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C-type lectin-mediated modulation of IRF5-induced interferon-β for Th17 regulation

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Type I interferon (IFN-I) responses are crucial in antiviral immunity but little is known about their role in bacterial or fungal infections. Here we have identified an important role for IFN-I effector responses in antifungal Th17 immunity. Exposure of human primary dendritic cells (DCs) to non-virulent Candida albicans resulted in strong induction of IFN-β expression and IFN-I-stimulated gene (ISG) products. These activities were controlled by C-type lectin receptor dectin-1, which induced IFNB1 transcription via Syk- and CARD9-Bcl-10-MALT1-dependent activation of transcription factor IFN regulatory factor 5 (IRF5). Strikingly, whereas we demonstrate that blockade of IFN-I signaling enhanced human DC-driven Th17 polarization, highly virulent Fonsecaea fungi triggered mincle signaling for abrogation of dectin-1-induced type I IFNs. By inducing the proteasome-mediated degradation of IRF5, mincle perturbed IRF5 nuclear accumulation
C-type lectin-mediated IFN-β modulation for Th17 regulation

INTRODUCTION

Dendritic cells (DCs) are the principal immune cells detecting microbial evasion and are instrumental for subsequent activation of immediate innate defense mechanisms as well as cell-mediated acquired immunity\(^{1,2}\). An elaborate repertoire of pattern recognition receptors (PRRs) provide the means by which DCs can provoke these responses\(^3\). PRRs facilitate antigen presentation to T lymphocytes and their intracellular signaling orchestrates gene transcription for secretion of antimicrobial peptides and cytokines\(^4\). Many classes of transmembrane and cytosolic PRRs have been identified, such as Toll-like receptors (TLRs)\(^5\), C-type lectin receptors (CLRs)\(^6\) and NOD-like receptors (NLRs)\(^7\), each detecting a different type of microbial signature molecule, allowing tailored and pathogen-specific responses.

Polysaccharides predominate the fungal cell wall and carbohydrate-sensing CLRs have been identified as main effectors in the human antifungal immune response. Dectin-1 is amongst the most well-characterized of CLRs, and key to generation of antifungal immune defenses\(^8\)\(^9\), owing to its ability to control phagocytosis\(^10\) and cytokine transcription and maturation for activation of two functionally distinct CD4\(^+\) T helper (T\(_{\text{H}}\)) cell populations\(^11\)\(^12\). It is well documented that immunity mediated by both T\(_{\text{H}}\)1 and interleukin (IL)-17-producing T\(_{\text{H}}\)17 cells confers immune protection to fungal infection\(^13\)\(^14\): T\(_{\text{H}}\)1 cells are required for activation of phagocytic effector mechanisms\(^15\), whereas T\(_{\text{H}}\)17 cells are fundamental for neutrophil accumulation and maintenance of mucosal defense.
C-type lectin-mediated IFN-β modulation for Th17 regulation

Chapter five

barrier immunity. Dectin-1 signaling, through Syk- and CARD9-Bcl-10-MALT1-mediated transcription factor NF-kB activation, elicits expression of Th1-polarizing IL-12p70 as well as key cytokines for instruction and maintenance of human Th17 cells: IL-6, IL-23 and IL-1β. A growing list of human CLRs are, however, being characterized as antifungal immune sensors with a markedly different effector potential. These CLRs, including amongst others dectin-2 and mincle, respond cooperatively and ‘modulate’ responses induced by other PRRs, by directly acting on gene transcription. As such, these modulating receptors influence the extent and type of immune response generated: whereas dectin-2 specifically amplifies Th17 polarization via MALT1-dependent activation of NF-kB subunit c-Rel, mincle suppresses Th1 responses and simultaneously amplifies Th17 immunity via PI(3)K-PKB-mediated activation of E3 ligase Mdm2 (Chapters 3 and 4). A single fungal pathogen is recognized by multiple PRRs at the same time, and such cooperative PRR signaling allows DCs to cope with the large diversity of fungal microbes and to ‘scale’ an immune response according to the pathogenic potential of the encountered fungus.

Evidently, receptors that strongly skew T helper responses away from protective immunity (that is combined Th1 and Th17 immunity) can be targeted by virulent fungi for immune evasion, as exemplified by the predomination of mincle in shaping the overall immune response to fungi which, for example, cause chromoblastomycosis or exacerbate psoriasis (ref and Chapters 3 and 4). Thus the outcome of fungal infection is determined by the cooperative recognition and cross-interference of signaling by CLRs and other immune receptors, yet the precise molecular mechanisms of interference are mostly unclear.

Cytokines of the type I interferon (IFN-I) family, including the various IFN-α and IFN-β molecules, have established roles in defense to intracellular viruses, bacteria and protozoa, and provoke robust expression of IFN-stimulated gene products (ISGs). However, while numerous IFN-I effectors have been unequivocally classified as potent antiviral effectors, actions of the vast majority remain uncharacterized. Not surprisingly therefore, IFN-I...
responses appear to have a much broader physiological role than was originally anticipated\(^2\). Type I IFNs are now emerging as potential players in antifungal immunity\(^29-31\), a notion supported very recently by the finding that murine dectin-1 controls IFNβ transcription through Syk-mediated activation of transcription factor IFN regulatory factor 5 (IRF5)\(^32\). The specific function of IFN-β and IFN-I-stimulated gene products during fungal-directed inflammation remain elusive, while they are known to strongly inhibit IL-1β processing and maturation\(^33,34\) as well as IL-17-producing Th17 cells\(^35-38\). Gain-of-function polymorphisms in human STAT1 impair IL-17 immunity and underlie chronic mucocutaneous candidiasis (CMC)\(^39,40\), with enhanced IFN-α and/or IFN-β responses referred to as part of the underlying mechanism\(^39\). These findings suggest a possible link between IFN-I responses and CLR-mediated control of acquired human antifungal immunity.

In this study we demonstrate that a type I IFN response contributes to human DC-mediated antifungal Th17 immunity. Specifically, we observed strong induction of IFN-β responses along with expression of IFN-I-stimulated gene products upon DC stimulation with *C. albicans*. Instrumental for induction of this antifungal IFN-I program was dectin-1, and its signaling was found to directly trigger IFN-β expression through activation of transcription factor IRF5. Strikingly, whereas we found that blockade of IFN-I receptor signaling enhanced the capacity of dectin-1 to drive Th17 polarization, highly pathogenic fungi suppressed type I IFN induction by cotriggering mincle signaling. Activation of the Mincle-PKB-Mdm2 axis antagonized IFN-β expression by interfering with IRF5 nuclear abundance through a proteasome-dependent degradation process. Thus, our data uncover a link between IFN-I responses and DC-induced immune defenses to fungal infection, and a novel mechanism by which CLRs control the effector functions of fungus-specific Th17 cells. That virulent fungi, via mincle, severely suppress type I IFN signaling, may indicate that the ability of dectin-1 to confer optimal immune protection to fungal infection correlates with its ability to induce such an ‘antiviral’ type I IFN program.

**RESULTS**

*C. albicans* induce type I IFN responses via dectin-1. Here, we investigated a role for IFN-I responses in human DC-induced antifungal immunity. Stimulation of human primary monocyte-derived DCs with heat-killed *Candida albicans* conidia (strains CBS8781 and CBS2712)\(^17\) induced a potent IFN-β mRNA response (Figure 1a). The *C. albicans*-triggered IFN-β response was immediate and peaked at 2 hours post fungal DC stimulation (Figure 1a). We observed no induction of IFN-α mRNA expression (data not shown). Type I IFNs act in an autocrine and paracrine manner to induce expression of IFN-I-stimulated gene products (ISGs), including myxovirus resistance-1 (MxA) and 2-5-oligoadenylate synthase 1 (OAS1) transcripts, by triggering IFN-I receptor signaling\(^28\). We observed accumulation of
MxA and ISG15 mRNA transcripts, starting 2 h post stimulation with *C. albicans* spp. (Figure 1b). These data strongly indicate that human DCs mount an efficient IFN-I response upon sensing fungi.

C-type lectin receptor dectin-1 is important for human DC-mediated antifungal immune defenses to *C. albicans* by inducing Syk-dependent signaling for activation of cytokine gene transcription. We addressed whether dectin-1 was similarly required for IFN-I responses to *C. albicans*. The induction of IFN-β mRNA and the ISGs MxA and ISG15 by the *C. albicans* strains was almost completely abolished after treatment of DCs with blocking anti-dectin-1 antibodies (Figure 2a-d), suggesting that dectin-1 activity is required for induction of IFN-I responses by *C. albicans*. Consistent with these observations, dectin-1-specific triggering with dectin-1 agonist curdlan induced synthesis of IFN-β mRNA (Figure 2e) as well as ISGs MxA and ISG15 at later time points (Figure 2f). Next, we assessed involvement of Syk in the activation of both IFN-β and IFN-I-stimulated gene products by dectin-1. Inhibition of Syk,
via pre-treatment with small molecule Syk inhibitor piceatannol, completely abrogated synthesis of IFN-β transcripts (Figure 2e) as well as IFN-I-inducible MxA and ISG15 responses to curdlan (Figure 2f). Thus, Syk signaling plays a primary role in dectin-1-induced IFN-β responses. Taken together, these observations strongly suggest that dectin-1 triggering by *C. albicans* leads to Syk-dependent IFN-I responses.

**Human dectin-1-induced IFN-β requires transcription factor IRF5 activity.** To examine further the dectin-1-dependent control of IFN-β expression, and accompanying IFN-I-stimulated gene products, we investigated the requirements for productive *IFNB1* gene transcription by dectin-1. IRF transcription factors bind IFN-stimulated elements (ISREs) in the *IFNB1* gene promoter and, as such, function as master transcriptional regulators of IFN-I responses. IRF5 has been recently implicated in IFN-β expression mediated by dectin-1 in mice. We investigated whether IRF5 was involved in dectin-1-triggered IFN-β in human DCs, and silenced IRF5 expression by RNA interference (RNAi; Figure 3a). We observed that induction of IFN-β mRNA by curdlan was completely abolished upon silencing of IRF5 expression (Figure 3b), supporting involvement of IRF5. Furthermore, induction of ISGs MxA, ISG15 and OAS1 by dectin-1 was abrogated after silencing of IRF5 (Figure 3c). Since

**Figure 3. Dectin-1-dependent IFN-β expression requires transcription factor IRF5 activity.** (a) Quantitative real-time PCR of IRF5 mRNA expression to confirm IRF5 silencing by RNA interference (siRNA) in DCs treated with IRF5 SMARTpool siRNA or non-targeting siRNA as a control. Expression is normalized to GAPDH and set at 1 in control siRNA-treated cells. (b,c) Quantitative real-time PCR of IFN-β (b), and IFN-I-stimulated gene products MxA, ISG15 and OAS1 (c) mRNA in DCs stimulated for 2 (b) or 6 h (c) with curdlan, or after IRF5 silencing. Expression is normalized to GAPDH and set at 1 in curdlan-stimulated cells. *P < 0.05; **P < 0.01 (Student’s paired t-test). Data are representative of at least two (c; for ISG15), three (c; MxA and OAS1), four (a) or five (b) independent experiments (mean and s.d. in a-c).
Figure 4. **Mincle suppresses dectin-1-induced IFN-β, via a PI(3)K-PKB Mdm2-dependent pathway.** (a-g) Quantitative real-time PCR of IFN-β (a,c,e,f,g) and IFN-I-stimulated gene products MxA and ISG15 (b,d) mRNA in DCs stimulated for 2 (a,c) or 6 h (b,d-g) with *C. albicans* CBS2712, *Fonsecaea monophora*, *F. pedrosoi*, curdlan and/or TDB, in the absence or presence of PKB inhibitor triciribine (e) or PI(3)K inhibitor wortmannin (g), or after mincle, PKB, Mdm2 or Trim28 silencing (f,g). Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells in (f,g). **P < 0.01 (Student’s paired t-test).** Data are representative of at least one (c,e), two (a,b; ISG15, d), three (b; MxA, f,g) independent experiments (mean and s.d. in a,b,d,f,g).

Figure 5. **Mincle blocks IRF5 nuclear accumulation via a proteasome-dependent mechanism.** (a) Fluorescence staining of IRF5 (red) and nucleus (blue, DAPI) in DCs left unstimulated or stimulated with curdlan and/or TDB. (b,c) ELISA of IRF5 in nuclear extracts of DCs stimulated with curdlan and/or TDB, in the absence or presence of proteasome inhibitor MG-132. (d) ChIP assay of IRF5 recruitment to ISRE binding site(s) of the *IFNB1* promoter in DCs stimulated with curdlan and/or TDB. IgG indicates a negative control. Levels are normalized with respect to the ‘input DNA’ sample, which had not undergone immunoprecipitation; results are expressed as the % input DNA. Data are representative of at least one (c,d), two (a,b) independent experiments (mean and s.d. of duplo measurements in d).

TLR4 relies on transcription factor IRF3 for IFN-β expression\(^1\), IRF5 silencing had no effect on TLR4-induced IFN-β mRNA (Supplementary Figure 2). These results strongly suggest that selective induction of IRF5 activity is crucial for dectin-1 to trigger IFN-β synthesis and mount an IFN-I response in human DCs.
Mincle suppresses IFN-β expression through PKB-Mdm2-Trim28. We determined whether type I IFN responses were induced upon DC recognition of Fonsecaea spp., highly pathogenic fungi that cause chromoblastomycosis. Notably, challenging human DCs with F. monophora (CBS269.37) or F. pedrosoi (CBS271.37) conidia did not induce IFN-β mRNA expression (Figure 4a). Consistent with that observation, we were unable to detect mRNA expression of IFN-I-inducible effectors MxA and ISG15 upon DC stimulation with these fungi (Figure 4b). Different subsets of innate DC receptors are activated by different fungi, for tailored host immune defenses, and besides dectin-1, CLR mincle is cotriggered upon DC recognition of Fonsecaea spp. We therefore investigated contribution of mincle and found that stimulation of DCs with mincle agonist trehalose dibehenate (TDB) failed to induce IFN-β responses and subsequent expression of MxA and ISG15 mRNA (Figure 4c,d). Since mincle signaling cross-interferes with signaling from other PRRs for direct modulation of cytokine gene expression (Chapter 3 and 4), we studied the ability of mincle to affect induction of IFN-β by dectin-1 and performed dual stimulations with curdlan and TDB. Notably,
C-type lectin-mediated IFN-β modulation for Th17 regulation

162

Chapter five

dectin-1-induced IFN-β and MxA and ISG15 mRNA responses were completely abrogated upon mincle costimulation with TDB (Figure 4c,d). In addition, silencing of mincle abrogated the suppression of IFN-β mRNA transcripts upon stimulation with *F. monophora* but not with curdlan or TLR4 agonist LPS (Figure 4e). These data strongly indicate that *F. monophora* suppresses IFN-I responses through mincle signaling.

We next explored the mechanism(s) underlying mincle-dependent IFN-I modulation. Our previous studies have shown that mincle modulates cytokine gene transcription by activating Syk-PI(3)K-PKB signaling that culminates in nuclear translocation of E3 ubiquitin ligase Mdm2 (*Chapter 3* and *4*). Pre-treatment of DCs with small-molecule PKB inhibitor triciribine attenuated the mincle-mediated suppression of dectin-1-induced IFN-β mRNA (Figure 4f). Inhibition of PI(3)K activity by small molecule inhibitor wortmannin or silencing of PKB and Mdm2 expression also restored IFN-I responses to DC stimulation with *F. monophora* (Figure 4f). These results indicate that both PKB and the E3 ubiquitin ligase Mdm2 are involved in suppression of IFN-β-dependent responses by mincle.

Functioning as an effector downstream mincle-PKB signaling, Mdm2 acts on cytokine gene transcription via two independent molecular mechanisms: adaptor protein Trim28 facilitates Mdm2/IRF1 complex formation and links Mdm2 to IRF1 protein degradation (*Chapter 4*). We investigated whether Trim28 was involved in Mdm2-dependent IFN-β suppression by mincle and silenced Trim28 expression. Strikingly, Trim28 silencing by RNAi completely abrogated TDB-driven inhibition of IFN-β expression levels and, accordingly, MxA and ISG15 transcripts after curdlan stimulation (Figure 4f). Consistent with those observations, we were able to detect induction of IFN-β mRNA in response to *F. monophora* in Trim28-silenced DCs (Figure 4f). Of note, TDB costimulation similarly abrogated TLR4-induced IFN-β mRNA, the suppression of which required PI(3)K-PKB signaling as well as contribution of Mdm2 and Trim28 (Supplementary Figure 1), confirming the suppressive effect of the mincle-PKB-Mdm2 axis on IFN-β expression. Thus, whereas dectin-1 induces IFN-I responses upon fungal recognition, activation of mincle signaling suppresses induction of IFN-β mRNA through PKB-dependent activation of Mdm2/Trim28.

**Mincle suppresses IFN by degrading nuclear IRF5.** Aside from bridging nuclear Mdm2-IRF1 interactions upon activation of mincle-PKB signaling, Trim28 has been characterized an interaction partner and modulator of IRF5<sup>43</sup>, we speculated that mincle might directly interfere with nuclear IRFS activities. IRF5 is retained inactive in the cytoplasm and undergoes nuclear localization upon activation<sup>41</sup>; we examined the ability of TDB to alter nuclear localization of IRF5. Dectin-1 triggering by curdlan led to nuclear translocation of IRF5 (Figure 5a,b), which was in agreement with its ability to induce an IFN-β response (Figure 1c). Upon mincle costimulation with TDB, however, curdlan-activated and nuclear localized IRF5 was almost completely blocked (Figure 5a,b). These data strongly indicate that mincle interferes with nuclear localization of IRF5.
Further analysis strongly suggested a proteasome-dependent regulatory mechanism, similar to what has been reported for IRF1. Treatment of DCs with proteasome inhibitor MG132 rescued dectin-1-induced IRF5 nuclear translocation from mincle-mediated suppression (Figure 5c). To confirm these findings, we analyzed IRF5 transcriptional activities at the IFNB1 gene promoter and performed chromatin immunoprecipitation (ChIP) experiments. Activation of dectin-1 signaling triggered recruitment of IRF5 to the IFNB1 promoter ISRE (Figure 5d). In contrast, IRF5 occupation of the IFNB1 promoter was completely abrogated in response to mincle costimulation with TDB (Figure 5d). Collectively, these results indicate that mincle suppresses IRF5 transcriptional activity, and hence synthesis of IFN-β mRNA, by degrading nuclear localized IRF5.

Type I IFN responses attenuate DC-induced antifungal Th17 immunity. Differential regulation of IFN-I responses by dectin-1 and mincle led us to address whether activation of an IFN-I response interfered with human DC-driven Th17 polarization. For inhibition of auto- and paracrine IFN-I signaling, we pre-treated DCs with a blocking antibody against the IFN-α/β receptor chain 2 (IFNAR2). Induction of MxA and OAS1 mRNA synthesis in response to curdlan as well as C. albicans strain CBS2712 was attenuated upon blocking IFN-I receptor responsiveness in human DCs (Figure 6a). Triggering human dectin-1 signaling allows generation of an antifungal Th17 response. Accordingly, both curdlan- and C. albicans-primed DCs activated Th17 polarization and induced IL-17 production by CD4⁺ T cells (Figure 6b,c). Notably, substantially higher proportions of CD4⁺ T cells that produced IL-17 were found when blocking IFN-I signaling upon stimulation with curdlan and C. albicans (Fig 6b,c), indicating that induction of a type I IFN response impairs DC-driven Th17 polarization. As DC-derived cytokines IL-1β, IL-6 and IL-23 are key instigators of Th17 polarization, we investigated the possibility that IFN-β acted directly at the level of IL-1β, IL-6 and IL-23 expression for suppression of Th17. Both silencing of IRF5 and blocking IFN-I receptor signaling substantially amplified curdlan-induced expression of IL-1β and IL-23 subunit p19 mRNA (Figure 5e). In contrast, whereas neither IRF5 silencing nor blocking IFN-I responsiveness had an effect on synthesis of the p40 subunit of IL-23, we found suppression of curdlan-induced IL-6 mRNA levels (Figure 5d,e). This indicated that IFN-I responses might act on DC-driven Th17 polarization, at least in part, via modulation of Th17-cytokine expression. Taken together, our results show that a type I response plays a role in human antifungal Th17 immunity, and indicate that transcriptional modulation of IFN-β expression represents an important mechanism that allows CLRs to ‘fine-tune’ Th17 immunity to fungal infection.
Figure 6. IFN-I signaling limits DC-driven T\textsubscript{h}17 polarization during fungal infection. (a,d,e) Quantitative real-time PCR of IFN-I-stimulated gene products MxA and OAS1 (a), IL-1\textbeta, IL-23p19, IL-12p40 or IL-6 (d,e) mRNA in DCs stimulated with curdlan or \textit{C. albicans} CBS2712 in the absence or presence of blocking IFNAR2 antibodies to abrogate auto- and paracrine IFN-I signaling (a,d) or after IRF5 silencing (e). Expression is normalized to GAPDH and set at 1 in curdlan-stimulated cells. (b,c) T helper polarization was assessed by flow cytometry analysis (FI, fluorescence intensity) by staining for intracellular IFN-\textbeta (T\textsubscript{h}1) and IL-17A (T\textsubscript{h}17) expression at day 12-17 after PMA plus ionomycin restimulation after coculture of memory CD4\textsuperscript{+} T cells with DCs that were left unstimulated (iDC) or primed with curdlan or \textit{C. albicans} CBS2712, in the absence or presence of blocking IFNAR2 antibodies to abrogate IFN-I signaling. In (c), the percentages of IL-4- and IFN-\textbeta-producing T cells are shown, corresponding to the upper left and right quadrants of (b). Data are representative of at least one (a,d) or two (b,c,e) independent experiments (mean and s.d. of duplo measurements in c; mean and s.d. in e).
DISCUSSION

Human host control of fungal infection is complex and incompletely characterized. Dendritic cells are key to T₉ lineage differentiation and establishment of antifungal immunity, for which they rely on transmembrane CLRs. Collaborative signaling by CLRs orchestrates cytokine production and underlies subsequent DC-driven activation of T₉1 and T₉17 subsets. Further understanding of how induction of T₉17 responses to fungal infection are regulated at the molecular level, but also their magnitude and duration, will therefore aid in understanding disease pathogenesis and development of new antifungal therapies and vaccines. Here, we demonstrate contribution of a type I IFN response -a classic antiviral immune effector program- to human antifungal inflammation. Human primary DCs mount a dectin-1-driven IFN-β response upon sensing C. albicans that controls T₉17 immunity. Dectin-1 triggering induced Syk-dependent IRF5 activation, leading to induction of type I IFNs. Blocking these responses led to enhanced antifungal T₉17 polarization, suggesting that type I IFNs are required to limit T₉17 immunity. Notably, highly virulent Fonsecaea fungi suppressed IFN-I responses by DCs, and triggered mincle signaling leading to proteasomal degradation of IRF5 and subsequent abrogation of IFNB transcription. These data strongly suggest that type I IFN responses are an integral part of protective host responses to fungal infections to balance T₉17 responses and this might be targeted by virulent fungi to dysregulate human antifungal immunity.

Dectin-1 promotes host protection from fungal infection by regulating expression and maturation of cytokines implicated in T₉1 and T₉17 polarization. In agreement with recent studies in the mouse, we demonstrate that dectin-1 also directs IFN-β, but not IFN-α, expression, and subsequent ISG expression, upon C. albicans binding. Paradoxically, we found that IFN-I responses had a strong antagonizing effect on the instruction of T₉17 immunity by DCs. Thus, dectin-1 signals for induction of protective T₉1 and T₉17 responses, while at the same time inducing IFN-β production that suppresses T₉17 immunity via IFN-I receptor signaling. While the reason for this seeming discrepancy is unclear, the previous findings and our observation that highly virulent fungal strains abrogate type I IFN production via mincle, strongly point towards a protective function for type IFNs. This could reflect, for example, a self-limiting mechanism to keep T₉17 inflammation in check and prevent T₉17-mediated immunopathology. Uncontrolled or exaggerated T₉17 immunity, has a high host-tissue damaging potential, implicates failure to confer protection against fungal invasion, and is a common theme in chronic inflammatory disorders and autoimmunity. Hence, the human immune system is equipped with numerous mechanisms to avoid immunopathology, irrespective of conferring protection against microbial invasion. Not excluding, that type I IFNs may serve a beneficial role by controlling the antifungal properties of (an) other immune cell type(s): NK cells -again part of the classic antiviral defense program- are now emerging as antifungal effectors, with their activation controlled by DC-derived
type I IFN\textsuperscript{50,51}. In addition, it will be of interest to delineate whether dectin-1-induced IFN-\(\beta\) cross-interferes with Th1 cell frequencies, or, instead, controls activation of natural killer T cells (NKT cells), \(\gamma\delta\) T cells, innate lymphoid cells, and the recently identified IL-17-producing neutrophils\textsuperscript{52} to confer optimal protection to fungal infection.

We demonstrate that transcription factor IRF5 is required for induction of IFN-\(\beta\) expression by dectin-1, and thus identify a second IRF family member acting downstream dectin-1. Our data indicate that the dectin-1-induced IFN-\(\beta\) response is mediated via signaling through the Syk-CARD9-Bcl-10-MALT1 axis - central as well to activation of transcription factor NF-\(\kappa\)B by dectin-1\textsuperscript{11,12}. How further signaling (i.e. downstream the CBM scaffold) culminates in activation and nuclear translocation of IRFs is still unknown. TRAF proteins are likely contributors, with TRAF6 reported to be required for IRF5 nuclear translocation induced by TLR7/9-dependent Myd88 signaling\textsuperscript{53}.

Multiple DC PRRs are triggered upon encountering a fungal pathogen, and their functional collaboration determines the final outcome of an immune response. Evidently, whereas we found a predomination for dectin-1 activities, blocking dectin-1 function did not completely impair the IFN-I response mounted to \textit{C. albicans}. This might indicate (partial) contribution of other receptors. Indeed, results from the mice study support this notion by demonstrating that also dectin-2 contributes to IFN-\(\beta\) induction in response to \textit{C. albicans}\textsuperscript{32}.

A key finding of the present study is that CLR mincle negatively interfered with, and severely suppressed, induction of type I IFN responses by human DCs. Mincle, in contrast to dectin-1, interacts in particular with pathogenic and virulent fungi (Chapter 3 and 4), and has been characterized a distinct ‘modulator’ of DC-mediated antifungal immunity. Mincle signaling directly interferes with signals emanating from other PRRs leading to abrogation of Th1 polarization (Chapter 3) and strong amplification of Th17 immune responses (Chapter 4), an effector program presumably instrumental in the evasion of protective immunity by \textit{Fonsecaea} and \textit{Malassezia} spp. For the impact on Th17 differentiation, mincle signaling enhances transcription of IL-1\(\beta\) and IL-23p19 mRNA and suppresses IL-27 protein synthesis in human DCs. Here we corroborate our earlier findings by demonstrating that mincle signaling suppresses dectin-1-induced IFN-\(\beta\) production, through interfering with IRF5 activation. In light of our present observation that IFN-I responses suppress Th17 polarization, it is likely that mincle-induced IRF5 degradation represents an alternative mechanism by which mincle can promote Th17 immunity. Clearly, mincle regulates innate signal transduction pathways and induction of T helper responses during antifungal inflammation through several avenues.

Mincle alters dectin-1-induced synthesis of IFN-\(\beta\) through a nontranscriptional mechanism and selectively suppresses the nuclear abundance of transcription factor IRF5. Analogous to IRF1 (Chapter 3), we demonstrate that this ability of mincle can be attributed to a proteasome-dependent degradation process. Our preliminary data indicate that mincle-induced IRF5 degradation might be dependent on the concerted action of E3 ubiquitin
ligase Mdm2 and nuclear adaptor protein Trim28, the effector proteins similarly required for mincle-induced IRF1 ubiquitination and proteolysis, leading to IL-12p35 and IL-27p28 suppression. While Trim28 is a binding partner for IRF5, undermining IRF5 function, evidence supporting direct physical and functional interaction between Mdm2 and IRF5 has not yet been obtained. With numerous new Mdm2 substrates continuously being identified, it is not unlikely that Mdm2 will be characterized an E3 ubiquitin ligase for IRF5. However, an indirect effect of Mdm2 and Trim28 on IRF5 protein degradation and, likewise, contribution of other factors, could not be ruled out. Further studies are needed to clarify these issues.

Regardless the specific manner of regulation, it is of interest that the proteasome-dependent degradation of IFN-regulatory factors IRF1 and IRF5 is central to mincle activity. As stated above, besides dectin-1, other DC receptors are likely to contribute to induction of an antifungal IFN-I response. Most notable, analysis of the effects of mincle on TLR4-induced IFN-β and IFN-I-stimulated gene products revealed that mincle also abrogated these responses. Since TLR4 mediated IFN-β induction (in human DCs) is dependent on IRF3 activities, but not on IRF1 or IRF5 (Supplementary Fig. 1), this infers that mincle might be a broad-spectrum antagonist of IRF transcription factors. A possibility not unlikely given the high structural similarities between IRF3 and IRF5 as well as IRF7. It will therefore be of most interest to delineate if mincle acts on multiple members of the IRF family for the modulation of (IFN-I-) cytokine responses and establishment of an antifungal immune state.

The IFN-I effector response to fungal infection dampened DC-mediated instruction of T(h)17 immunity. Functionally, it remains unclear how T(h)17 development is held in check by type I IFNs. Whereas we did observe alterations at mRNA expression levels of T(h)17 polarizing cytokines IL-1β and IL-23 subunit p19, other possibilities regarding the mechanism(s) through which type II IFNs control T(h)17 responses are possible. First, IFN-I effectors might suppress maturation and release of IL-1β by directly acting on inflammasomes. Furthermore, involvement of immunosuppressive factors IL-10 and IL-27 is conceivable: both IL-10 and IL-27 (in)directly inhibit T(h)17 development (Chapter 4 and), while their expression is strongly enhanced upon activation IFN-I receptor signaling. Whether these or other cellular events contribute to regulation of T(h)17 development by type I IFN effectors is under current investigation.

In conclusion, the study here indicates that a type I IFN response is activated in human DCs upon sensing fungal infection and controls induction of T(h)17 immunity. With expression of antifungal IFN-β subject to differential regulation by CLRss dectin-1 and mincle, and hence corresponding to fungal pathogenicity, our results could identify the type I IFN axis as a critical part of the host-protective pathway that mediates resistance to fungal invasion. These data improve our understanding of the complex mechanisms underlying human antifungal immunity, and further investigation into this area might aid in development of candidate human fungal vaccines.
EXPERIMENTAL PROCEDURES

Cells, stimuli, inhibitors and RNA interference. CD14⁺ monocytes from healthy volunteer blood donors were isolated, cultured and differentiated into immature DCs as described and used for experiments at day 6 or 7. Donors were routinely screened for dectin-1 single nucleotide polymorphism rs16910526 using a TaqMan Genotyping Assay (Assay ID C_33748481_10; Applied Biosystems); only dectin-1 wild-type DCs were used for experiments. This study was done in accordance with ethical guidelines of the Academic Medical Center. DCs were stimulated with curdlan (10 μg/ml), lipopolysaccharide from Salmonella typhosa (10 ng/ml; both Sigma), and/or strains from Candida albicans, or Fonsecaea spp. (heat-inactivated organisms) at a multiplicity of infection (MOI) 5, or trehalose-6,6-dibehenate as previously described (50 mg/well; Avanti Polar Lipids). Cells were pre-incubated with inhibitor or blocking antibodies for 1 or 2 h, respectively, with 40 μM piceatannol (Syk inhibitor; Tocris), 50 μM z-VRPR-FMK (MALT1 inhibitor; Alexis), 5 μM triciribine (PKB inhibitor), 0.1 μM MG-132 (proteasome inhibitor; both Calbiochem), 20 mg/ml anti-dectin-1 (MAB1859; R&D Systems), or 20 mg/ml anti-IFNAR2 (MMHAR-2; PBL Interferon Source). DCs were transfected with 25 nM siRNA through the use of transfection reagents DF4 (Dharmacon) and were used for experiments 72 h after transfection. The following SMARTpool siRNAs were used (all from Dharmacon): Mincle (M-021374-02), IRF1 (M-011704-01), IRF5 (M-011706-00), PKB (M-003000-03), Mdm2 (M-003279-04), Trim28 (M-005046-01) and non-targeting siRNA (D-001206-13) as a control. This protocol resulted in nearly 100% transfection efficiency as determined by flow cytometry analysis of cells transfected with siGLO-RISC free-siRNA (D-001600-01) and did not induce IFN responses, as determined by quantitative real-time PCR analysis. Silencing of expression was verified by real-time PCR and flow cytometry for each experiment (Chapter 3 and 4 of this thesis).

Fungal strains. Candida strains were grown in Sabouraud dextrose broth and incubated for 3 days at 25 °C, while shaking. Conidia were dislodged from slants by gentle tapping and then were resuspended in 0.1% (vol/vol) Tween-80 in PBS. Fonsecaea strains were grown for 7 days at 37 °C on Oatmeal agar culture plates. 0.1% Tween-80 in PBS was used to remove and resuspend the grown conidia. Hyphal contamination was removed by straining of the cell solutions through a glass filter. Swollen germinating conidia were obtained by incubation for 6 h at 37 °C, with shaking, in 0.1% (vol/vol) Tween-80 in PBS. Fungi were inactivated by being heated for 1 h at 56 °C.

Quantitative real-time PCR. Isolation of mRNA from DCs stimulated for 2 or 6 h, synthesis of cDNA and amplification by PCR with the SYBR Green method with the ABI 7500 Fast PCR detection system (Applied Biosystems) were performed as described. Specific primers were designed with Primer Express 2.0 (Applied Biosystems; Supplementary Table 1). The Cycling threshold (Ct) value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold value. For each sample, the normalized amount of target mRNA (Nt) was calculated from the obtained Ct values for both target and GAPDH mRNA with the following equation: Nt = 2^(-ΔΔCt) of GAPDH - Ct of target. Relative mRNA expression was obtained by setting of Nt in curdlan-stimulated samples as 1 within one experiment and for each donor, unless otherwise stated.

IRF5 localization. Nuclear and cytoplasmic extracts of DCs were prepared after 30 min of stimulation using the NucBuster protein extraction.
kit (Novagen). Total nuclear levels of IRF5 was detected by ELISA (USCN Life Science). Cellular localization of IRF5 was further determined by immunofluorescence staining: DCs were stimulated for 1 h, fixed with 4% para-formaldehyde, then permeabilized with 0.2% (vol/vol) Triton X-100 in PBS, and stained with anti-IRF5 (ab124792, Abcam). Incubation of Alexa Fluor 594-conjugated goat anti-rabbit (A11072, Molecular Probes) was followed by staining of nuclei with DAPI (Molecular Probes). IRF5 localization was visualized with a Leica DMR A microscope.

**Chromatin immunoprecipitation (ChIP) assay.** The ChIP-IT Express Enzymatic kit (Active Motif) was used for ChIP assays to determine occupancy of the proximal *IFNB1* promoters by IRF5. After 30 min of stimulation, cells were fixed with 1% (vol/vol) formaldehyde. Nuclei were isolated and chromatin DNA was fragmented by enzymatic shearing (10 min at 37 °C). Protein-DNA complexes were immunoprecipitated overnight at 4 °C with anti-IRF5 (ab2932, Abcam) or normal rabbit IgG (negative control; sc-2027, Santa Cruz) and protein G-coated magnetic beads. Input and immunoprecipitated DNA was purified after reversal of crosslinks. Real-time PCR was done with primer sets spanning the ISRE sites (PRDIII/I region, primer sequences Supplementary Table 1). Primers spanning genomic DNA at cytogenetic location 12 p13.3 (Active Motif) were used as a negative control. To normalize for DNA input, a sample for each condition was taken along that had not undergone immunoprecipitation (input DNA); results are presented as the % of input DNA.

**T helper cell differentiation assays.** Memory CD4+ T cells were isolated with MACS beads isolation as described previously64. DCs were preincubated with blocking antibodies as indicated and subsequently activated with curdlan and/or heat-killed fungal strains. After 48 h, cells were washed extensively and cocultured with CD4+ T cells (20,000 T cells/5000 DCs) in the presence of *Staphylococcus aureus* enterotoxin B (10 pg/ml, Sigma). DCs primed with LPS plus IFN-γ (1000 U/ml, U-CyTech) or curdlan were used as positive controls for Th1 or Th17 differentiation, respectively. After 5 days of coculture, cells were further cultured in the presence of IL-2 (10 U/ml, Chiron). Resting cells were restimulated after 12-17 days with PMA (100 ng/ml) and ionomycin (1 mg/ml) for 6 h, the last 4 h in the presence of brefeldin A (10 mg/ml, all Sigma). Intracellular cytokine expression was analyzed by staining with FITC-conjugated mouse anti-IFN-γ (25723.11) and APC-conjugated mouse anti-IL-17A (Clone eBio64DEC17, 17-7179, eBioscience).
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Figure S1. Mincle signaling suppresses IRF1/IRF5-independent type I IFN responses induced by TLR4.
(a–e) Quantitative real-time PCR of IFN-β mRNA in DCs stimulated with LPS and/or TDB, in the absence or presence of PI(3)K inhibitor wortmannin (d), or after IRF1, IRF5, PKB, Mdm2 or Trim28 silencing (a,b,e).
Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. **P < 0.01 (Student’s paired t-test). Data are representative of at least two (a,c), three (d,e) or four (b) independent experiments (mean and s.d. in a–e).
SUPPLEMENTAL TABLE S1

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Author contributions: B.A.W. and A.D. conceived ideas, designed, performed and interpreted most experiments and prepared the manuscript. T.M.K. helped with T cell differentiation assays, performed IRF5 ELISAs, microscopy analyses, and prepared cellular extracts. B.T. and TB prepared the fungal strains. T.B.H.G. and S.I.G. supervised study design, execution and interpretation, and manuscript preparation.