C-type lectin signaling in dendritic cells: molecular control of antifungal inflammation
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Party crashing

Frozen brussel sprouts
six. The C-type lectin orchestra: dectin-2 and mincle set the tone
-a general discussion

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In this thesis, we have addressed the roles of C-type lectin receptors (CLRs) dectin-1, dectin-2 and mincle and how these receptors collaborate in ‘shaping’ an ensuing antifungal immune response. These studies have revealed markedly distinct modes of ‘action’ during polarization of the effector CD4⁺ T cell response. From our studies a model is emerging in which dectin-1 as an ‘inducer’ fuels T helper (Th) polarization, but that the overall outcome of the inflammatory response—whether protective or not—is dictated by ‘modulating’ receptors such as dectin-2 and mincle, which by themselves are incapable of inducing the response. Our attempt to understand (part of) the molecular events that underlie their functional specialization has led to novel insights into how expression of T helper cell-polarizing cytokines by dendritic cells (DCs) is transcriptionally regulated or dysregulated, but also to the characterization of the signal transduction pathways and molecular mechanisms operating downstream dectin-2 and mincle. In this final chapter, I will highlight our studies’ main findings and discuss how studying these receptors has provided new insights into human antifungal immunity.

Brigitte Wevers
Amsterdam, 2014
TURNING ON ANTIFUNGAL INFLAMMATION

The human host is continuously being exposed to a tremendous amount of potentially pathogenic fungi, in the form of commensal strains that colonize our skin and gut, as well as (virulent) strains abundantly present in the environment. When fungi succeed in breaching our physical barriers of protection (i.e. skin and mucosal epithelia) and establish an infection, an adaptive response consisting of both T helper type I (Th1) and Th17 effector CD4+ T cells is considered to confer optimal protection, while limiting tissue damage. Specifically, Th17-derived IL-17 is involved in immediate recruitment of neutrophils, which have an essential role clearing the invading fungi, and by producing IL-22, Th17 cells contribute to maintenance of mucosal tissue homeostasis. Chief among the functions of Th1 cells is production of IFN-γ for optimal activation of phagocytic effector function (e.g. release of nitric oxide and production of reactive oxygen intermediates). Dendritic cells are responsible for instruction of the T helper cell response, as they sense a particular threat in the periphery -in the form of invading pathogens as well as damaged-self molecules, and subsequently convey information about the type of danger involved to the responding T cell population in the lymphoid organs. While antigen presentation and costimulatory signals are sufficient for general activation of the naive CD4+ T cell population, cytokines produced by DCs are decisive for polarization into pathogen-specific effector T cell subsets. These processes are elicited and controlled by germ-line encoded pattern recognition receptors (PRRs). Of the many classes of PRRs, receptors from the CLR family are emerging as key players when it comes to instruction of the immune system upon detection of an invading, and thus pathogenic, fungus.

An important regulator of the antifungal immune response is CLR dectin-1, identified more than a decade ago as the long-sought receptor for β-glucan, fungal-derived carbohydrate polymers with immune modulating ability. Since then, the functional characterization of dectin-1 has been the subject of intensive investigation; its role in antifungal immunity is now known to be multifaceted, and includes uptake and processing of fungal components for antigen presentation, induction of a complex network of signaling pathways that coordinates expression of chemokine and cytokine genes, as well as activation of the caspase-1 and caspase-8-dependent inflammasomes for maturation and expression of IL-1β protein. Earlier studies have indicated that dectin-1 conveys intracellular signals via the kinase Syk and assembly of a signaling scaffold comprising adaptors CARD9, Bcl-10 and MALT1, downstream converging with a separate, Raf-1-dependent, signaling pathway for activation of transcription factor NF-kB (Chapter 1; Figure 2). As such, dectin-1 on human DCs is specialized in propagation of combined Th1 and Th17 responses. In this thesis we extended these findings, by providing novel insights into the molecular processes utilized by dectin-1 to drive the Th1-polarizing (IL-12) and Th17-polarizing (IL-1β, IL-6 and IL-23) cytokine profiles.
Spotlight on dectin-1 transcriptional activities. Transcription factors from the NF-κB family (heterodimers composed of p65, RelB, c-Rel, p50 and p52 subunits) are well-established regulators of cytokine responses induced by dectin-1. Activation of both canonical (p65- and c-Rel-containing) and noncanonical (RelB-containing) NF-κB subunits by the CARD9/Bcl-10/MALT1 signaling scaffold underlies the characteristic dectin-1-induced Th1/Th17 cytokine signature. We have now demonstrated that individual members of the CARD9/Bcl-10/MALT1 scaffold differentially activate p65, RelB and c-Rel upon activation of dectin-1 signaling (Chapter 2). CARD9 and Bcl-10 are involved in the recruitment of all subunits, whereas the paracaspase MALT1 has a specialized function and, through its proteolytic activity, engages an alternative pathway which activates c-Rel-containing NF-κB dimers. By the selective targeting of c-Rel, MALT1 controls a transcriptional subprogram which efficiently induces IL23A and IL1B gene transcription. Thus, by regulating IL-23p19 and pro-IL-1β expression, MALT1 is crucial for optimal Th17 effector responses induced by dectin-1 (Figure 1). Remarkably, this unique MALT1-dependent signaling pathway is a defining feature of the modulating actions of CLR dectin-2 (Chapter 2). MALT1-dependent signaling is initiated by dectin-2 for upregulation of IL-1β and IL-23p19 expression and amplification Th17 immunity in the context of fungal infection (see further below).

Figure 1. C-type lectin receptor crosstalk in human dendritic cells (DCs) tailors human antifungal host defense. (a) Dectin-1, as an ‘inducing’ receptor, provides all transcriptional signals for induction of host protective immunity - consisting of both Th1 and Th17 effector cell subsets. While NF-κB c-Rel controls IL-1β and IL-23p19 transcription, and hence Th17 polarization, transcription factor IRF1 is essential for IL12A transcription and IL-12-driven Th1 immunity. Moreover, dectin-1 induces expression of IFN-β via transcription factor IRF5. Whereas type I IFNs inhibit Th17 immunity, IFN-β-together with DC-derived IL-12-activates NK-cells for induction of neutrophil-mediated fungal clearance. (b+c) Dectin-2 and mincle are ‘modulating’ receptors that promote or repress transcriptional signals from dectin-1 (or any other innate receptor) for instruction of pathogen-specific CD4+ T helper responses. Dectin-2 activates Syk-dependent signal transduction, which results in the assembly of a complete CARD9/MALT1/Bcl-10 scaffold, yet selectively activates c-Rel-containing NF-κB dimers (through proteolytic activity of MALT1). This leads to preferential transcription of IL1B and IL23A genes and upregulation of Th17 polarization upon recognition Candida albicans. Mincle recognizes virulent Fonsecaea fungi and Malassezia spp., and its signaling via Syk and CARD9/MALT1/Bcl-10 couples to a PI(3)K-PKB pathway for nuclear translocation of Mdm2. Activation of this cascade attenuates IL-12p35, IL-27p28 and IFN-β production, via MDM2-dependent degradation of IRF1 and IRF5, but upregulates IL-1β and IL-23p19 expression in an Mdm2-dependent manner. Note that mincle, in contrast to dectin-2, modulates Th17 immunity independent of NF-κB and does not activate IL1B and IL23A transcription on its own. Overall, triggering of mincle signaling leads to abrogation of protective Th1, antifungal immunity, and Th2-skewed responses, yet strong amplification of Th17 immunity. Ab, antibody; MHC II, major histocompatibility complex II.
The C-type lectin orchestra: dectin-2 and mincle set the tone

a. Inducing receptor

Dectin-1

Syk

CARD9

Bcl-10

MALT1

RelB

p65

c-Rel

IRF1

IRF5

IL-6

IL-12p40

IL-1-23p19

IL-27p28

b. Modulating receptors

Dectin-2

Syk

CARD9

Bcl-10

MALT1

p65

c-Rel

IL-1-1β

IL-23p19

IL-12p35

IL-27p28

Mincle

CARD9

Bcl-10

MALT1

PKB

Mdm2

Mdm2

Trim28

IL-1-1β

IL-23p19

Mucosal homeostasis

Antimicrobial peptides

Neutrophils

Mobilization

Crosstalk with dectin-2

Dectin-1

Dectin-2

Candida

TH1 cell

TH17

Crosstalk with mincle

Fonsecaea

Malassezia

Dectin-1

Mincle

TH1 cell

TH17

TH17

TH17

TH17

Neutrophil
Second, our study presented in Chapter 3 has shown that cytokine gene transcription by dectin-1 is more heterogeneously regulated than previously anticipated and extends beyond the NF-κB ensemble. Acting downstream dectin-1, transcription factor IRF1 is fundamental for synthesis of IL-12 subunit p35, and hence secretion of bioactive IL-12 protein. Prior studies have shown that transcription of the gene encoding IL-12p35, *IL12A*, is stringently controlled and that repositioning of a nucleosome (nuc-2) at the proximal promoter region is required for transcription initiation[33-35]. Nevertheless, the factor(s) controlling these transcriptional events remained enigmatic for a long time. In Chapter 3, we have identified IRF1 as the crucial regulator (Figure 2). This in agreement with other studies showing that transcription factors as ‘pioneering factors’ might serve pivotal role in these nuclear processes, typically via recruitment of chromatin remodeling factors[36], while transcription factors with intrinsic remodeling activity have been identified[37]. The realization that IRF1 activity underlies *IL12A* remodeling came from our observation that CLR mincle suppresses IL-12p35 expression by counteracting the activation of IRF1 via ubiquitin-dependent degradation (discussed below). Also, we have demonstrated that TLR4 similarly depends on IRF1 for *IL12A* remodeling, and hence IL-12p35 transcription (Chapter 3), we hypothesize therefore that IRF1 is more generally required for *IL12A* transcription in human DCs.

Concluding, these results indicate that the activation of the two arms of the antifungal effector CD4⁺ T cell response by dectin-1 relies on transcriptional activities of c-Rel and IRF1, with c-Rel crucial for induction of Th17 polarization, while IRF1 is indispensable for instruction of Th1 immunity (Figure 1). This conclusion is strengthened by our subsequent findings showing that Th polarization is subject to distinct modulation by dectin-2 and mincle, through interference with c-Rel and IRF1 activities, respectively.

**Antiviral effectors in antifungal immunity.** Another IRF family member, IRF5, is key to IFN-β expression controlled by dectin-1, thereby formally demonstrating the involvement of a type I IFN response—a program classically mounted upon viral infection—in human antifungal immunity (Chapter 5). Gaps knowledge remain regarding the precise function of type I IFNs in the general context of fungal inflammation. Paradoxically, there is a substantial amount of literature suggesting a negative influence of type I IFNs on protective antifungal immunity, such that it would challenge the protective antifungal activities ascribed to dectin-1 so far. Not only antagonize type I IFNs pro-IL-1β and IL-23p19 production, which is in agreement with our findings, they also interfere with inflammasome activation and inhibit Th17 polarization[38-43]. Still, these effects do not exclude the idea of a protective function for type I IFNs in the overall antifungal response[25,26]. Inflammation, irrespective of effective eradication of a harmful pathogen or other type of threat, but must be stringently controlled and timely terminated to avoid potential host tissue damage; often this concerns induction of responses that do not always favor, and might even directly antagonize, host immunity[44]. This in particular holds true for controlling Th17 immunity[13,45-47], and therapeutic targeting of type IFN
production has proven beneficial for treatment of autoimmune conditions characterized by exaggerated Th17 inflammation. Apart from such a regulatory mechanism, type I IFNs might serve a beneficial role by controlling the functional competence of immune cells with direct antiviral effector function yet unrecognized antifungal activities. Indeed, NK cells—their activation regulated by DC-derived type I IFNs and IL-12—now manifest themselves as antifungal effectors by promoting the antifungal activity of neutrophils. Clues for a protective function for type I IFNs are further provided by mincle, for which severe suppression of type I IFN responses arguably fits both into its strong Th17-amplifying capacity and its detrimental role in the overall response to virulent fungi. Overall, while our knowledge on how IFN-I responses provide functional benefit to the protective responses induced by dectin-1 is still limited, these new insights emphasize a much more versatile role for dectin-1 in regulating fungal inflammation than previously suspected.

MODULATORS ENTER THE STAGE

Despite the numerous roles of dectin-1 in the human immune response to fungi, it gradually became clear that signaling for antifungal immunity is a CLR orchestra rather than a single musician. CARD9-deficient individuals are more susceptible to fungal infection, and display more severe clinical symptoms, with CARD9 SNPs even linked to systemic candidiasis, than those patients with inborn errors in dectin-1. Observations in mouse models of fungal infection indicated that dectin-1 cannot not fully account, or is even dispensable, for instruction of protective immunity. On the basis of numerous studies, participation of CARD9-coupled receptors dectin-2 and mincle had been suggested, yet their involvement in human antifungal immunity and their precise mechanism of signaling had not been investigated. In this thesis we have characterized dectin-2 and mincle and provide evidence that their functionality markedly influences the overall outcome of the Th effector cell response. A crucial aspect of their unique function, however, is that both receptors are incapable of instructing the Th response autonomously, for which they rely on other PRRs instead. Hence we denote them as 'modulators'.

Dectin-2: signaling for Th17 amplification. Dectin-2 is a signaling CLR that relies on the common FcRγ-chain for transduction of ITAM-coupled signaling. Human DC-expressed dectin-2, in stark contrast to dectin-1, does not activate all NF-κB subunits, having major consequences for the instruction of Th17 cell polarization. Human dectin-2-Syk signaling culminates in the induction of the MALT1-c-Rel axis for preferential IL1B and IL23A transcription, in a manner analogous to dectin-1. The reason behind the lack of canonical p65 activation by dectin-2 is still uncertain. Dectin-2, similar to dectin-1, induces assembly of the complete CARD9/Bcl-10/MALT1 scaffold, and relies as much on CARD9...
Figure 2. Mincle signaling modulates antifungal inflammation. C-type lectin receptor (CLR) dectin-1 and Toll-like receptor 4 (TLR4) control IL-12-driven T helper cell type 1 (Th1) polarization by triggering nuclear accumulation of transcription factor interferon-regulatory factor 1 (IRF1), whose activity is crucial for the nucleosome remodeling events preceding IL12A transcription. In contrast, the Fc receptor common γ-chain (FcRγ)-coupled CLR mincle acts as a repressor of IL-12p70 biosynthesis by directing the proteolytic breakdown of nuclear IRF1. Mincle via Syk couples CARD9-Bcl-10-MALT1-mediated signaling to activation of a PI(3)K-PKB cascade which culminates in the phosphorylation and nuclear translocation of E3 ubiquitin ligase Mdm2. Within the nucleus, Mdm2 subsequently catalyzes IRF1 polyubiquitination (poly Ub) to directly target IRF1 for degradation by the 26S proteasome; Trim28 functions as an adaptor protein and facilitates Mdm2/IRF1 nuclear complex formation. Notably, mincle signaling is exploited by Fonsecaea monophora and other chromoblastomycosis-associated fungi to attenuate dectin-1-induced IL-12 production, thereby adversely affecting antifungal Th1 immunity. Second, mincle-Mdm2 signaling promotes Th17 polarization, through suppression of dectin-1-induced IFN-β production, via protea-
and Bcl-10 for c-Rel activation as it does on MALT1 paracaspase activity. Differential use of effector molecules downstream the CARD9/Bcl-10/MALT1 triad by both dectin receptors might explain this discrepancy, with the TRAF proteins as likely candidates. Even though MALT1 is central to dectin-2 signaling, dectin-2 does not couple to the caspase-8 inflammasome for pro-IL-1β processing (unpublished observations). Instead, the classical NLRP3 inflammasome is held responsible for generation of mature IL-1β by dectin-2. In sum, transcriptional activation by dectin-2 strictly generates release of Th17 polarizing cytokines IL-1β and IL-23 subunit p19. Importantly, however, activation and maintenance of Th17 cells requires secretion of IL-1β together with IL-6 and IL-23. This implies that dectin-2 specifically favors Th17 responses, being incapable of inducing production of IL-6 and mature IL-23, due to the absence of IL-12 subunit p40. Simultaneous engagement of other PRRs is therefore an absolute requirement for dectin-2 to enhance a Th17 response, signifying a ‘modulating’ role for dectin-2 in the antifungal immune response (Figure 1).

**Mincle: a degrading view on Th17 polarization.** While being similarly dependent on the common FcRγ-chain for transduction of ITAM-coupled signaling, very distinctive—that is, not necessarily protective—responses are induced by mincle. Intriguingly, we identified the E3 ubiquitin ligase Mdm2 as the central driving force behind the mechanisms of mincle action (Chapters 3, 4 and 5). Mdm2 is a classical DNA damage response effector involved in the regulation of numerous tumor suppressors, including p53 and FOXO transcription factors.

Mincle does neither signal for NF-κB nor IRF activation and translocation (Chapters 3, 4 and 5); instead it targets multiple key transcriptional regulators for proteasomal degradation, including IRF1 and IRF5. Although there is a great complexity involved, and multiple cytokines interfered with, the overall outcome of mincle signaling is characterized by abrogated Th1 immunity, but amplified antifungal Th17 responses (Figure 2).

As illustrated above, when PRRs signal for IL-12p35 expression, and hence DC-induced Th1 immunity, this should involve activation and nuclear translocation of IRF1 for induction of IL12A nucleosome remodeling and transcription initiation. In Chapter 3, we have demonstrated that mincle facilitates the rapid and specific proteasome-mediated degradation of nuclear IRF1. This is driven by a signaling cascade operating downstream the CARD9/Bcl-10/MALT1 scaffold, initiated by PI(3)K-mediated phosphorylation of PKB (or AKT), that...
leads to the activation and nuclear translocation of Mdm2. Subsequently, the degradation of activated IRF1 by Mdm2 is facilitated by adaptor protein Trim28 (also known as KAP1 or TIF1β), which mediates Mdm2-IRF1 interactions, allowing IRF1 ubiquitination and proteolysis (Chapter 4). Thus, mincle signaling prevents expression of IL-12 protein and instruction of the Th1 effector arm of antifungal immunity.

In defining the participation of mincle in antifungal immunity, in Chapter 4 we noted that induction of Th17 polarization to various (virulent) fungi did involve mincle activities; in line with prior studies demonstrating that mincle mediates the Th17 adjuvancy of mycobacterial cord factor (TDM). Specifically, mincle signaling, via PI3(K)-PKB-Mdm2, triggers transcriptional upregulation of the Th17 polarizing cytokines pro-IL-1β and IL-23 subunit p19. In addition to synthesis of pro-IL-1β, mincle signals for processing of pro-IL-1β and generation of mature IL-1β (Chapter 4), involving caspase-1 and caspase-8-dependent mechanisms (Intermezzo). Since PKB and Mdm2, so far, touch on every aspect of the mincle response it would be interesting to explore their role in the setting of inflammasome assembly. Thus, human DC-expressed mincle seems to function as an amplifier of Th17 immune responses, in analogy to dectin-2 (Figure 1).

IL-1β and IL-23p19 mRNA upregulation by mincle occurs independent of Trim28 nuclear activities, a separate pathway must therefore exist that induces the proteasomes-mediated degradation of an as-yet-unidentified transcriptional or posttranscriptional repressor(s). In addition to a direct transcriptional repressor, putative candidates being FOXO transcription factors, microRNAs might play a role, a class of gene expression regulators which act by translational inhibition and decay of target mRNA. miRNAs are thought to be part of an intrinsic terminating signal induced after receptor ligation, and target signal transduction molecules and cytokines. For example, miR-29 is activated by NOD2, a CARD9-containing cytoplasmic sensor, and has been demonstrated to mediate IL-23p19 degradation, while miR-155 in human DCs diminishes IL-23p19 and IL-1β, as well as caspase-1 expression. Concerning the latter, it is of particular interest to note that miR-155 also functions as a negative regulator of the PI(3)K-PKB pathway. Hence, mincle may have evolved a mechanism to counteract such negative regulation by miRNA(s), coinciding with, among others, enhanced IL-1β and IL-23p19 expression levels. Indeed, PKB signaling has been shown to repress miR-155 expression in macrophages.

Besides induction and release of Th17 polarizing cytokines, mincle also conveys ‘degrading’ signals for a second level of Th17 modulation, by interfering with expression of cytokines that counteract or antagonize Th17 function: IL-27 and type I IFNs (Chapter 4). That particular mechanism involves a Trim-28-dependent pathway and is described in the following section.

**Plan B for Th17 modulation.** We (Chapter 4 and Chapter 5) and others have shown that IL-27 and type I IFNs limit Th17 polarization. These responses have been ascribed a regul-
The C-type lectin orchestra: dectin-2 and mincle set the tone

The regulatory role to keep in check exaggerated and potentially tissue destructive Th17 immunity, and their abrogation would therefore aid in strong ‘focusing’ of host-Th17 immunity. Our data demonstrate that mincle abrogates synthesis of both IL-27 subunit p28 and IFN-β, and subsequently IFN-I response gene products, obviously fitting its function as a Th17 amplifier (Figure 2).

Similar to IL12A, we found that IRF1 is the key facilitator of IL27 transcription (Chapter 4). As stated above, mincle signaling governs the proteasome-mediated degradation of nuclear IRF1, and hence IRF1 transcriptional activities; the abrogation of IL-27p28 expression by mincle is therefore directly attributable to these activities. Of particular note is that co-factors such as p65 as well as RNA polymerase II are not recruited in the absence of functional IRF1, which suggest limited accessibility of the proximal promoter binding sites. Since IL12A and IL27 promoter structures display profound similarities, this raises the possibility that IL-27p28 expression similarly relies on nucleosome remodeling processes controlled by IRF1.

Next to perturbed IL-27 expression, the suppression of dectin-1-induced IFN-β (Chapter 5) further supports that mincle functions to promote Th17 immunity in the context of fungal infection. As such, Mincle links the PKB-Mdm2-Trim28 pathway directly to inhibition of IFN-β expression by dectin-1, via abrogation of IRF5 nuclear abundance. IRF5, as mentioned earlier, is crucial for dectin-1 dependent IFN-β expression. Intriguingly, mincle appears to interfere with these IRF5 activities in a proteasome-dependent manner. With an ever-growing list of Mdm2 substrates and Trim28 described as an interaction partner and inhibitor of IRF5, Mdm2-dependent IRF5 degradation is to be expected.

Transcription of the IFNB1 gene is very heterogeneously regulated. Depending on the receptor involved, numerous transcription factors can contribute, though IRFs are crucially involved. For instance, expression of IFN-β by TLR4 occurs through IRF3, whereas TLR7 and TLR9 activate IRF7 to induce IFN-β. Adding to the complexity of mincle function, our preliminary data suggest that mincle deregulates type I IFN responses, irrespective of the IFN-β-inducing receptor that is co-triggered. More specific, mincle was found to inhibit TLR4- and TLR9-induced (Chapter 5 and data not shown), implying concomitant degradation of multiple IRFs. This would be in analogy to the evasion strategy of rotaviruses, which have developed a mechanism to antagonize IRF function via simultaneous proteasome-mediated degradation of IRF3, IRF5 and IRF7, possibly a direct result of the high structural similarity among these IRFs. In this scenario, Trim28 might function as an adaptor molecule dedicated to degradation of IRF transcription factors by Mdm2.

In sum, mincle modulates Th17 immunity at multiple levels (i.e. Th17-polarizing and Th17-antagonizing cytokines) by interfering with the activity of multiple key transcriptional regulators (including IRF1 and IRF5). Presumably, these combined mechanisms enable stringent, even sustained, amplification of an ensuing Th17 effector responses (Figure 1).
**Modulators are not created equal.** Recapitulating our current knowledge, an unexpected picture is emerging: whereas both dectin-2 and mincle transduce ITAM-coupled signaling via Syk and the CARD9/Bcl-10/MALT1 complex (refs\(^\text{77,92}\) and Chapter 3), and signal for amplification of Th17 responses, the underlying transcriptional mechanisms appear to be substantially divergent. Dectin-2 autonomously induces expression of polarizing cytokines (i.e. IL-1β and IL-23p19) via selective activation of NF-κB subunit c-Rel. Contrary, mincle is unable to induce NF-κB nuclear translocation and can only interfere with the transcriptional signals induced by other PRRs, again independent of NF-κB. The opposite holds true for pro-IL-1β processing: dectin-2 does not share the capacity of mincle to mediate IL-1β maturation in a caspase-8-dependent manner (unpublished data). Evidently, this raises a number of questions and issues to be addressed. We are tempted to suggest that differences in the composition of adaptor molecules directly operating downstream the CARD9/Bcl-10/MALT1 scaffold, such as TRAF proteins, account for the differences in signaling outcome\(^\text{93,94}\). Not excluding, as mentioned earlier, that miRNAs might be involved: miR-155 indirectly targets TRAF6 activity resulting in downmodulation of the NF-κB pathway\(^\text{75}\). Variation in terms of functionality can also be determined further upstream, by the magnitude (avidity) and duration of the receptor-ligand interaction\(^\text{95,96}\), a known phenomenon among ITAM-associated receptors. In this regard, and contrary to what their name might imply (ITAM denotes immunoreceptor tyrosine-based activation motif), some receptors clearly act a dual role and generate synergistic and inhibitory signals, with FcRγ-associated receptor for immunoglobulin A (FcaR) and DAP12-coupled TREM proteins notable examples\(^\text{97-99}\). Also, it is plausible that functionality of either one is a result of dimerization and synergistic interactions with divergent other DC receptors, such as has been demonstrated for dectin-1 and galactin-3\(^\text{100}\). With CLR dectin-3 an unlikely candidate, as it heterodimerizes with both dectin-2 and mincle\(^\text{101,102}\).

Overall, our findings argue against the common view that CLR-induced Syk/CARD9 signaling cascades share characteristics and have NF-κB activation as a common principle. Although the proximal signaling events may seem identical, the resulting gene expression programs can be markedly different. Whether human dectin-2, similar to mincle, interferes with Th1 immunity and aberrantly induces Th2 immunity, as has been proposed\(^\text{103,104}\), is still unclear, but might thus not be the most obvious possibility. Despite the unknowns, the striking differential ability of dectin-2 and mincle to dictate DC cytokine responses and instruction of the T helper response likely implicates tailored immune responses to the encountered fungus. In the remaining sections of this chapter, we will discuss how our findings connect to the ‘bigger picture’: the antifungal immune response.
The innate immune system has a great degree of redundancy: pathogens or other types of danger are recognized by multiple receptors at the same time. Indeed, this also applies to CLR-mediated recognition of fungal pathogens: Table 1 summarizes the binding specificity of human CLRs towards a set of fungal pathogens. Each individual receptor contributes to a specific aspect of the immune response, so that the spectrum of host receptors utilized, but also the cell types involved, tunes the exact nature of the immune response. Additional specificity is provided by the functional interaction of PRRs; crosstalk occurs at levels of pattern recognition and phagocytosis, but also transduction of intracellular signaling for inflammasome activation and gene expression control\textsuperscript{105,106}. Our studies not only emphasized signal transduction crosstalk, but also indicate that the collaboration extends further and involves the synergistic instruction of adaptive immunity.

With regard to dectin-2 and mincle, crosstalk with these Th\textsubscript{17}-amplifying receptors would be expected to tailor an ensuing Th\textsubscript{17} response in a pathogen-specific manner, for several reasons. First, one might argue that their crosstalk with a given ‘inducing’ receptor, for instance dectin-1, involves the regulation of the Th\textsubscript{17} response both in magnitude and duration. A key point is, however, that both neutrophils and activated Th\textsubscript{17} cells have a high host tissue-damaging potential\textsuperscript{13,45,107}, and it is therefore tempting to speculate that ‘modulators’ such as dectin-2 and mincle ensure also that a threshold for Th\textsubscript{17} activation is exceeded only when necessary. In other words, it may reflect a mechanism that allows effective Th\textsubscript{17} activation only to real pathogenic events, while preventing unnecessary
responses to harmless commensal fungi (see below). The differential binding potential of the different CLRs adds to this second hypothesis: while human dectin-1 broadly recognizes different types of fungal species, hence β-1,3-glucans are abundant in cell walls of nearly all fungi\textsuperscript{108}, dectin-2 and mincle respond in particular to pathogenic subtypes: strains associated with opportunistic infection (for dectin-2) or which are highly virulent in nature (mincle) (Table 1). This is evident also in light of the fact that the cell wall composition of fungi is dynamic and, as part of their infection strategy, changes upon host tissue invasion\textsuperscript{109-111}, increasing the likelihood that another or additional set of PRR is triggered when an infection is established. Indeed, invading fungi trigger other types of immune responses than do their colonizing or resting counterparts\textsuperscript{53,112}, while numerous studies in mice now also point towards an essential role for dectin-2 in the protection to invasive fungal infection\textsuperscript{50,61,113}.

Third, their modulating capacity might also become apparent in activation of a T\textsubscript{H}17 cell subset with different pathogenic potential; a growing body of evidence indicates that cells from the T\textsubscript{H}17 effector population vary in terms of their effector function and cytokine expression profile (either IFN-γ or IL-10)\textsuperscript{114,115}. Generation of more terminally differentiated IFN-γ-producing (inflammatory) T\textsubscript{H}17 cells is dependent on exposure to IL-23 and IL-1β; IL-17/IFN-γ double-producing T cells have been strongly linked to chronic inflammation but also autoimmunity\textsuperscript{115,116}. A relative lack of these cytokines induces an IL-10-producing (anti-inflammatory) T\textsubscript{H}17 effector phenotype\textsuperscript{56,117,119}, possibly less beneficial in clearing infection and serving a regulatory role by preventing excessive inflammation\textsuperscript{42,120}. Whether or not these two types are functionally specialized and, not the least, their relevance in antifungal immunity, is unclear, but their induction indeed seems to be pathogen-tailored\textsuperscript{56}.

Notwithstanding the fact that other immune cell types with putative antifungal effector function exist, including CD8\textsuperscript{+} cytotoxic T cells\textsuperscript{121}, and are continuously being defined, among many others skin-homing CD4\textsuperscript{+} T\textsubscript{H}22 cells\textsuperscript{122,123}, IL-17-producing neutrophils\textsuperscript{124}, and NK cells\textsuperscript{51-55}. The studies described here emphasized on the functionality of CLRs during instruction of antifungal T\textsubscript{H}1 and T\textsubscript{H}17 immunity, and their impact on other immune cell types will be worthwhile investigating in the future. This might even reveal that we have underestimated the functional specialization of ‘modulating’ receptors such as dectin-2 and mincle. Hence, mincle suppresses production of both dectin-1-induced IL-12 and type I IFNs, and as such might directly antagonize the activation and antifungal effector function of NK cells\textsuperscript{51}.

**Tolerance versus responsiveness.** Prevention of unnecessary immune responses to harmless fungi is in particular evident at mucosal surfaces of skin and gastrointestinal tract where commensal fungi reside, as part of the normal microbiome (referred to as the mycobiome)\textsuperscript{1,125,126}. Here, immune cells must tolerate the commensal fungi and bacteria, but also need to detect and eradicate pathogenic microbes. Intriguingly, recent data suggest that
dectin-1 has a role in the establishment of a state of immunological tolerance: the absence of dectin-1 leads to fungal dysbiosis and heightened aberrant intestinal inflammation in the murine host. Also, there has been found strong association between severe ulcerative colitis and a single nucleotide polymorphism (SNP) in the human dectin-1 gene. One might also hypothesize that dectin-1 has an important role in dampening chronic inflammation to quiescent persistent fungi, while cooperative signaling with modulators such as dectin-2 and mincle generates effector responses required for restricting growth of pathogenic fungi. The concept that PRRs themselves can discriminate commensal from potentially pathogenic microbes is emerging; the cytosolic sensor NLRC4 in intestinal phagocytes has been found to respond (and trigger IL-1β processing) only to pathogenic but not commensal bacteria.

When mincle-induced Th17 inflammation doesn’t stop. While our findings seem to strengthen the paradigm that integrating receptor signaling allows for appropriate or robust antifungal immune responses, a cautionary note with respect to those responses involving mincle is needed. Various virulent Fonsecaea strains establish chronic disease in the human host and cause severe tissue-destructing skin infections (chromoblastomycosis). Our studies (Chapters 3, 4 and 5) identify mincle as the crucial determinant in the responses to fungi associated with chromoblastomycosis. As previously mentioned, unrestrained and exaggerated Th17 inflammation is well known to have severe pathological, tissue-damaging consequences. The responses triggered by mincle — that is absence of protective antifungal Th1 immunity, possibly NK cell activity, at the one hand and strong and sustained activation of Th17 immunity on the other — might well be host-deleterious during Fonsecaea infection. Importantly, mincle signaling concomitantly skews responses towards Th2 immunity (Chapter 2), likely because of limited IL-12/Th12. Th2 cells have been found to predominate the skin lesions with high fungal load, and oppose fungal elimination in general, as shown in experimental models. Thus, it is reasonable to suggest that, by inducing such detrimental responses, mincle provides the opportunity for these virulent fungi to prolong their survival and establish chronic infection.

Of particular interest, a characteristic histopathological feature of chromoblastomycosis is the formation of granulomatous lesions; a common theme also in tuberculosis (TB). These mycobacteria-containing granuloma structures — compact aggregates of immune cells — are beneficial for the host by avoiding bacterial dissemination, but at the same time provide an environment in which the mycobacteria are protected from immune-mediated killing. Although the question remains whether mincle in an analogous manner might facilitate chromoblastomycosis chronicity, mincle agonist TDM (or cordfactor), the most abundant mycobacterial cell wall glycolipid, has been recognized for decades for its granulomagenic and toxic properties. In turn, our results might aid in understanding the complex pathogenesis of tuberculosis: most recent data associates active TB with unchecked Th17 responses and neutrophil influx, neutrophil-driven lung tissue damage...
and, possibly, failing Th1 immunity\textsuperscript{139-142}.

There is much to be learnt about mincle; further unravelling the striking functional characteristics of mincle, and its interplay with host protective immunity but also fungal evasion strategies will bring many more advances in the field of antifungal immunity. Hence, not only increasing our knowledge on the processes that govern host resistance, but also identification of the mechanisms underlying host susceptibility to fungal infection is central to successful development of targeted therapeutic interventions.

CONCLUDING REMARKS

DC-expressed CLRs and their intricate interplay control human antifungal immunity. In this thesis we have deciphered several of the key transcriptional events and intracellular signaling pathways induced by dectin-1, dectin-2 and mincle that underlie their functional crosstalk. This provides intriguing new mechanistic insight into how the human immune system mounts a response to fungal infection. One of the most important concepts to arise from our data is the realization that receptors dectin-2 and mincle are uniquely specialized in modulating adaptive antifungal immunity. Development of targeted therapies should therefore take into account their distinct and not necessarily host-protective function. Emerging evidence indicates that intrinsic mechanisms of host cell physiology, with most notable examples autophagy\textsuperscript{143} and the circadian clock\textsuperscript{144}, have fundamental role in regulating the course of inflammation. The identification of a set of DNA damage response molecules in the transcriptional processes controlled by mincle will undoubtedly provide a useful basis for future research and further understanding the complicated host mechanisms that control fungal elimination and also inflammation in general.
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