Cell Host & Microbe
Article

Fungal Engagement of the C-Type Lectin Mincle Suppresses Dectin-1-Induced Antifungal Immunity

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http://dx.doi.org/10.1016/j.chom.2014.03.008

SUMMARY

Recognition of fungal pathogens by C-type lectin receptor (CLR) dectin-1 on human dendritic cells is essential for triggering protective antifungal T_H1 and T_H17 immune responses. We show that *Fonsecaea monophora*, a causative agent of chromoblastomycosis, a chronic fungal skin infection, evades these antifungal responses by engaging CLR mincle and suppressing IL-12, which drives T_H1 differentiation. Dectin-1 triggering by *F. monophora* activates transcription factor IRF1, which is crucial for *IL12A* transcription via nucleosome remodeling. However, simultaneous *F. monophora* binding to mincle induces an E3 ubiquitin ligase Mdm2-dependent degradation pathway, via Syk-CARD9-mediated PKB signaling, that leads to loss of nuclear IRF1 activity, hence blocking *IL12A* transcription. The absence of IL-12 leads to impaired T_H1 responses and promotes T_H2 polarization. Notably, mincle is similarly exploited by other chromoblastomycosis-associated fungi to redirect T_H responses. Thus, mincle is a fungal receptor that can suppress antifungal immunity and, as such, is a potential therapeutic target.

INTRODUCTION

Fungal species are ubiquitously present and pose considerable risk to human health. While opportunistic fungi cause disease in vulnerable patient groups, particular fungal strains are virulent regardless of host immunocompetence. Dendritic cells (DCs) have a key role in the generation of protective antifungal immunity by orchestrating activation and expansion of $CD4^+$ effector T cell populations that restrict fungal growth and enable phagocytic clearance (Roy and Klein, 2012). Efficient host protection to fungi requires a coordinated immune response consisting of T helper cell (T_H) type 1 and T_H17 cells (Romani, 2011; Wüthrich et al., 2012). Both T_H1 and T_H17 cells are involved in chemotaxis and activation of phagocytes, particularly macrophages and neutrophils, via secretion of interferon-γ (IFN-γ) and IL-17, respectively (De Luca et al., 2010; Korn et al., 2009). T_H1-produced IFN-γ is essential for optimal activation of phagocytic effector cell functions (e.g., release of nitric oxide and production of reactive oxygen intermediates) to combat fungal persistence (Schroder et al., 2004). The critical role of T_H1 cells is underscored by the susceptibility of IFN-γ knockout mice to fungal infections (Káposzta et al., 1998) and the successful use of IFN-γ therapy in controlling human mycoses (Stevens et al., 2006).

Chromoblastomycosis is a chronic progressive fungal infection of skin and subcutaneous tissue that occurs worldwide and is caused by traumatic inoculation of a specific group of dematiaceous fungi, most commonly *Fonsecaea*, *Cladophialophora*, and *Phialophora* species (Queiroz-Telles et al., 2009). Fungal strains that cause chromoblastomycosis are highly pathogenic and affect immunocompetent hosts (Sousa et al., 2011). Lesions of chromoblastomycosis patients that manifest a high fungal load are characterized by the presence of T_H2 cells (d'Avila et al., 2003). T_H2 cells are detrimental for antifungal defense as they oppose fungal elimination (Romani, 2011), suggesting that these fungi have evolved to escape or manipulate innate and adaptive immune responses.

C-type lectin receptors (CLRs), a prominent class of pathogen recognition receptors (PRRs), expressed on DCs couple innate recognition of fungal carbohydrates to expression of cytokines involved in T_H polarization (Wevers et al., 2013). Interleukin (IL)-12p70 is crucial for T_H1 differentiation (Moser and Murphy, 2000), while activation and maintenance of T_H17 cells requires secretion of IL-1β, IL-6, and IL-23 by DCs (Korn et al., 2009; Zielinski et al., 2012). CLR-induced signaling pathways that induce human antifungal T_H polarization programs are now being defined. Dectin-1 is an important fungal sensor, and dectin-1 triggering activates Syk, which induces assembly of a signaling complex consisting of CARD9, Bcl-10, and MALT1 (Gross et al., 2006), resulting in activation of both classical and noncanonical NF-κB pathways to induce expression of T_H1- and T_H17-polarizing cytokines (Gringhuis et al., 2009). CLR mincle is also involved in host responses against several fungi, including *Malassezia*, some *Candida* species, and *Fonsecaea pedrosoi* (Sousa et al., 2011; Wells et al., 2008; Yamasaki et al., 2009); however, its contribution to antifungal immunity remains unclear. Mincle transduces Syk-CARD9 signaling via the paired Fc receptor common γ-chain (FcRγ) adaptor (Ishikawa et al., 2009; Strasser et al., 2012; Yamasaki et al., 2008). Mincle can heterodimerize with another CLR, macrophage C-type lectin (MCL), forming a functional trimeric receptor complex with FcRγ

(Lobato-Pascual et al., 2013). In mice, mincle agonists promote induction of T_H1 and T_H17 responses (Schoenen et al., 2010). However, how human mincle affects cytokine transcription and subsequently T_H differentiation remains unclear.

Here, we identify mincle as a suppressor of antifungal defenses by suppressing IL-12. We show that interferon regulatory factor 1 (IRF1) is crucial for IL-12 production by inducing nucleosome remodeling of *IL12A*. Dectin-1 triggering by *F. monophora* induced IRF1-dependent IL-12p35 mRNA expression, but our study demonstrates that *F. monophora* simultaneously triggered mincle, which led to specific degradation of IRF1, thereby suppressing *IL12A* transcription. Mincle signaling led to E3 ubiquitin ligase Mdm2-dependent proteasomal degradation of IRF1 in the nucleus. IL-12p70 suppression redirected T_H differentiation from T_H1 to T_H2 responses, thereby adversely affecting antifungal defense mechanisms. Thus, human mincle is a prominent modulator of antifungal immunity and immune suppressor and might be targeted to treat chromoblastomycosis as well as disorders characterized by aberrant IL-12-driven inflammation, such as autoimmune diabetes.

RESULTS

F. monophora Selectively Represses IL-12p35 Production in Human DCs

Stimulation of primary human DCs with heat-killed conidia from chromoblastomycosis isolate *F. monophora* induced expression of maturation markers CD80, CD86, CD83, and HLA-DR (Figure S1 available online) as well as expression of IL-6, IL-1β, IL-23, and IL-12p40, but not IL-12p70 (Figure 1A). We next examined the lack of IL-12p70 expression. Whereas TLR4 triggering by lipopolysaccharide (LPS) induced strong IL-12p70 secretion, simultaneous stimulation with *F. monophora* severely suppressed LPS-mediated IL-12p70 release (Figure 1B). IL-12p70 comprises of IL-12p35 and IL-12p40 subunits (Hunter, 2005). *F. monophora*-stimulated DCs produced IL-12p40 as well as IL-23, which consists of IL-12p40 and IL-23p19 subunits (Figure 1A), indicating that IL-12p70 expression is restricted at the level of IL-12p35. *F. monophora* alone failed to trigger IL-12p35 expression, while LPS-induced IL-12p35 mRNA levels were significantly blocked after coexposure to *F. monophora* (Figure 1C). *F. monophora*-mediated suppression of LPS-induced IL-12p35 mRNA expression was even more evident with live fungi (Figure 1C). In contrast, *Candida albicans* strongly induced IL-12p35 and enhanced LPS-induced IL-12p35 mRNA expression (Figure 1D). These data strongly suggest that *F. monophora* actively suppresses IL-12p35 production.

F. monophora Targets Mincle to Inhibit IL-12p35 Expression

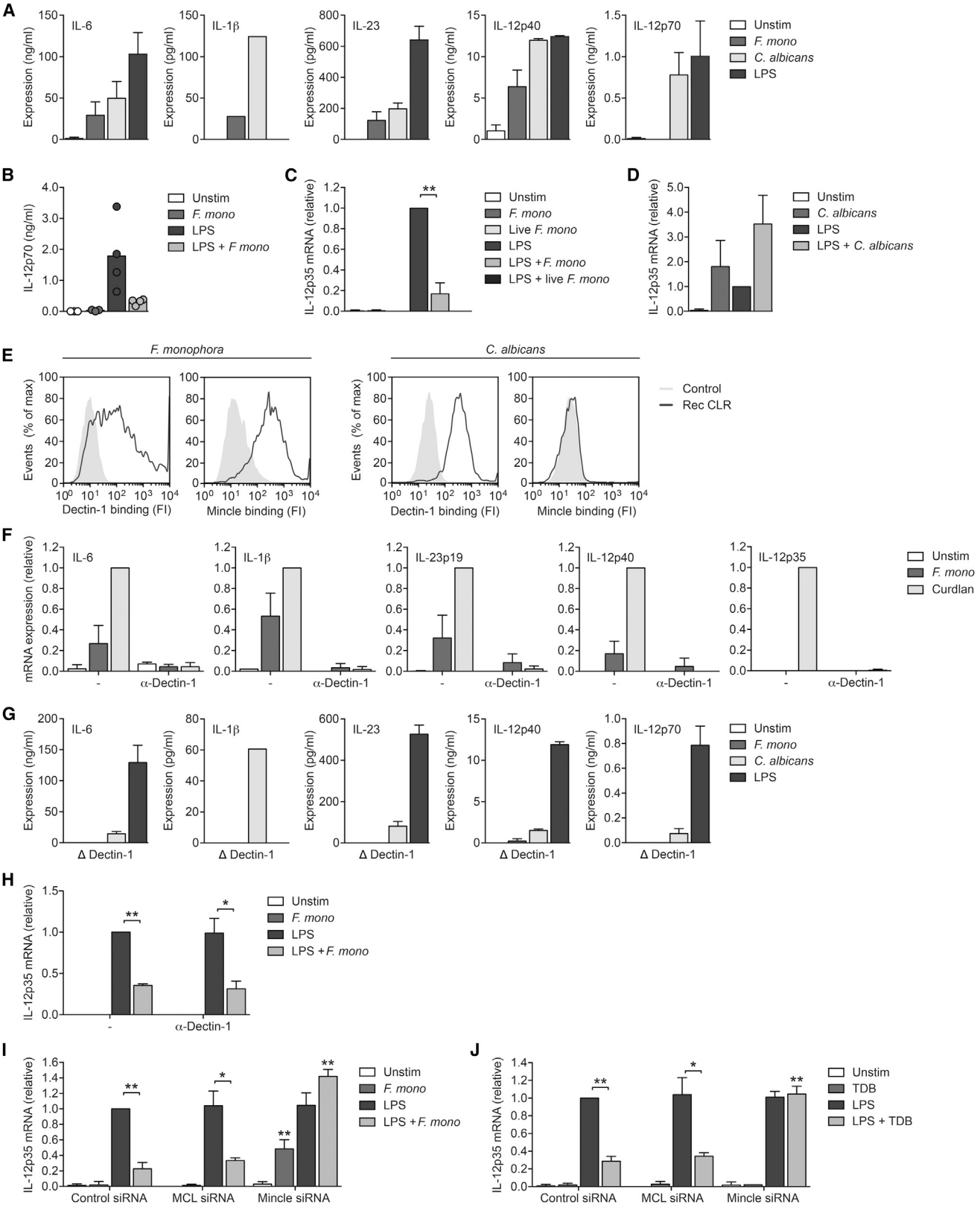
We next set out to identify the innate receptor(s) involved in *F. monophora* responses and IL-12p35 suppression. Recombinant human dectin-1 interacted with *F. monophora* as well as *C. albicans* (Figure 1E). We found that blocking antibodies against dectin-1 abrogated *F.-monophora*-induced IL-6, IL-1β, IL-23p19, and IL-12p40 mRNA expression, as were the responses induced by dectin-1 agonist curdlan (Figure 1F). Moreover, DCs derived from a donor carrying a homozygous Y238X dectin-1 mutation, hence lacking functional dectin-1 expression (Ferwerda et al., 2009) (Figure S2), did not produce IL-6, IL-1β, IL-23, and IL-12p70 after *F. monophora* stimulation (Figures 1G and S2). In contrast, both LPS and *C. albicans* (strain CBS2712, which is not strictly dependent on dectin-1 for triggering cytokine responses) (Gringhuis et al., 2011) induced cytokines in the absence of functional dectin-1 (Figure 1G). Furthermore, blocking dectin-1 signaling did not interfere with *F. monophora*-mediated suppression of LPS-induced IL-12p35 mRNA expression (Figure 1H). These data indicate that dectin-1 recognition of *F. monophora* is crucial for induction of cytokine gene transcription, whereas another innate receptor is responsible for suppression of IL-12p35.

We found that *F. monophora*, but not *C. albicans*, interacted with recombinant human mincle (Figure 1E). As mincle is constitutively expressed on human immature DCs (Figure S2), we investigated whether mincle is involved in IL-12p35 suppression by *F. monophora* by silencing mincle expression by RNAi (Figure S3). Strikingly, mincle silencing restored IL-12p35 mRNA and IL-12p70 protein expression in response to *F. monophora*, without affecting curdlan-induced IL-12p35 synthesis (Figures 1I and S4). We next determined whether mincle-mediated IL-12p35 suppression required the presence of MCL, which can form functional complexes with mincle and FcRγ (Lobato-Pascual et al., 2013). MCL silencing did not interfere with mincle surface expression, while, vice versa, mincle silencing only slightly affected cell surface expression of MCL (Figure S3). Importantly, MCL silencing did not interfere with IL-12p35 suppression by *F. monophora* (Figure 1I), indicating that the suppression is independent of MCL.

To further examine the involvement of mincle in IL-12p35 suppression, we used trehalose-6,6-dibehenate (TDB), a known mincle and MCL agonist (Ishikawa et al., 2009; Miyake et al., 2013). Similar to *F. monophora*, triggering of DCs by TDB alone failed to induce IL-12p35 but suppressed LPS-induced IL-12p35 mRNA expression (Figure 1J). Mincle, but not MCL, silencing effectively prevented suppression of LPS-induced IL-12p35 mRNA expression by TDB (Figure 1J). Taken together, these data demonstrate that dectin-1 induces cytokine expression in response to *F. monophora*, whereas mincle signaling suppresses IL-12p35 transcription.

Mincle Suppresses IL-12p35 Expression via PKB Activation

We set out to elucidate the mechanism behind mincle-mediated IL-12p35 suppression. As both dectin-1 and mincle signal via Syk-CARD9 (Gross et al., 2006; Yamasaki et al., 2008), we combined TLR4 triggering with selective mincle stimulation by TDB to distinguish between dectin-1- and mincle-mediated effects. Silencing of Syk, CARD9, Bcl-10, or MALT1 abrogated the suppressive effects of TDB on LPS-induced IL-12p35 expression (Figure 2A), demonstrating that mincle suppresses IL-12p35 via Syk-CARD9-Bcl-10-MALT1-mediated signaling. To further identify mincle signaling components, we utilized a panel of small molecule inhibitors. We noted in particular that pretreatment of DCs with wortmannin, an irreversible PI3K inhibitor, abrogated IL-12p35 suppression by TDB and restored expression to LPS-induced levels (Figure 2B). Similarly, inhibition of PKB (or Akt), a well-established effector of PI3K, by triciribine



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(Figure 2B) or PKB silencing (Figure 2C) blocked TDB-mediated suppression of IL-12p35. Thus, the PI3K-PKB cascade plays a primary role in mincle-mediated IL-12p35 suppression. Indeed, TDB, in contrast to curdlan or LPS, strongly induced PKB kinase activity (Figure 2D). PKB activation requires phosphorylation at Thr308 and Ser473, which is controlled by PI3K via kinases PDK1 and mTORC2, respectively (Sarbassov et al., 2005). Mincle stimulation led to PKB phosphorylation at both Thr308 and Ser473 (Figure 2E), while LPS and curdlan had no effect (Figure S5), correlating with PKB activity (Figure 2D). PKB phosphorylation after TDB stimulation was dependent on PI3K activity (Figure 2E) as well as mincle and Syk-CARD9-Bcl-10-MALT1-mediated signaling as silencing of these proteins inhibited TDB-induced PKB phosphorylation (Figure 2F). Furthermore, we found that *F. monophora* induced PI3K-dependent PKB phosphorylation via mincle (Figures 2E and 2F), which relied on Syk and the CARD9-Bcl-10-MALT1 scaffold (Figure 2F). These results indicate that mincle suppresses dectin-1-induced IL-12p35 expression in response to *F. monophora* by activation of PI3K-PKB signaling, through a Syk-CARD9-Bcl-10-MALT1-dependent pathway.

Mincle Impairs *IL12A* Nucleosome Remodeling

IL12A transcription is rigidly controlled; in resting cells, the *IL12A* promoter is assembled into stable nucleosomes, whereas upon stimulation, repositioning of nucleosome 2 (nuc-2) allows binding of, among others, NF-κB and subsequent transcriptional initiation by RNA polymerase II (RNAPII) (Goriely et al., 2003). We observed that, although *F. monophora* interfered with NF-κB p65 recruitment to the *IL12A* promoter (Figure 3A), fungal stimulation did not abrogate nuclear translocation of p65 (Figure 3B). Indeed, *F. monophora* alone induced nuclear translocation of p65, c-Rel, and RelB (Figure 3B), a hallmark of dectin-1 signaling (Gringhuis et al., 2009). Moreover, RNAPII recruitment to the *IL12A* promoter was almost completely abolished (Figure 3A). Since these data suggest that mincle signaling interferes with *IL12A* transcription prior to RNAPII and NF-κB binding, we next examined whether *F. monophora* interferes with *IL12A* nucleosome remodeling. *F. monophora* did not induce *IL12A* nucleosome remodeling, whereas curdlan and LPS evoked complete remodeling (Figure 3C). Notably, LPS-induced *IL12A* nucleosome remodeling was attenuated after costimulation with *F. monophora* (Figure 3C), while mincle silencing restored LPS-induced *IL12A* nucleosome remodeling (Figure 3D). These results strongly suggest that *F.-monophora*-induced mincle signaling inhibits IL-12p35 expression by interfering with nucleosome remodeling events indispensable for transcriptional activation at the *IL12A* promoter.

Mincle-Induced PKB Signaling Blocks Nuclear IRF1 Activity

The nuc-2 promoter region of *IL12A* contains an IFN-stimulated response element (ISRE) that can be bound by IRF1 (Liu et al., 2003). We found that IRF1 silencing abrogated IL-12p35 expression in response to curdlan or LPS stimulation (Figure 4A). In accordance, both dectin-1 and TLR4 triggering induced IRF1 recruitment to the ISRE site of the *IL12A* promoter (Figure 4B). Strikingly, IRF1 silencing completely abrogated *IL12A* nucleosome remodeling induced by dectin-1 and TLR4 signaling (Figure 4C), which coincided with impaired recruitment of RNAPII and p65 to the *IL12A* promoter (Figure 4D). These data show that IRF1 plays an essential role in *IL12A* transcription in response to both dectin-1 and TLR4 via nucleosome remodeling.

We next investigated whether mincle triggering affects *IL12A* nucleosome remodeling by interfering with IRF1 activation. We found that both curdlan and LPS induced nuclear translocation of IRF1 (Figure 4E). Remarkably, simultaneous stimulation with *F. monophora* completely abrogated LPS-induced nuclear IRF1 accumulation (Figure 4F). *F. monophora* alone did not induce nuclear IRF1 accumulation (Figure 4F). Also, we observed a complete absence of nuclear IRF1 after TLR4 and mincle costimulation (Figures 4G and 4H). Silencing of either mincle or downstream effector PKB restored nuclear IRF1 accumulation after LPS and TDB costimulation, as well as *F. monophora* stimulation, without affecting LPS-induced nuclear IRF1 accumulation or cytoplasmic IRF1 expression (Figure 4I). These data show that PKB-mediated mincle signaling restricts nuclear accumulation of IRF1, suggesting that this mechanism underlies the block in *IL12A* nucleosomal remodeling and subsequent transcription in response to *F. monophora*.

Mincle Directs Proteasomal Degradation of Nuclear IRF1 via Mdm2

IRF1 steady-state levels are tightly controlled by a balance between synthesis and ubiquitin-mediated proteolysis (Landré et al., 2013; Nakagawa and Yokosawa, 2000). We next investigated whether mincle interferes with IRF1 by inducing its degradation. Treatment with proteasome inhibitor MG-132 reversed the block in nuclear IRF1 accumulation after LPS-TDB costimulation (Figure 5A), indicating that IRF1 is indeed targeted for proteasomal degradation by mincle. Since PKB can induce the E3 ubiquitin ligase activity of Mdm2, which recently was found to aid in IRF1 proteasomal degradation (Landré et al., 2013; Mayo and Donner, 2001; Zhou et al., 2001), we explored a possible Mdm2 contribution. Remarkably, Mdm2 silencing completely abrogated mincle-mediated IL-12p35 suppression

Figure 1. *Fonsecaea monophora* Induces Cytokine Expression via Dectin-1, while Mincle Signaling Selectively Suppresses IL-12p35 Expression

(A, B, and G) Cytokine secretion in supernatants of DCs 24 hr after stimulation with LPS and/or heat-killed *F. monophora* or *C. albicans* measured by ELISA. In (G), DCs are from a donor lacking functional dectin-1 due to a homozygous dectin-1 Y238X mutation. In (A) and (G), data are presented as mean ±SD of duplicate samples.

(C, D, F, H, I, and J) Cytokine mRNA expression by DCs 6 hr after stimulation with curdlan, LPS, and/or heat-killed or live *F. monophora*, heat-killed *C. albicans,* or TDB in the absence or presence of blocking dectin-1 antibodies (F) or after mincle or MCL silencing ([H] and [I]) by RNA interference (siRNA), measured by real-time PCR, normalized to GAPDH, and set at 1 in LPS- ([C], [D], [H], [I], and [J]) or curdlan (F)-stimulated cells. Data are presented as mean ±SD. *$p < 0.05$; **$p < 0.01$.

(E) Binding of recombinant C-type lectins (rec CLR) dectin-1 or mincle protein to heat-killed *F. monophora* or *C. albicans* conidia, determined by flow cytometry (FI, fluorescence intensity). Data are representative of at least two ([A], [E], and [G]), three ([D], [F], [H], [I], and [J]), four (B), or six (C) independent experiments. See also Figures S1, S2, S3, and S4.

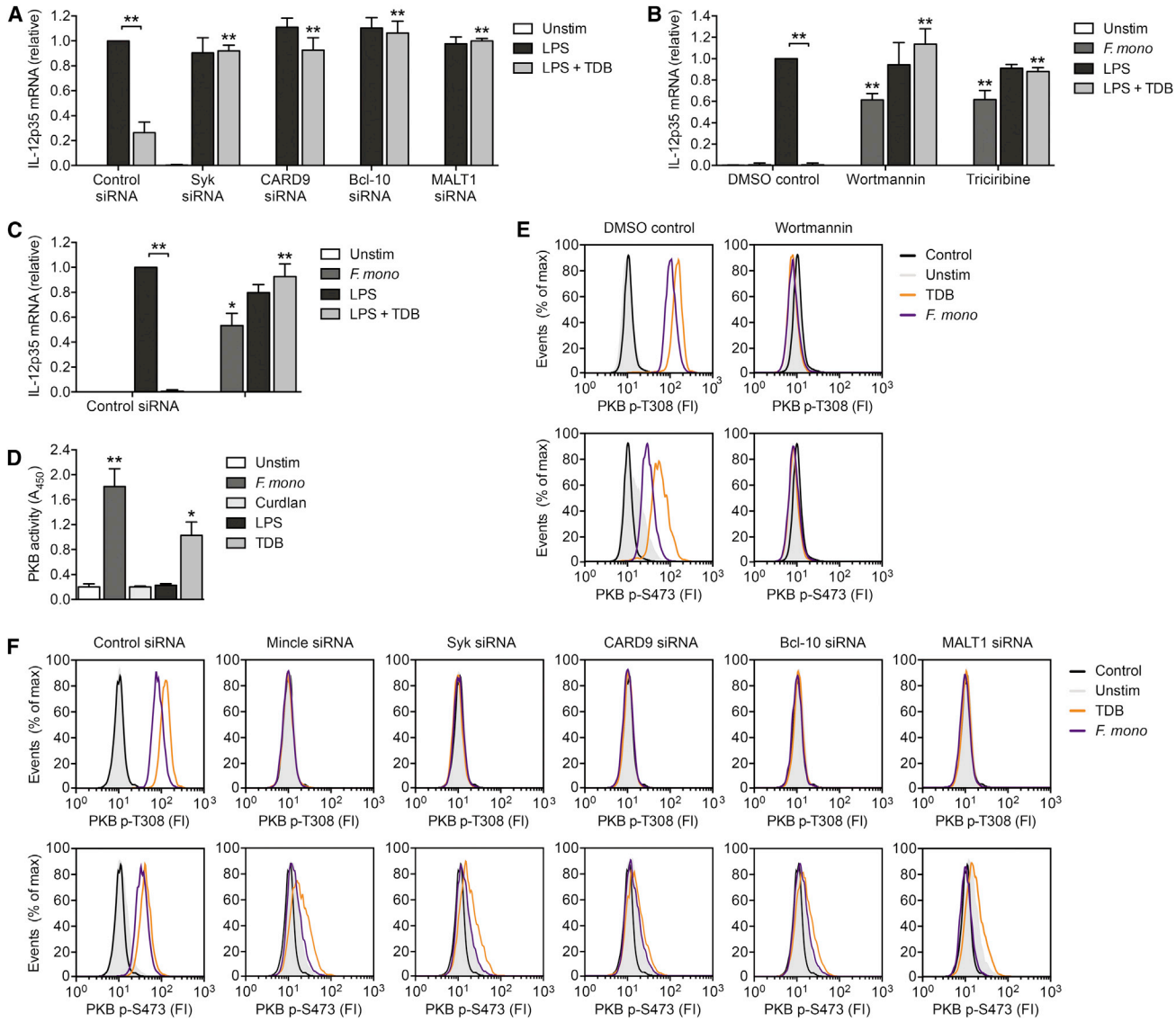

Figure 2. Mincle Signaling Suppresses IL-12p35 Expression via PI3K and PKB

(A–C) IL-12p35 mRNA expression by DCs 6 hr after stimulation with LPS and/or TDB or *F. monophora* after Syk, CARD9, Bcl-10, MALT1, or PKB silencing ([A] and [C]) or in the absence or presence of PI3K inhibitor wortmannin or PKB inhibitor triciribine (B), measured by real-time PCR, normalized to GAPDH, and set at 1 in LPS-stimulated cells. Data are presented as mean ±SD. *$p < 0.05$; **$p < 0.01$.

(D) PKB kinase activity in whole-cell lysates of DCs after 30 min of stimulation with curdlan, *F. monophora*, LPS, and/or TDB. Data are presented as mean ±SD. *$p < 0.05$; **$p < 0.01$.

(E and F) PKB phosphorylation at Thr308 or Ser473 in DCs left unstimulated (*gray*) or 20 min after stimulation with TDB (*orange*) or *F. monophora* (*purple*); in the absence or presence of PI3K inhibitor wortmannin (E); or after mincle, Syk, CARD9, Bcl-10, or MALT1 silencing (F), determined by flow cytometry (FI, fluorescence intensity). Data are representative of at least three ([A]–[F]) independent experiments. See also Figures S3 and S5.

in response to TDB and *F. monophora*—without affecting LPS-induced IL-12p35 expression (Figure 5B). To regulate IRF1, Mdm2 must gain nuclear entry, a process which requires phosphorylation of Mdm2 at Ser166 by PKB (Mayo and Donner, 2001; Zhou et al., 2001). We found that mincle triggering by both TDB and *F. monophora*, but not TLR4 triggering, induced nuclear translocation of Mdm2 (Figure 5C). Only Mdm2 present within the nucleus was phosphorylated at key residue Ser166 (Figure 5C). Mdm2 Ser166 phosphorylation following TDB and *F. monophora* stimulation was abrogated by silencing of mincle, Syk, CARD9, Bcl-10, and MALT1, or chemical inhibition of PI3K or PKB (Figures 5D and S5). Similarly, mincle and PKB silencing blocked Mdm2 nuclear translocation (Figure 5C). TDB-induced Mdm2 phosphorylation and nuclear translocation was not affected by LPS stimulation (Figures 5C and S5). Since Mdm2 ubiquitin ligase activity relies on association with its substrates (Haupt et al., 1997; Yang et al., 2008), we prepared nuclear extracts in the presence of MG-132 to block degradation, allowing us to determine whether Mdm2 interacts with IRF1 within the nucleus. We found that nuclear IRF1

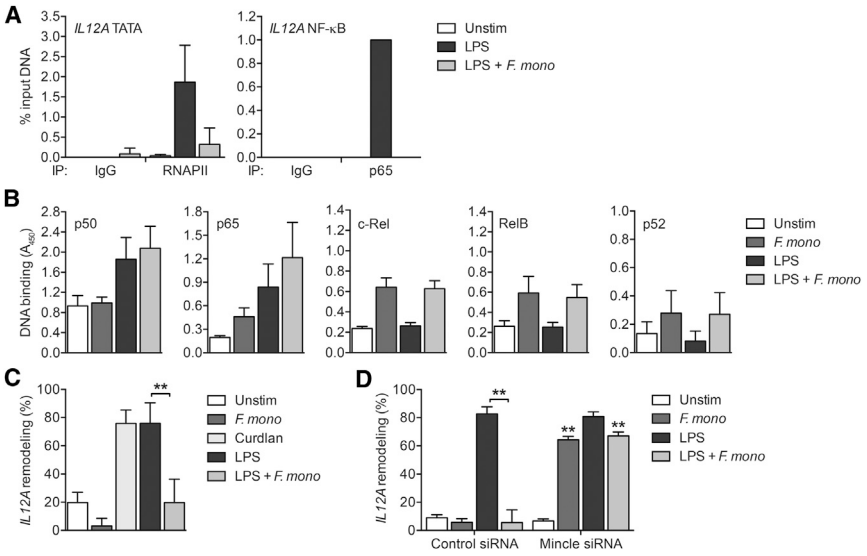

Figure 3. Mincle Signaling Blocks *IL12A* Transcription via PKB by Inhibition of Nucleosome Remodeling

(A) RNA polymerase II (RNAPII) and p65 recruitment to TATA box and NF-κB binding motifs of the *IL12A* promoter in DCs 2 hr after stimulation with LPS and/or *F. monophora*, determined by ChIP assay. IgG indicates a negative control. Data are expressed as percent input DNA and are presented as mean ±SD.

(B) NF-κB subunit activation in nuclear extracts of DCs 2 hr after stimulation with LPS and/or *F. monophora*, measured by DNA binding ELISA. Data are presented as mean ±SD.

(C and D) *IL12A* nucleosome remodeling in DCs 3 hr after stimulation with curdlan, LPS, and/or *F. monophora* or TDB, after mincle silencing (D), determined by ChART assay and normalized to GAPDH. Data are expressed as percent remodeling and presented as mean ±SD. $^{**}p < 0.01$. Data are representative of at least four ([A]; p65) or three (A RNAPII; [B]–[D]) independent experiments. See also Figure S3.

immunoprecipitated together with Mdm2 in both TDB-stimulated and *F. monophora*-stimulated, but not LPS-stimulated, DCs (Figure 5E). The importance of Mdm2 in IRF1 regulation was further supported by data showing that Mdm2 silencing prevented IRF1 degradation after mincle triggering (Figure 5F). Taken together, these results demonstrate that mincle signaling induces Mdm2 activation and nuclear translocation in a PKB-dependent manner to target IRF1 for proteasomal degradation, accounting for the IL-12p35 antagonism observed after *F. monophora* stimulation.

Mincle Signaling Suppresses Antifungal T_H1 Responses

IL-12 is crucial in antifungal T_H1 differentiation. We next investigated how mincle-induced PKB-Mdm2-mediated IRF1 degradation affects T_H differentiation. LPS-primed DCs directed T_H polarization toward T_H1 responses, whereas IRF1 silencing led to severely diminished T_H1 responses after LPS stimulation (Figures 6A and 6B). Neutralizing antibodies against IL-12 impaired T_H1 cell differentiation by LPS-primed DCs (Figures 6C and 6D), underlining the crucial role for IL-12 in T_H1 polarization. *F. monophora*-primed DCs failed to induce T_H1 responses but promoted potent T_H2 polarization (Figures 6E and 6F), consistent with IL-12p70 suppression by *F. monophora*. Moreover, *F. monophora* strongly impaired LPS-induced T_H1 differentiation, actively skewing polarization toward a T_H2-dominated response (Figures 6E and 6F). Notably, mincle silencing completely restored T_H1 polarization by LPS plus *F.-monophora*-primed DCs (Figure 6G). Thus, IL-12p70 suppression after mincle triggering by *F. monophora* is fundamental in redirecting antifungal T_H1 responses to T_H2 immunity.

Various fungi, highly related to *F. monophora*, are also causative agents of chromoblastomycosis in humans (Queiroz-Telles et al., 2009). We next investigated whether these fungi also target mincle to suppress antifungal immune responses. All tested strains (i.e., *F. pedrosoi*, *F. compacta*, and *Cladophialophora carrionii*) were recognized by recombinant mincle protein (Figure 7A) and, strikingly, strongly suppressed LPS-induced T_H1 differentiation and skewed T_H polarization toward a T_H2 response (Figure 7B). These virulent strains repressed IL-12p35 expression similar to *F. monophora* (Figure 7C), accounting for the observed T_H redirection. Mincle silencing reversed skewing of T_H2 differentiation of LPS-primed DCs toward T_H1 polarization for all strains tested (Figure 7D). Collectively, these data demonstrate that virulent fungal pathogens involved in chromoblastomycosis exploit mincle on human DCs to attenuate T_H1 responses and induce T_H2 immunity via suppression of *IL12A* transcription, which might allow these fungi to establish chronic infection.

DISCUSSION

An essential aspect of host resistance to fungal infection is the requirement for T_H1 and T_H17 responses (Romani, 2011; Wüthrich et al., 2012). Here, we have identified mincle as a suppressor of proinflammatory cytokine IL-12, which redirects immunity to fungal pathogens causing chromoblastomycosis from antifungal T_H1 responses toward T_H2 differentiation. At the molecular level, we identified IRF1 as the crucial factor for nucleosome remodeling at the proximal *IL12A* promoter that was induced by dectin-1. *F. monophora* fungi binding to mincle led to activation of a PI3K-PKB signaling cascade that directed Mdm2-mediated proteasomal degradation of IRF1. Loss of IRF1 interfered with *IL12A* nucleosome remodeling, thereby suppressing IL-12p70. Thus, mincle antagonizes dectin-1 signaling in response to chromoblastomycosis-related fungi to suppress protective T_H1 immunity, which might contribute in establishing chronic subcutaneous infection.

In recent years, dectin-1 has been revealed as the primary PRR in antifungal immunity, capable of orchestrating T_H1 and T_H17 immune responses independently of other PRRs (Gringhuis et al., 2009; LeibundGut-Landmann et al., 2007). Here we found that chromoblastomycosis isolate *F. monophora* also triggered dectin-1 signaling, which was a prerequisite for classical and noncanonical NF-κB activation and, consequently, expression of T_H1- and T_H17-polarizing cytokines. However, *F. monophora* induced a second pathway through mincle that interfered with

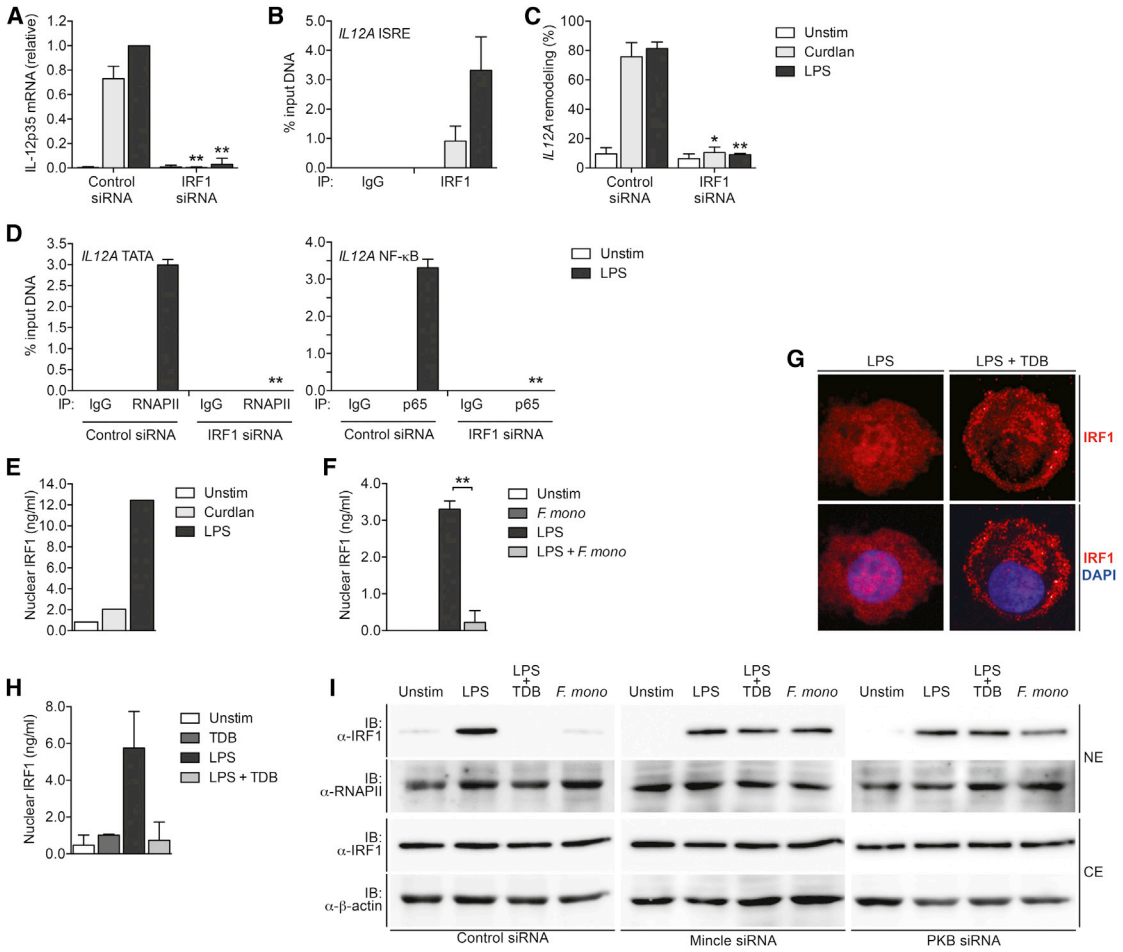

Figure 4. Mincle Signaling Suppresses Nuclear IRF1 to Block *IL12A* Nucleosome Remodeling

(A) IL-12p35 mRNA expression by DCs 6 hr after stimulation with curdlan or LPS, after IRF1 silencing, measured by real-time PCR and normalized to GAPDH and set at 1 in LPS-stimulated cells. Data are presented as mean ±SD. **p < 0.01.

(B and D) IRF1 (B), RNAPII, and p65 (D) recruitment to ISRE binding site (B); TATA box or NF-κB binding motif (D) of the *IL12A* promoter in DCs 2 hr after stimulation with curdlan or LPS ([B] and [D]); and after IRF1 silencing (D), determined by ChIP assay. IgG indicates a negative control. Data are expressed as percent input DNA and presented as mean ±SD. **p < 0.01.

(C) *IL12A* nucleosome remodeling in DCs 3 hr after stimulation with curdlan or LPS, after IRF1 silencing, determined by ChART assay and normalized to GAPDH. Data are expressed as percent remodeling and presented as mean ±SD. *p < 0.05; **p < 0.01.

(E–I) IRF1 nuclear translocation in DCs 2 hr after stimulation with curdlan, LPS, and/or TDB or *F. monophora*, after mincle or PKB silencing (I), determined by ELISA on nuclear extracts ([E], [F], and [H]), immunofluorescence microscopy ([G]; IRF1, *red*; DAPI, *blue*) or immunoblotting ([I]; nuclear, NE; cytoplasmic, CE extracts). In (I), RNAPII and β-actin served as loading controls for NE and CE, respectively. Data in ([E], [F], and [H]) are presented as mean ±SD. *p < 0.05. Data are representative of at least three ([A], [C], [D], [F], and [H]), two ([B], [G], and [I]), one ([E]; curdlan), or five ([E]; LPS) independent experiments. See also Figure S3.

dectin-1-induced IL-12p70 expression. The combined activation of dectin-1 and mincle by *F. monophora* led to a cytokine expression profile that prevented generation of protective T_H1 immune responses, instead inducing adverse T_H2 responses. Thus, the overall immune response to *F. monophora* is determined by cooperation between dectin-1 and mincle, with dectin-1 acting as the general instigator and mincle as the crucial determinant or director.

Deficiency of T_H1 effector responses due to mincle-mediated suppression of IL-12p35 expression in human DCs was not restricted to infection with *F. monophora*, but extended to chromoblastomycosis isolates *F. pedrosoi*, *C. carrionii*, and *F. compacta*, suggesting a common mode of immune evasion in humans. In mice, mincle is involved in innate recognition of *F. pedrosoi*, yet it is incapable of controlling protective immunity; in this mouse model of chromoblastomycosis, TLR engagement is sufficient to revert immune dysfunction (Sousa et al., 2011). In contrast, in our human model, we observed that mincle suppressed TLR4-induced IL-12p70 expression. This discrepancy might reflect differences in host defense between mice and humans. Indeed, we observed several differences between expression and function of human and murine mincle. While mincle has been described as an inducible receptor on mouse bone-marrow-derived DCs, via triggering of MCL (Miyake et al., 2013), we detected constitutive expression of mincle on human immature DCs. Furthermore, while mincle and MCL

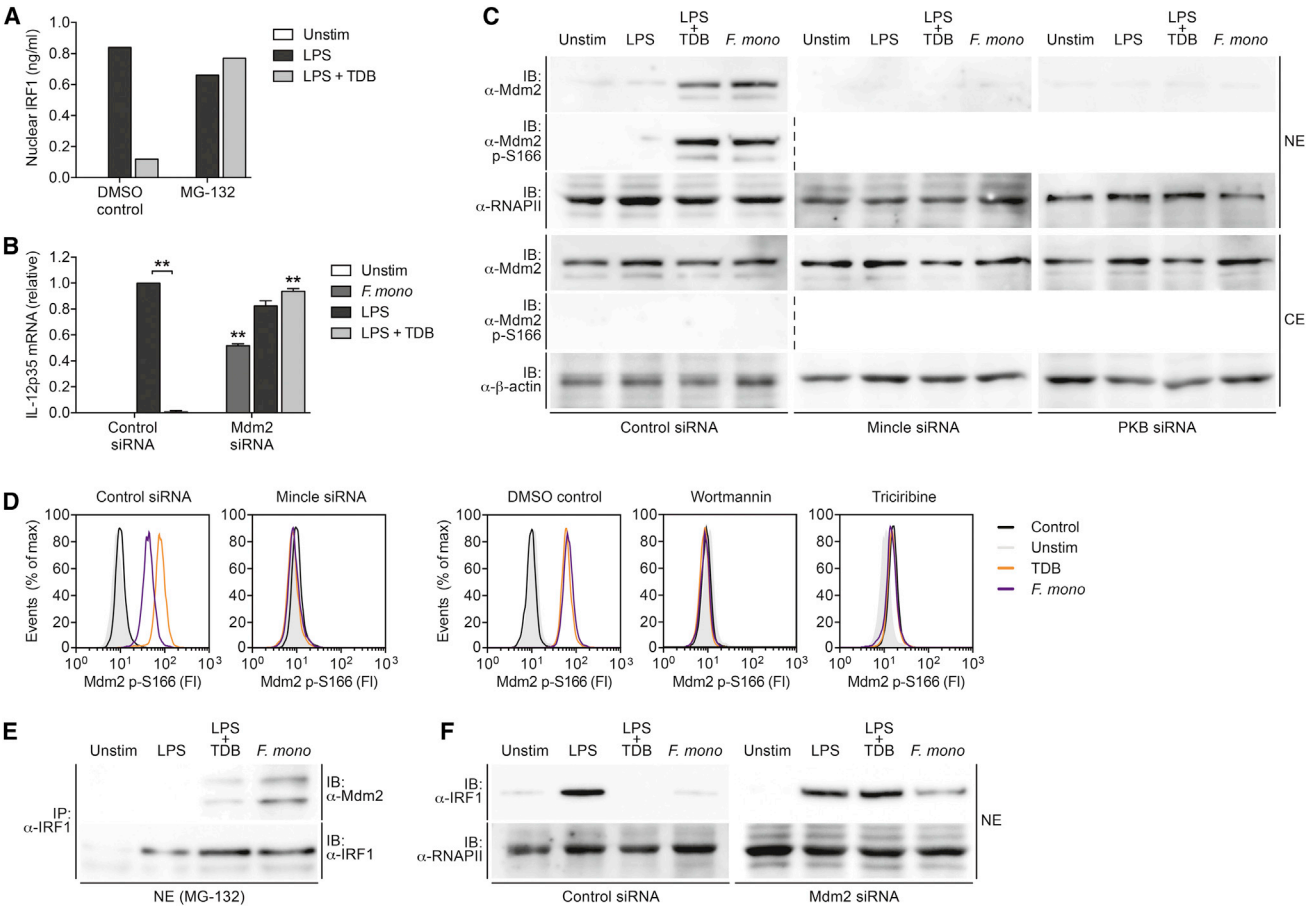

Figure 5. Mincle-PKB Signaling Induces Proteasomal Degradation of Nuclear IRF1 via Mdm2

(A) IRF1 expression in nuclear extracts of DCs 2 hr after stimulation with LPS and/or TDB or *F. monophora*, in the absence or presence of proteasome inhibitor MG-132, measured by ELISA.

(B) IL-12p35 mRNA expression by DCs 6 hr after stimulation with LPS and/or TDB or *F. monophora*, after Mdm2 silencing, measured by real-time PCR, normalized to GAPDH and set at 1 in LPS-stimulated cells. Data are presented as mean ±SD. **$p < 0.01$.

(C and F) Mdm2 (C) and IRF1 (F) cellular localization and Mdm2 phosphorylation at Ser166 (C) in DCs 2 hr after stimulation with LPS and/or TDB or *F. monophora*, after mincle, PKB (C), or Mdm2 silencing (F), determined by immunoblotting (nuclear, NE; cytoplasmic, CE extracts). RNAPII and β-actin served as loading controls for NE and CE, respectively.

(D) Mdm2 phosphorylation at Ser166 in DCs left unstimulated (*gray*) or 20 min after stimulation with TDB (*orange*) or *F. monophora* (*purple*), after mincle silencing, or in the absence or presence of PI3K inhibitor wortmannin or PKB inhibitor triciribine, determined by flow cytometry (FI, fluorescence intensity).

(E) Mdm2 immunoprecipitated together with IRF1 (IP) from nuclear extracts of DCs 2 hr after stimulation with LPS and/or TDB, or *F. monophora*, determined by immunoblotting (IB). NE were prepared in the presence of proteasome inhibitor MG-132 to block protein degradation. RNAPII served as loading control. Data are representative of at least two ([A], [C], [E], and [F]) or three ([B] and [D]) independent experiments. See also Figures S3 and S5.

form functional complexes with FcRγ in rat primary cells (Lobato-Pascual et al., 2013), we found that the presence of MCL was neither required for mincle expression, surface localization, nor mincle-mediated signaling induced by either *F. monophora* or TDB to suppress *IL12A* transcription. These studies suggest that mincle has evolved different functions in defense against fungi, and further studies are required to understand the differences between its functions in mice and humans.

Mincle acts as a key regulator for therapeutic T_H17 adjuvanticity of mycobacterial glycolipid TDM and its synthetic derivate TDB (Schoenen et al., 2010). In these murine models, in addition to T_H17 immunity, mincle-mediated adjuvanticity translates into robust T_H1 responses (Schoenen et al., 2010). Even though a TDB-containing *M. tuberculosis* subunit vaccine (Agger et al., 2008) entered clinical trial, the stimulatory actions of TDB and TDM have never been tested extensively in the human setting (Lang et al., 2011). Our finding that mincle suppressed human T_H1 immunity implies that utilizing mincle agonists as vaccine adjuvants should be considered with caution.

Another important finding of our study is the crucial and nonredundant role of IRF1 in *IL12A* nucleosome remodeling and IL-12p35 synthesis by different PRRs. We found that IRF1 was crucial for not only nucleosome remodeling of the proximal *IL12A* promoter in response to dectin-1 triggering but also TLR4 ligation. IRF1-mediated *IL12A* nucleosome remodeling allows NF-κB subunit p65 and RNAPII recruitment—and hence productive *IL12A* transcription. Despite tight regulation of IRF1 transcriptional activity, with IRF1 steady-

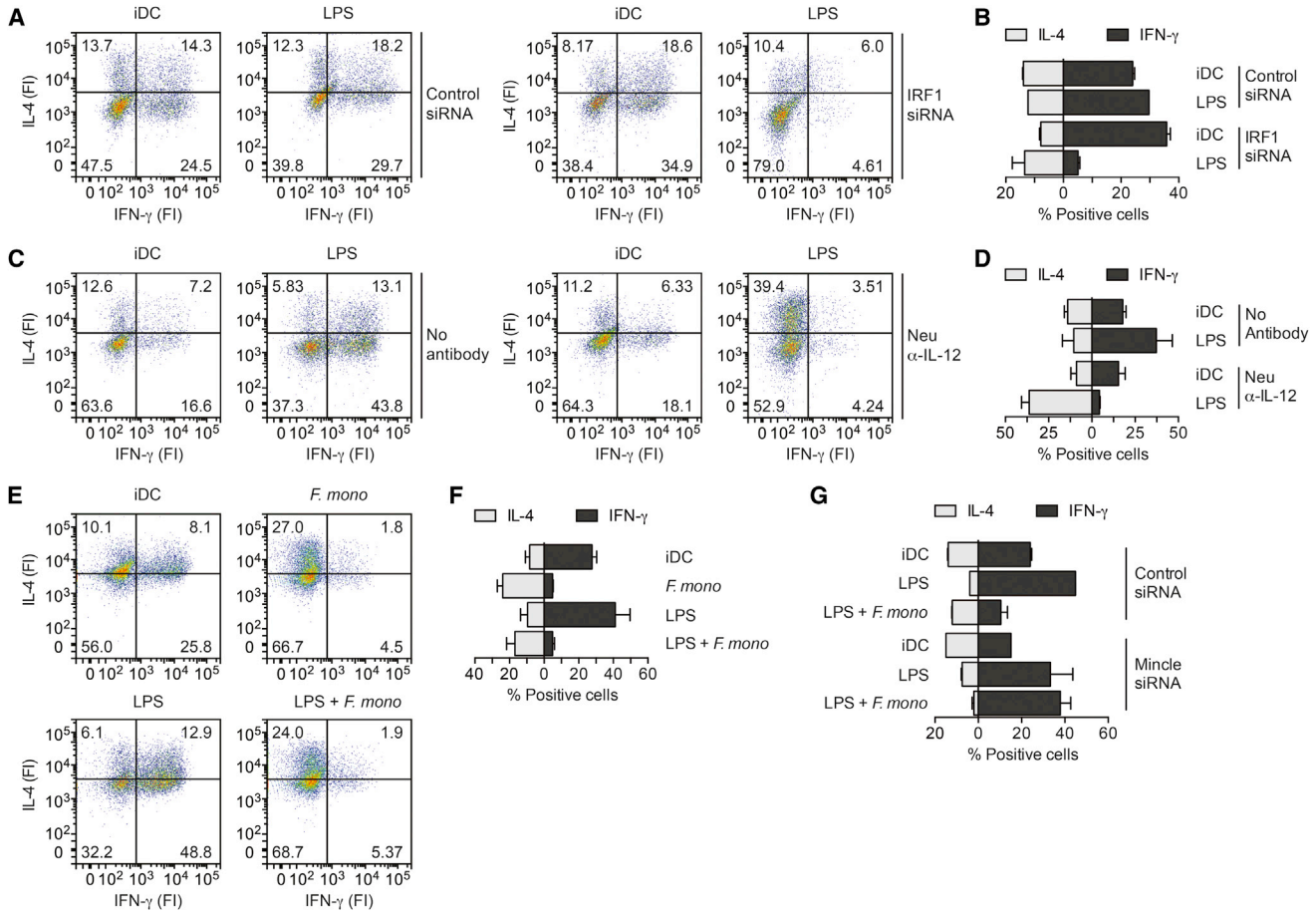

Figure 6. *F. monophora* Targets Mincle-Induced IRF1 Degradation to Redirect Antifungal T_H1 Polarization to T_H2 Responses

(A–G) T helper cell polarization was determined by flow cytometry by staining for intracellular IL-4 (T_H2) or IFN-γ (T_H1) expression after coculture of naive $CD4^+$ T cells with DCs that were left unstimulated (iDC) or primed for 48 hr with curdlan, *C. albicans*, LPS, and/or *F. monophora*. In (A) and (B), DC-induced IL-12 secretion was abrogated by IRF1 silencing, while in (C) and (D), T cell stimulation by IL-12 was blocked by neutralizing IL-12 antibodies during DC-T cell coculture. In (B), (D), and (F), the percent IL-4- and IFN-γ-producing T cells are shown, corresponding to the upper left and lower right quadrants of (A), (C), and (E), respectively. Data are presented as mean ±SD of duplicates (B), (D), (F), and (G). Data are representative of at least two ([A], [B], and [G]), three ([C] and [D]), or eight ([E] and [F]) independent experiments. See also Figure S3.

state levels continuously restricted by ubiquitin-mediated proteolysis (Landré et al., 2013; Nakagawa and Yokosawa, 2000), and excess activation linked to cellular oncogenicity (Tamura et al., 2008), the molecular aspects of IRF1 activation are yet to be delineated. TLR4-mediated IRF1 activation might be dependent on adaptor protein MyD88 (Negishi et al., 2006), yet how dectin-1 triggering led to IRF1 activation remains to be resolved.

Innate signaling by mincle interfered with nuclear IRF1 activity and abrogated IRF1-mediated *IL12A* remodeling. Our findings revealed that mincle exerted its suppressive effect by directing proteolytic breakdown of activated and nuclear localized IRF1 via Syk-CARD9-Bcl-10-MALT1-dependent activation of a PI3K-PKB cascade. We identified E3 ubiquitin ligase Mdm2 as the downstream effector: mincle-induced PKB activation led directly to Mdm2 phosphorylation and nuclear translocation. Mdm2 is a major negative regulator of numerous proteins involved in transcriptional regulation, most notably p53 and FOXO tumor suppressors, under conditions of physiological stress (Haupt et al., 1997; Yang et al., 2008). We found that the association of Mdm2 with IRF1 within the nucleus targeted IRF1 for proteasomal degradation.

Activation of Syk to induce assembly of the CARD9-Bcl-10-MALT1 or related CARD11-Bcl-10-MALT1 module is a common occurrence after receptor ligation. However, whereas dectin-1 and dectin-2 triggering induces NF-κB activation via this protein scaffold (Gringhuis et al., 2011; Gross et al., 2006), mincle triggering uniquely coupled CARD9-Bcl-10-MALT1-mediated signaling to a PI3K-PKB cascade without triggering NF-κB activation (unpublished data). The mechanisms underlying the fundamental differences between dectin-1, dectin-2, and mincle signaling, despite the use of common signaling modules, remain elusive. PKB is also involved in antigen receptor signaling, where it modulates CARD11-Bcl-10-MALT1-induced NF-κB activation (Cheng et al., 2011). Our data now show the existence of a bidirectional relation between CARD9/CARD11-Bcl-10-MALT1 and PKB signaling that regulates immune responses.

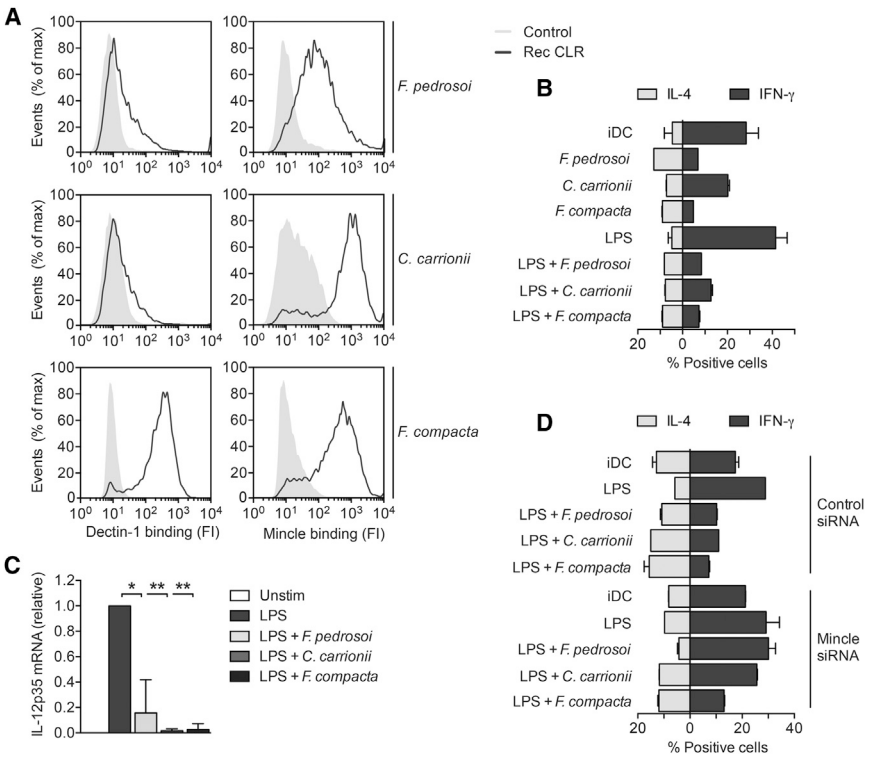

Figure 7. Pathogenic Chromoblastomycosis-Related Fungi Redirect T_H Polarization via Mincle

(A) Binding of recombinant C-type lectins (rec CLR) dectin-1 or mincle protein to heat-killed *F. pedrosoi*, *C. carrionii*, or *F. compacta* conidia, determined by flow cytometry (FI, fluorescence intensity).

(B and D) T helper cell polarization was determined by flow cytometry by staining for intracellular IL-4 (T_H2) or IFN-γ (T_H1) expression after coculture of naive $CD4^+$ T cells with DCs that were left unstimulated (iDC) or primed for 48 hr with LPS and/or *F. pedrosoi*, *C. carrionii*, or *F. compacta*, after mincle silencing. Data are presented as mean ±SD of duplicates.

(C) IL-12p35 mRNA expression in DCs 6 hr after stimulation with LPS and/or *F. pedrosoi*, *C. carrionii*, or *F. compacta*, measured by real-time PCR, normalized to GAPDH, and set at 1 in LPS-stimulated cells. Data are presented as mean ±SD. *$p < 0.05$; **$p < 0.01$. Data are representative of at least two ([A], [B], and [D]) or three (C) independent experiments. See also Figure S3.

Our results define IRF1 as a crucial player in antifungal T_H1 responses induced by human DCs via its role in IL-12p70 biosynthesis. Intriguingly, we found that mincle functioned as a direct inhibitor of nuclear IRF1 activity and as such was commonly targeted by fungi causing chromoblastomycosis to restrict generation of protective T_H1 immunity. In fact, the adaptive response induced by these virulent fungi was dominated by T_H2 cells. T_H2-biased immunity causes severe suppression of phagocytic effector cell function, and hence has adverse effects on fungal infections (Lionakis and Kontoyiannis, 2003; Wüthrich et al., 2012). Therefore, T_H2 predominance and impaired T_H1 responses might represent a central immunological "defect" in chromoblastomycosis that contributes to the establishment of chronic subcutaneous infections.

Overall, our study has identified an essential role for mincle in shaping overall adaptive immunity to virulent fungi associated with chromoblastomycosis. Therapeutic targeting of mincle may therefore prove beneficial not only in treatment of chromoblastomycosis but also in diseases marked by uncontrolled IL-12-driven inflammation.

EXPERIMENTAL PROCEDURES

Cells, Stimuli, RNAi, and Maturation

$CD14^+$ monocytes from healthy volunteer blood donors were isolated, cultured, and differentiated into immature DCs as described (Gringhuis et al., 2009) and used at day 6 or 7 for experiments. Donors were routinely screened for dectin-1 single nucleotide polymorphism rs16910526 using TaqMan genotyping Assays (Assay ID C_33748481_10; Applied Biosystems); only dectin-1 wild-type DCs were used for experiments, unless otherwise indicated. This study was done in accordance with ethical guidelines of the Academic Medical Center. Cell surface expression of CLRs and maturation markers was determined as described in Supplemental Experimental Procedures. Cells were stimulated with TLR and CLR ligands and fungal pathogens in the presence or absence of signaling inhibitors as described in Supplemental Experimental Procedures. DCs were transfected with 25 nM siRNA using transfection reagent DF4 (Dharmacon) as described (Gringhuis et al., 2009). Details on the used SMARTpool siRNAs are in Supplemental Experimental Procedures. Silencing of expression was verified by real-time PCR and flow cytometry (Figure S3) (Gringhuis et al., 2011).

CLR-Fungi Binding

Recombinant human His-tagged dectin-1 (1859-DC-50; R&D Systems) and DDK-tagged mincle (TP300244; Origene) were incubated with heat-killed fungal conidia and subsequently labeled with anti-His (sc-8036; Santa Cruz) or anti-DDK (TA50011-100; Origene), respectively, followed by incubation with Alexa Fluor 488-conjugated anti-mouse (A11029; Invitrogen). CLR-fungi binding was analyzed on a FACS Calibur (BD).

Cytokine Production

Cell culture supernatants were harvested after 24 hr of stimulation, and concentrations of IL-6, IL-23, IL-1β, IL-12p40 (Invitrogen), and IL-12p70 (eBioscience) were determined by ELISA.

Quantitative Real-Time PCR

mRNA isolation from DCs stimulated for 6 hr, cDNA synthesis, and PCR amplification with the SYBR Green method in an ABI 7500 Fast PCR detection system (Applied Biosystems) were performed as described (Gringhuis et al., 2009). Specific primers were designed with Primer Express 2.0 (Applied Biosystems) (Table S1). The C_t value is defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value. For each sample, the normalized amount of target mRNA N_t was calculated from the obtained C_t values for both target and GAPDH mRNA with $N_t = 2^{Ct\ (GAPDH)-Ct\ (target)}$. The relative mRNA expression was obtained by setting N_t in LPS- or curdlan-stimulated samples at 1 within one experiment and for each donor.

NF-κB DNA Binding

Nuclear and cytoplasmic extracts of DCs were prepared after 2 hr of stimulation using NucBuster protein extraction kit (Novagen). NF-κB DNA binding was determined using TransAM NF-κB family kit (Active Motif).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express Enzymatic Shearing and ChIP-IT Express HT kits (both from Active Motif). Briefly, cells were fixed with 1% (vol/vol) *para*-formaldehyde after 2 hr of stimulation, nuclei were isolated, and chromatin DNA was fragmented by enzymatic shearing (10 min; 37°C). Protein-DNA complexes were immunoprecipitated, and DNA was purified after reversal of crosslinks. Details are described in Supplemental Experimental Procedures. Real-time PCR reactions were performed with primer sets spanning TATA box, NF-κB, and ISRE binding sites (Table S1). 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 which had not undergone immunoprecipitation ("input DNA"); results are expressed as percent input DNA.

Chromatin Accessibility Real-Time PCR Assay

Chromatin accessibility real-time (ChART) assays were performed to measure *IL12A* nucleosome remodeling as described previously (Hovius et al., 2008). Details are described in Supplemental Experimental Procedures. Briefly, nuclei were isolated after 3 hr of stimulation, digested with *Bst*XI or EcoRI, and DNA was purified. Real-time PCR reactions were performed with primer sets spanning (A) *Bst*XI site located at nt −298 that becomes available after nuc-2 remodeling, (B) *Bst*XI site located at nt 456 that is not subject to chromatin alterations as an internal control (Goriely et al., 2004), and (C) GAPDH to normalize for DNA input (Table S1). Results are expressed as percent remodeling observed in the EcoRI-digested sample for each cell treatment using the formula $(Nt_{EcoRI} - Nt_{BstXI})/Nt_{EcoRI} \times 100\%$, with $Nt = 2^{Ct(C)-Ct(A)}$.

Cellular Localization and Association of Mdm2 and IRF1

Nuclear and cytoplasmic extracts of DCs were prepared after 2 hr of stimulation as described above. For detection of association between Mdm2 and IRF1, extracts were prepared from DCs stimulated in the presence of proteasome inhibitor MG-132. Mdm2-IRF1 complexes were immunoprecipitated from 20 μg of nuclear extract with anti-IRF1 (sc-497; Santa Cruz) coated on protein A/G-PLUS agarose beads (Santa Cruz). Proteins were resolved by SDS-PAGE, and Mdm2 and IRF1 were detected by immunoblotting. Details are described in Supplemental Experimental Procedures. IRF1 was also detected in extracts by ELISA (USCN Life Science). IRF1 localization was further determined by immunofluorescense staining; DCs were stimulated, fixed with 4% *para*-formaldehyde, then permeabilized with 0.2% (vol/vol) Triton X-100 in PBS, and stained with anti-IRF1. Incubation of Alexa Fluor 594-conjugated anti-rabbit (A11072; Molecular Probes) was followed by staining of nuclei with DAPI (Molecular Probes). IRF1 localization was visualized with a Leica DMR A microscope.

PKB and Mdm2 Phosphorylation

Phosphorylation of PKB and Mdm2 after 20 min of stimulation was detected by flow cytometry and analyzed on a FACS Calibur. Details are described in Supplemental Experimental Procedures. Phosphorylated Mdm2 was also detected by immunoblotting as described above.

PKB Activity Assay

Whole cell extracts were prepared after 30 min of stimulation in kinase activity lysis buffer as described (Gringhuis et al., 2007). PKB kinase activity was measured using PKB kinase activity kit (Enzo Life Science); details are described in Supplemental Experimental Procedures.

T Cell Differentiation Assay

DCs were silenced for indicated proteins, activated for 48 hr with LPS and/or heat-killed fungal strains and subsequently cocultured with naive $CD4^+$ T cells as described (de Jong et al., 2002). Neutralizing IL-12 antibodies (MAB219; R&D) were added at this point as indicated. Details are described in Supplemental Experimental Procedures. After restimulation with 100 ng/ml PMA (Sigma) and 1 μg/ml ionomycin (Sigma), intracellular cytokine expression was analyzed by flow cytometry by staining with APC-conjugated rat anti-IL-4 (MP4-25D2) and FITC-conjugated mouse anti-IFN-γ (25723.11; both BD).

Statistical Analysis

Statistical analyses were performed using the Student's t test for paired observations. Statistical significance was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.03.008.

ACKNOWLEDGMENTS

This work was supported by the Netherlands Organisation for Scientific Research (Vici 918.10.619 to T.B.H.G.; NGI 40-41009-98-8057 to S.I.G.).

Received: November 27, 2013
Revised: February 13, 2014
Accepted: March 17, 2014
Published: April 9, 2014

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