CMI-2011-0177R

Dectin-1 isoforms contribute to distinct Th1/Th17 cell activation in mucosal candidiasis

Agostinho Carvalho,[1,2,3] Gloria Giovannini,[1] Antonella De Luca,[1] Carmen D'Angelo,[1] Andrea Casagrande,[1] Rossana G. Iannitti,[1] Giovanni Ricci[1], Cristina Cunha[1] and Luigina Romani,[1]

[1]Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy; [2]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; [3]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal.

Correspondence: Luigina Romani, Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06126 Perugia, Italy. Telephone and Fax: +39 075 585 7411. E-mail: lromani@unipg.it.

Abstract

Recognition of β-glucans by dectin-1 has been shown to mediate cell activation, cytokine production and a variety of antifungal responses. Here, we report that the functional activity of dectin-1 in mucosal immunity to *Candida albicans* is influenced by the genetic background of the host. Dectin-1 was required for the proper control of gastrointestinal and vaginal candidiasis in C57BL/6 but not BALB/c mice, the latter actually showing increased resistance in the absence of dectin-1. Susceptibility of dectin-1-deficient C57BL/6 mice to infection was associated with defective IL-17A, aryl hydrocarbon receptor-dependent IL-22 production as well as adaptive Th1 responses. In contrast, resistance of dectin-1-deficient BALB/c mice was associated with increased IL-17A and IL-22 production, and the skewing towards Th1/Treg immune responses that provide immunological memory. Disparate canonical/noncanonical NF-κB signaling pathways downstream dectin-1were activated in the two different mouse strains. Thus, the net activity of dectin-1 in antifungal mucosal immunity is dependent on the host's genetic background that affects both the innate cytokine production as well as the adaptive Th1/Th17 cell activation upon dectin-1 signaling.

Introduction

Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors, particularly dectin-1, are essential determinants of host antifungal immunity [1-3]. Dectin-1 is a major β-glucan receptor expressed on the surface of a variety of cells, including myeloid [4] and epithelial cells [5-6]. It recognizes β-1,3-glucans exposed on particles such as zymosan and many fungi, including species of *Candida*, *Aspergillus* and *Pneumocystis* [7-9] either alone or in conjunction with other PRRs, most notably TLR2 and the mannose receptor [10]. As the main non-opsonic receptor involved in fungal uptake [11], dectin-1 engagement mediates cell activation, cytokine production and a variety of antifungal responses through the spleen tyrosine kinase (Syk)/ caspase recruitment domain-containing protein 9 (CARD9)-dependent pathway [12]. Recently, dectin-1 was demonstrated to signal also through Raf-1 and both Syk- and Raf-1-dependent pathways converging at the level of NF-κB activation to control adaptive immunity to fungi [13].

Although the crucial role of dectin-1 in antifungal immunity is undisputed both in mice and humans, the precise mechanisms by which dectin-1signalling contributes to innate and adaptive immune resistance to mucosal and systemic candidiasis are not completely clarified. The recent discovery of a genetic polymorphism in the human *DECTIN1* gene, Y238X, which generates a truncated protein with impaired cell surface expression and decreased ligand binding ability, points to the important antifungal function of dectin-1 in humans [14]. Indeed, Y238X carriers were more susceptible to mucocutaneous candidiasis [14] and, when undergoing allogeneic stem cell transplantation, displayed increased frequency of oral and gastrointestinal colonization with *Candida* species [15]. Interestingly, the Y238X polymorphism had no associated risk with systemic candidiasis, likely due to unimpaired phagocytosis and killing of *C. albicans* by host leukocytes [15]. Thus, both in humans and mice [16], dectin-1 appears to be crucially involved in the control of mucosal candidiasis, while discrepant results were obtained on its role in the systemic infection.

One most important mechanism of dectin-1-mediated immune resistance relies on the activation of Th1 and Th17 cells. Th17 responses are thought to be important in the defense against *C. albicans*, as humans with diseases characterized by defective Th17 responses (e.g. chronic mucocutaneous candidiasis, hyper IgE syndrome, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) show increased susceptibility to mucosal candidiasis [14, 17]. While murine studies have failed to show an unequivocal role for dectin-1 in IL-17 production during systemic candidiasis [7-8, 12, 18], the role of dectin-1 in the Th1/Th17 cell skewing in experimental mucosal candidiasis has never been directly addressed. Ultimately, the contrasting results in the different models may be related to the site-specific requirement of Th17 cells that are central to the control of mucosal, rather than systemic, infection [19].

In the present study, we have directly assessed the contribution of the dectin-1/Th17 axis in different models of mucosal candidiasis by using a side-by-side comparison of dectin-1 functional deficiency in genetically unrelated strains of mice. The contribution of dectin-1-mediated

mechanisms of antifungal resistance was indeed found to depend to some extent on the genetic background of the host [5, 20]. Macrophages from BALB/c and genetically related strains have been reported to express the full-length dectin-1A and the stalkless dectin-1B isoforms at comparable levels, whereas macrophages from the C57BL/6 background and related mice predominantly expressed the smaller isoform [21]. The results of the present study demonstrate that the functional activity of dectin-1 in both gastrointestinal and vaginal candidiasis is contingent upon the host genetic background and affects both the innate cytokine production as well as the adaptive Th1/Th17 cell activation.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice, 8 to 10 weeks old, were purchased from Charles River laboratories (Calco, Italy). Homozygous *Dectin-1*$^{-/-}$ mice on both C57BL/6 and BALB/c backgrounds were kindly provided by Emiko Kazama, University of Tokyo, Japan. All mice were housed under specific pathogen-free facilities at the Animal Facility of Perugia University, Perugia, Italy and were used in accordance with protocols approved by Animal Welfare Assurance A-3143-01.

Fungal strains

The wild-type *C. albicans* MKY378 [22] and the isogenic strains obtained by mutagenesis of the parental strain 3153A and capable (referred to as virulent Vir^{3}) or not (low-virulence Vir^{-3}) of yeast-to-hyphae transition, as assessed by germ-tube formation in vitro [23], were used. Yeast cells were obtained by harvesting at the end of the exponential growth phase.

Gastrointestinal infection

Unless stated otherwise, gastrointestinal infection was performed by inoculating mice intragastrically (i.g.) with 1×10^{8} Vir^{3} or Vir^{-3}cells in 200 µl of saline using a 18-gauge 4 cm-long plastic catheter. Re-infection was performed 14 days after the primary i.g. infection by intravenous (i.v.) inoculation of 5×10^{5} Vir^{3}cells. Quantification of fungal burden in the stomach, colon and kidneys of infected mice was performed at different days post-infection (dpi) by plating triplicate serial dilutions of homogenized organs in Sabouraud dextrose agar and results were expressed as colony-forming units (CFUs) per organ (mean ± SE). For histology, paraffin-embedded tissue sections (3-4 µm) of stomach were stained with periodic acid-Schiff (PAS) reagent. Histology sections were observed using a BX51 microscope (Olympus, Milan, Italy) and images were captured using a high-resolution DP71 camera (Olympus).

Vaginal infection

Vaginal infection was performed by inoculating mice intravaginally with 5×10^6 Vir[3] cells in 10 μl of saline. Seventy-two hours prior to infection, mice were injected subcutaneously with 0.1 mg of estradiol benzoate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 ml sesame oil. Estrogen treatments were continued at weekly intervals thereafter. Estrogen-treated control mice were treated with estrogen as described above and given saline intravaginally. Re-infection was performed by intravaginal inoculation of 5×10^6 Vir[3] cells in intravaginally-infected mice, 3 weeks after the primary infection. To quantify vaginal fungal burden at different dpi, 100 μl of vaginal lavages were directly plated onto Sabouraud dextrose agar plates supplemented with gentamicin (Sigma-Aldrich). After incubation at 37°C for 48 h, CFUs were enumerated and results were expressed as mean ± SE. Cytospin preparations of the lavage fluids were stained with May-Grünwald-Giemsa and analyzed for polymorphonuclear (PMN) cell recruitment using a BX51 microscope equipped with a high-resolution DP71 camera (Olympus).

In vitro cultures

Peyer's patches (PP) cells from naïve mice were stimulated for 18 h in vitro with 10 μg/ml β-glucan (Sigma-Aldrich) prior to assess *Tnfa, p35, p19* and *Il10* gene expression by real-time PCR. Expression of *Il22* was assessed following stimulation with β-glucan as above or with 20 nM 6-formylindolo[3,2-b]carbazole (FICZ, Enzo Life Sciences, Vonci-Biochem, Italy).

Canonical and noncanonical NF–κB on dendritic cells (DCs)

Murine DCs were obtained by culturing bone marrow cells in RPMI medium containing 10% filtered bovine serum, penicillin, streptomycin, 2mM L-glutamine in the presence of (20 ng/ml) mouse rGM-CSF (PROSPECbio, Prodotti Gianni S.p.A. Milan, Italy) and (10 ng/ml) rIL-4 (PROSPECbio) for 7 days to obtain CD11b+ DCs . Cells were stimulated with *C. albicans* Vir[3] (1:1 ratio) for 30 min at 37°C. We used an ELISA-based TransAM ™Flexi NF–κB Family Kit (Active Motif) to monitor activity of NF–κB family members on nuclear extracts (Nuclear Extract Kit, TransAM ™ Flexi NF–κB Family Kit).

Real time RT-PCR

Real-time RT-PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry (Agilent Technologies, Milan, Italy). Total RNA was extracted from $CD4^+$ T cells purified the mesenteric lymph nodes (MLN) of infected animals using RNeasy Mini Kit (QIAGEN, Milan, Italy) and was reverse transcribed with Sensiscript Reverse Transcriptase (QIAGEN) according to the manufacturer's instructions. PCR primers were as described [23]. The sense/antisense primer for *Ahr* was as follows: sense 5'-TCCATCCTGGAAATTCGAACC–3'; antisense 5'-

TCTTCATGCGTCAGTGGTCTC –3'. The thermal profile for real-time PCR was 95°C for 3 min, followed by 45 cycles of denaturation for 1 min at 95°C, annealing for 1 min at the appropriate temperature and extension for 30 sec at 72°C. Amplification efficiencies were validated and normalized against *Gapdh*. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as fold increase over day zero.

ELISA assay

Cytokine content was determined by enzyme-linked immunosorbent assays (R&D Systems, Milan, Italy) on stomach homogenates or vaginal lavage fluids. The detection limits ($pg.ml^{-1}$) of the assays were <10 for IFN-γ, IL-17A, IL-17F and IL-17E, and <3.2 for IL-22.

Statistical analysis

Statistical significance was assessed by one-way analysis of variance (ANOVA) or unpaired Student's T-test with Bonferroni's correction using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). P values ≤0.05 were considered statistically significant. Data are representative of at least two independent experiments or pooled from three to five experiments. The in vivo groups consisted of 6-8 mice/group.

Results

The susceptibility of Dectin-1$^{-/-}$ mice to gastrointestinal candidiasis depends on host's genetic background

Given that distinct expression patterns of dectin-1 isoforms have been described among genetically unrelated strains of mice [21], we assessed whether and how the host's genetic background influenced dectin-1 activity in mucosal candidiasis. For this purpose, we infected dectin-1-deficient mice on both C57BL/6 and BALB/c backgrounds with two different strains of C. *albicans* intragastrically and assessed the pattern of susceptibility and/or resistance to infection in terms of fungal burden, inflammatory pathology, innate and adaptive immunity. We found an opposite role for dectin-1 in antifungal resistance, depending on the host's genetic background. Indeed, the susceptibility to the gastrointestinal infection was increased in C57BL/6 *Dectin-1$^{-/-}$* mice with both strains of *C. albicans*, as judged by the higher fungal burden in the stomach and colon at 4 and 7 dpi, as well as the dissemination to the kidneys (Fig. 1*A*). A similar susceptibility phenotype was observed using a lower inoculum (5×10^6) of the Vir^3 cells or with the low-virulence Vir^{-3} strain (Fig. 1*B*). Although both wild-type and *Dectin-1$^{-/-}$* mice eventually cleared the infection (Fig. 1*A*), histological analysis revealed a predominant superficial infection with limited submucosal inflammation and inflammatory cell recruitment in the stomach of wild-type mice as opposed to *Dectin-1$^{-/-}$* mice who showed instead signs

of massive inflammatory infiltrates with extensive tissue invasion and parakeratosis, as well as hyphae penetrating the mucosal barrier (Fig. 1*C*). In contrast to what observed in C57BL/6 mice, BALB/c *Dectin-1*$^{-/-}$ mice were more resistant to the infection with both strains of *C. albicans* than wild-type mice, as judged by the decreased fungal burden, lack of peripheral dissemination (Fig. 2*A*) and limited signs of inflammatory cell recruitment and mucosal hyperplasia (Fig. 2*C*). Expectedly, *Dectin-1*$^{-/-}$ mice were also more resistant to infection with the lower inoculum of the Vir^{3} or with the low-virulence Vir^{-3} strain (Fig. 2*B*). These results clearly show that the susceptibility of *Dectin-1*$^{-/-}$ mice to gastrointestinal candidiasis depends on the host's genetic background and less on fungal strains, at least with the fungal strains we have used.

To assess whether the susceptibility phenotypes of *Dectin-1*$^{-/-}$ mice on both the C57BL/6 and BALB/c backgrounds were retained in vaginal candidiasis, we intravaginally infected each strain of mice with the Vir^{3} *C. albicans* cells. Similarly to what observed in the gastrointestinal infection, C57BL/6 *Dectin-1*$^{-/-}$ mice were more susceptible than wild-type mice to the infection as judged by the higher fungal burdens in the vagina (Fig. 3*A*) and the prominent inflammatory cell recruitment in the vaginal fluids (Fig. 3*C* inset) and vagina. BALB/c *Dectin-1*$^{-/-}$ mice were instead more resistant to the infection than their wild-type counterpart, as judged by the decreased fungal burden (Fig. 3*B*), limited signs of inflammation in the vagina (Fig. 3*D*) and of inflammatory cell recruitment in the vaginal fluid (Fig. 3*D* inset).

Dectin-1 promotes distinct cytokine profiles in the different mouse strains

The divergent effects of dectin-1 deficiency in C57BL/6 and BALB/c mice on susceptibility to the infection predict that distinct cytokine profiles are activated following dectin-1 engagement that critically defines the phenotypes observed. We measured the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-6, as well as cytokines of the IL-17 family, such as IL-17A, IL-17F and IL-17E, known to be crucial for mucosal antifungal defense [23-24], in the stomach of mice with gastrointestinal candidiasis. We found that the levels of TNF-α and IL-6 were higher in C57BL/6 *Dectin-1*$^{-/-}$ mice and lower in BALB/c *Dectin-1*$^{-/-}$ mice (Fig. 4*A*) as compared to the respective wild-type control. A different pattern of production was observed with members of the IL-17 family, being IL-17A, IL-17F and IL-17E production greatly reduced in C57BL/6 *Dectin-1*$^{-/-}$ mice and significantly increased in BALB/c *Dectin-1*$^{-/-}$ mice (Fig. 4*B*) as compared to the respective wild-type strains. The cytokine profiles in the vaginal fluids mirrored those in the stomach homogenates of gastrointestinal-infected mice as IL-17A, IL-17F and IL-22 were almost completely absent in C57BL/6 *Dectin-1*$^{-/-}$ mice and significantly increased in BALB/c *Dectin-1*$^{-/-}$ mice (Fig. 4*C*) as compared to the respective controls. Altogether, these findings point to similar susceptibility phenotypes to both vaginal and gastrointestinal candidiasis in condition of dectin-1 deficiency.

To further prove that the different isoforms promote distinct cytokine profiles, we assessed cytokine gene expression in Peyer's patches (PP) following stimulation with β-glucan. We found that (Fig. 5*A-B*) *Tnfa* induction was critically dependent on dectin-1, irrespective of the genetic background; the expressions of *IL-12p35*, *IL-23p19* and *Il10* were instead differently affected in condition of dectin-1 deficiency. The expressions of *IL-12p19* was particularly reduced in PP from C57BL/6 compared to BALB/c *Dectin-1*$^{-/-}$ mice while the expressions of *IL-12p35* and *Il10* were particularly reduced in BALB/c compared to C57BL/6 *Dectin-1*$^{-/-}$ mice. Altogether, these results indicate that the impact of dectin-1 function on cytokine response is contingent upon the genetic background and presumably on distinct dectin-1 isoform expression.

Dectin-1 differentially impacts on the Ahr/IL-22 axis in the different mouse strains

IL−22 plays a crucial role in the innate immune defense and mucosal protection from damage in mucosal candidiasis [23]. Produced by NK22 cells expressing the aryl hydrocarbon receptor (AhR), IL−22 directly targeted gut epithelial cells (ECs) to induce signal transducer and activator of transcription 3 phosphorylation and the release of S100A8 and S100A9 peptides known to have anticandidal activity and anti−inflammatory effects [3, 23]. We looked for *Ahr* expression and IL-22 production in vivo, as well as on PP cells comparatively stimulated in vitro with either β-glucan or the AhR agonist FICZ. We found that C57BL/6 *Dectin-1*$^{-/-}$ mice failed to up-regulate *Ahr* expression and IL-22 production in infection as opposed to wild-type control (Fig. 6*A*). In vitro, *Il22* expression was not up-regulated in PP from C57BL/6 *Dectin-1*$^{-/-}$ mice upon stimulation with either β-glucan or FICZ (Fig. 6*B*). An opposite pattern of *Ahr* expression and IL-22 production was observed in BALB/c *Dectin-1*$^{-/-}$ mice who showed a significant increased *Ahr* expression and concomitant IL-22 production in vivo (Fig. 6*C*) and in vitro after stimulation with FICZ (Fig. 6*D*). These data suggest that dectin-1 signaling differentially impacts on the functional activity of the AhR/IL-22 axis in gastrointestinal candidiasis.

Dectin-1 promotes distinct adaptive memory Th immune responses depending on mouse genetic background

Stimulation of dectin-1 on DCs efficiently generates Th1 and Th17 responses [25]. We evaluated the activation of distinct Th cell subsets in vaccine-induced resistance to *C. albicans*. We subjected intragastrically-infected mice to intravenous re-infection with the fungus two weeks later and evaluated parameters of fungal growth in the kidneys and activation of $CD4^+$ Th cells in the MLN. We found that C57BL/6 *Dectin-1*$^{-/-}$ mice were unable to resist re-infection, as opposed to wild-type mice (Fig. 7*A*). In contrast, both wild-type and BALB/c *Dectin-1*$^{-/-}$ mice resisted the re-infection (Fig. 7*B*). Actually, resistance to re-infection occurred despite the high susceptibility to the primary disseminated infection exhibited by BALB/c *Dectin-1*$^{-/-}$ mice, a finding pointing to the ability of these mice to mount strong protective memory responses to the fungus. Similar to the above findings,

intravaginally-infected C57BL/6 *Dectin-1*$^{-/-}$ mice were unable to resist re-infection, as opposed to BALB/c *Dectin-1*$^{-/-}$ mice (Fig. 7*C-D*). On assessing the quality of the Th cell responses, we found a decreased expression of *Ifng* and *Il10* and the corresponding transcription factors *Tbet* and *Foxp3* in MLN of C57BL/6 *Dectin-1*$^{-/-}$ mice as compared to the wild-type (Fig. 7*E*). This was associated with an increased expression of *Il17a* and *Rorc* (Fig. 7*E*), suggesting that Th1, more than Th17, cell activation is dependent on dectin-1 in C57BL/6 mice. In BALB/c *Dectin-1*$^{-/-}$ mice, resistance to re-infection was instead associated with a robust Th1/Treg response, associated with levels of *Il17a/Rorc* expression that were actually lower than those of wild-type mice (Fig. 7*F*), suggesting that Th17, more than Th1, cell activation is dependent on dectin-1 on BALB/c . These results indicate that the role of Dectin-1 in shaping antifungal memory Th cell responses also depends on the genetic background of the host.

NF-κB signaling pathways are differently affected by dectin-1 deficiency depending on mouse strain

In addition to the classical Syk-dependent pathway leading to canonical NF-κB p65 and c-Rel subunit activation and TNF-α /IL-10 production [26], the Raf-1-dependent pathway inhibiting the expression of the noncanonical NF-κB RelB subunits and crucially promoting *Il12b* transcription [13] has also been described. We assessed whether either one or both of these pathways were altered in the absence of dectin-1 in DCs from C57BL/6 and BALB/c mice exposed to *C. albicans*. We found that the nuclear translocation of c-Rel was impaired in *Dectin-1*$^{-/-}$ mice on the C57BL/6 more than BALB/c background in response to the fungus (Fig. 8*A*). In contrast, the nuclear translocation of RelB was greatly promoted in *Dectin-1*$^{-/-}$ mice on the BALB/c more than C57BL/6 background (Fig. 8*B*). These findings suggest that the Syk and the Raf-1 pathways are differently affected in the absence of dectin-1 signaling in the two mouse strains.

Discussion

Although the dectin-1/inflammasome host immune pathway drives protective Th17 responses and distinguishes between colonization and tissue invasion by *C. albicans* [27], the present study shows that function of dectin-1 in mucosal antifungal immunity extend beyond Th17 cell activation and is critically dependent on the genetic background of the host. This has been previously demonstrated for infections with *A. fumigatus* [5] and *Coccidioides* spp. [20], in which mechanisms of antifungal resistance were to some extent determined by host's genetics. It has been suggested that dectin-1 responses may be dependent on fungal strains[1] as well on the physical status of β-glucans, soluble or particulate [28]. Although we have obtained similar findings using two distinct *C. albicans* strains, we cannot rule out that these responses are also contingent upon the fungal strains. The present study is the first to show a side-by-side comparison of dectin-1 functional deficiency in mucosal candidiasis driven by a given fungal strain in genetically unrelated strains of mice. Consistent with

the distinct downstream signaling pathways that are activated upon dectin-1 engagement to regulate immunity to fungi [13], we found that dectin-1 signaling is either required or dispensable in mucosal candidiasis, depending on mouse genetics. Dectin-1 was required for the control of fungal colonization at mucosal surfaces, both in the gastrointestinal and vaginal tracts, in C57BL/6 mice and was required for the production of IL-17A, IL-17F and IL-22 at sites of infection. In contrast, dectin-1 was dispensable in BALB/c mice in which resistance to infection was associated with the production of IL-17A, IL-17F and IL-22. In terms of adaptive memory Th responses, Dectin-1 was apparently required for the activation of Th1/Treg memory responses in C57BL/6 mice and for Th17 memory responses in BALB/c mice. It has been shown that dectin-1 may influence cytokine production in DCs, leading to Th1/Th17 cell activation, by affecting the balance between canonical/noncanonical NF-κB activation on DCs [13]. Although we found a different pattern of canonical/noncanonical NF-κB activation in DCs from either type of mice, the exact molecular pathways linking DC activation to Th skewing in infection, in condition of dectin-1deficiency on the different genetic backgrounds need further evaluation. Additional studies aimed at selectively inhibiting the Syk or Raf-1 pathway in the two strains of mice are required to provide the causal association between signaling pathways activated in vitro and susceptibility/resistance to infection in vivo.

It is plausible to hypothesize that these two extremely divergent phenotypes may rely on the differential expression of dectin-1 isoforms. Indeed, these alternatively spliced isoforms have been found to lead to the production of different levels of TNF upon zymosan recognition [21], a finding suggesting that the structure of the receptor and its ability to form dimmers through the stalk region may influence cytokine production. It is therefore not surprising that dectin-1 deficiency in C57BL/6 and BALB/c mice leads to disparate cytokine profiles upon mucosal infection with *C. albicans*. In addition, and despite no functional consequences have been observed upon zymosan recognition, nonsynonymous single nucleotide polymorphisms have been identified in C57BL/6 mice by genetic comparison with the BALB/c background [21], suggesting additional functional variability of dectin-1 among these strains. In this regard, as cooperative signaling between dectin-1 and other PRRs is crucial for efficient recognition of *C. albicans* cells [10], the differences in TLR2 and MR expression seen among different inbred strain of mice [30-31] may offer an additional plausible explanation.

One interesting observation of the present study is the intriguing relationship between dectin-1 and AhR. Originally recognized as causing immunosuppression after binding dioxin, mammalian AhR is now known to crucially affect IL-22 production [32] as well as the balance of T cell differentiation into Th1/Treg *vs*. Th17 cells [33]. In this regard, IL-22 was recently found to be produced in response to *A. fumigatus* by a dectin-1-dependent mechanism [34]. In combination with IL-17A, IL-22 has been found to be crucially involved in the control of *Candida* growth in the gastrointestinal tract in conditions of Th1 and Th17 deficiency [23]. Moreover, vaginal ECs also produced S100A8 and S100A9

following interaction with *Candida* [35] suggesting the possible involvement of IL-22 in vaginal candidiasis. Thus, IL-22$^+$ cells, employing ancient effector mechanisms of immunity, may represent a primitive mechanism of resistance against the fungus under conditions of limited inflammation. The finding that the AhR/ IL-22 axis was impaired in C57BL/6 *Dectin-1*$^{-/-}$ mice but not in BALB/c *Dectin-1*$^{-/-}$ mice indicates that dectin-1 receptor cooperativity may go beyond PRRs to include receptors involved in cell cycle and metabolism [36].

Regardless of the mechanisms of this cooperative signaling, our study clearly shows that dectin-1 crucially contributes to the balance of Th1/Th17/Treg CD4$^+$ T cell populations during infection. At variance with what observed in murine aspergillosis [37], dectin-1 deficiency disproportionally increases both Th1/Treg (in BALB/c mice) and Th17 (in C57BL/6 mice) cell responses after *C. albicans* infection, a finding showing that dectin-1 signaling is involved in either Th1 or Th17 cell differentiation. This may explain the relative ability of *Dectin-1*$^{-/-}$ mice on either background to eventually control the infection. However, as BALB/ c, more than C57BL/6 mice, showed resistance to re-infection, Th1, more than Th17 cells, are endowed with long-term immune protection to the fungus, as already shown [23]. In the early phase of the infection, in contrast, IL-17A, and likely IL-17F, production clearly associates with a better control of the infection at both the gastrointestinal and vaginal sites and both productions are influenced by dectin-1. The mechanisms by which dectin-1 regulates IL-17A/IL-17F production by which innate immune cells in candidiasis is not known, but it is of interest that both neutrophils [38] and gammadelta T cells [39] produce IL-17A via dectin-1.

Altogether, our data indicate that the net activity of dectin-1 in antifungal mucosal immunity is dependent on the host's genetic background that affects both the innate production of IL-17A, IL-17F and IL-22 as well as the regulation of the Th1/Th17/Treg balance in adaptive immunity.

Acknowledgements

We thank Dr. Cristina Massi Benedetti for digital art and editing. The studies were supported by the Specific Targeted Research Project "ALLFUN" (FP7−HEALTH−2009 contract number 260338 to LR) and the Italian Project AIDS 2010 by ISS (Istituto Superiore di Sanità - contract number 40H40 to LR) and Fondazione Cassa di Risparmio di Perugia Project n. 2011.0124.021. AC and CC were financially supported by fellowships from Fundação para a Ciência e Tecnologia, Portugal (contracts SFRH/BPD/46292/2008 and SFRH/BD/65962/2009, respectively).

References

1. Drummond RA, Brown GD. The role of Dectin-1 in the host defence against fungal infections. *Curr Opin Microbiol* 2011.

2. Brown GD. Innate antifungal immunity: the key role of phagocytes. *Annu Rev Immunol* 2011; **29:** 1-21.

3. Romani L. Immunity to fungal infections. *Nat Rev Immunol* 2011; **11**(4)**:** 275-288.

4. Taylor PR, Brown GD, Reid DM, Willment JA, Martinez-Pomares L, Gordon S *et al.* The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *J Immunol* 2002; **169**(7)**:** 3876-3882.

5. Cunha C, Di Ianni M, Bozza S, Giovannini G, Zagarella S, Zelante T *et al.* Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood* 2010; **116**(24)**:** 5394-5402.

6. Lee HM, Yuk JM, Shin DM, Jo EK. Dectin-1 is inducible and plays an essential role for mycobacteria-induced innate immune responses in airway epithelial cells. *J Clin Immunol* 2009; **29**(6)**:** 795-805.

7. Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K *et al.* Dectin-1 is required for host defense against Pneumocystis carinii but not against *Candida albicans*. *Nat Immunol* 2007; **8**(1)**:** 39-46.

8. Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H *et al.* Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 2007; **8**(1)**:** 31-38.

9. Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, Schwiebert LM *et al.* Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. *J Immunol* 2009; **182**(8)**:** 4938-4946.

10. Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G *et al.* Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J Clin Invest* 2006; **116**(6)**:** 1642-1650.

11. Heinsbroek SE, Taylor PR, Martinez FO, Martinez-Pomares L, Brown GD, Gordon S. Stage-specific sampling by pattern recognition receptors during *Candida albicans* phagocytosis. *PLoS Pathog* 2008; **4**(11)**:** e1000218.

12. LeibundGut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV *et al.* Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 2007; **8**(6)**:** 630-638.

13. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Wevers B, Bruijns SC *et al.* Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. *Nat Immunol* 2009; **10**(2)**:** 203-213.

14. Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, Venselaar H *et al.* Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med* 2009; **361**(18)**:** 1760-1767.

15. Plantinga TS, van der Velden WJ, Ferwerda B, van Spriel AB, Adema G, Feuth T *et al.* Early stop polymorphism in human DECTIN-1 is associated with increased candida colonization in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 2009; **49**(5)**:** 724-732.

16. Gales A, Conduche A, Bernad J, Lefevre L, Olagnier D, Beraud M *et al.* PPARgamma controls dectin-1 expression required for host antifungal defense against *Candida albicans*. *PLoS Pathog* 2010; **6**(1)**:** e1000714.

17. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK *et al.* Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 2011; **332**(6025)**:** 65-68.

18. Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A *et al.* Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* 2010; **32**(5)**:** 681-691.

19. Zelante T, Luca A, Romani L. TH17 Cells in Fungal Infections. In: Jiang S (ed). *TH17 Cells in Health and Disease*. Springer New York, 2011, pp 299-317.

20. del Pilar Jimenez AM, Viriyakosol S, Walls L, Datta SK, Kirkland T, Heinsbroek SE *et al.* Susceptibility to Coccidioides species in C57BL/6 mice is associated with expression of a truncated splice variant of Dectin-1 (Clec7a). *Genes Immun* 2008; **9**(4)**:** 338-348.

21. Heinsbroek SE, Taylor PR, Rosas M, Willment JA, Williams DL, Gordon S *et al.* Expression of functionally different dectin-1 isoforms by murine macrophages. *J Immunol* 2006; **176**(9)**:** 5513-5518.

22. Karababa M, Valentino E, Pardini G, Coste AT, Bille J, Sanglard D. CRZ1, a target of the calcineurin pathway in Candida albicans. *Mol Microbiol* 2006; **59**(5)**:** 1429-1451.

23. De Luca A, Zelante T, D'Angelo C, Zagarella S, Fallarino F, Spreca A *et al.* IL-22 defines a novel immune pathway of antifungal resistance. *Mucosal Immunol* 2010; **3**(4)**:** 361-373.

24. Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ *et al.* Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 2009; **206**(2)**:** 299-311.

25. Agrawal S, Gupta S, Agrawal A. Human dendritic cells activated via dectin-1 are efficient at priming Th17, cytotoxic CD8 T and B cell responses. *PLoS One* 2010; **5**(10)**:** e13418.

26. Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E *et al.* Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 2005; **22**(4)**:** 507-517.

27. Cheng SC, van de Veerdonk FL, Lenardon M, Stoffels M, Plantinga T, Smeekens S *et al.* The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*. *J Leukoc Biol* 2011; **90**(2)**:** 357-366.

28. Qi C, Cai Y, Gunn L, Ding C, Li B, Kloecker G *et al.* Differential pathways regulating innate and adaptive antitumor immune responses by particulate and soluble yeast-derived beta-glucans. *Blood* 2011; **117**(25)**:** 6825-6836.

29. Bonifazi P, D'Angelo C, Zagarella S, Zelante T, Bozza S, De Luca A *et al.* Intranasally delivered siRNA targeting PI3K/Akt/mTOR inflammatory pathways protects from aspergillosis. *Mucosal Immunol* 2010; **3**(2)**:** 193-205.

30. Autenrieth SE, Autenrieth IB. Variable antigen uptake due to different expression of the macrophage mannose receptor by dendritic cells in various inbred mouse strains. *Immunology* 2009; **127**(4)**:** 523-529.

31. Liu T, Matsuguchi T, Tsuboi N, Yajima T, Yoshikai Y. Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. *Infect Immun* 2002; **70**(12)**:** 6638-6645.

32. Ramirez JM, Brembilla NC, Sorg O, Chicheportiche R, Matthes T, Dayer JM *et al.* Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells. *Eur J Immunol* 2010; **40**(9)**:** 2450-2459.

33. Stockinger B, Hirota K, Duarte J, Veldhoen M. External influences on the immune system via activation of the aryl hydrocarbon receptor. *Semin Immunol* 2011; **23**(2)**:** 99-105.

34. Gessner MA, Werner JL, Lilly LM, Nelson MP, Metz AE, Dunaway CW *et al.* Dectin-1 dependent IL-22 contributes to early innate lung defense against *Aspergillus fumigatus*. *Infect Immun* 2011.

35. Yano J, Lilly E, Barousse M, Fidel PL, Jr. Epithelial cell-derived S100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during *Candida* vaginitis. *Infect Immun* 2010; **78**(12)**:** 5126-5137.

36. Stejskalova L, Dvorak Z, Pavek P. Endogenous and exogenous ligands of aryl hydrocarbon receptor: current state of art. *Curr Drug Metab* 2011; **12**(2)**:** 198-212.

37. Rivera A, Hohl TM, Collins N, Leiner I, Gallegos A, Saijo S *et al.* Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *J Exp Med* 2011; **208**(2)**:** 369-381.

38. Werner JL, Gessner MA, Lilly LM, Nelson MP, Metz AE, Horn D *et al.* Neutrophils produce IL-17A in a Dectin-1 and IL-23 dependent manner during invasive fungal infection. *Infect Immun* 2011.

39. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 2009; **31**(2)**:** 321-330.

Figure legends

FIGURE 1. C57BL/6 *Dectin-1*$^{-/-}$ mice are susceptible to gastrointestinal candidiasis. *A*, Fungal growth (CFU±SE) at 4, 7, 14 and 21 days post-infection (dpi) in the stomach, colon and kidneys of wild-type (black diamonds) or *Dectin-1*$^{-/-}$ (white diamonds) mice infected intragastrically with 1×10^8 cells of Vir^3 or MKY378 strains of *C. albicans*. Data is representative of two to four independent experiments. *B*, Fungal growth (CFU±SE) at 4 dpi in the stomach of wild-type (black diamonds) or *Dectin-1*$^{-/-}$ (white diamonds) mice infected intragastrically with 5×10^6 Vir^3 cells or 1×10^8 low-virulence Vir^{-3}cells, respectively. Data are representative of two to four independent experiments. *C*, Histological analysis of the stomach from wild-type or *Dectin-1*$^{-/-}$ mice infected intragastrically with 1×10^8 *C. albicans* Vir^3 or MKY378 cells. PAS-stained stomach sections (at 4 dpi) showing fungal elements penetrating the mucosal barrier and inflammatory cell recruitment in *Dectin-1*$^{-/-}$ mice. Representative images of two to four independent experiments are shown. * P≤0.05, ** P≤0.01 and *** P≤0.001, wild-type C57BL/6 *vs Dectin-1*$^{-/-}$ mice.

FIGURE 2. BALB/c *Dectin-1*$^{-/-}$ mice are resistant to gastrointestinal candidiasis. *A*, Fungal growth (CFU±SE) at 4, 7, 14 and 21 days post-infection (dpi) in the stomach, colon and kidneys of wild-type (black dots) or *Dectin-1*$^{-/}$ (white dots) mice infected intragastrically with 1×10^8 cells of Vir^3 or MKY378 strains of *C. albicans*. Data is representative of two to four independent experiments. *B*, Fungal growth at 4 dpi in the stomach of wild-type (black dots) or *Dectin-1*$^{-/-}$ (white dots) mice infected intragastrically with 5×10^6 Vir^3 cells or 1×10^8 low-virulence Vir^{-3}cells, respectively. Data are representative of two to four independent experiments. *C*, Histological analysis of the stomach from wild-type or *Dectin-1*$^{-/-}$ mice infected intragastrically with 1×10^8 cells of *C. albicans* Vir^3 or MKY378. PAS-stained stomach sections (at 4 dpi) showing absence of mucosal damage and inflammatory cell recruitment in *Dectin-1*$^{-/-}$ mice. Representative images of two to four independent experiments are shown. * P≤0.05, ** P≤0.01 and *** P≤0.001, wild-type BALB/c *vs Dectin-1*$^{-/-}$ mice.

FIGURE 3. Dectin-1 deficiency affects susceptibility to vaginal candidiasis. Fungal growth (CFU±SE) at 2, 7, 14 and 21 days post-infection (dpi) in the vagina of *A*, C57BL/6 (black diamonds) or *Dectin-1* $^{-/-}$ (white diamonds) and *B*, BALB/c (black dots) or *Dectin-1* $^{-/-}$ (white dots) mice infected intravaginally with 5×10^6 cells of *C. albicans* Vir^3. Data are representative of two to four independent experiments. Histological analysis of the vagina from *C*, C57BL/6 or *D*, BALB/c wild-type or *Dectin-1*$^{-/-}$ mice, infected intravaginally with 5×10^6 cells of *C. albicans* Vir^3, as indicated. PAS-stained vaginal fluids (at 2 dpi) showing hyphal growth and inflammatory cell recruitment in C57BL/6 *Dectin-1*$^{-/-}$ mice, but not in BALB/c *Dectin-1*$^{-/-}$ mice. Representative images of two to four independent experiments are shown. * P≤0.05, ** P≤0.01, wild-type *vs. Dectin-1*$^{-/-}$ mice in either background, as indicated.

FIGURE 4. Dectin-1 promotes distinct cytokine profiles in the different mouse strains. Production of *A*, TNF-α and IL-6 and *B*, IL-17A, IL-17F and IL-17E at 4 dpi in the stomach of wild-type (black bars) and *Dectin-1*$^{-/-}$ (white bars) mice from C57BL/6 or BALB/c background infected intragastrically with 1×10^8 cells of *C. albicans* Vir^3. *C*, Production of IL-17A, IL-17F and IL-22 at 2 dpi in the vaginal fluid of wild-type (black bars) or *Dectin-1*$^{-/-}$ (white bars) mice from C57BL/6 or BALB/c background infected intravaginally (VG) with 5×10^6 cells of *C. albicans* Vir^3. Data represent the mean ± SE of three independent experiments. * P≤0.05, ** P≤0.01 and *** P≤0.001, uninfected vs. infected mice or infected wild-type vs. infected *Dectin-1*$^{-/-}$ mice in either background, as indicated.

FIGURE 5. Dectin-1 deficiency differently affects cytokine induction in response to β-glucan vitro. Expression of *Tnfa*, *Il12p35*, *Il23p19* and *Il10* in ex-vivo Peyer's patches from naïve wild-type and *Dectin-1*$^{-/-}$ mice from *A*, C57BL/6 or *B*, BALB/c backgrounds, either unstimulated (-) or stimulated (+) in vitro with β-glucan for 18 h. * P≤0.05, ** P≤0.01 and *** P≤0.001, unstimulated *vs.* stimulated Peyer's patches from either background, as indicated.

FIGURE 6. Dectin-1 differentially impacts on the AhR/IL-22 axis in the different mouse strains. *A* and *C*, *Ahr* expression and IL-22 production in vivo or *B* and *D*, in vitro in wild-type and *Dectin-1*$^{-/-}$ mice from the C57BL/6 *(A* and *B)* or BALB/c *(C* and *D)* mice infected intragastrically with *C. albicans* Vir^3 4 days before. *Ahr* and *Il22* expression (real time RT-PCR) and IL-22 production (ELISA) were done on ex-vivo Peyer's patches cells from naïve (-) or infected (+) mice or in cells from naïve mice stimulated in vitro with β-glucan or FICZ for 18 h. * P≤0.05, ** P≤0.01 and *** P≤0.001, wild-type *vs Dectin-1*$^{-/-}$ mice in either background, as indicated.

FIGURE 7. Dectin-1 deficiency associates with distinct adaptive Th responses. Fungal growth (CFU±SE) in the kidneys of *A*, C57BL/6 (black diamonds) or *Dectin-1*$^{-/-}$ (white diamonds) and *B*, BALB/c (black dots) or *Dectin-1*$^{-/-}$ (white dots) mice subjected to an intravenous rechallenge (2^{nd}) with 5×10^6 cells of *C. albicans* Vir^3 14 days after the primary i.g. infection. Control mice were subjected to a primary i.v. infection (1^{st}) in the same conditions. Data is representative of two to four independent experiments. * P≤0.05 and *** P≤0.001, wild-type control vs. rechallenged mice, as indicated. Fungal growth (CFU±SE) in the vagina of *C*, C57BL/6 (black diamonds) or *Dectin-1*$^{-/-}$ (white diamonds) and *D*, BALB/c (black dots) or *Dectin-1*$^{-/}$ (white dots) mice subjected to an intravaginal rechallenge with 5×10^6 cells of *C. albicans* Vir^3 at 14 dpi following the primary infection. Data is representative of two to four independent experiments. Gene expression of *Ifng*, *Il17a*, *Il10*, *Tbet*, *Rorc* and *Foxp3* at 0, 7 and 14 dpi in MLN from *E*, C57BL/6 (black diamonds) or *Dectin-1*$^{-/-}$ (white diamonds) and *F*, BALB/c (black dots) or *Dectin-1*$^{-/-}$ (white dots) mice infected intragastrically

with 1×10^8 cells of *C. albicans* Vir[3]. Data is representative of two to four independent experiments. * $P\leq0.05$ and ** $P\leq0.01$, wild-type vs. *Dectin-1*$^{-/-}$ mice in either background, as indicated.

FIGURE 8. Dectin-1 deficiency associates with distinct activation of NF–κB subunits. Activation of *A*, c-Rel or *B*, RelB subnuits of NF–κB was assessed by ELISA on dendritic cells from wild-type or *Dectin-1*$^{-/-}$ mice untreated (–) or stimulated (+) with *C. albicans* Vir[3] (1:1 ratio) for 30 min. Results are expressed as transcriptional activity levels of NF–κB determined by measuring absorbance at 450 nm (A_{450}). Data represents mean ± SE of three independent experiments. *P< 0.05, wild-type *vs. Dectin-1*$^{-/-}$ mice in either background, as indicated.