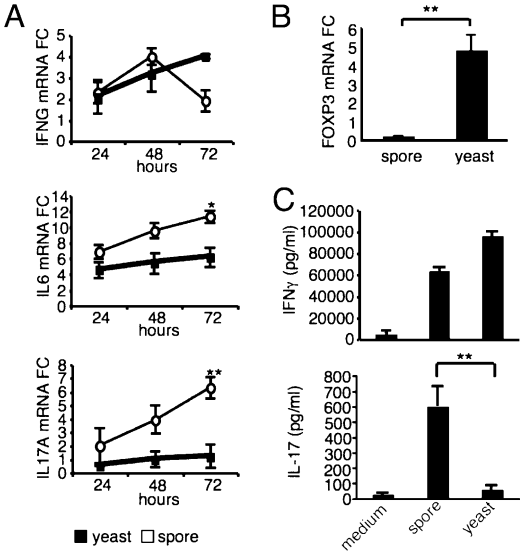

FIGURE 5. Effect of spore-stimulated DCs or yeast-stimulated DCs on $CD4^+$ effector T cell differentiation. $CD4^+$ cells were cocultured in a ratio of 20:1 with autologous moDCs, which matured for 12 h with *S. cerevisiae* or *C. albicans*. *A*, *IL6, IL17A,* and *IFNγ* gene expression assessed by real-time PCR in $CD4^+$ T cells exposed to spores (○) or yeast cell (■) for 24, 48, and 72 h. *B*, *FOXP3* gene expression assessed by real-time PCR in $CD4^+$ T cells exposed to spores or yeast cell for 5 d. *C*, IL-17 and IFN-γ measurement in 5-d culture supernatants of $CD4^+$ T cells produced in response to yeast- and spore-stimulated DCs assessed by ELISA. Data are representative of three (*A*, *B*, mean ± SD) and seven (*C*, mean ± SD) separate experiments. Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

binding of *C. albicans* to DCs (9). In contrast, in *S. cerevisiae*, the *OCH1* gene is required not only for proper mannosylation but also for masking the β-glucan and immune recognition by the Dectin-1 pathway (42) involved in Th17 priming (33). This prompted us to further investigate the effects of the absence of exposed mannan in

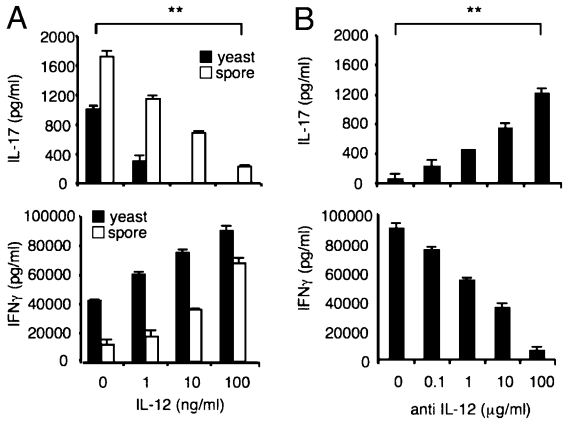

FIGURE 6. IL-12p70 drives the balancing between Th1 and Th17 response. *A*, Human rIL-12p70 rescues Th1 polarization upon *S. cerevisiae* spore and *C. albicans* hyphae stimulation in a dose-dependent fashion. DCs were treated with spores or *C. albicans* (4:1, stimuli/DC ratio) for 8 h in the presence of human rIL12-p70 at different concentrations, and the ability of DCs to polarize a Th1 or a Th17 response was assessed by IFN-γ and IL-17 measurement, respectively, by ELISA in 5-d culture supernatants of $CD4^+$ T cells. *B*, Blocking IL-12p70 activity, using an Ab specific for IL-12, results in a loss of IFN-γ induction and promotes IL-17 production in 5-d culture supernatants of $CD4^+$ T cells in response to yeast- stimulated DCs (4:1, stimuli/DC ratio). Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

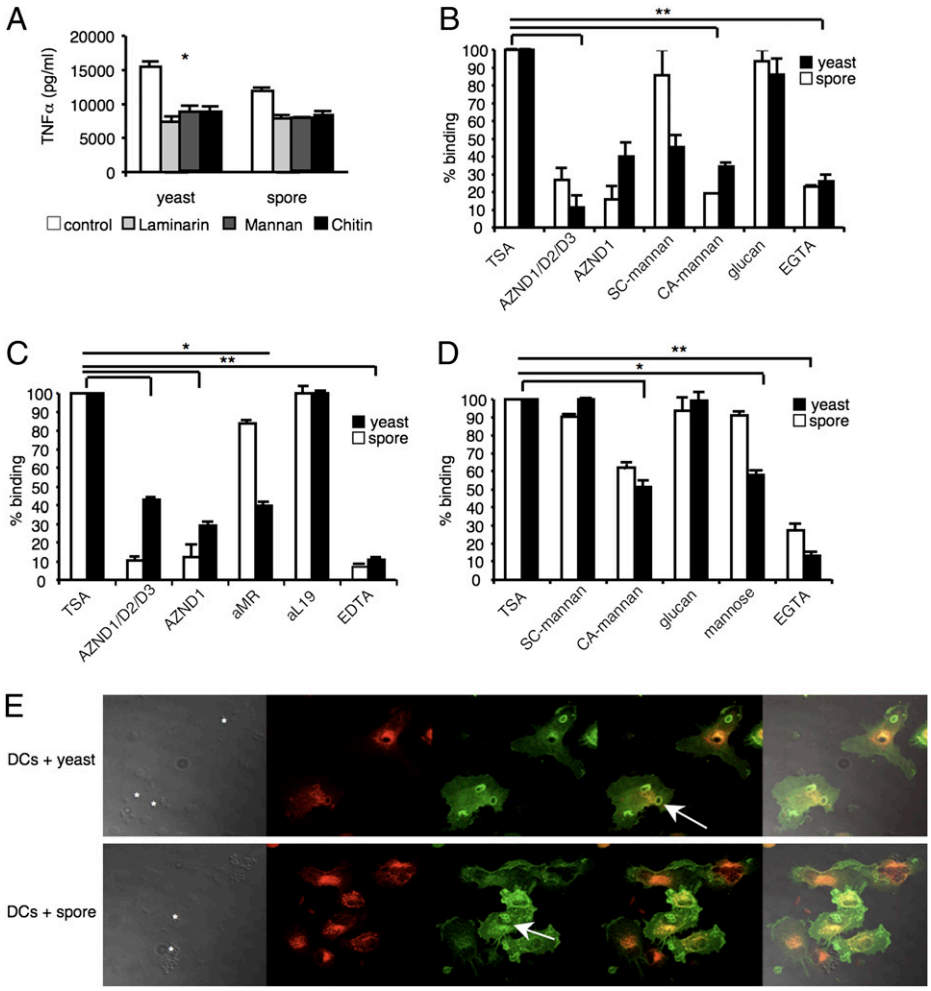

FIGURE 7. MR and DC-SIGN involvement in *S. cerevisiae* recognition. *A*, Untreated DCs or DCs pre-exposed to receptor agonists, laminarin, mannan, and chitin, were incubated with yeasts and spores for 6 h. TNF-α production was inhibited by receptor agonist treatment only in yeast-stimulated DCs. *B*, 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 β_2-integrin, respectively. Addition of EGTA showed that DC-SIGN–mediated binding is Ca^{2+} dependent. *C*, *D*, Immature DCs bind *S. cerevisiae* through 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–β_2-integrin Ab as control or SC-mannan, CA-mannan, β-glucan, or mannose. Basal binding (TSA) is set as 100%. *E*, Labeling of MR (in red) and DC-SIGN (in green) shows considerable colocalization with yeast cells (asterisk) as indicated by the white arrow (*first panel*). Spores are internalized by DC-SIGN–containing vesicle only as indicated by the white arrow (*second panel*). All data are presented as mean ± SD ($n = 3$). Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

Th17 induction by *S. cerevisiae* spores. Cytokine production by DCs and by $CD4^+$ T cells stimulated with spore-, yeast-, or *C. albicans* hyphae-matured DCs was measured. Stimulation with *S. cerevisiae och1* mutant yeast cells polarized a Th1/Th17 population toward the type of responses induced by spores (Fig. 8*B*). Stimulation with *C. albicans* hyphae polarized a Th17 response, as previously demonstrated (10). These data demonstrate that the presence of exposed mannan on the cell wall influences the IR mounted by the DCs to properly respond and tolerate *S. cerevisiae* yeast cells, in a different fashion from what occurs with *C. albicans* hyphae. We demonstrated that spores are recognized by DC-SIGN, 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 exposed DCs to blocking Abs 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 cell stimulation, whereas blocking of DC-SIGN showed a slight effect on IL-17 production (Fig. 8*C*). Thus, we show that in contrast to *C. albicans* hyphae stimulation, *S. cerevisiae* cell interaction with DCs, exerted by mannan, favors the IL-12/IFN-γ axis, whereas the absence of exposed mannan in spores induces a Th17 response.

Discussion

Host–fungal interactions have so far been studied only for pathogenic fungi, such as *C. albicans*; 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. This is the first report that addresses the mechanism by which the host immune system discriminates friends from foes in the interplay with microorganisms, demonstrating that the IR to spores is closer to the response to the well-known pathogen *C. albicans*.

Whole-genome transcriptional analysis of moDCs stimulated with *S. cerevisiae* cells or spores and *C. albicans* hyphae confirms the presence of a peculiar transcriptional program governing the recognition of fungi, be they pathogenic or commensal. The DC transcriptional response to pathogenic fungi has been previously addressed, in part. The transcriptional report of DCs stimulated with a *C. albicans* strain defective in filamentous development in experimental conditions largely superimposable on those used in our paper was offered by Huang et al. (43). 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. In fact, some of the genes (e.g., *CD83*, *IL12A*, *TNFA*, *IL6*, *IL1B,* and more) found to be differentially regulated by Huang et al. were also found to be differentially regulated in a similar manner in our experiment, using DCs stimulated with *S. cerevisiae* cells.

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 (44). Although pathogenic fungi have been thoroughly investigated, to the best of our knowledge this is the first study demonstrating that different forms of the same nonpathogenic

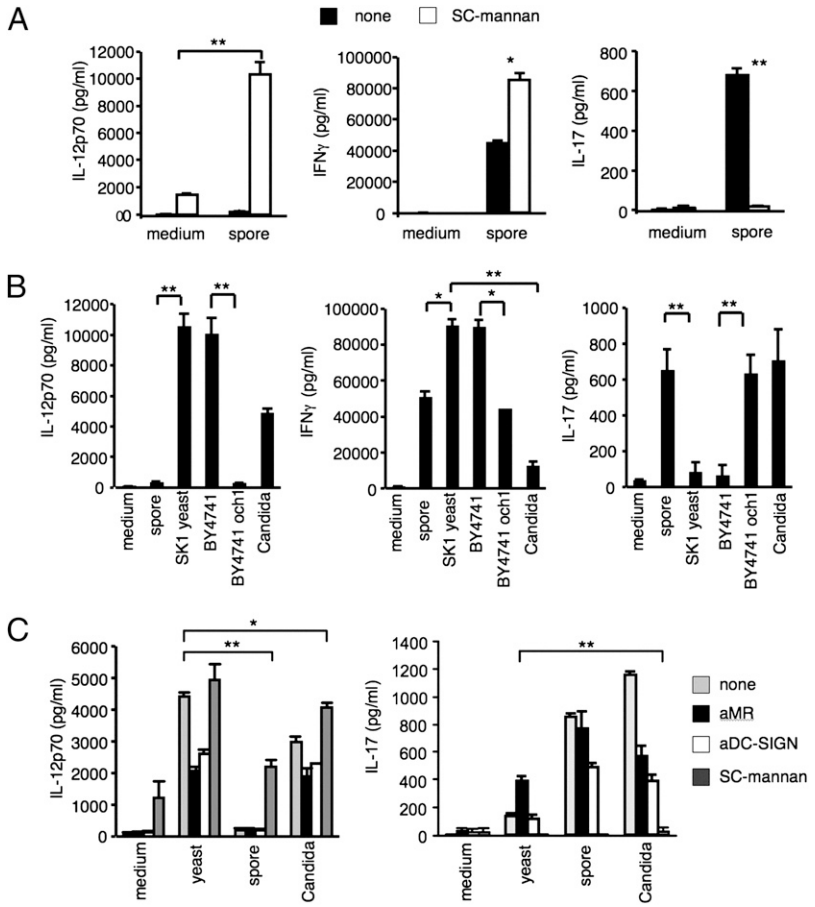

FIGURE 8. Role of exposed mannan in defining IRs to *S. cerevisiae*. *A*, SC-mannan inhibits Th17 response induced by spores. DCs were exposed (white bars) or not (black bars) to an excess of SC-mannan (100 mM) for 2 h and stimulated with spores (4:1, stimuli/DC ratio) for 8 h. Thus, the coculture with $CD4^+$ T cells was performed. IL-12p70, IFN-γ, and IL-17 were measured. *B*, Absence of 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 (4:1, stimuli/DC ratio) for 12 h, and their capacity to prime Th17 or Th1 polarization of T cells was assessed by IL-12, IL-17, and IFN-γ measurement by ELISA. *C*, DCs were exposed to *S. cerevisiae* cells and spores and to *C. albicans* (light gray bars). mAbs were used to block MR (black bars) or DC-SIGN (white bars), or exposure to SC-mannan (dark gray bars) was used. After 12 h, IL-12-p70 was measured, and after 5 d of coculture, IL-17 was assessed on T cell supernatants. All data are presented as mean ± SD ($n = 3$). Statistical comparisons between samples are represented as horizontal bars. $*p < 0.05$; $**p < 0.01$.

microorganism stimulate profoundly different IRs and that skewing in the use of PRRs dictated by differences in cell wall composition is a requirement for pathogenicity. Our results demonstrate that, despite the lack of IL-12p70 production by DCs, spores, a peculiar form of *S. cerevisiae*, induce a Th1/Th17 polarization of $CD4^+$ T cells. In contrast, the activation of suppressive mechanisms, such as the increased expression of the *IDO* gene in DCs and the induction of a $CD4^+$ $FOXP3^+$ regulatory T cell 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 NADPH oxidase, inducing a poor fungicidal activity of these cells, as previously reported for *C. albicans* (38). Further investigation into the nature of this possible oxidative damage escape mechanism will be carried out in follow-up studies. Thus, far from being inactive microbes, yeast cells activate a defined pathway of IRs in moDCs, balancing each other to avoid potentially harmful inflammatory processes.

Similar to what was reported for *C. albicans* (9), we report that interaction between *S. cerevisiae* yeast and DCs is mediated by mannan, rather than other fungal cell wall components. Although 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 the spore wall could hide important carbohydrates from DCs, influencing immune detection by eluding important PAMPs.

Our findings 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 Th17 induction by the spores.

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

The differential recognition of mannan from *C. albicans* and *S. cerevisiae* is most likely related to the different branching of these structures. CA-mannan induces potent Th17 responses (10), whereas SC-mannan does not, suggesting that MR could be differently engaged by *C. albicans* and *S. cerevisiae.* The relative contribution of DC-SIGN in Th17 priming is under investigation.

Although *S. cerevisiae* cannot qualify as a human saprophyte or commensal, throughout human civilization the consumption of bread and fermented beverages has exposed our immune system to this yeast on a daily basis, without harmful effect—on the contrary, with potential beneficial effects. 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 IRs and tolerance. Both Th1 (33) and Th17 (45–47) 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 (48) recruited by Th17 cells. Th1 cells express CXCR3 and migrate toward IFN-dependent chemokines, such as CXCL10 and CXCL11, which are preferentially induced in response to the yeast. In turn, Th17 cells express CCR6 and are recruited in response to CCL20, which is preferentially expressed in the presence of hyphae.

This observation is consistent with the differential chemokine profile we observed in cells challenged with the *S. cerevisiae* yeast or *C. albicans* hyphae. Accordingly, 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 favor a Th17 response, which might favor spore expulsion. Our finding may have relevance for autoimmune diseases, such as inflammatory bowel disease (IBD), in which *S. cerevisiae* spores could play a role in the inflammation process mediated by a mixed Th1/Th17 response. Interestingly, anti-*S. cerevisiae* Abs can be detected in a subset of patients with Crohn's disease and are considered markers of active disease (49). The capacity of *S. cerevisiae* spores to promote IL-17 release could contribute to the pathogenesis of this disease; 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 with yeast cells (50), and that spores are thus more resistant to innate mechanisms of protection.

In vivo preliminary results (C. De Filippo, L. Rizzetto, and D. Cavalieri, unpublished observations) suggested that spores have a stronger capacity to survive through the gut. The different pathway of IR activated by spores compared with yeast cells in DCs could be involved in such increased survival. Further investigations are needed to clarify this issue.

Reports of spores in mammalian intestine are rare, probably owing to the habit of culturing fecal samples rather than observing them by microscopy. Our findings open new avenues for further investigation into the in vivo nature of the IR in animal models and in patients with IBD in future studies.

Tolerance requires the ability of the microbe to circumvent inflammatory signals evolved in response to potentially pathogenic microorganisms. We demonstrate that differential recognition of specific mannan structures is one of the major regulators of discriminating between harmful and harmless fungi, emphasizing the importance of deeper insight into the specific contribution of each receptor in fungal recognition.

The higher capacity of SC-mannan to induce a IL-12/Th1 protective response is likely to play a major role in the nonpathogenicity 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 IR. This trait may be advantageous and may explain the maintenance of the ability to sporulate, as indicated by the high sporulation rates of many of the naturally existing strains (51). The yeast ecological cycle could therefore possibly include mammals as hosts and vectors.

Our results underline the importance of differences in cell wall structure that occur during the life stages of *S. cerevisiae* in conveying anti-inflammatory signals. 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 IBD.

Acknowledgments

We thank the Wellcome Trust Centre for Human Genetics (University of Oxford), for performing microarray experiments, Enrica Calura (University of Firenze) for support in transcriptional data analysis, Elaine Tritto (Novartis Vaccine) and Ben Joosten (Nijmegen Centre for Molecular Life Science) for technical help with confocal microscopy, and AnnMaria Di Clemente (University of Firenze) for support in quantitative real-time PCR experiments. We thank Prof. Neil Gow for providing the *Saccharomyces och1* strain. We thank Iliana Ferrero Fortunati (University of Parma) for insight and inspiration.

Disclosures

The authors have no financial conflicts of interest.

References

1. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449: 819–826.
2. Richardson, M. D. 2005. Changing patterns and trends in systemic fungal infections. *J. Antimicrob. Chemother.* 56(Suppl 1): i5–i11.
3. Cavalieri, D., P. E. McGovern, D. L. Hartl, R. Mortimer, and M. Polsinelli. 2003. Evidence for *S. cerevisiae* fermentation in ancient wine. *J. Mol. Evol.* 57(Suppl 1): S226–S232.
4. Salonen, J. H., M. D. Richardson, K. Gallacher, J. Issakainen, H. Helenius, O. P. Lehtonen, and J. Nikoskelainen. 2000. Fungal colonization of haematological patients receiving cytotoxic chemotherapy: emergence of azole-resistant *Saccharomyces cerevisiae. J. Hosp. Infect.* 45: 293–301.
5. Muñoz, P., E. Bouza, M. Cuenca-Estrella, J. M. Eiros, M. J. Pérez, M. Sánchez-Somolinos, C. Rincón, J. Hortal, and T. Peláez. 2005. *Saccharomyces cerevisiae* fungemia: an emerging infectious disease. *Clin. Infect. Dis.* 40: 1625–1634.
6. Sethi, N., and W. Mandell. 1988. Saccharomyces fungemia in a patient with AIDS. *N. Y. State J. Med.* 88: 278–279.
7. Hernanz-Falcón, P., O. Joffre, D. L. Williams, and C. Reis e Sousa. 2009. Internalization of Dectin-1 terminates induction of inflammatory responses. *Eur. J. Immunol.* 39: 507–513.
8. Newman, S. L., and A. Holly. 2001. *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect. Immun.* 69: 6813–6822.
9. Cambi, A., M. G. Netea, H. M. Mora-Montes, N. A. Gow, S. V. Hato, D. W. Lowman, B. J. Kullberg, R. Torensma, D. L. Williams, and C. G. Figdor. 2008. Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J. Biol. Chem.* 283: 20590–20599.
10. van der Veerdonk, F. L., R. J. Marijnissen, B. J. Kullberg, H. J. Koenen, S. C. Cheng, I. Joosten, W. B. van den Berg, D. L. Williams, J. W. van der Meer, L. A. Joosten, and M. G. Netea. 2009. The macrophage mannose receptor induces IL-17 response to *Candida albicans. Cell Host Microbe* 5: 1–12.
11. d'Ostiani, C. F., G. Del Sero, A. Bacci, C. Montagnoli, A. Spreca, A. Mencacci, P. Ricciardi-Castagnoli, and L. Romani. 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.* 191: 1661–1674.
12. Gantner, B. N., R. M. Simmons, and D. M. Underhill. 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. *EMBO J.* 24: 1277–1286.
13. Kreger-Van Rij, N. J. 1978. Electron microscopy of germinating ascospores of *Saccharomyces cerevisiae. Arch. Microbiol.* 117: 73–77.
14. Coluccio, A., E. Bogengruber, M. N. Conrad, M. E. Dresser, P. Briza, and A. M. Neiman. 2004. Morphogenetic pathway of spore wall assembly in *Saccharomyces cerevisiae. Eukaryot. Cell* 3: 1464–1475.
15. Chorvatovicová, D., E. Machová, J. Sandula, and G. Kogan. 1999. Protective effect of the yeast glucomannan against cyclophosphamide-induced mutagenicity. *G. Mutat. Res* 21: 117–122.
16. Lowman, D. W., D. A. Ferguson, and D. L. Williams. 2003. Structural characterization of (1→3)-beta-D-glucans isolated from blastospore and hyphal forms of Candida albicans. *Carbohydr. Res.* 338: 1491–1496.
17. Geijtenbeek, T. B., R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, G. J. Adema, Y. van Kooyk, and C. G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100: 575–585.
18. Casalone, E., C. Barberio, D. Cavalieri, I. Ceccarelli, M. Riparbelli, S. Ugolini, and M. Polsinelli. 1999. Disruption and phenotypic analysis of six novel genes from chromosome IV of *Saccharomyces cerevisiae* reveal YDL060w as an essential gene for vegetative growth. *Yeast* 15: 1691–1701.
19. Sallusto, F., and A. Lanzavecchia. 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. *J. Exp. Med.* 179: 1109–1118.
20. Choi, H. S., J. W. Kim, Y. N. Cha, and C. Kim. 2006. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J. Immunoassay Immunochem.* 27: 31–44.
21. Cambi, A., K. Gijzen, J. M. de Vries, R. Torensma, B. Joosten, G. J. Adema, M. G. Netea, B. J. Kullberg, L. Romani, and C. G. Figdor. 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.
22. Du, P., W. A. Kibbe, and S. M. Lin. 2007. nuID: a universal naming scheme of oligonucleotides for illumina, affymetrix, and other microarrays. *Biol. Direct* 2:16.
23. Du, P., W. A. Kibbe, and S. M. Lin. 2008. lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 24: 1547–1548.
24. Hong, F., R. Breitling, C. W. McEntee, B. S. Wittner, J. L. Nemhauser, and J. Chory. 2006. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* 22: 2825–2827.

25. Cavalieri, D., C. Castagnini, S. Toti, K. Maciag, T. Kelder, L. Gambineri, S. Angioli, and P. Dolara. 2007. Eu.Gene Analyzer a tool for integrating gene expression data with pathway databases. *Bioinformatics* 23: 2631–2632.
26. Beltrame, L., L. Rizzetto, R. Paola, P. Rocca-Serra, L. Gambineri, C. Battaglia, and D. Cavalieri. 2009. Using pathway signatures as means of identifying similarities among microarray experiments. *PLoS One* 4: e4128.
27. Robinson, M. J., F. Osorio, M. Rosas, R. P. Freitas, E. Schweighoffer, O. Gross, J. S. Verbeek, J. Ruland, V. Tybulewiez, G. G. Beown, et al. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 206: 2037–2051.
28. Romani, L. 2008. Cell mediated immunity to fungi: a reassessment. *Med. Mycol.* 46: 515–529.
29. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21: 685–711.
30. Romani, L., and P. Puccetti. 2007. Controlling pathogenic inflammation to fungi. *Expert Rev. Anti Infect. Ther.* 5: 1007–1017.
31. Reske, A., G. Pollara, C. Krummenacher, D. R. Katz, and B. M. Chain. 2008 Glycoprotein-dependent and TLR2-independent innate immune recognition of herpes simplex virus-1 by dendritic cells. *J. Immunol.* 180: 7525-7536.
32. Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187: 129–134.
33. Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8: 639–646.
34. Hirota, K, H. Yoshitomi, M. Hashimoto, S. Maeda, S. Teradaira, N. Sugimoto, T. Yamaguchi, T. Nomura, H. Ito, T. Nakamura, et al. 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J. Exp. Med.* 204: 2803–2812.
35. Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat. Immunol.* 10: 514–523.
36. Chiani, P., C. Bromuro, and A. Torosantucci. 2000. Defective induction of interleukin-12 in human monocytes by germ-tube forms of *Candida albicans*. *Infect. Immun.* 68: 5628–5634.
37. Vulcano, M., S. Dusi, D. Lissandrini, R. Badolato, P. Mazzi, E. Borroni, E. Calleri, M. Donini, A. Plebani, L. Notarangelo, et al. 2004. Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *J. Immunol.* 173: 5749–5756.
38. Donini, M., E. Zenaro, N. Tamassia, and S. Dusi. 2007. NADPH oxidase of human dendritic cells: role in *Candida albicans* killing and regulation by interferons, dectin-1 and CD206. *Eur. J. Immunol.* 37: 1194–1203.
39. Bettelli, E., T. Korn, M. Oukka, and V. K. Kuchroo. 2008. Induction and effector functions of T(H)17 cells. *Nature* 453: 1051–1057.
40. Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. Gow. 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Natl. Rev.* 6: 67–78.
41. Cambi, A., M. Koopman, and C. G. Figdor. 2005. How C-type lectins detect pathogens. *Cell. Microbiol.* 7: 481–488.
42. Wheeler, R. T., and G. R. Fink. 2006. A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog.* 2: e35.
43. Huang, Q., D. Liu, P. Majewski, L. C. Schulte, J. M. Korn, R. A. Young, E. S. Lander, and N. Hacohen. 2001. The plasticity of dendritic cell responses to pathogens and their components. *Science* 294: 870–875.
44. Liti, G., D. M. Carter, A. M. Moses, J. Warringer, L. Parts, S. A. James, R. P. Davey, I. N. Roberts, A. Burt, V. Koufopanou, et al. 2009. Population genomics of domestic and wild yeasts. *Nature* 458: 337–341.
45. Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger. 2004. Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. *J. Infect. Dis.* 190: 624–631.
46. Milner, J. D., J. M. Brenchley, A. Laurence, A. F. Freeman, B. J. Hill, K. M. Elias, Y. Kanno, C. Spalding, H. Z. Elloumi, M. L. Paulson, et al. 2008. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452: 773–776.
47. Conti, H. R., F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. H. Hai, J. J. Yu, J. W. Jung, et al. 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* 16: 299–311.
48. Urban, C. F., U. Reichard, V. Brinkmann, and A. Zychlinsky. 2008. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell. Microbiol.* 8: 668–676.
49. Standaert-Vitse, A., T. Jouault, P. Vandewalle, C. Mille, M. Seddik, B. Sendid, J. M. Mallet, J. F. Colombel, and D. Poulain. 2006. *Candida albicans* is an immunogen for anti-*Saccharomyces cerevisiae* antibody markers of Crohn's disease. *Gastroenterology* 130: 1764–1775.
50. Coluccio, A. E., R. K. Rodriguez, M. J. Kernan, and A. M. Neiman. 2008. The yeast spore wall enables spores to survive passage through the digestive tract of Drosophila. *PLoS One* 3: e2873.
51. Mortimer, R. K. 2000. Evolution and variation of the yeast (Saccharomyces) genome. *Genome Res.* 10: 403–409.