JGH Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12701

GASTROENTEROLOGY

Differential stimulation of peripheral blood mononuclear cells in Crohn's disease by fungal glycans

Liran Baram,*,† Sarit Cohen-Kedar,*,† Lior Spektor,*,† Hofit Elad,*,† Hanan Guzner-Gur*,‡ and Iris Dotan*,†

*IBD Center, Department of Gastroenterology and Liver Diseases and The Research Center for Digestive Tract and Liver Diseases and ‡Internal Medicine B, Tel Aviv Medical Center, and †Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Key words
β-glucans, anti-glycan antibodies, Crohn's disease, cytokines, mannan.

Accepted for publication 20 April 2014.

Correspondence
Dr Iris Dotan, IBD Center, Department of Gastroenterology and Liver Diseases, Tel Aviv Medical Center, 6 Weizmann Street, Tel Aviv 64239, Israel. Email: irisd@tasmc.health.gov.il

Abstract

Background and Aim: Crohn's disease (CD) is characterized by loss of tolerance to intestinal microorganisms. This is reflected by serological responses to fungal glycans such as mannan and β-glucans. Fungal glycans have various effects on immune cells. However, the evidence for their effects in CD is vague. This study aimed to assess the effects of fungal cell wall glycans on human peripheral blood mononuclear cells (PBMCs) from CD and control patients.

Methods: Human PBMCs from CD and control patients were stimulated by fungal cell wall glycans. Cytokine secretion was detected by ELISA and glycan receptor expression by flow cytometry.

Results: Mannan, β-glucans (curdlan), chitosan, and zymosan induced the secretion of interleukin (IL)-1β, IL-6, IL-23, IL-10, and tumor necrosis factor-α by PBMCs. Spleen tyrosin kinase and Src tyrosine kinase were involved in the response to mannan and β-glucans. Mannan and whole yeast cells induced a significantly higher pro-inflammatory cytokine response in CD compared with control patients.

Conclusions: The results may suggest that CD is characterized by hyperresponsiveness to fungal glycans. Thus, glycans may potentially be triggering or perpetuating inflammation.

Introduction

The pathogenesis of inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) involves interactions between the immune system and intestinal microorganisms in genetically susceptible individuals.[1–3] Hence, upon a yet unknown trigger, probably a component of the intestinal microflora, inappropriate activation of the intestinal mucosal immune system occurs in susceptible subjects. This loss of tolerance may be reflected by the production of antibodies directed against components of the intestinal flora. Our group and others have described the presence of specific antibodies directed against bacterial and fungal antigens in the sera of IBD patients, specifically those with CD.[4–8] The identification of these antibodies has contributed to more accurate diagnoses of CD[9–12] and enabled better stratification of CD into more *versus* less aggressive phenotypes.[5,12] Most CD-related antibodies are directed against various glycans that characterize the fungal cell wall, including commensal yeast such as *Saccharomyces cerevisiae* and *Candida albicans*. The cell wall of these and other fungi is made up of a complex network of glycans, whose layers are mostly comprised of mannan (outermost), β-glucans, and chitin/chitosan (innermost).[13,14] Thus, these glycans are the first to encounter the host immune system.

The antifungal immune response is carried out primarily by innate immune cells and is mediated by cell surface pattern recognition receptors (reviewed in Brown[15]), mainly by members of the C-type lectin receptor (CLR) family that are able to recognize fungal cell wall glycans (reviewed in Hardison and Brown[16]). These receptors include dendritic cell-associated c-type lectin (Dectin)-1 (CLEC7A),[17–19] Dectin-2 (CLEC6A),[18,20,21] and the mannose receptor (MR).[22]

Previous studies have shown that β-glucans, mannan, and chitin have various immunomodulating effects[23–27] such as the stimulation of reactive oxygen species production,[28–31] phagocytosis,[28,30,31] and the secretion of various cytokines[23,24,31,32] by human and mouse innate immune cells. However, the evidence for their effects in CD is vague.

We hypothesized that commensal fungi, specifically their glycans, may be able to stimulate the immune system and that this could be a step in the immunopathogenesis of CD. Hence, this study aimed to investigate the inflammatory response of human peripheral blood mononuclear cells (PBMCs) challenged with

glycans and fungi, and its mechanism. We further asked whether a differential response existed in CD patients.

Patients and methods

Population. Peripheral blood was drawn from IBD patients and healthy controls recruited at a tertiary IBD center, after obtaining informed consent. The study was approved by the local Ethics Committee.

Isolation of PBMCs. Human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Venous blood of IBD patients and Healthy Controls (NL) controls was drawn into citrate tubes, and the mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:3 in PBS over Ficoll-Hypaque (GE-Healthcare, Uppsala, Sweden). Cells were washed twice in PBS and suspended in RPMI 1640 culture medium (Biological Industries, Beit HaEmek, Israel) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at a concentration of 5×10^6 cells/mL.

Cell activation and cytokine secretion. Freshly isolated PBMCs (5×10^5 cells at a final volume of 0.5 mL) were cultured for 24 h at 37°C in a 5% CO_2 humidified atmosphere with zymosan, a cell wall preparation of *S. cerevisiae* (10 μg/mL), curdlan (1→3)-β-D-glucan from *Alcaligenes faecalis* (10 μg/mL), mannan (1–3)-α-linked mannose from *S. cerevisiae* (500 μg/mL), and chitosan lactate from crab shells (> 90% deacetylation, 500 μg/mL), all purchased from Sigma-Aldrich (Sigma-Aldrich Israel Ltd., Rehovot, Israel), or Lipopolysaccharide (LPS) (10 ng/mL) (Invivogen, San-Diego, CA, USA). Cell-free supernatants were obtained, and the concentration of the cytokines (interleukin (IL)-1β, IL-6, IL-23, IL-10, Interferon-γ [IFN-γ] and tumor necrosis factor-α[TNF-α]) was determined by ELISA (R&D Systems, Minneapolis, MN, USA). For inhibition assays, cells were preincubated for 30 min with 0.1–2 μM Src inhibitor PP2 or its control peptide PP3, 20–50 μM spleen tyrosin kinase (Syk) inhibitor piceatannol, Syk inhibitor II (Calbiochem, San Diego, CA, USA), or 0–200 μg/mL laminarin from *Laminaria digitata* (Sigma-Aldrich).

PBMC stimulation by heat killed yeast cells. *Candida albicans* strain CBS-563 (kindly supplied by Dr Nir Osherov from the Department of Clinical Microbiology and Immunology, The Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel) and *S. cerevisiae* strain BYα (kindly supplied by Dr Martin Kupiek from the Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv, Israel) were used for PBMCs stimulation.

Yeasts (*S. cerevisiae* and *C. albicans*) were seeded into Difco Sabouraud dextrose agar (Becton Dickinson, Sparks, MD, USA) plates and incubated at 28/37°C, respectively. After 48 h, a typical colony was chosen and suspended in PBS. Yeast cells were counted and resuspended to a density of 1×10^8 cells/mL before heat killing at 100°C for 30 min. PBMCs (5×10^5 cells at a final volume of 0.5 mL) were cultured with 1×10^5 heat-killed yeast cells for 24 h at 37°C in a 5% CO_2 humidified atmosphere, cell-free supernatants were obtained, and the concentration of cytokines was determined by ELISA as indicated.

Detection of surface glycan receptors. Glycan receptors were detected using immunostaining with the appropriate fluorophore-conjugated antibodies, namely antihuman Dectin-1 (AbD Serotec, Oxford, UK), antihuman Dectin-2 (R&D Systems), antihuman MR (Biolegend, San Diego, CA, USA), and anti-CD14 (Becton Dickinson, San Diego, CA, USA) and their matched isotype controls. Cells were washed and acquisition was performed on a FACSCanto flow cytometer (Becton Dickinson) with FlowJo analysis software, version 6.0 (Treestar, Ashland, OR, USA) for data analysis.

Statistical analysis. Statistical analysis used IBM SPSS statistics 20 (IBM SPSS Statistics, Chicago, IL, USA). Data are reported as the mean ± standard error of the mean of at least three independent experiments. Differences in values were tested for significance by a Student's *t*-test or a Mann–Whitney rank sum test, depending on whether the data were normally distributed. A value of $P \leq 0.05$ was considered significant.

Results

Twenty-eight subjects (18 IBD and 10 healthy controls) were recruited. Of the IBD patients, 13 had CD (female : male 5:8) and 5five UC (femal : male 1:4). Their mean age was 37.4 ± 13 (range: 26–73) and 41.4 ± 15.9 (range: 24–66) years for CD and UC, respectively. All IBD patients had non-active or minimally active disease (Crohn's Disease Activity Index < 170, Mayo score ≤ 1) (Table 1) during blood sampling.

Of the 10 healthy subjects recruited, six were males, and the mean age was 33.3 ± 5.4 (range: 27–46) years (P = Non Significant (NS) *vs* IBD patients).

Yeast induces higher TNF-α and lower IL-10 secretion by CD compared with NL PBMCs. Antifungal glycan serological responses characterize CD patients to a greater extent than either UC patients or healthy controls. We hence investigated whether differential responses to fungal stimulation existed in CD patients. To this end, we used heat-killed *S. cerevisiae* and *C. albicans* yeast cells to stimulate PBMCs from CD and UC patients and healthy controls (NL), and cytokine secretion was assessed using ELISA. Higher TNF-α secretion by CD compared with NL PBMCs was induced by *C. albicans* (5876 ± 1543.0 *vs* 2469 ± 3355.2 pg/mL, $P = 0.03$) and *S. cerevisiae* (5328 ± 1596 *vs* 3026 ± 716.9 pg/mL, $P = 0.29$). In contrast, lower IL-10 secretion by CD compared with NL PBMCs in response to *S. cerevisiae* (56 ± 18.7 *vs* 253 ± 54.6 pg/mL, $P = 0.007$) and *C. albicans* (297 ± 115.7 *vs* 440 ± 191.3 pg/mL, $P = 0.52$) was observed (Fig. 1). Cytokine secretion by UC PMBCs was comparable with NL. Of note, comparable LPS-induced TNF-α and IL-10 secretion by NL, CD, and UC PBMCs were detected (TNF-α: 1737 ± 270.1, 1691 ± 301.9, 1661 ± 200.7; IL-10: 1643 ± 150.9, 1710 ± 292.4, 968 ± 664.8 in NL, CD, and UC respectively). Taken together, this suggests that in CD, yeasts induce a skewed pro-inflammatory response.

Table 1 Patient characteristics

		CD (n = 13)	UC (n = 5)	Healthy controls (n = 10)
Gender (F/M)		5/8	1/4	6/4
Age at sample date (mean years ± SD, range)		37.4 ± 13.0 (26–73)	41.4 ± 15.9 (24–66)	33.3 ± 5.4 (27–46)
Disease location	Ileal	5	—	—
	Colonic	1	5	—
	Ileocolonic	7	—	—
Disease behavior	Non-penetrating Non-stricturing	4	—	—
	Penetrating	3	—	—
	Stricturing	6	—	—
Treatment	None	3	0	10
	Anti-TNF-α	1	0	—
	AZA/6MP	9	1	—
	5-ASA	2	5	—

5-ASA, 5-aminosalicylic acid; AZA/6MP, Azathioprine/6-mercaptopurine; CD, Crohn's disease; SD, standard deviation; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

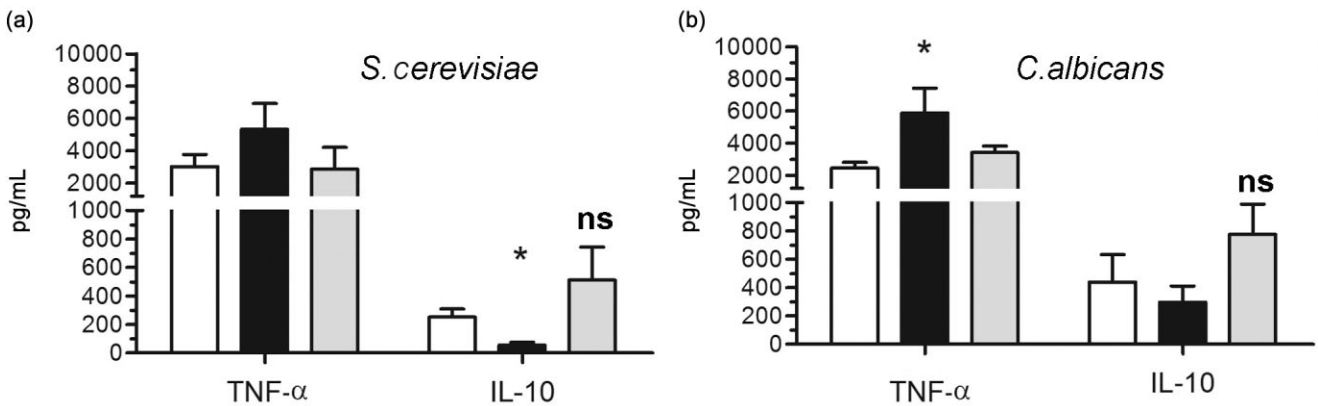

Figure 1 Yeast induce higher tumor necrosis factor-α (TNF-α) and lower interleukin (IL)-10 secretion by Crohn's disease (CD) compared with NL peripheral blood mononuclear cells (PBMCs). PBMCs from CD (n = 4, black columns) and ulcerative colitis (UC) (n = 3, grey columns) patients as well as NL controls (n = 3, white columns) were stimulated with heat-killed (a) *Saccharomyces cerevisiae* or (b) *Candida albicans* (10^6 cells/mL). Cells were incubated for 20 h, and the secretion of TNF-α and IL-10 was determined in cell culture supernatants by ELISA. Data are presented as mean ± standard error (SE) of cytokine concentration (pg/mL), $*P \leq 0.05$, ns—non-significant *versus* NL, Mann–Whitney rank sum test. ▭, NL; ■, CD; ▭, UC.

Fungal glycans induce cytokine secretion by human PBMC. To examine the specific effect of different fungal cell-wall glycans, we first asked whether fungal glycans would activate PBMCs from normal controls (NL). NL PBMCs (n = 10) were cultured with fungal glycans (concentrations are indicated in Fig. 2 legend) and cytokine (TNF-α, IL-10, IFN-γ) secretion to cell culture supernatants after 24 h of incubation was measured using ELISA. As illustrated in Figure 2, mannan, curdlan, and zymosan induced significant TNF-α and IL-10 secretion by NL PBMCs ($P < 0.05$ compared to untreated PBMCs). In contrast, chitosan induced only minor IL-10 secretion. IFN-γ secretion in response to all glycans tested was non-significant (data not shown).

The glycan receptors Dectin-1, Dectin-2, and MR are expressed by human peripheral monocytes. As antifungal immune response has been reported primarily in myeloid cells,[33,34] we next asked whether human monocytes expressed the major receptors involved in fungal recognition and response.[15,35,36] To this end, freshly isolated PBMCs were stained with antibody to the human monocyte marker CD14 together with antibodies to the glycan receptors Dectin-1, Dectin-2, or MR. Expression was detected using flow cytometry. As seen in Figure 3, Dectin-1, Dectin-2, and MR expression by CD14+ peripheral monocytes was observed.

Glycan-induced cytokine secretion is Syk- and Src-dependent. The β-glucan receptor Dectin-1 and the mannan receptor Dectin-2 signal via Syk, followed by downstream activation of Src.[21,28,37] To determine whether glycan activation of PBMCs involved this signaling pathway, we assessed the effect of Syk and Src inhibition on glycan-induced cytokine secretion by PBMCs. Significant (> 80%, $P < 0.05$ *vs* no inhibition) inhibition of mannan and β-glucan, but not chitosan-induced TNF-α secretion was detected when PBMCs were preincubated with Syk inhibitors (piceatannol, Fig. 4a, and Syk inhibitor cat. 574711 Calbiochem, Merck-Millipore, Darmstadt, Germany; data not shown). Significant (> 85%) inhibition was detected when PBMCs were preincubated with the Src inhibitor PP2 (Fig. 4b) as well

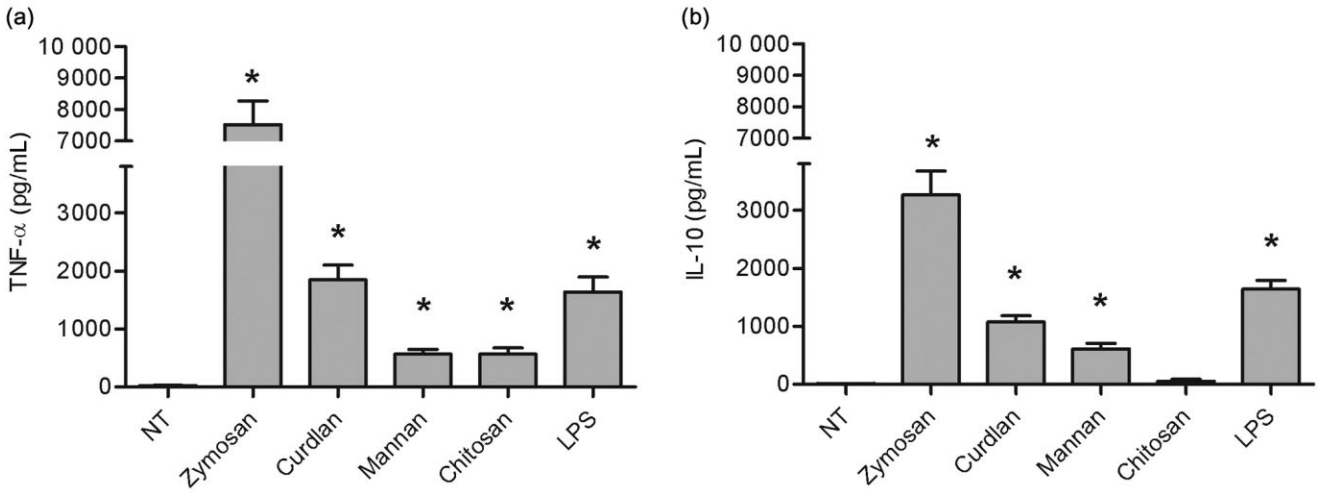

Figure 2 Fungal glycans induce cytokine secretion by peripheral blood mononuclear cells (PBMCs). PBMCs from NL controls ($n = 10$) were stimulated with 10 μg/mL zymosan, 10 μg/mL curdlan, 500 μg/mL chitosan, 500 μg/mL mannan, or 10 ng/mL LPS as a positive control. Non-treated (NT) PBMCs served as negative control. Cells were incubated for 20 h, supernatants were collected, and the secretion of (a) tumor necrosis factor-α (TNF-α) and (b) interleukin (IL)-10 was determined by ELISA. Data are presented as the mean ± standard error (SE) ($n = 10$) of cytokine concentration (pg/mL), $*P \leq 0.001$ *versus* NT, Student's *t*-test.

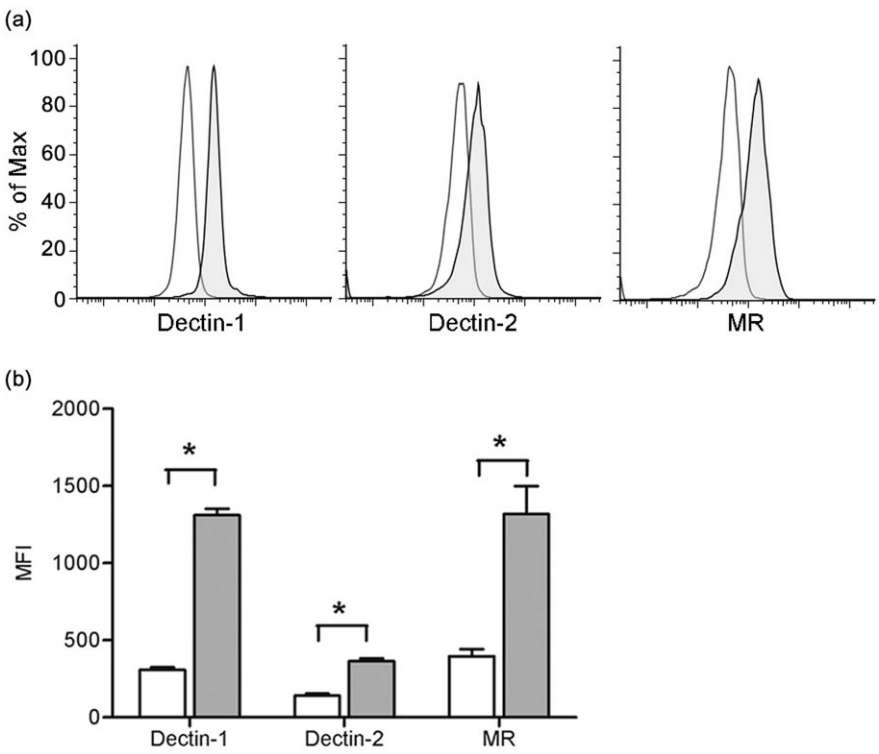

Figure 3 Glycan receptors are expressed by human peripheral blood monocytes. Peripheral blood mononuclear cells (PBMCs) from NL controls were double stained with anti-CD14 and antidendritic cell-associated c-type lectin (Dectin)-1, Dectin-2, and mannose receptor (MR) (gray-filled histograms and columns) or isotype-matched control Ab (white histograms and columns). Expression was assessed by flow cytometry. (a) Representative histograms of at least three experiments of glycan receptor expression by CD14+ cells. (b) Mean fluorescence intensity (MFI) of each receptor, presented as average of 3 experiments ± standard error (SE), $*P \leq 0.01$ *versus* control Ab, Student's *t*-test.

($P < 0.05$ *vs* no inhibition). This not only suggested that Syk and Src participate in glycan-induced signaling, but further supported our finding that Dectin-1 and Dectin-2 mediate glycan effects.

β-glucan-induced cytokine secretion is Dectin-1 dependent. We then examined whether Dectin-1, the β-glucan receptor, mediated glycan stimulation of PBMCs. The Dectin-1 antagonist laminarin was incubated with PBMCs for 30 min before adding curdlan or zymosan. Importantly, laminarin inhibited zymosan and curdlan-induced TNF-α secretion by PBMCs in a dose-dependent manner (Fig. 5). The resulting inhibition of curdlan- and zymosan-induced TNF-α secretion was 64% and 74%, respectively, suggesting that Dectin-1 is indeed involved in β-glucan-induced PBMC activation.

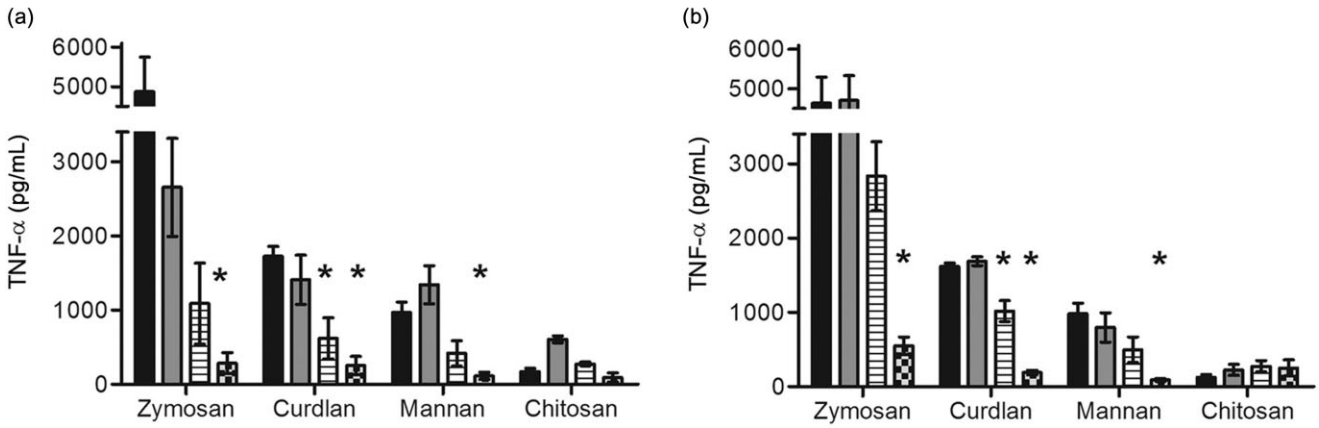

Figure 4 Glycan-induced cytokine secretion is Syk and Src dependent. Peripheral blood mononuclear cells (PBMCs) from NL controls ($n = 3$) were preincubated with/without (a) the Syk inhibitor piceatannol (20–50 μM) or (b) the Src inhibitor PP2 for 30 min before addition of glycans: zymosan (10 μg/mL), curdlan (10 μg/mL), mannan (500 μg/mL), and chitosan (500 μg/mL). Cells were incubated for 20 h, and the secretion of tumor necrosis factor-α (TNF-α) was determined in cell culture supernatants by ELISA. Data are presented as mean of all experiments ± standard error (SE) of cytokine concentration (pg/mL), $*P \leq 0.05$ *versus* (a) no inhibitor (▬, no inhibitor; ▬, Syk inhibitor 20 μM; ▭, Syk inhibitor 40 μM; ▬, Syk inhibitor 50 μM) or (b) *versus* PP3 (▬, PP3 2 μM; ▬, PP2 0.1 μM; ▭, PP2 0.5 μM; ▬, PP2 2 μM) (control peptide for Src inhibitor), Student's *t*-test.

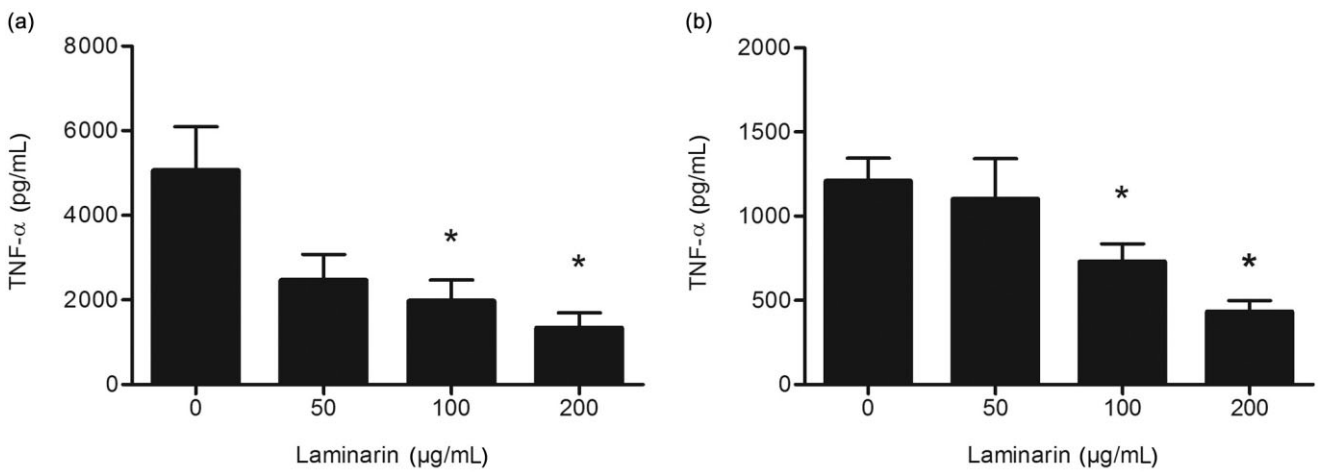

Figure 5 β-glucan-induced cytokine secretion is dendritic cell-associated c-type lectin (Dectin-1) dependent. Peripheral blood mononuclear cells (PBMCs) from healthy controls ($n = 3$) were preincubated with/without laminarin (0–200 μg/mL) for 30 min prior to the addition of 10 μg/mL (a) zymosan (▬) or (b) curdlan (▬). Cells were incubated for 20 h, and the secretion of tumor necrosis factor-α (TNF-α) into cell culture supernatants was determined by ELISA. Data are presented as the mean of experiments ± standard error (SE) of cytokine concentration (pg/mL) $*P \leq 0.05$ *versus* no inhibitor, Student's *t*-test.

Mannan induces higher pro-inflammatory cytokine secretion by CD compared with NL PBMCs. We next investigated whether differential responses to specific glycan stimulation existed in CD patients. To this end, PBMCs from CD and UC patients and normal controls (NL) were stimulated with glycans, and cytokine secretion was assessed using ELISA. Interestingly, mannan induced significantly higher levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-23 by CD, but not UC patients, compared with NL (Fig. 6). In contrast, zymosan-, curdlan-, and chitosan-induced cytokine secretion by CD, UC, and NL PBMCs was comparable. All glycans induced comparable IL-10 secretion by CD, UC, and NL PBMCs.

Discussion

Glycans, specifically mannan, β-glucans, and chitin are abundant fungal cell wall components. As fungi are abundant environmental (including intestinal) residents, their glycans may be the first to trigger immune responses. This may explain phenomena such as the existence of antifungal immune responses in CD patients. The immunological effects of fungal glycans have been observed in

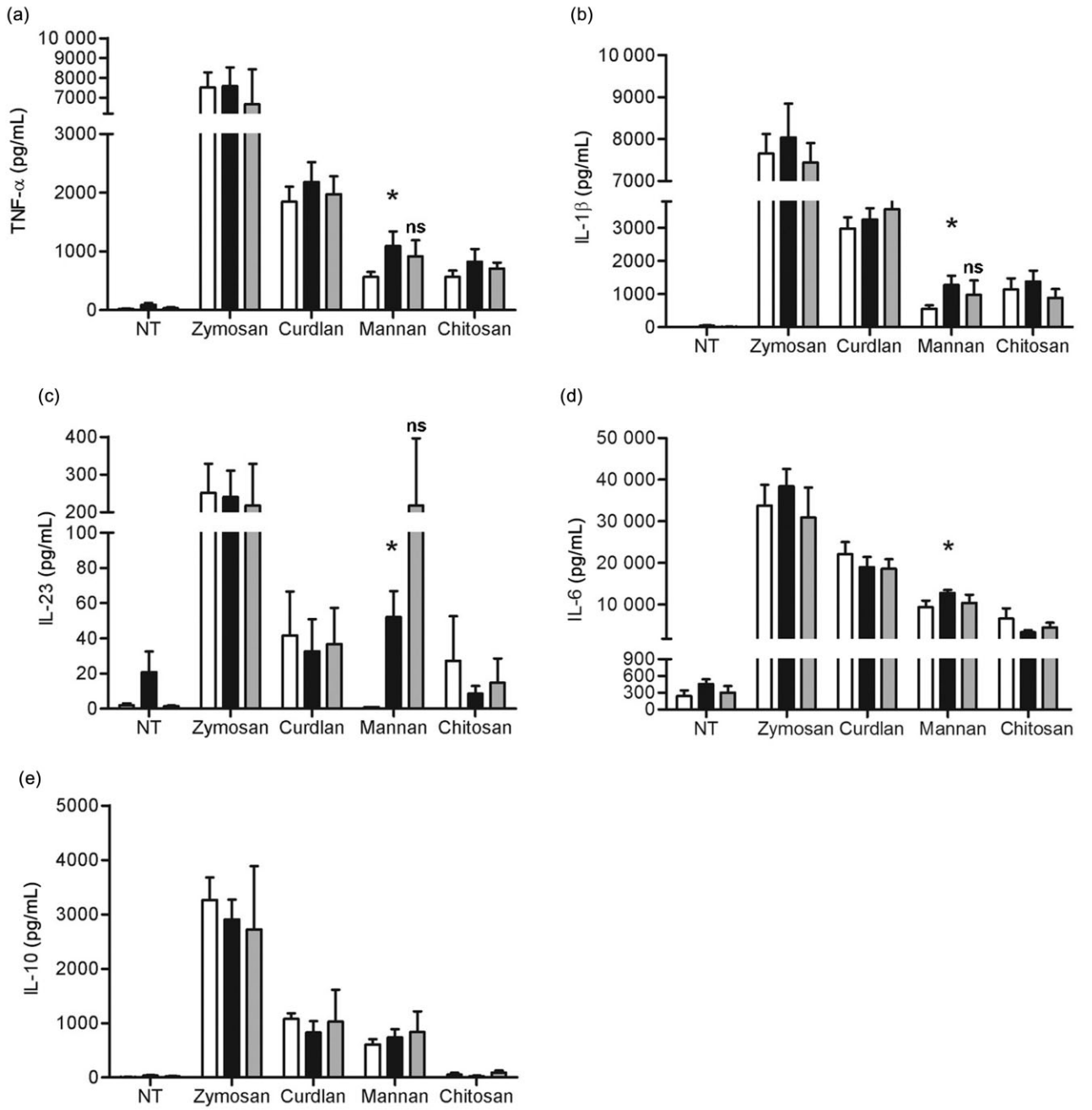

Figure 6 Mannan induces higher pro-inflammatory cytokine secretion by Crohn's disease (CD) compared with NL PBMCs. Peripheral blood mononuclear cells (PBMCs) from patients with CD (black columns, $n = 11$) and ulcerative colitis (UC) (gray columns, $n = 5$) patients as well as NL controls (white columns, $n = 10$) were stimulated or not (NT) with zymosan (10 μg/mL), curdlan (10 μg/mL), mannan (500 μg/mL), and chitosan (500 μg/mL). Cells were incubated for 20 h, and the secretion of tumor necrosis factor-α (TNF-α), inerleukin (IL)-1β, IL-23, IL-6, and IL-10 was determined in cell culture supernatants by ELISA. Data are presented as mean ± standard error (SE) of cytokine concentration (pg/mL), $*P \leq 0.05$, ns—non-significant *versus* NL, Mann–Whitney rank sum test. ▭, NL; ▬, CD; ▭, UC.

experimental murine models[23,26,27,29–32] and in human cells.[24,27,28] However, data on the effects of glycans in CD patients are scarce. We hypothesized that commensal fungi, specifically their glycans, can stimulate the immune system and that this may be a step in the immunopathogenesis of CD.

We hereby show that yeast-induced TNF-α secretion was higher and IL-10 secretion was lower by CD compared with NL PBMCs. PBMCs express the glycan receptors Dectin-1, Dectin-2, and MR, and respond to fungal glycans by secretion of various cytokines in a Syk and Src dependent pathway. Finally, the secretion of

mannan-induced pro-inflammatory cytokines was higher in CD than in NL PBMCs.

Fungi are recognized by a number of immune receptors, among which Dectin-1 has emerged as key mediator for phagocytosis and of fungal killing by phagocytes. Dectin-1 is a CLR that recognizes β-(1, 3)-glucans, a major constituent of fungal cell walls.[14] We confirmed that the effects of β-glucans (both curdlan and zymosan) can be blocked using the Dectin-1 antagonist laminarin.

β-Glucans stimulated PBMCs to secrete pro-inflammatory and regulatory cytokines in a Syk- and Src-dependent pathway. These results are consistent with data reported by Elsori *et al*.[28] showing that Dectin-1 is the primary receptor for zymosan-induced O_2^- production by human monocytes, and identified Syk and Src as signaling components downstream of Dectin-1. Furthermore, these results are consistent with findings reported in Kankkunen *et al*.[24] and Skrzypek *et al*.[38] indicating that Dectin-1 is important for cytokine production, as well as phagocytosis, oxidative burst in response to β-glucans/*C. albicans*, and that this response was Syk-dependent.

Our data that mannan activated PBMCs in a Syk- and Src-dependent pathway are specifically intriguing. Among the receptors recognizing α-mannan, Dectin-2 is known to act via Syk signaling.[20] The inhibition of mannan-induced TNF-α secretion following Syk or Src inhibition are indicative of the role of Dectin-2 in the response of PBMCs to mannan. As shown in Figure 3, human monocytes express Dectin-2. Dectin-2 expression is consistent with a previous report by Gavino *et al*., who showed that human monocytes constitutively expressed Dectin-2 mRNA.[39] Recently, Zhu *et al*. found that similarly to Dectin-2, a newly characterized CLR, Dectin-3, recognizes α-mannans on *C. albicans* cell surface and induces Syk-mediated activation. These authors also showed that Dectin-3 forms a heterodimeric complex with Dectin-2, enabling innate immune cells to sense fungal infection.[40] While Dectin-3 expression by PBMCs was not assessed in the current study, its combined activity with Dectin-2 suggests that it might be present on these cells.

The observation that chitosan-induced TNF-α secretion was Syk and Src independent indicates that chitosan is not recognized by either Dectin-1 or Dectin-2. Furthermore, this may suggest that chitosan effects are mediated by MR, a CLR known to recognize *N*-acetylglucosamine residues (the building blocks of chitin/chitosan) via a signaling pathway that is still unknown (reviewed in Taylor *et al*.[41]).

Evidence has been accumulating regarding the mechanisms that are used by CLRs to drive adaptive immunity, such as stimulating TH17 differentiation (reviewed in Hardison and Brown[16]). While the current study focused on direct glycan recognition and innate immune responses, specific T-cell populations may have a role in glycan-induced immunity. Finally, the combination of cytokines secreted after fungal glycans activation suggests that the engagement of lectin receptors can induce innate as well as adaptive immune responses.

The central function of Syk and Src signaling in the differential responses of CD patients to mannan highlights the potential importance of this signaling pathway in CD. It may also suggest that substances modulating this pathway, such as the Syk inhibitor Fostamatinib/R788, recently suggested as therapy in other inflammatory diseases,[42] may be effective in CD as well.

Mannan, chitosan, and β-glucans are all yeast antigens and widespread components of food. A proportion of CD patients develop antibodies against these antigens, such as ASCA (anti-*S. cerevisiae* antibodies), ALCA (antilaminarobioside carbohydrate antibodies), and antichitobiose carbohydrate antibodies directed against mannan, β-glucan, and chitosan moieties, respectively.[4,7,43] The presence of these antibodies may reflect specific loss of tolerance toward commensal fungi such as *S. cerevisiae* and *C. albicans*. However, little is known about the interaction of fungi with the mucosal immune system in states of mucosal homeostasis and inflammation such as the one observed in CD. Interestingly, Ruutu *et al*. reported that β-glucan triggers spondylarthritis and CD-like ileitis in SKG mice.[44] Moreover, deficiencies in Dectin-1 result in enhanced susceptibility to pathogenic fungal infections in humans and mice.[19,45] These findings, together with the strong association of ASCA and ALCA with CD,[4,7,43] suggest a possible link between immune responses to commensal fungi and intestinal inflammation.

In support of this, higher secretion of pro-inflammatory cytokines in response to mannan was induced in CD (but not UC) patients. These results may thus be a step in the loss of immune tolerance toward gut microflora ascribed to CD patients and reflected by the characteristic serological response to mannan (ASCA) commonly found in CD patients.

Seibold *et al*. reported an association between mannan-binding lectin (MBL) deficiency and ASCA prevalence in CD.[46] MBL is considered to be part of the innate immune response, specifically the lectin activation pathway of complement.[16] Muller *et al*. showed that lack of MBL resulted in the acceleration of experimental colitis.[47] Taken together, this may indicate that impaired innate recognition/reaction to mannan may lead to an inadequate adaptive immune response toward mannan-bearing microorganisms.

The absence of a differential response to β-glucans or chitosan in CD compared with NL PBMCs may be due to the predominance of mannan on the surface of intact yeast cells, masking the β-glucan and chitin layers beneath it.[13,14] Wheeler and Fink identified a network of genes involved in masking β-glucans from the immune system. These authors showed that heat inactivation as well as other environmental conditions may alter the expression of these genes and consequently affect the exposure of β-glucans to immune cells, resulting in their increased ability for immune stimulation.[48]

Candida albicans and β-glucan have been shown to induce priming of human PBMCs *in vitro*, as reflected by enhanced cytokine production following stimulation with TLR ligands and bacterial commensals.[49] Here, we found increased TNF-α along with decreased IL-10 secretion in CD patients compared with controls in response to heat-killed *C. albicans* and *S. cerevisiae*. This response is the outcome not only of mannan stimulation, but also of a network of fungal cell wall glycans including β-glucans and chitin that were exposed by heat inactivation.

CD patients had no or minimal inflammatory activity. Importantly, they also minimally used therapies. This enabled us to focus on intrinsic immune responses to glycans, as patients with more active disease are usually treated with immunomodulation and biologic therapies that may potentially skew the results. Thus, one of the strengths of our study is showing that hyperresponsiveness to mannan is an intrinsic characteristic of CD patients as the results were not influenced by disease flare up or medical therapy.

As CD affects primarily the intestinal mucosa, and mucosal cells are the first to encounter intestinal antigens, whether bacterial or fungal, a major question is whether mucosal immune responses to fungi will be skewed similarly to peripheral ones. The issue of comparing and contrasting mucosal and peripheral immune responses to glycans is the focus of further studies. Noticeably, if fungal glycans would have a similar pro-inflammatory effect in the lamina propria, interventions aimed to ameliorate this effect could be planned.

Overall, our findings indicate that fungal glycans and whole yeast cells induce the secretion of various cytokines by PBMCs. Furthermore, mannan-induced pro-inflammatory cytokine secretion was significantly higher in CD. Given these data, it is plausible that there is a distorted chronic stimulation of immune cells by fungal antigens in CD, which is reflected by antibodies to specific fungal cell wall components. This may account for the differences between CD and NL PBMCs described in this article, and potentially represent comparable effects in mononuclear cells in the intestinal lamina propria.

Acknowledgments

This study was partially supported by a generous grant from the Leona M and Harry B. Helmsley Charitable Trust.

References

1 Gionchetti P, Rizzello F, Lammers KM *et al*. Antibiotics and probiotics in treatment of inflammatory bowel disease. *World J. Gastroenterol.* 2006; **12**: 3306–13.
2 Sartor RB. The influence of normal microbial flora on the development of chronic mucosal inflammation. *Res. Immunol.* 1997; **148**: 567–76.
3 Sellon RK, Tonkonogy S, Schultz M *et al*. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect. Immun.* 1998; **66**: 5224–31.
4 Dotan I, Fishman S, Dgani Y *et al*. Antibodies against laminaribioside and chitobioside are novel serologic markers in Crohn's disease. *Gastroenterology* 2006; **131**: 366–78.
5 Ferrante M, Henckaerts L, Joossens M *et al*. New serological markers in inflammatory bowel disease are associated with complicated disease behaviour. *Gut* 2007; **56**: 1394–403.
6 Sendid B, Quinton JF, Charrier G *et al*. Anti-*Saccharomyces cerevisiae* mannan antibodies in familial Crohn's disease. *Am. J. Gastroenterol.* 1998; **93**: 1306–10.
7 Seow CH, Stempak JM, Xu W *et al*. Novel anti-glycan antibodies related to inflammatory bowel disease diagnosis and phenotype. *Am. J. Gastroenterol.* 2009; **104**: 1426–34.
8 Israeli E, Grotto I, Gilburd B *et al*. Anti-*Saccharomyces cerevisiae* and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease. *Gut* 2005; **54**: 1232–6.
9 Dotan I. New serologic markers for inflammatory bowel disease diagnosis. *Dig. Dis.* 2010; **28**: 418–23.
10 Konrad A, Rutten C, Flogerzi B, Styner M, Goke B, Seibold F. Immune sensitization to yeast antigens in ASCA-positive patients with Crohn's disease. *Inflamm. Bowel Dis.* 2004; **10**: 97–105.
11 Rieder F, Hahn P, Finsterhoelzl L *et al*. Clinical utility of anti-glycan antibodies in pediatric Crohn's disease in comparison with an adult cohort. *Inflamm. Bowel Dis.* 2012; **18**: 1221–31.
12 Rieder F, Schleder S, Wolf A *et al*. Association of the novel serologic anti-glycan antibodies anti-laminarin and anti-chitin with complicated Crohn's disease behavior. *Inflamm. Bowel Dis.* 2010; **16**: 263–74.
13 Chaffin WL. *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* 2008; **72**: 495–544.
14 Free SJ. Fungal cell wall organization and biosynthesis. *Adv. Genet.* 2013; **81**: 33–82.
15 Brown GD. Innate antifungal immunity: the key role of phagocytes. *Annu. Rev. Immunol.* 2011; **29**: 1–21.
16 Hardison SE, Brown GD. C-type lectin receptors orchestrate antifungal immunity. *Nat. Immunol.* 2012; **13**: 817–22.
17 Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 2003; **197**: 1119–24.
18 Gringhuis SI, Wevers BA, Kaptein TM *et al*. Selective C-Rel activation via Malt1 controls anti-fungal T(H)-17 immunity by dectin-1 and dectin-2. *PLoS Pathog.* 2011; **7**: e1001259.
19 Taylor PR, Tsoni SV, Willment JA *et al*. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 2007; **8**: 31–8.
20 Robinson MJ, Osorio F, Rosas M *et al*. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 2009; **206**: 2037–51.
21 Sato K, Yang XL, Yudate T *et al*. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J. Biol. Chem.* 2006; **281**: 38854–66.
22 van de Veerdonk FL, Marijnissen RJ, Kullberg BJ *et al*. The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe* 2009; **5**: 329–40.
23 Garner RE, Rubanowice K, Sawyer RT, Hudson JA. Secretion of TNF-alpha by alveolar macrophages in response to *Candida albicans* mannan. *J. Leukoc. Biol.* 1994; **55**: 161–8.
24 Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J. Immunol.* 2010; **184**: 6335–42.
25 Muzzarelli RA. Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Mar. Drugs* 2010; **8**: 292–312.
26 Rao TS, Currie JL, Shaffer AF, Isakson PC. In vivo characterization of zymosan-induced mouse peritoneal inflammation. *J. Pharmacol. Exp. Ther.* 1994; **269**: 917–25.
27 Rizzetto L, Kuka M, De Filippo C *et al*. Differential IL-17 production and mannan recognition contribute to fungal pathogenicity and commensalism. *J. Immunol.* 2010; **184**: 4258–68.
28 Elsori DH, Yakubenko VP, Roome T *et al*. Protein kinase Cdelta is a critical component of Dectin-1 signaling in primary human monocytes. *J. Leukoc. Biol.* 2011; **90**: 599–611.
29 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; **106**: 2543–50.
30 Shah VB, Huang Y, Keshwara R, Ozment-Skelton T, Williams DL, Keshvara L. Beta-glucan activates microglia without inducing cytokine production in Dectin-1-dependent manner. *J. Immunol.* 2008; **180**: 2777–85.
31 Goodridge HS, Reyes CN, Becker CA *et al*. Activation of the innate immune receptor Dectin-1 upon formation of a "phagocytic synapse". *Nature* 2011; **472**: 471–5.
32 Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J. Immunol.* 2009; **182**: 3573–82.
33 Drummond RA, Brown GD. The role of Dectin-1 in the host defence against fungal infections. *Curr. Opin. Microbiol.* 2011; **14**: 392–9.
34 Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* 2011; **34**: 651–64.

35 Roy RM, Klein BS. Dendritic cells in antifungal immunity and vaccine design. *Cell Host Microbe* 2012; **11**: 436–46.
36 Sancho D, Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu. Rev. Immunol.* 2012; **30**: 491–529.
37 Drummond RA, Saijo S, Iwakura Y, Brown GD. The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. *Eur. J. Immunol.* 2011; **41**: 276–81.
38 Skrzypek F, Cenci E, Pietrella D, Rachini A, Bistoni F, Vecchiarelli A. Dectin-1 is required for human dendritic cells to initiate immune response to *Candida albicans* through Syk activation. *Microbes Infect.* 2009; **11**: 661–70.
39 Gavino AC, Chung JS, Sato K, Ariizumi K, Cruz PD Jr. Identification and expression profiling of a human C-type lectin, structurally homologous to mouse dectin-2. *Exp. Dermatol.* 2005; **14**: 281–8.
40 Zhu LL, Zhao XQ, Jiang C *et al*. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. *Immunity* 2013; **39**(2): 324–34.
41 Taylor PR, Gordon S, Martinez-Pomares L. The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol.* 2005; **26**: 104–10.
42 Weinblatt ME, Kavanaugh A, Genovese MC, Musser TK, Grossbard EB, Magilavy DB. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. *N. Engl. J. Med.* 2010; **363**: 1303–12.
43 Reese GE, Constantinides VA, Simillis C *et al*. Diagnostic precision of anti-*Saccharomyces cerevisiae* antibodies and perinuclear antineutrophil cytoplasmic antibodies in inflammatory bowel disease. *Am. J. Gastroenterol.* 2006; **101**: 2410–22.
44 Ruutu M, Thomas G, Steck R *et al*. beta-glucan triggers spondylarthritis and Crohn's disease-like ileitis in SKG mice. *Arthritis Rheum.* 2012; **64**: 2211–22.
45 Ferwerda B, Ferwerda G, Plantinga TS *et al*. Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* 2009; **361**: 1760–7.
46 Seibold F, Boldt AB, Seibold-Schmid B *et al*. Deficiency for mannan-binding lectin is associated with antibodies to *Saccharomyces cerevisiae* in patients with Crohn's disease and their relatives. *Gut* 2007; **56**: 152.
47 Muller S, Schaffer T, Flogerzi B *et al*. Mannan-binding lectin deficiency results in unusual antibody production and excessive experimental colitis in response to mannose-expressing mild gut pathogens. *Gut* 2010; **59**: 1493–500.
48 Wheeler RT, Fink GR. A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog.* 2006; **2**: e35.
49 Ifrim DC, Joosten LA, Kullberg BJ *et al*. *Candida albicans* primes TLR cytokine responses through a Dectin-1/Raf-1-mediated pathway. *J. Immunol.* 2013; **190**: 4129–35.