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Conventional Dendritic Cells Mount a Type I IFN Response against *Candida* spp. Requiring Novel Phagosomal TLR7-Mediated IFN-β Signaling

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Human fungal pathogens such as the dimorphic *Candida albicans* or the yeast-like *Candida glabrata* can cause systemic candidiasis of high mortality in immunocompromised individuals. Innate immune cells such as dendritic cells and macrophages establish the first line of defense against microbial pathogens and largely determine the outcome of infections. Among other cytokines, they produce type I IFNs (IFNs-I), which are important modulators of the host immune response. Whereas an IFN-I response is a hallmark immune response to bacteria and viruses, a function in fungal pathogenesis has remained unknown. In this study, we demonstrate a novel mechanism mediating a strong IFN-β response in mouse conventional dendritic cells challenged by *Candida* spp., subsequently orchestrating IFN-α/β receptor 1-dependent intracellular STAT1 activation and IFN regulatory factor (IRF) 7 expression. Interestingly, the initial IFN-β release bypasses the TLR 4 and TLR2, the TLR adaptor Toll/IL-1R domain-containing adapter-inducing IFN-β and the β-glucan/phagocytic receptors dectin-1 and CD11b. Notably, *Candida*-induced IFN-β release is strongly impaired by Src and Syk family kinase inhibitors and strictly requires completion of phagocytosis as well as phagosomal maturation. Strikingly, TLR7, MyD88, and IRF1 are essential for IFN-β signaling. Furthermore, in a mouse model of disseminated candidiasis we show that IFN-I signaling promotes persistence of *C. glabrata* in the host. Our data uncover for the first time a pivotal role for endosomal TLR7 signaling in fungal pathogen recognition and highlight the importance of IFNs-I in modulating the host immune response to *C. glabrata*. *The Journal of Immunology*, 2011, 186: 3104–3112.

Invasive *Candida* infections are life-threatening clinical conditions, primarily affecting immunosuppressed patient cohorts and patients with defects in cellular immunity (1). Mortalities associated with disseminated candidemia can exceed 30–40%, despite appropriate antifungal treatment. The dimorphic *Candida albicans* and the inherently drug-tolerant yeast-like *Candida glabrata* constitute the most frequent causes of fungal infections in humans (1). *C. albicans* can switch between a yeast and a filamentous (hyphae) form upon host or environmental stimuli.

Initial colonization and subsequent development of disseminated diseases are determined by the nature of the interaction of *Candida* spp. with host immune cells and tissues (2). The rate of clearance by the host immune surveillance versus the fungal fitness and growth in organs and tissues determines the outcome such as fungal clearance or host death. Early recognition of pathogens by immune cells is mediated by dedicated pattern recognition receptors (PRRs), including TLRs and C-type lectins expressed at the surface of innate cells, mainly monocytes, macrophages, neutrophils, and dendritic cells (DCs) (2). PRRs recognize microbe-specific pathogen-associated molecular patterns (PAMPs) and trigger several intracellular signaling pathways to orchestrate a pathogen-specific as well as cell-type–specific host immune response (3, 4).

The sugar polymers (e.g., chitin, β-D-glucans, and mannan) and proteins forming the fungal cell surface are considered the prime source of fungal PAMPs. Notably, β-D-glucans seem to be preferential ligands for the dectin-1 receptor, which mediates fungal recognition and signaling alone, as well as with the phospholipomannan receptor TLR2 as a coreceptor. By contrast, mannose-sensing receptors include the mannan receptor, TLR4, dectin-2, Mincle, the SIGNR receptor family, and galectin3, whereas TLR6 in association with TLR2 mediates zymosan-induced signaling (reviewed in Ref. 2). Deficiency in certain PRRs, including TLR4,

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Abbreviations used in this article: BM-DC, bone marrow-derived conventional dendritic cell; BMDM, bone marrow-derived macrophage; DC, dendritic cell; IFNAR, IFN-α/β receptor; IFN-I, type I IFN; iNOS, inducible NO synthase; IRF, IFN regulatory factor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN-β; WT, wild-type.

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TLR2, Mincle, dectin-1, or their intracellular signaling adaptors such as MyD88 and CARD9 strongly impairs survival of mice to *Candida* infections, emphasizing the essential role of early pathogen recognition for mounting efficient host immune responses (4). Nevertheless, conflicting reports on individual contributions of certain PRRs (reviewed in Refs. 2, 4) point to the enormous underlying complexity.

In addition to cell surface PAMPs, nucleic acids from *Candida* may also stimulate or modulate the dynamic host response during infection. Indeed, dsDNA from *C. albicans* elicits cytokine release in mice (5, 6) in a TLR9-dependent fashion (6). Moreover, ssRNA induces a Th1 response normally associated with a protective role (7).

Binding of fungal PAMPs to PRRs preludes phagocytosis and stimulates the release of extracellular reactive oxygen species (8) and specific cytokines, ultimately activating innate effector cells. Among others, DCs are instrumental in relaying pathogen information from innate immunity to the adaptive response through their ability to act as professional APCs. PAMP recognition stimulates DCs to produce signal cytokines, including type I IFNs (IFNs-I) through the so-called first-wave response (9). Extracellular IFN-β subsequently activates its cognate receptor, IFN-α/β receptor (IFNAR), in an autocrine/paracrine fashion, driving the second-wave of IFN-α/β, which is considered a hallmark response against many viral and bacterial pathogens (9). This massive release of IFN-I triggers the subsequent expression of IFN-I–stimulated genes, many of which drive maturation of DCs both in vivo and in vitro, thereby modulating the Th cell differentiation in a pathogen-specific manner (9).

A lack of the functional IFNAR increases susceptibility of mice to a number of viral and bacterial pathogens. However, in certain cases, IFN-β can also cause deleterious effects for the host, creating a "yin-yang" situation for both the host and the pathogen (10). Interestingly, Flt3-induced DCs release IFNs-I in response to *C. albicans* (11), and unmethylated CpG motifs from *Aspergillus fumigatus* induce IFN-α production through TLR9 activation in human DCs (12). A recent report also suggests that IFNs-I contribute to the response of mice to *Cryptococcus neoformans* and *Histoplasma capsulatum* in vivo (13, 14). However, the molecular mechanisms by which *Candida* spp. induce the IFN-I response remain ill-defined.

Here, we show that bone marrow-derived conventional dendritic cells (BM-DCs) challenged with *Candida* spp. release high levels of IFN-β, which subsequently drives an IFNAR1-dependent activation of intracellular STAT1 and IRF7 expression. Further, IFN-β release by BM-DCs requires dynamin-dependent phagocytosis of fungal cells, maturation of phagolysosomes, the recognition of fungal PAMPs by the endosomal TLR7, activation of the TLR-specific cytoplasmic MyD88 adaptor, and intracellular Src/Syk family kinase signaling pathways. To our knowledge, this is the first demonstration that a TLR7-dependent pathway is involved in the recognition of *Candida* spp. and mediates activation of IFN-I signaling by a fungal pathogen. Furthermore, in a mouse model of disseminated candidiasis we show that IFN-I signaling promotes persistence of *C. glabrata* in the host. Our results highlight the importance of IFNs-I in modulating the host immune response to this opportunistic pathogen.

Materials and Methods

Ethics statement

All of the animal experiments were discussed and approved through the University of Veterinary Medicine Vienna institutional ethics committee and carried out in accordance with animal experimentation protocols approved by the Austrian law (GZ 680 205/67-BrGt/2003).

Fungal strains and growth conditions

C. albicans clinical isolate SC5314 (15), *C. glabrata* clinical isolate ATCC2001 (CBS138) (16), and *Candida dubliniensis* clinical isolate CD36 (17) were used in this study. Fungal cells were grown to the logarithmic growth phase in single-use, pyrogen- and endotoxin-free sterile flasks as described previously (8). UV-treated *Candida* cell suspensions were prepared by treating an aliquot of the *Candida* infection suspension with 999 μJ/cm^2 in a Stratalinker (Stratagene, La Jolla, CA).

Mouse models and genetic backgrounds

All of the mice were derived from the C57BL/6 background and housed under specific pathogen-free conditions according to Federation of Laboratory Animal Science Associations guidelines (18). IFNAR1$^{-/-}$ (19), Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) (lps2)$^{-/-}$ (20), TLR2$^{-/-}$ (21), TLR4$^{-/-}$ (22), TLR9$^{-/-}$ (23), MyD88$^{-/-}$ (24), and IRF1$^{-/-}$ (25) mice were housed at the animal facility of the University of Veterinary Medicine Vienna. TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, and MyD88$^{-/-}$ mice were kindly provided by Dr. Shizuo Akira, Osaka University, Osaka, Japan. Bone marrow from dectin-1$^{-/-}$ (26) and corresponding wild-type (WT) mice were generously supplied by Dr. G. Brown, University of Aberdeen, Aberdeen, U.K. WT C57BL/6 mice used as controls and CD11b$^{-/-}$ (27) and TLR7$^{-/-}$ (28) mice were purchased from The Jackson Laboratory.

Cell culture of primary innate immune cells differentiated from bone marrow

BM-DCs and bone marrow-derived macrophages (BMDMs) were differentiated from mouse bone marrow cultured for 7–8 d in GM-CSF as described previously (29), and cell surface markers of the BM-DC preparations were assessed by flow cytometry for expression of CD4, CD8, CD11b, Ly-6c, CD11c, MHC class II, CCR2, Mac3, and CX_3CR_1. (Supplemental Table I for results). More than 95% of the cells were CD11b$^+$ but CD8$^-$, CD4$^-$, CX3CR1$^-$, Mac3$^-$, and CCR2$^-$. Our GM-CSF–driven bone marrow culture contains ~64% DCs or Ly-6C$^+$ monocytes, precursors of DCs (33% conventional DCs, 6% monocyte-derived inflammatory DCs, and 25% inflammatory monocytes). The remaining 27% of the cells were monocytes/macrophages (Ly-6C$^-$/CD11c$^-$/CD11b$^+$/), which we show fail to release IFN-β or to modulate expression of IFN-I–regulated genes such as inducible NO synthase (iNOS) in response to *Candida* (Supplemental Fig. 1*B*). In agreement with previous reports (30), we did not obtain any contaminating plasmacytoid DCs under these differentiation conditions.

Coculture of innate immune cells with fungi or cell wall extracts

Fungal–mammalian cell coculture was performed at a target-to-effector ratio of 2:1, exactly as described previously (29). Pretreatment of BM-DCs with inhibitor molecules (dynasore, cytochalasin D, PP2, PP3, R406, bafilomycin A1, or chloroquine) or vehicle was carried out at 37˚C (5% CO_2) for 30 min prior to stimulation. Inhibitor final concentrations were 80 μM for dynasore and 8 μM for cytochalasin D (Sigma-Aldrich, St. Louis, MO), 25 μM for PP2 and PP3 (Calbiochem, La Jolla, CA), 4 μM for R406 (a kind gift from Rigel Pharmaceuticals, San Francisco, CA), 25 or 50 nM for bafilomycin A1 (Sigma-Aldrich), and 10 or 50 μM for chloroquine (Sigma-Aldrich). Pretreatment of BM-DCs with the indicated concentrations of synthetic oligodeoxynucleotides (IRS661 and IRS954) or a nonspecific oligonucleotide control (CTRL_IRS) were carried out at 37˚C for 60 min prior to stimulation. IRS661, IRS954, and CTRL_IRS were synthesized by TIB Molbiol (Berlin, Germany) as described previously (31). Stimulation of BM-DCs with cell wall components were performed with 100 μg/ml β-glucans from *Saccharomyces cerevisiae* (Calbiochem), 100 μg/ml Curdlan, a high-m.w. β-1,3-glucan from *Alcaligenes faecalis* (WAKO Chemicals, Neuss, Germany), or 0.1 μg/ml "TLR-grade" LPS from *Salmonella minnesota* (Sigma-Aldrich). All of the treatments with *Candida* or fungal cell wall extracts were carried out in the presence of 30 μg/ml polymyxin B (Sigma-Aldrich) to neutralize endotoxins (32).

Reverse transcription and real-time PCR analysis

RNA sample preparation, reverse transcription, and real-time PCR were performed as described previously (29) using the following primers: mouse GAPDH, forward 5′-CATGGCCTTCCGTGTTCCTA-3′ and reverse 5′-GCGGCACGTCAGATCCA-3′ (RTPrimerDB, http://medgen.ugent.be/rtprimerdb/index.php); mouse IFN-β, forward 5′-TCAGAATGAGTGGTGGTTGC-3′ and reverse 5′-GACCTTTCAAATGCAGTA GAT TCA-3′ (33); mouse iNOS, forward 5′-GTTCTCAGCCCAACAATACAAGA-3′ and reverse 5′-GTGGACGGGTCGATGTCAC-3′ (Harvard Primer database, http://pga.mgh.harvard.edu/primerbank/); mouse IRF7, forward 5′-CTGGAGCCATGGGTATGCA-3′

and reverse 5′-AAGCACAAGCCGAGACTGCT-3′ as determined using the sequence analysis software Vector NTI (Invitrogen, Carlsbad, CA).

For relative quantification purposes, efficiencies of the individual PCR reactions were determined by the LinReg method (34). Results are expressed as the fold expression (R) of the gene of interest (IFN-β) versus the expression of a housekeeping gene (GAPDH) in treated (t) versus untreated (ut) conditions. The equation used for normalization was: $R = (E_{GOI(ut)}^{CtGOI(ut)}/E_{GAPDH(ut)}^{CtGAPDH(ut)})/(E_{GOI(t)}^{CtGOI(t)}/E_{GAPDH(t)}^{CtGAPDH(t)})$, where E is the PCR efficiency and Ct is the number of cycles to the threshold fluorescence.

Immunodetection

Sample preparation and immunoblotting were performed as described previously (29). Blots were probed with anti-STAT1 Abs recognizing phospho-Tyr^{701}, anti–phospho-ERK Abs (Cell Signaling, Danvers, MA), anti-IRF3 and anti-p38 Abs, (Santa Cruz, Santa Cruz, CA), or anti–C-terminal STAT1 sera, a kind gift from Pavel Kovarik (35). Immune complexes were detected with an infrared-labeled secondary Ab (LI-Cor, Lincoln, NE). Analysis was performed using the infrared imaging system Odyssey (LI-Cor) according to conditions recommended by the manufacturer.

Cytokine measurements by ELISA and phagocytosis assays

The amount of IFN-β released in cell culture supernatants was assayed using the VeriKine mouse IFN-β ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Phagocytosis assays were performed as described previously (36) using the following modifications. Briefly, *C. glabrata* cells were labeled with 10 mM Alexa Fluor 488 C5 maleimide (Invitrogen) in 100 mM HEPES buffer (pH 7.5) for 15 min at room temperature. Labeled *C. glabrata* were washed in HEPES buffer and kept until use. BM-DCs were treated with inhibitors or vehicle at 37°C (5% CO_2) for 30 min prior to the assay and then precooled on ice. After 20 min, Alexa Fluor 488-labeled *C. glabrata* in ice-cold DMEM was added at a target-to-effector ratio of 2:1, and samples were incubated at 37° C (5% CO_2) for 45 min to allow for phagocytosis to occur. Phagocytosis was terminated by chilling plates on ice, where they remained during detaching and fixation in 1% formaldehyde. Fluorescence of extracellular *C. glabrata* was quenched by addition of 0.4% trypan blue. Negative controls for phagocytosis were left on ice during the whole process. Duplicate samples were subjected to flow cytometry analysis, gating on Alexa Fluor 488/BM-DC populations with internalized *C. glabrata*. The percentage of phagocytosis was determined as follows: (inhibitor-treated BM-DCs with *C. glabrata* at 37°C − inhibitor-treated BM-DCs with *C. glabrata* at 4°C)/(vehicle-treated BM-DCs with *C. glabrata* at 37°C − vehicle-treated BM-DCs with *C. glabrata* at 4°C) × 100. Results are expressed as the mean ± SD of the percentage of ingestion (the percentage of BM-DCs containing one or more yeast cells).

Animal model of disseminated candidiasis

WT (C57BL/6) mice and IFNAR1$^{-/-}$ mice were injected i.v. with 5×10^7 CFU *C. glabrata* per 25 g of mouse weight. Infected mice were euthanized at days 7, 14, and 21 postinfection ($n = 5$ mice per time point) to determine the fungal burden in spleen, liver, kidney, and brain. Organs were aseptically collected and homogenized in sterile PBS. Homogenate dilutions were plated in triplicate on yeast peptone dextrose agar plates containing antibiotics. After a 2-d incubation at 30°C, *C. glabrata* colonies were counted. Results are expressed as CFU per gram of tissues.

Statistical analysis

Statistical analysis of data was performed using the Prism graphing and analysis software. Comparison of two groups was done with the Student t test (in vitro experiments) or with the nonparametric t test (Mann–Whitney U test) for animal experiments. A p value < 0.05 was considered significant.

Results

Candida *spp. trigger IFN-β release in BM-DCs and induce IFN-I–specific genes*

Phagocytes of the innate immune system such as macrophages and DCs can release IFNs-I in response to various microbial pathogens (9, 10). To investigate the molecular mechanisms of IFN-I response elicited by *Candida* spp., we used in vitro cell culture models of primary mouse BM-DCs or BMDMs challenged with *Candida* spp. We used *C. albicans* and *C. dubliniensis*, two dimorphic *Candida* spp., or *C. glabrata*, existing exclusively in the yeast form. mRNA levels as well as IFN-β protein release were measured by quantitative real-time PCR or ELISA, respectively. All three *Candida* spp. strongly stimulated IFN-β mRNA expression in BM-DCs after 3 h, when compared with that in unstimulated BM-DCs (Fig. 1*A*). ELISA assays confirmed that the increase in IFN-β expression correlated with the release of IFN-β by *Candida*-infected BM-DCs (Fig. 1*B*). Notably, both UV-inactivated *C. albicans* cells and the yeast *S. cerevisiae* (Supplemental Fig. 1*A*) triggered IFN-β production. Interestingly, however, the response was cell-type–specific and restricted to certain innate immune cells, because we failed to detect IFN-β mRNA induction in BMDMs cocultured with *C. glabrata*, although they still induced IFN-β and iNOS mRNA, an IFN-I–regulated gene, in response to LPS (Supplemental Fig. 1*Ba*, 1*Bb*). Peritoneal macrophages, neutrophils, or splenic DCs also did not release IFN-β in response to *C. glabrata* challenge (Supplemental Fig. 1*C*). Remarkably, *C. glabrata* consistently showed the highest potency in stimulating IFN-β mRNA expression and subsequent cytokine release (Fig. 1*A*, 1*B*). Hence, we chose *C. glabrata* cells as the main pathogen stimulus in further experiments to investigate the mechanisms of IFN-I response to *Candida* spp. by BM-DCs.

A hallmark of the IFN-I response is its ability to induce expression of a large number of effector genes (i.e., IFN-I–stimulated genes). Indeed, IFN-β released in the first wave binds to its own receptor IFNAR, activating the STAT1 and STAT2 transcription factors and thereby triggering expression of typical IFN-I target genes such as IRF7.

Because mouse BM-DCs express IFNAR, we asked whether the initial IFN-β release observed upon *Candida* spp. challenge can induce IFN-I signaling in an autocrine/paracrine fashion. Thus, WT BM-DCs or BM-DCs lacking the IFNAR1 subunit of IFNAR (IFNAR1$^{-/-}$) were cocultured with *Candida* spp. or left unstimulated for 2 h, after which protein extracts were prepared and

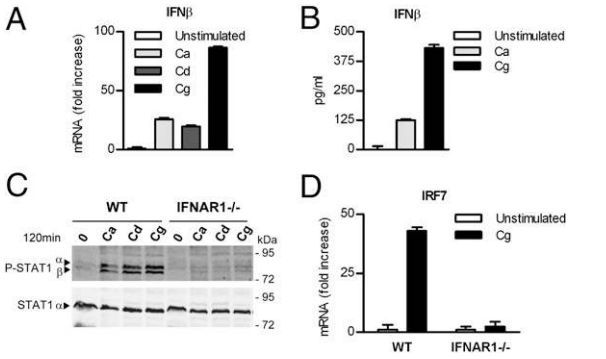

FIGURE 1. *Candida* species induce a IFN-I response in mouse BM-DCs. *A* and *D*, WT BM-DCs or BM-DCs lacking the IFNAR1 subunit of the IFN-I receptor (IFNAR1$^{-/-}$) were infected with the indicated *Candida* spp. or left untreated for the indicated time, after which cell lysates were harvested and RNA or protein extracts were prepared. *A* and *B*, IFN-β expression was measured by real-time PCR after 3 h of coculture (*A*) or by ELISA after 24 h of coculture (*B*). *C*, Phosphorylated STAT1 was detected by immunoblotting of protein extracts prepared after 2 h of *Candida* spp.–BM-DC coculture (*upper panel*), and blots were reprobed with polyclonal anti-STAT1 Abs to assess equal loading between lanes (*lower panel*). *D*, IRF7 gene expression was measured by real-time PCR after 24 h of coculture. Real-time PCR results are expressed as fold increase of mRNA expression over untreated BM-DCs. ELISA results are expressed as picograms of IFN-β per milliliter of cell culture supernatant. Data presented are from one experiment representative of at least three independent experiments.

STAT1 activation was verified by immunodetection using phospho-specific Abs. In WT BM-DCs cocultured with *Candida*, IFN-β release strictly correlated with STAT1 phosphorylation (Fig. 1*C*) as well as with the activation of IRF7 transcription, a typical IFN-I–regulated gene (Fig. 1*D*). By contrast, in IFNAR1$^{-/-}$ BM-DCs stimulated with *C. glabrata*, no STAT1 phosphorylation was seen (Fig. 1*C*), and IRF7 transcription was not induced (Fig. 1*D*). Consistent with the absence of IFN-β release in BMDMs challenged with *C. glabrata*, no STAT1 activation was observed (Supplemental Fig. 1*Bc*). As expected, STAT1 activation was still detected in BM-DCs lacking the IFN-γ gene (IFN-γ$^{-/-}$), which can signal through STAT1 phosphorylation (Supplemental Fig. 1*D*). All together, these data demonstrate that *Candida* spp. trigger a first wave of IFN-β in BM-DCs, thereby activating an IFN-I response in an IFNAR1-dependent fashion.

Candida-*induced IFN-β release is partially dependent on Src/Syk kinase signaling*

The main PRRs involved in *Candida* spp. recognition belong to the C-type lectin and TLR families (3). C-type lectins, like other ITAM-bearing receptors, signal through intracellular Syk and Src family kinases. We thus reasoned that PP2, an inhibitor of Src family kinases, and R406, a highly specific inhibitor of the Syk kinase, may impair the IFN-β response to *Candida* spp. Indeed, pretreatment of BM-DCs with R406 prior to *C. glabrata* stimulation significantly decreased the IFN-β release by ~80% (Fig. 2*A*, *left panel*). Likewise, PP2 strongly inhibited the *C. glabrata*-triggered IFN-β release to ~12% of the level observed when BM-DCs were pretreated with DMSO vehicle alone; as a control, PP3, the inactive analogue of PP2, had no significant effect (Fig. 2*A*, *right panel*). Thus, these results unequivocally show that both intracellular Src and Syk signaling pathways are required to elicit the IFN-β release in response to fungal cells. Moreover, the data imply the involvement of ITAM-bearing receptors in this signaling process.

The C-type lectin dectin-1, a β-1,3-glucan receptor, has been known as one of the major receptors mediating *Candida* spp. recognition (37). Surprisingly, however, BM-DCs lacking the dectin-1 receptor still released IFN-β upon *C. glabrata* challenge (Fig. 2*B*, *left panel*). Thus, these results show that dectin-1 is not involved in mediating the *Candida*-induced IFN-β release. In addition to dectin-1, CD11b (integrin α_M) can also bind *C. albicans* β-glucans, is present in the phagosome upon *Candida* phagocytosis, and also activates the Src family and Syk kinases (38). Thus, we investigated the potential role of CD11b in the IFN-β response by challenging CD11b-deficient BM-DCs with *C. glabrata*. Even in the absence of CD11b, IFN-β release was observed, albeit slightly reduced, suggesting that this β-glucan receptor is not the main receptor involved in triggering the initial IFN-β release in BM-DCs infected with *C. glabrata*. Furthermore, no significant IFN-β release was observed when BM-DCs were pretreated with β-glucan extract from *S. cerevisiae* or Curdlan (Fig. 2*C*). However, these cells readily responded to LPS, which was used as a positive control for active PRR signaling via TLR4. Additional potential PAMPs such as chitin or mannan failed to induce detectable IFN-β levels in BM-DCs (data not shown). Thus, our data show that neither dectin-1 nor CD11b or other PRRs for known cell wall PAMPs mediate the initial IFN-β release upon *Candida* spp. challenge. Nevertheless, it still required activation of intracellular Src family and Syk kinase signaling pathways.

Beside their role as intracellular adaptors for certain PRRs, Src family and Syk kinases are also involved in mediating phagocytic processes. Therefore, we assayed their involvement in phagocytosis using a flow cytometry-based analysis of the percentage of BM-DCs having phagocytosed at least one Alexa Fluor 480-labeled *C. glabrata* with or without inhibitor pretreatment. Pretreatment with the Src kinase inhibitor PP2 had no significant effect on the phagocytic properties of BM-DCs (data not shown). By contrast, only 60% of the BM-DCs pretreated with the Syk inhibitor R406 contained at least one *C. glabrata* cell when compared with 100% of BM-DCs having engulfed at least one *C. glabrata* in vehicle (DMSO)-treated BM-DCs (Fig. 2*D*). Therefore, these data suggest that Syk kinase activation promotes IFN-β release by contributing to the invasion process in BM-DCs phagocytosing fungal pathogens.

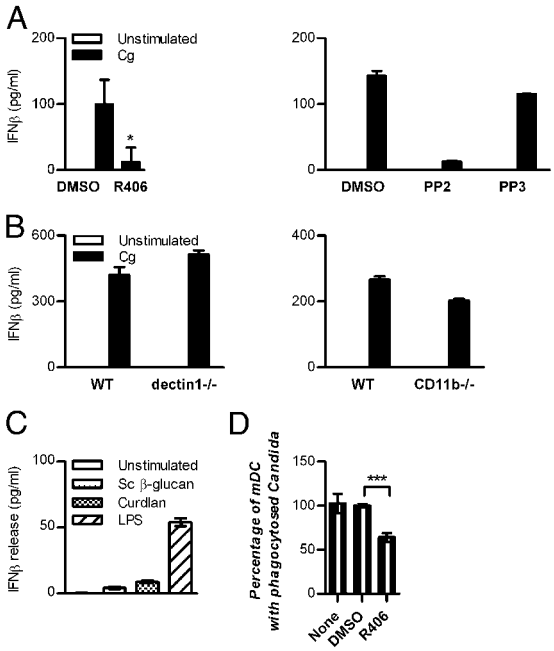

FIGURE 2. Role of Syk/Src kinases and β-glucan receptors. *A*, WT BM-DCs were preincubated with a Syk kinase inhibitor (R406), an inhibitor of Src family kinases (PP2), and an inactive homologue (PP3) or vehicle (DMSO) at 37°C for 30 min prior to coculture with *C. glabrata* or media alone for 6 h. IFN-β release into the cell culture medium was measured by ELISA. *B*, WT BM-DCs or BM-DCs lacking dectin-1 (dectin-1$^{-/-}$) or CD11b (CD11b$^{-/-}$) were cocultured for 6 h with *C. glabrata* or left unstimulated. IFN-β release was measured by ELISA. *C*, BM-DCs were stimulated with either β-glucan preparations (*S. cerevisiae* β-glucans) or Curdlan in media containing polymyxin B, or with LPS for 4 h, or left untreated as a control. IFN-β release into the cell culture medium was measured by ELISA. ELISA results are expressed as picograms of IFN-β per milliliter of supernatant. Data presented are from one experiment representative of three independent experiments. *D*, WT BM-DCs were preincubated with a Syk kinase inhibitor (R406) or vehicle (DMSO) prior to coculture with Alexa Fluor 480-labeled *C. glabrata* at 37°C or 4°C (adherence control) for 45 min. Cells were collected, and the number of BM-DCs containing at least one *C. glabrata* was analyzed by flow cytometry. Results are expressed as percentage of ingestion (the percentage of BM-DCs containing one or more yeast cells). *B* and *C*, Data presented are from one experiment representative of at least three independent experimental repeats. *A* and *D*, Data presented are the mean ± SD of data from three independent experiments. $*p < 0.05$, $***p \leq 0.0005$, unpaired, two-tailed *t* test.

Candida-*induced IFN-β release is TLR2- and TLR4-independent*

The second main class of PRRs involved in sensing and recognizing *Candida* spp. is the TLR family. In particular, TLR2 and TLR4 are thought to be involved in *Candida* recognition and reported to be essential for survival to *Candida* infections in mice (2). Thus, we

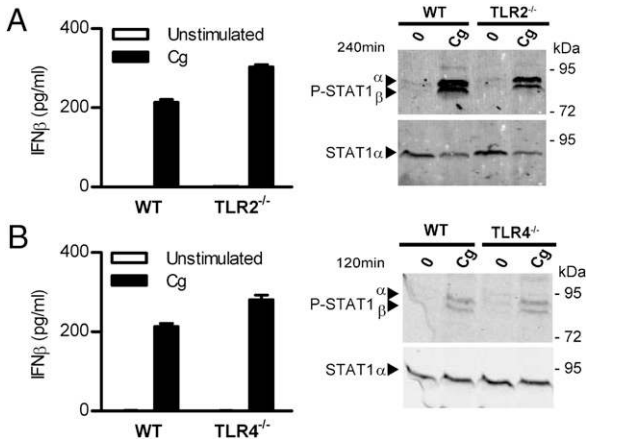

FIGURE 3. IFN-β release triggered by *C. glabrata* is TLR2- and TLR4-independent. *A* and *B*, WT BM-DCs or BM-DCs lacking TLR2 (TLR2$^{-/-}$) or TLR4 (TLR4$^{-/-}$) were cocultured for 4 h with *C. glabrata* or left unstimulated, and IFN-β release was measured by ELISA (*left panel*) or phosphorylated STAT1 was detected by immunoblotting of extracts prepared after 2 h of *C. glabrata*–BM-DC coculture; blots were reprobed with polyclonal anti-STAT1 Abs to verify equal loading (*right panel*). ELISA results are expressed as picograms of IFN-β per milliliter of cell culture medium. Data presented are from one experiment representative of three independent experiments.

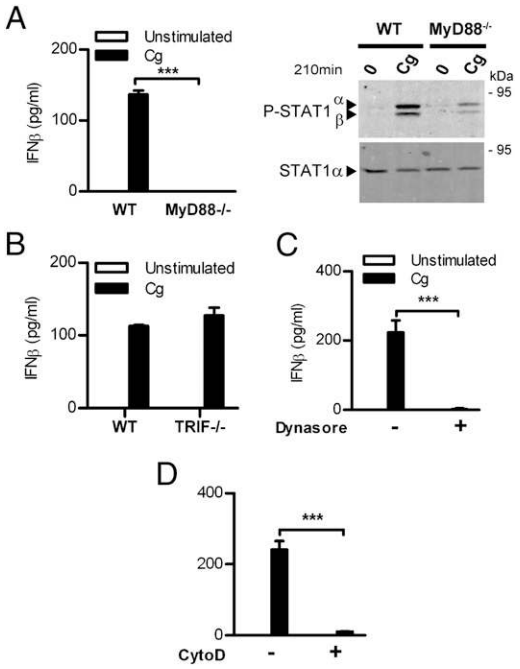

FIGURE 4. IFN-β release requires phagocytosis and MyD88 activation. *A*, WT BM-DCs or BM-DCs lacking the MyD88 signaling adaptor (MyD88$^{-/-}$) were cocultured for 4 h with *C. glabrata* or left unstimulated, IFN-β release was measured by ELISA (*left panel*) or phosphorylated STAT1 was detected by immunoblotting of protein extracts prepared after 3.5 h of *C. glabrata*–BM-DC coculture, and blots were reprobed with polyclonal anti-STAT1 Abs to assess equal loading between lanes (*right panel*). *B*, WT BM-DCs or BM-DCs lacking the TRIF signaling adaptor (TRIF$^{-/-}$) were cocultured with *C. glabrata* or left unstimulated. IFN-β release was measured by ELISA after 4 h. *C* and *D*, WT BM-DCs pretreated with either dynasore (*C*), cytochalasin D (*D*), or vehicle (DMSO) for 30 min were cocultured with *C. glabrata* or left untreated; IFN-β production was measured by ELISA after 4 h. ELISA results are expressed as picograms of IFN-β per milliliter of cell culture medium. Western blot data presented are from one experiment representative of at least three independent experiments. ELISA data presented are the mean ± SD of data from three independent experiments. ***$p \leq 0.0005$, unpaired, two-tailed t test.

investigated whether these receptors were involved in the IFN-β response to *Candida* using BM-DCs lacking either TLR2 (TLR2$^{-/-}$) or TLR4 (TLR4$^{-/-}$). Surprisingly, when WT and mutant BM-DCs were infected with *C. glabrata*, IFN-β release was stimulated to similar or even higher levels (Fig. 3*A*, 3*B*, *left panels*). Accordingly, a strong STAT1 activation was observed in both WT and mutant BM-DCs (Fig. 3*B*, *right panel*), demonstrating that the IFN-I response to *Candida* spp. is bypassing the known TLR2 or TLR4 signaling pathways.

IFN-β release of BM-DCs requires phagocytosis and MyD88 activation

TLRs use either MyD88 (e.g., TLR1, TLR2, TLR6, TLR7, TLR8, and TLR9), TRIF (in the case of TLR3), or both, as in the case of TLR4 (39), as intracellular signaling adaptors. To further investigate the possible involvement of TLR signaling in the IFN-β release, we used BM-DCs lacking either one of these signaling adaptors. Remarkably, BM-DCs lacking MyD88 (MyD88$^{-/-}$) failed to release any IFN-β after *C. glabrata* (Fig. 4*A*, *left panel*) or *C. albicans* (Supplemental Fig. 2*E*) challenge. Accordingly, MyD88$^{-/-}$ BM-DCs cocultured with *C. glabrata* also failed to mount the subsequent IFN-I response, as evident from a much weaker STAT1 phosphorylation when compared with that of the WT BM-DCs (Fig. 4*A*, *right panel*). By sharp contrast, a lack of TRIF did not impair the IFN-β release after *Candida* challenge (Fig. 4*B*). These data exclude a role for TLR3 but unequivocally demonstrate a strict requirement for a TLR/MyD88 signaling mechanism in sparking the first wave of IFN-β release in BM-DCs facing fungal invasion.

In mice, both surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) and phagosomal TLRs (TLR7–9) signal through the MyD88 adaptor. To further narrow down the list of candidate TLRs recognizing *Candida* spp. for triggering IFN-β, we used dynasore, a specific and potent small-molecule inhibitor of the GTP-binding protein dynamin required for proper constriction of clathrin-coated vesicles during endocytosis and phagocytosis (40). Strikingly, dynasore pretreatment of BM-DCs prior to *Candida* addition completely abolished IFN-β release to the level of unstimulated BM-DCs. No inhibition was observed in BM-DCs pretreated with vehicle (DMSO) only (Fig. 4*C*). The effect of dynasore treatment was not caused by loss of cell viability, as verified by life-staining (data not shown). It was also specific as confirmed by the remaining ERK activation upon fungal challenge in treated BM-DCs (Supplemental Fig. 2*A*), the latter being a hallmark of cell surface PRR activation on immune cells upon *Candida* recognition. Cytochalasin D, a well-known inhibitor of actin polymerization blocking endocytosis, also prevented release of IFN-β in *C. glabrata*-challenged BM-DCs (Fig. 4*D*). Thus, IFN-β induction by *Candida* spp. in BM-DCs requires dynamin-dependent phagocytosis, strongly suggesting that the activation of MyD88 demands PAMP recognition by phagosomal TLRs.

Candida-*induced IFN-β release requires phagosome acidification and TLR7*

Endosomal maturation through acidification is required for activation of intracellular TLRs by their specific ligands and subsequent stimulation of their adaptor MyD88 in the presence of PAMPs (41). Thus, we asked whether bafilomycin A1 or the antimalarial drug chloroquine, both compounds specifically inhibiting endosome acidification, would also block *Candida*-induced IFN-β release. Strikingly, pretreatment of BM-DCs with 10 μM chloroquine or 25 nM bafilomycin A1 strongly inhibited IFN-β re-

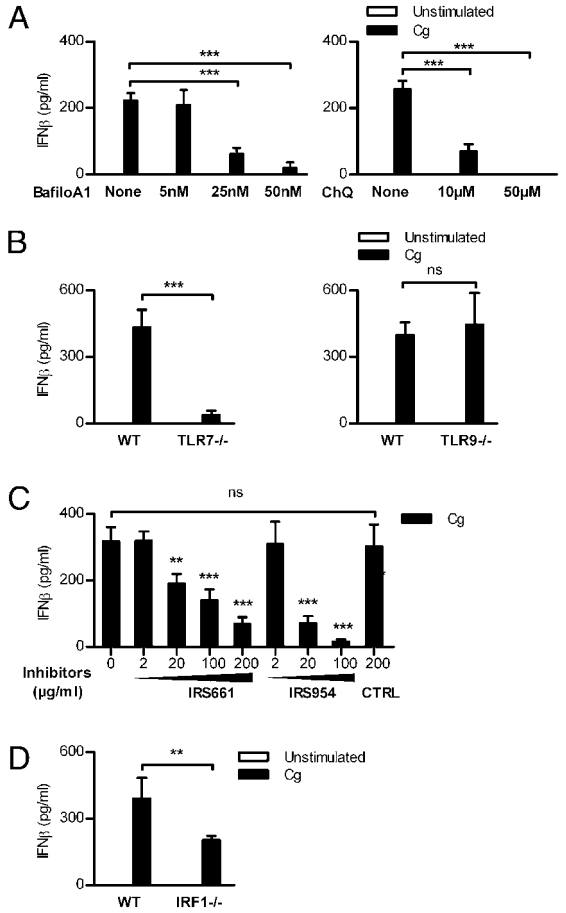

FIGURE 5. IFN-β release requires activation of phagosomal TLR7. *A*, BM-DCs pretreated for 30 min with either bafilomycin A1 (bafiloA1), chloroquine (ChQ), or vehicle alone were cocultured with *C. glabrata* for 6 h or left untreated. IFN-β release was measured by ELISA, and results are expressed as picograms of IFN-β per milliliter of cell culture medium. *B*, WT BM-DCs or BM-DCs lacking either TLR7 (TLR7$^{-/-}$) or TLR9 (TLR9$^{-/-}$) were cocultured for 6 h with *C. glabrata* or left unstimulated, and IFN-β release was measured by ELISA. *C*, BM-DCs were pretreated for 60 min with the indicated concentrations of synthetic inhibitory oligodeoxynucleotides (IRS661 or IRS954) or with an unspecific oligodeoxynucleotide (CTRL_IRS) as a control. Subsequently, BM-DCs were cocultured for 6 h with *C. glabrata* or left unstimulated. IFN-β release was measured by ELISA, and results are expressed as picograms of IFN-β per milliliter of cell culture medium. *D*, WT BM-DCs or BM-DCs lacking the IRF1 transcription factor (IRF1$^{-/-}$) were cocultured for 6 h with *C. glabrata* or left unstimulated, and IFN-β release was measured by ELISA. *A–D*, Values represent the mean ± SD of three independent experiments performed in triplicate. $^{**}p \leq 0.005$, $^{***}p \leq 0.0001$, unpaired, two-tailed *t* test.

lease upon stimulation with *C. glabrata* ($^{***}p \leq 0.0001$) (Fig. 5*A*), demonstrating a pivotal role for endosomal TLRs in IFN-β release in BM-DCs. The effect of bafilomycin A1 or chloroquine treatment was not caused by loss of cell viability, as verified by life-staining (data not shown). Moreover, BM-DCs still activated intracellular ERK upon *C. glabrata* challenge (Supplemental Fig. 2*B*) (a typical response of innate immune cells to *Candida* challenge originating from activated cell surface PRRs) or released TNF-α upon *Candida* challenge (Supplemental Fig. 2*C*).

In mice, the endosomal TLRs requiring MyD88 to recognize PAMPs comprise TLR7, TLR8, and TLR9, but TLR7 and TLR9 seem to account for most of the signaling. To investigate the contribution of these PRRs, we used BM-DCs lacking either TLR7 (TLR7$^{-/-}$) or TLR9 (TLR9$^{-/-}$). Surprisingly, the IFN-β release was fully abrogated in BM-DCs lacking TLR7 (Fig. 5*B*, *left panel*) but was stimulated to levels similar to those of WT cells in BM-DCs lacking TLR9 (Fig. 5*B*, *right panel*). The absence of response to *C. glabrata* in TLR7-deficient BM-DCs was not due to a general defect in IFN-β production, because these cells still released IFN-β to WT comparable levels upon LPS stimulation (Supplemental Fig. 2*D*). Furthermore, specifically blocking TLR7 activation using synthetic oligodeoxynucleotides (e.g., IRS661, an antagonist of TLR7, or IRS954, which inhibits both TLR7 and TLR9) strongly decreased IFN-β production upon fungal challenge in a dose-dependent fashion (Fig. 5*C*). The unspecific control oligodeoxynucleotide CTRL_IRS had no detectable or significant effect on IFN-β production (Fig. 5*C*). To further identify the transcription factors involved in the *Candida*-induced IFN-β release, we used BM-DCs lacking either IRF1, IRF3, or IRF7. The IFN-β release was only significantly reduced in the absence of IRF1 (Fig. 5*D*) but was unaffected in BM-DCs lacking IRF3 or IRF7 (data not shown).

Integrated model of IFN-I response to fungal pathogens in BM-DCs

On the basis of our collective data, we propose the following model for the *Candida*-induced IFN-β signaling pathway operating in BM-DCs (Fig. 6). Adhesion and recognition of *Candida* spp. at the surface of innate immune cells initiates dynamin-dependent phagocytosis. Maturation and acidification of the phagolysosome allow for processing of TLR7, which is subsequently activated by its cognate PAMP ligands, most likely fungal-specific RNAs, to recruit and activate cytoplasmic MyD88 and subsequently the IRF1 transcription factor, ultimately triggering the IFN-β release. Other as yet unknown pathways acting through Syk/Src family kinase signaling may also contribute to the induction of IFN-I response in BM-DCs. Taken together, the results provide the first demonstration of an IFN-β release by BM-DCs in response to phagosomal *Candida* recognition, hence revealing a novel role for endosomal TLRs in fungal recognition.

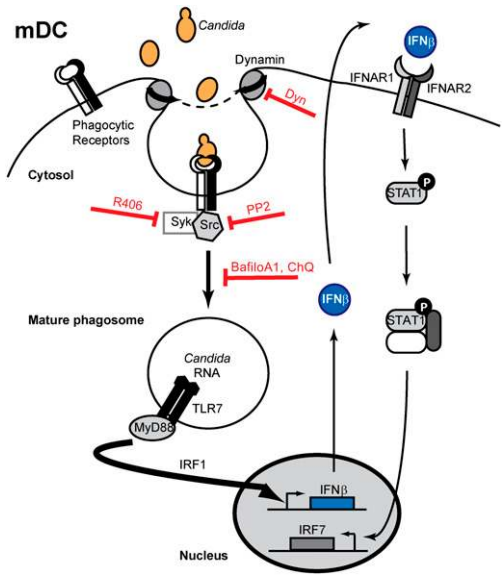

FIGURE 6. Model of IFN-β response to *Candida* spp. in mouse BM-DCs. In BM-DCs, a dynamin-dependent phagocytosis step, Syk/Src-dependent-signaling, followed by endosome acidification, leads to the activation of TLR7, probably by *Candida* RNAs, thereby stimulating a MyD88–IRF1-dependent signaling pathway required for the initial release of IFN-β and the subsequent induction of an IFN-I response to fungal infection. Actions of inhibitors (dynasore, R406, PP2, bafilomycin A1, and chloroquine) used in this study are indicated on their specific targets.

IFN-I response promotes C. glabrata *persistence in host tissues*

To investigate the role of this IFN-I in the host response to *Candida*, we used a model of disseminated candidiasis and compared the outcome of *C. glabrata* infections in WT mice versus those in IFNAR1$^{-/-}$ mice, which are unresponsive to IFN-I. Because systemic *C. glabrata* infection is normally not pathogenic for immunocompetent mice (42, 43), we used tissue fungal burden to assess persistence. At day 7 postinfection, CFU counts in spleen, liver, and brain of IFNAR1$^{-/-}$ mice were significantly lower than those in WT organs (Fig. 7*A*). No significant difference in *C. glabrata* CFU counts was observed in kidney of WT versus IFNAR1$^{-/-}$ mice (data not shown). At days 14 and 28 postinfection, CFU counts were still detectable in organs of infected animals, although without statistically significant differences between WT and IFNAR1$^{-/-}$ tissues (data not shown). Interestingly, a significantly more pronounced splenomegaly was observed in infected IFNAR1$^{-/-}$ mice in comparison with that of infected WT mice at day 7 (Fig. 7*B*), suggesting a stronger immune response in the spleen of IFNAR1$^{-/-}$ mice than in the spleen of WT animals at day 7 postinfection.

Discussion

In this report, we show for the first time, to our knowledge, that *Candida* spp. trigger an IFN-I response in mouse BM-DCs. We identify TLR7 as the essential PRR activating this immune response in a MyD88-dependent fashion from within maturing phagolysosomes. Because of the phagocytic and microbicidal properties, innate immune cells are the first line of defense against many microbial pathogens. In addition, they are producers of IFNs-I, a family of cytokines specialized in coordinating the cross talk between the innate and adaptive immune responses to microbial or viral infections (9). Therefore, we studied the capabilities of several innate immune cell types such as bone marrow-derived neutrophils or macrophages, peritoneal macrophages, and bone marrow or splenic DCs to respond to fungal challenges by producing IFN-β using an in vitro coculture interaction system. However, only mouse BM-DCs are able to release IFN-β but not IFN-α (data not shown) in response to *Candida* spp. Notably, others also observed detectable IFN-β induction upon challenge with *C. albicans* in *flt3*-differentiated DCs but only a weak but

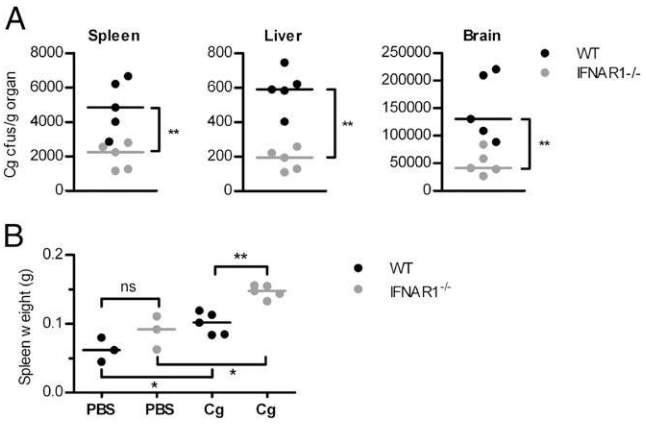

FIGURE 7. IFN-I signaling promotes *C. glabrata* persistence in host tissues. WT and IFNAR1$^{-/-}$ mice were i.v. infected with 5×10^7 CFU *C. glabrata* on day 0. *A*, On day 7 postinfection, fungal burdens were determined by culture from tissue homogenates of five animals per group. *B*, Average spleen weight of WT and IFNAR1$^{-/-}$ mice on day 7 postinfection with *C. glabrata*. Spleen weights of three mice injected with PBS only are shown as controls. Nonparametric *t* test (Mann–Whitney *U* test) was performed on data sets. $*p < 0.05$, $**p < 0.01$.

significant induction of IFN-α gene transcription in BM-DCs stimulated with the yeast form of *C. albicans*. This discrepancy may be caused by differences in the protocols used to prepare the BM-DCs (11). Markedly, both BMDMs and BM-DCs release IFN-β when challenged with another prominent fungal pathogen, *Cryptococcus neoformans* (13). Under our experimental conditions, the IFN-I response to *Candida* is highly cell-type–specific within innate immune cells, because peritoneal or BMDMs as well as neutrophils or splenic DCs challenged by *Candida* fail to release detectable amounts of IFN-β. Thus, our results are consistent with reports about distinct and cell-type–specific cytokine responses between BMDMs and myeloid dendritic cells (44–46), suggesting that different innate immune cells may have distinct repertoires to sense and to respond to microbial PAMPs, depending on the differentiation procedure used to obtain the cells in vitro or the host tissue environment in vivo. This may help to fine-tune the host defense and immune surveillance.

We show in this study that other *Candida* spp. such as *C. dubliniensis.* and *C. glabrata* also spark IFN-β release. Interestingly, *C. glabrata*, a nondimorphic yeast-like species, appears as a much better trigger for IFN-β than the pleomorphic filamentous species *C. albicans* or *C. dubliniensis*. This may relate to the fact that *C. glabrata* can persist in the host for prolonged periods (42, 47), whereas *C. albicans* normally efficiently kills host cells after a few hours or escapes from the phagosome (48, 49). Hence, it seems feasible that the ability of *C. albicans* to escape the phagosome, subsequently causing host cell lysis, may explain the weaker induction of IFN-I response versus *C. glabrata*, the latter leading to strong IFN-β release due to its persistence in the host immune cells.

β-Glucan preparations stimulate the release of a number of cytokines such as TNF-α, IL-2, or IL-12 from innate immune cells (38), and they can induce BM-DC maturation in humans (50) as well as in mice (51). β-Glucan drives BM-DC maturation at least in part through dectin-1, which is considered a major PRR for glucans (37). However, in this study we show that the *C. glabrata*-induced IFN-β release is independent of dectin-1 and CD11b, both acting as β-glucan receptors accumulating at the site of *Candida* uptake by macrophage phagocytosis (52). Furthermore, we tend to exclude the involvement of other unknown β-glucan receptors, because all of the β-glucan preparations that we used, namely, β-1,3- and β-1,6-glucan extracts obtained from the *S. cerevisiae* cell wall, and Curdlan, a linear β-1,3-glucan polymer, fail to trigger an IFN-β release in BM-DCs. Likewise, mannan and chitin are also inactive under these conditions. Hence, in contrast to LPS from the cell wall of Gram-negative bacteria, fungal cell wall extracts do not induce IFN-β release in innate immune cells, despite being a rich source of fungal PAMPs for other cytokine responses (2). However, care has to be taken when performing these experiments, because many commercial and custom-made cell wall preparations contain minute LPS contamination, which may lead to conflicting interpretations of results. Therefore, all of our cytokine experiments in primary cells were carried out in the presence of polymyxin B, which alleviates the LPS contamination problem (32)

By specifically inhibiting dynamin, we demonstrate that fungal phagocytosis is a mandatory prerequisite for IFN-β release. Similarly, inhibition of Src family kinases also strongly blocks IFN-β release. Notably, phosphorylation of cortactin and dynamin by Src kinase is required for activation of endocytosis in epithelial cells (53), and both cortactin and dynamin are essential for *Candida* internalization in epithelial cells (54). Furthermore, Syk kinase activation is also in part necessary for IFN-β production. Although in this context, Syk function on IFN-β release is not dectin-1– or CD11b-dependent, it is relevant for mediating

Candida phagocytosis. Whether it occurs through its role in integrin-mediated signal transduction (55), as signaling adaptor of ITAM-bearing receptors, or as mediator of phagocytic processes (56) is under investigation. Nonetheless, our data emphasize the pivotal importance of phagocytosis and thus compartmentalized ligand–receptor interactions for orchestrating the IFN-I host response to *Candida* spp. as for other microbial pathogens (41).

Using BM-DCs lacking the TLR adaptors TRIF or MyD88, we demonstrate that the *C. glabrata*-induced IFN-β release strictly requires MyD88 signaling. Hence, our findings are consistent with the model of a MyD88 pathway mediating the cytokine response of inflammatory DCs to yeast (11). Our data further strengthen the importance of inflammatory DCs in the response against *Candida* infection as major producers of IFN-β. Surprisingly, however, neither lack of TLR2 nor TLR4 impairs the IFN-β release, although these PRRs appear to be critical for survival to *C. albicans* dissemination in mouse models (2).

Most interestingly, our results demonstrate that TLR7 is the main phagosomal PRR triggering an initial IFN-β release upon *Candida* recognition by BM-DCs. They are in agreement with the notion that endolysosomes are unique IFN-inducible organelles (41). Spatial recognition of microbial ssRNA by TLR7 within endosomes was first observed for viruses (9). More recently, this mechanism was found to apply to bacterial recognition in mice and humans (46, 57). However, this is the first report, to our knowledge, of fungal recognition by TLR7, thus underscoring the importance of the TLR7–MyD88–IRF1 pathway for endolysosomal recognition of pathogens in BM-DCs. A potential role of TLR7 in survival to *C. albicans* or any fungal microbe has yet to be further explored. Because TLR7 recognizes ssRNA, the present report also hints the importance of *Candida*-derived RNAs as potential PAMP sources driving host immune cell activation through the IFN-I response. Indeed, recognition of *Candida* DNA by TLR9 triggers release of IL-12p40 in BM-DCs (6). Interestingly, *Candida* RNA-pulsed bone marrow and spleen DCs undergo activation and confer protection against systemic *C. albicans* infection in mice (7). Whether these properties resulted from activation of an IFN-I response in these innate immune cells is unknown.

Activation of the IFN-I response is critical for the maturation of DCs into professional APCs that help to shape the magnitude and duration of the adaptive immune response by inducing the differentiation of Th cells (9). In general, IFNs-I can function both as proinflammatory and anti-inflammatory cytokines, but the contribution of each property to the overall host response is not well understood (9, 58). To test the pathophysiological relevance of IFN-I signaling in the host response to *Candida* infection, we challenged mice lacking a functional IFNAR through tail vein injections with *C. glabrata*. Strikingly, we show that the IFN-I response promotes the persistence of *C. glabrata* in host tissues, thus suggesting that IFN-type I is detrimental for fungal clearance in a model of disseminated *Candida* infection. Notably, very little is known as yet about the mechanisms enabling *C. glabrata* to persist in host tissues, and we report in this study for the first time, to our knowledge, that IFN-I plays a role in this process. Treatment with neutralizing Abs against TNF-α indicates that this cytokine promotes *C. glabrata* clearance from host tissues in the early postinfection phase (43). When we measured TNF-α at day 7 postinfection in liver and spleen though, we were unable detect a statistically significant difference between WT and IFNAR1$^{-/-}$ mice (data not shown). The more pronounced splenomegaly observed in IFNAR1$^{-/-}$ versus WT mice in response to infections implies that the decrease in *C. glabrata* persistence in IFNAR1$^{-/-}$ mice could be due to a higher general immune response in the absence of IFN-I. In humans and mice, IFNs-I inhibit DC-mediated Th17 cell differentiation (59–62). Furthermore, by stimulating IL-10 expression and downregulating IL-12 production, IFNs-I appear to modulate Th1/Th2 polarization favoring reduced inflammation and host tissue damage, indicating a protective role (63). Further experiments are ongoing in our laboratory to decipher the molecular mechanisms of IFN-Is in promoting *C. glabrata* persistence or clearance.

Taken together, our results presented in this study hint to a crucial and as yet unrecognized role for TLR7 in fungal recognition and induction of IFN-I response in mouse BM-DCs challenged with *Candida* spp. Our work is entirely consistent with reports where fungal nucleic acids were recognized as PAMPs to trigger host immune responses (5–7, 12, 57). Importantly, our data are highly relevant for humans, because microbial RNAs induce IFN-α release in human DCs (57). Furthermore, our results stress the importance of IFN-I in modulating the host response to fungal pathogens such as *Candida* spp. and suggest a hitherto unrecognized general role of IFN-I in modulating fungal virulence.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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