Differential Control of Mincle-Dependent Cord Factor Recognition and Macrophage Responses by the Transcription Factors C/EBPβ and HIF1α

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Differential Control of Mincle-Dependent Cord Factor Recognition and Macrophage Responses by the Transcription Factors C/EBPβ and HIF1α

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Trehalose-6,6-dimycolate (TDM), the mycobacterial cord factor, and its synthetic analog Trehalose-6,6-dibehenate (TDB) bind to the C-type lectin receptors macrophage-inducible C-type lectin (Mincle) and Mcl to activate macrophages. Genetically, the transcriptional response to TDB/TDM has been defined to require FcRγ-Syk-Card9 signaling. However, TDB/TDM-triggered kinase activation has not been studied well, and it is largely unknown which transcriptional regulators bring about inflammatory gene expression. In this article, we report that TDB/TDM caused only weak Syk-phosphorylation in resting macrophages, consistent with low basal Mincle expression. However, LPS-priming caused MYD88-dependent upregulation of Mincle, resulting in enhanced TDB/TDM-induced kinase activation and more rapid inflammatory gene expression. TLR-induced Mincle expression partially circumvented the requirement for Mcl in the response to TDB/TDM. To dissect transcriptional responses to TDB/TDM, we mined microarray data and identified early growth response (Egr) family transcription factors as direct Mincle target genes, whereas upregulation of Cebpβ and Hif1α required new protein synthesis. Macrophages and dendritic cells lacking C/EBPβ showed nearly complete abrogation of TDB/TDM responsiveness, but also failed to upregulate Mincle. Retroviral rescue of Mincle expression in Cebpβ-deficient cells restored induction of Egr1, but not of G-CSF. This pattern of C/EBPβ dependence was also observed after stimulation with the Dectin-1 ligand Curdlan. Inducible expression of hypoxia-inducible factor 1α (HIF1α) also required C/EBPβ. In turn, HIF1α was not required for Mincle expression, kinase activation, and Egr1 or Csfr expression, but critically contributed to NO production. Taken together, we identify C/EBPβ as central hub in Mincle expression and inflammatory gene induction, whereas HIF1α controls Nos2 expression. C/EBPβ also connects TLR signals to cord factor responsiveness through MYD88-dependent upregulation of Mincle. The Journal of Immunology, 2014, 193: 000–000.

The intracellular pathogen Mycobacterium tuberculosis survives and multiplies in the phagosome of macrophages, which become activated and are recruited along with other cells to the site of infection to form typical granulomas. Mycobacteria are a rich source of pathogen-associated molecular patterns that include mycobacterial DNA triggering TLR9, the 19-kDa lipopeptide that activates TLR2, and several cell-wall lipids and glycolipids (1). The mycobacterial cord factor, Trehalose-6,6-dimycolate (TDM), is abundant in the mycobacterial cell and has long been known to be a major virulence factor of mycobacteria and a potent inducer of inflammation in animal models (2). Geisel et al. (3) demonstrated that TDM is the major cell-wall glycolipid inducing inflammatory responses by macrophages in vitro. Importantly, TDM per se is sufficient to induce formation of granulomas when injected in oil droplets (4). TDM (5) and its synthetic analog Trehalose-6,6-dibehenate (TDB) are potent adjuvants that efficiently induce Th1/Th17 responses to coadministered recombinant proteins (5–7).

Recently, the receptor(s) and signaling pathways required for cord factor recognition by macrophages have been identified. However, remarkably divergent results have been reported by different laboratories (8). On the one hand, Bowdish et al. (9) reported a requirement for the scavenger receptor MARCO and TLR2/TLR4/CD14 in recognition of TDM, followed by the demonstration that the substantial changes in gene expression induced by TDM are MYD88 dependent (10). In contrast, we and others observed intact in vitro activation of macrophages or granulocytes by TDM or TDB in the absence of MYD88, arguing against a role for TLR (7, 11, 12). In vivo, glycolipid adjuvant activity depends on MYD88 signaling (3, 6), which is at least partially due to IL-1R signaling but independent of TLR2/3/4/7/9 (13).

The C-type lectin receptor (CLR) macrophage-inducible C-type lectin (Mincle), encoded by Clec4e, directly binds to TDM and TDB, and recognition of TDM and TDB is completely abrogated in murine Mincle−/− macrophages (12, 14) and granulocytes (11).
The inflammatory response to whole mycobacteria was attenuated in Mincle-deficient phagocytes in vitro (11, 12, 14, 15), whereas control to mycobacterial infections was moderately impaired in two studies (11, 15), but not in another one (16). Recently, the CLR Mcl, encoded by the Clec4d gene located next to Clec4e, was identified as second receptor for TDB/TDM, whose main function may be to increase expression of Mincle for full responsiveness (17). Mcl is a phagocytic receptor (18) and can form heterodimers with Mincle (19). Downstream signaling of Mincle, which lacks an internal signaling motif, requires the adaptor FcR γ-chain (Fcerl1, FcRγ) (20). Macrophage activation in vitro and adjunctivity by TDB/TDM in vivo depends on the FcRγ/SYK/CARD9/BCL10/MALT1 signaling pathway (7, 14). Thus, the relative contribution of the TLR-MYD88 and the Mincle-FcRγ/SYK-CARD9 pathways to TDM-induced innate immune responses are controversial at present.

Activation of macrophages or dendritic cells (DCs) with ligands for TLR or CLR induces massive transcriptional reprogramming characterized by similarities but also differences in target gene expression (7). These are linked to pathway-specific biological effects such as divergent Th1 or Th17 differentiation by the TLR- and CLR-activating adjuvants (7, 21). Stimulation of macrophages with TLR ligands leads to the Myd88-dependent activation of multiple kinases, which, in turn, phosphorylate a plethora of substrates proteins (22), resulting in the activation of latent transcription factors (23). Several large-scale studies have addressed how these rapidly activated transcriptional regulators induce the ordered expression of hundreds of target genes, including secondary transcription factors and other regulators that amplify or downregulate TLR-triggered activation programs (24–26). In comparison, much less is known about the signaling networks involved in generating CLR-induced transcriptional programs. CLR-induced signaling has mostly been studied using the Dectin-1 ligands zymosan and curdlan (27–29). In contrast, the signaling events triggered by Mincle ligation with the cord factor TDM have received only limited attention (11). Upon CLR stimulation, SYK is recruited to ITAM-like motifs and phosphorylation is mediated by Src kinases (30). In turn, MAPKs are phosphorylated, assembly of the CARD9/BCL10/MALT1 complex and NF-kB activation is induced by protein kinase C δ (27), activation of NF-AT is mediated by phospholipase C γ 2 (31), and the NLRP3 inflammasome is activated (32, 33). Among the inducible transcription factors, only early growth response (Egr) family members have to date been implicated in mediating Dectin-1–triggered gene expression (34).

In this study, we focused on the characterization of transcription factors that are involved in cord factor recognition and responsiveness. To address the unresolved question whether the TLR-MYD88 pathway contributes to the APC activation by TDM/ TDB, we analyzed the consequences of Myd88 deficiency on Mincle expression levels and macrophage responsiveness in terms of intracellular signaling, and effector functions in vitro. We observed that LPS priming enhanced the responsiveness to TDB in a MYD88-dependent manner through upregulation of Mincle receptor expression. For a better understanding of the regulatory factors mediating cord factor–induced gene expression, we turned to the role of inducible transcription factors. We found that expression levels and kinetics of the transcription factors Egr1-3, Cebpβ, and Hif1α were distinct after stimulation via Mincle or TLR9. CEBPβ has a crucial and dual function in macrophage responses to TDM/TDB because it is required for inducible Mincle expression and for inflammatory gene expression. We identified Hif1α as a CEBPβ-dependent Mincle target gene with an essential function for Nos2 expression and NO production. Our findings show that TLR-MYD88–dependent signals can sensitize to cord factor responsiveness through CEBPβ-induced Mincle expression.

Materials and Methods

Animal housing and mouse strains

All mice used were on C57BL/6 background. Myd88−/− mice were used with the permission of Dr. Shizuo Akira (35). Mincle−/− mice were provided by Dr. Christine Wells (36). Clec4d−/− mice were obtained from the Consortium of Functional Glycomics. Hif1αfloxed (37) mice were provided by Dr. Jonathan Jantsch and crossed to Tie2Cre mice provided by Dr. Peter Murray (38). CEBPβlox/lox and Tie2Cre mice were maintained under specific pathogen-free conditions in the animal facility of the Istituto Oncologico Veneto. Tie2Cre and CEBPβfloxed mice were crossed to generate Tie2Cre and CEBPβ heterozygous (CEBPβ+/lox) mice, which were crossed with CEBPβlox/lox mice to obtain CEBPβ homozygous (Cebpβlox/lox) mice (39).

Generation and stimulation of bone marrow–derived macrophages and DCs

Bone marrow–derived macrophages (BMMs) and DCs were generated by M-CSF containing L cell conditioned media (40) and GM-CSF containing media from X63 Ag8 cells (41), respectively. Cells were stimulated with plate-coated compounds. Therefore, TDB (Avanti Polar Lipids) and TDM (Biolot) were dissolved in isopropanol by heating (60°C), added to the plates, and isopropanol was evaporated under the laminar flow. Curdlan (We, suspension in isopropanol) was evaporated by a suitable procedure without heating. Curdlan in suspension was prepared in PBS and diluted with cell culture media. Cpg (TIB Molbiol), LPS (Escherichia coli 055: B5; Sigma-Aldrich), and IFN-γ (Peprotech) were dissolved in PBS or cell culture media, and TDB and TDM were used at 5 µg/ml unless otherwise stated; Cpg and LPS were used at 0.1 µM and 100 ng/ml, respectively. Overnight prestimulation of macrophages with LPS (10 ng/ml) was performed in petri dishes.

Retroviral transduction of bone marrow–derived DCs

Vector containing virus particles were prepared by cotransfection of Phoenix cells with the packaging pCL-Eco (Imgenex) (42), and Mgr1/cGP (43) overexpression vector using the calcium phosphate method (44). For cloning of full-length Mincle from mouse cDNA into the Mgr1 vector, the following primers were used: 5'-ATATATATAGCTGCAATG-3' and 5'-ATATATCTGAGTTATGCCAGAAGCTTTTCTGCTATTAG-3' (forward) and 5'-ATATATCTGAGTTATGCCAGAAGCTTTTCTGCTATTAG-3' (reverse). For retroviral transduction (45), differentiating DCs were incubated with virus-containing supernatant plus Polybrene (6 µg/ml) for 4 h on day 2 of culture. On days 6 or 7, nonadherent cells were collected and G418− cells were sorted.

RNA isolation and quantitative RT-PCR

To isolate RNA, we plated 5 × 105 cells in a 48-well plate, and cellular lysates were prepared with Trifast (Peqlab) to perform phenol/chloroform isolation according to manufacturer’s protocol. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For quantitative RT-PCR (qRT-PCR), primers and probes were selected from the Universal Probe library (Roche) and are available upon request. Hprt was used as housekeeping control, and fold changes were calculated by the ΔΔCT method (46).

Griess assay and ELISA

To determine nitrates and cytokine concentrations, we collected supernatants from 250,000 cells in 96-well plates (triplicates) after 48 h. Concentration of nitrites was measured using Griess assay to assess NO production. Cytokine concentrations were assessed by DuoSet Sandwich ELISA (R&D Systems) according to the manufacturer’s protocol.

Western blot and PNGase digest

For Western blot analysis, cellular lysates in RIPA buffer containing proteinase and phosphatase inhibitors (Roche complete, 0.5 M sodium fluoride, 1 M β-glycerophosphate, 200 mM sodium orthovanadate) were prepared, and Western blot was performed by SDS-PAGE and wet blotting. For protein detection, the following Abs were used: anti-CEBPβ (Santa Cruz), anti-Egr1, anti-εnok (Thr202/Tyr204), anti-pSyk (Tyr525/526), anti-Syk, anti-ERK1/2 (Cell Signaling), anti-Ghr2 (BD), anti-Hif1α (Cayman), anti-Mincle (36), and HRP-conjugated secondary Abs (Jackson}

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Regulation and function of Mcl expression in TLR-induced priming of TDB/TDM responses

We next asked whether the expression of Mcl, the recently identified second receptor for TDM, is regulated by TDB or by TLR ligands. Confirming a previous report (17), basal levels of Mcl in macrophages were higher than those of Mincle and were only weakly phosphorylated and total SYK was performed. In this study, we first assessed expression of Mincle on the protein level in macrophages. To this end, we stimulated untreated and LPS-primed BMMs with TDB/TDM (Fig. 1B, bottom panel). Mincle protein was hardly detectable in resting BMMs, moderately induced by TDB stimulation, and strongly upregulated by LPS pretreatment alone or in combination with TDB stimulation (Fig. 1B), corroborating the mRNA expression data obtained earlier at the protein level. Because the Mincle-specific band had a higher m.w. than expected, we used the enzyme PNGase F to remove N-glycosylation and observed a shift of the Mincle-specific band to the expected m.w. of ~25 kDa, confirming that Mincle is indeed N-glycosylated as predicted earlier (49) (Fig. 1C).

We next compared TDB/TDM-induced SYK phosphorylation in resting and LPS-primed macrophages (Fig. 1B). Phospho-SYK was hardly detectable in nonprimed macrophages but was clearly visible 2 and 6 h after TDB/TDM stimulation of LPS-primed cells. Furthermore, SYK phosphorylation was Mincle dependent upon glycolipid stimulation, consistent with the requirement for Mincle and FcRγ in TDB/TDM-induced macrophage activation (12, 14).

MYD88 dependence of TLR-induced Mincle expression and sensitization to TDB/TDM responses

Next, we examined the influence of MYD88 on Mincle receptor expression. Stimulation with TDB substantially increased Mincle mRNA levels independent of MYD88 (Fig. 2A). In contrast, the marked upregulation of Mincle mRNA by the TLR9 ligand CpG ODN and by low-dose LPS pretreatment was abrogated in Myd88−/− macrophages (Fig. 2A). We next asked whether attenuated Mincle expression in primed Myd88-deficient macrophages would affect kinase activation and gene expression in response to Mincle ligation. Early TDB/TDM-induced levels of phosphorylated Syk and ERK1/2 were reduced in LPS-primed Myd88−/− macrophages compared with heterozygous controls, reflecting the reduction in Mincle protein expression in these cells (Fig. 2B). The TDB-induced expression of Nos2 (inducible NO synthase [iNOS]) and Csf3 (G-CSF; Fig. 2C, 2E), and production of NO and G-CSF (Fig. 2D, 2F) were not affected by Myd88 deficiency in unprimed macrophages, confirming our previous data (7). These responses were more strongly induced by TDB in LPS-primed cells, especially at an earlier time point. However, in LPS-primed Myd88−/− macrophages, TDB-mediated production of NO and expression of iNOS and G-CSF were significantly reduced, correlating with reduced Mincle receptor expression levels. As expected, iNOS expression, NO production, G-CSF expression, and release in Myd88-deficient macrophages were completely abrogated in response to CpG stimulation, whereas the response to LPS was only partially affected (Fig. 2D–F). Together, these data indicate that TLR signals can sensitize macrophages to the glycolipids TDB and TDM by inducing upregulation of the receptor Mincle. TLR priming caused much stronger Mincle-triggered phosphorylation of SYK and ERK1/2 (Fig. 2B), most likely by synchronizing kinase activation. In addition, LPS priming created an indirect and partial dependence of subsequent TDB/TDM responses on the adapter protein MYD88 through upregulation of the Mincle receptor.

**FIGURE 1.** Mincle-FcRγ-dependent kinase activation is primed by TLR-induced Mincle upregulation. (A) Macrophages from wt and Fcerg1−/− mice were stimulated with TDB, TDM, or CpG for 4 h, and phosphorylation of SYK and ERK was analyzed by WB. (B) wt and Mincle−/− BMMs were prestimulated with LPS overnight or left untreated and then stimulated with TDB or TDM as indicated. WB for Mincle and phosphorylated and total SYK was performed. (C) BMMs were prestimulated with LPS overnight and to further increase Mincle expression again stimulated with LPS for 3 h (±LPS), harvested in denaturation buffer, and deglycosylation was performed according to the protocol.
were much smaller than of Mincle, although still dependent on Myd88 (Fig. 3A). Mcl-deficient macrophages were used to determine its requirement for Mincle expression and TDB/TDM responsiveness. Although upregulation of Mincle mRNA in response to TDB was strongly, and to TDM partially, dependent on Mcl, the TLR ligand CpG ODN increased Mincle expression in Mcl<sup>2/2</sup> as in wild-type (WT) BMMs (Fig. 3B). Priming with LPS conferred robust Mincle protein expression in Mcl<sup>2/2</sup> BMMs, although the levels were somewhat lower than in WT, and conferred responsiveness to TDB/TDM stimulation at the level of ERK phosphorylation (Fig. 3C). TDB/TDM-induced G-CSF expression was reduced in Clec4d<sup>2/2</sup> BMMs but could be increased by priming with LPS, whereas Mincle-deficient BMMs did not respond to TDB/TDM irrespective of priming (Fig. 3D). Thus, TLR priming appears to reduce the requirement for Mcl through upregulation of Mincle expression.

Egr family members are direct TDB/TDM-induced Mincle target genes

TLR and CLR signals can synergize as shown earlier, but induce clearly different gene expression patterns (7). We reasoned that differential activation and/or induced expression of transcription factors may be the mechanism behind these characteristic gene expression programs. Therefore, we mined our existing microarray data sets of macrophages stimulated with TDB or CpG (accession no. GSE10530 in Gene Expression Omnibus at http://
TLR- or CLR-induced C/EBPβ transcription factors. Thus, expression of the direct target gene LPS-primed WT, and microarray data set, all three isoforms of C/EBPβ were strongly expressed in BMMs early after TDB stimulation, and hypoxia-inducible 1α (HIF1α). First, we confirmed by qRT-PCR that Egr1, Egr2, and Egr3 were strongly expressed in BMMs early after TDB stimulation, whereas CpG-mediated induction was comparatively weak (Fig. 4A). TDB-induced expression was Mincle dependent (Fig. 4A). EGR1 protein was strongly induced by both TDB and CpG, but with considerably distinct kinetics (Fig. 4B). Stimulation with TDB induced a delayed, but more sustained, expression of EGR1 compared with CpG, which was again dependent on Mincle (Fig. 4B).

Because Egr expression occurred early after macrophage stimulation, we checked whether the regulation required novel protein biosynthesis by adding cycloheximide (CHX). Even though CHX alone enhanced Egr expression already, a clear increase was detectable after TDB and CpG stimulation (Supplemental Fig. 1A). This direct transcriptional induction of Egrs may be mediated by the constitutively expressed NF-AT family members, as shown after Dectin-1 ligation (34). In contrast, relative mRNA induction of the constitutively expressed NF-AT family members, as shown after TDB and CpG stimulation (Supplemental Fig. 1A). This indicated that in the presence of CHX (Supplemental Fig. 1B), indicating that Csf3 is a secondary target gene regulated by inducible transcription factors. Thus, expression of the direct target gene Egr1 can be used to monitor early responses of TDB-stimulated cells.

TLR- or CLR-induced C/EBPβ is required for upregulation of Mincle

Consistent with the mRNA induction of Cebpβ observed in the microarray data set, all three isoforms of C/EBPβ (LAP*, 38 kDa; LAP, 35 kDa; LIP, 20 kDa) were strongly enhanced by stimulation of macrophages with TDB, and expression was sustained over a broad time period (Fig. 5A). As expected, C/EBPβ expression was Mincle dependent in TDB-stimulated, but not CpG-stimulated macrophages (Fig. 5B). Next, we used macrophages deficient in Cebp to analyze its role in TDB/TDM-induced macrophage activation (Fig. 5C). We observed that the induction of Mincle expression was abrogated in Cebpβ-deficient macrophages after stimulation of the cells with TDB and/or priming with LPS (Fig. 5D), corroborating and extending a previous report on C/EBPβ-dependent Mincle expression in response to LPS (49). Basal Mincle mRNA expression was detectable but reduced by 75% in the absence of C/EBPβ. Consistent with low levels of Mincle expression, Cebpβ−/− macrophages displayed only weak phosphorylation of SYK upon TDB stimulation (Fig. 5E), which was not enhanced by LPS prestimulation. In addition, induction of the direct target EGR1 was reduced but not abolished compared with heterozygous cells (Fig. 5F). Furthermore, Cebp−/− macrophages were severely compromised in iNOS and G-CSF mRNA expression (Fig. 5G, 5I), and in G-CSF production and NO release (Fig. 5H, 5J). Production of NO was also severely impaired in response to TLR stimulation (Fig. 5I). Although much weaker than after TDB/TDM treatment, CpG-induced Csf3 expression and LPS-induced G-CSF production were significantly reduced in Cebpβ-deficient cells (Fig. 5I, 5J), matching previous reports that TLR-triggered G-CSF expression depends on C/EBPβ (50).

Retroviral complementation of Mincle expression partially restores responsiveness of Cebpβ−/− APCs

The lack of robust Mincle expression in Cebpβ-deficient macrophages and DCs made it impossible to evaluate the role of
CONTROL OF MINCLE TARGET GENE EXPRESSION

TDB-induced expression of Egr family members is Mincle dependent. (A) Macrophages were prestimulated with LPS and then stimulated for 1 and 3 h as indicated. Expression levels of the Egr family members were determined by qRT-PCR and calibrated to the unstimulated wt control. Representative experiment of three shown as mean ± SD. *p < 0.05, **p < 0.01. (B) Nonprimed macrophages were stimulated with TDB (2.5 μg/ml) and CpG, and WB on EGR1 and GAPDH (loading control) was performed. Band intensities standardized to GAPDH and untreated WT for each time point are shown in the bottom panel.

C/EBPβ in Mincle-mediated gene regulation. To circumvent this hurdle, we genetically complemented Mincle receptor expression by retroviral transduction in Cebpb−/− DCs. The phenotype of Cebpb−/− DCs was comparable with that described for macrophages, including the absence of NO and G-CSF production, and reduced expression of the Mincle receptor in DCs (Supplemental Fig. 2A and data not shown). Although the restoration of Mincle expression by retroviral transduction in Cebpb−/− DCs was lower than in Mincle+/−, we were able to detect Mincle protein in mMincle transduced Cebpb−/− DCs (Supplemental Fig. 2A). Of note, the expression of Egr1 mRNA and protein in response to TDB stimulation was increased in Cebpb−/− and Mincle−/− DCs transduced with Mincle-Migr1 (Supplemental Fig. 2A, 2B). In contrast, retroviral Mincle expression in Cebpb−/− DCs did not restore NO production upon TDB stimulation, although iNOS mRNA induction was partially brought back (Supplemental Fig. 2C). Retroviral Mincle expression failed to confer G-CSF expression at the protein or mRNA level in Cebpb−/− DCs (Supplemental Fig. 2D). In contrast, retroviral Mincle expression in Mincle−/− DCs clearly enhanced NO production (Supplemental Fig. 2C) and restored TDB-induced iNOS and G-CSF expression (Supplemental Fig. 2D). Taken together, retroviral complementation of Mincle expression in Cebpb−/− DCs showed that the expression of the direct Mincle target gene Egr1 was independent of C/EBPβ; in contrast, C/EBPβ was important for full iNOS expression and absolutely required for G-CSF expression and production.

Requirement for C/EBPβ in Dectin-1 and Dectin-2 expression and responsiveness

The CLRs Dectin-1 (Clec7a), Dectin-2 (Clec4n), Mcl (Clec4d), and Mincle (Clec4e) are closely related and trigger similar gene expression programs in macrophages and DCs (7). If Dectin-1 and Dectin-2 expression were independent of C/EBPβ, then triggering of these related receptors in Cebpb−/− macrophages could be used to study the function of this transcription factor in CLR-induced gene expression. Similar to Mincle expression, basal and LPS-, TDB-, and CpG-induced expression of Dectin-2 were reduced in Cebpb−/− macrophages (Fig. 6A and data not shown). Thus, Dectin-2 shares with Mincle the requirement for C/EBPβ for robust expression. Increased Mcl expression in response to TDB or CpG also required C/EBPβ (Fig. 6A). In contrast, expression of Dectin-1 was comparable between Cebpb−/− and Cebpb+/− macrophages (Fig. 6B). We therefore used the Dectin-1 ligand Curdlan to investigate the requirement for C/EBPβ in SYK-CARD9–dependent gene expression. Expression of Nos2 in response to Curdlan was comparable between Cebpb heterozygous and knockout macrophages after 8 h, but reduced in the knockout after 24 h (Fig. 6C). In line with these findings, the production of NO was reduced but not abolished upon stimulation with Curdlan in Cebpb−/− (Fig. 6D). G-CSF mRNA and protein expression were strongly induced by Curdlan stimulation in heterozygous, but completely abrogated in Cebpb−/− cells (Fig. 6E, 6F). The early target gene Egr1 was moderately induced by Curdlan, and no difference between heterozygous and knockout macrophages was observed (Fig. 6G). To confirm the functionality of Cebpb-deficient macrophages, we analyzed the expression levels of non-C/EBPβ target genes Cxcl10 and Cxcl11 (51) in response to Curdlan stimulation. Expression levels for both were comparable between heterozygous and knockout cells after 8 h but reduced at the later time point (Fig. 6H, 6I). In summary, these data showed that macrophages are able to respond to Dectin-1 ligation in the absence of C/EBPβ by inducing Egr1 expression; however, C/EBPβ contributes to iNOS expression and is absolutely crucial for G-CSF expression. Thus, the data are reminiscent of the C/EBPβ requirements observed for TDB/TDM stimulation of DCs complemented with retroviral Mincle (Supplemental Fig. 2).

TDB-induced HIF1α selectively controls NO production

A role for HIF1α in inflammatory processes has emerged recently (52). However, increased HIF1α expression and its potential role in the response to CLR ligands has not been described yet. We...
confirmed the microarray-based finding that Hif1α mRNA was increased upon TDB and CpG stimulation by qRT-PCR, and validated Mincle dependence of TDB-induced expression (Fig. 7A). Expression of HIF1α protein reflected the mRNA expression pattern, as HIF1α was increased upon both stimuli in LPS-primed and nonprimed macrophages, with Mincle-dependent and more sustained induction after TDB (Fig. 7B). CHX prevented Hif1α induction by TDB (Supplemental Fig. 3A), indicating the requirement of inducible transcription factors for HIF1α transcription.

To assess the role of HIF1α in TDB-stimulated macrophages, we used Hif1α-deficient BMMs derived from Hif1α−/− mice, expressing the Cre-recombinase under regulation of the Tie2 promoter. Efficient deletion of the loxP-flanked exon was confirmed by Western blot showing truncated Hif1α protein (Fig. 7D) of the expected size lacking the dimerization and transactivation domain (37). Basal and inducible Mincle mRNA, and protein expression were not affected by the loss of HIF1α (Fig. 7E and Supplemental Fig. 3B). Furthermore, TDB-induced phosphorylation of SYK and ERK1/2 kinases (Fig. 7F), as well as the early induction of EGR1 (Fig. 7G), was intact in Hif1α-deficient cells.

In contrast, upon TDB stimulation, iNOS expression was significantly reduced in macrophages lacking HIF1α, whereas this effect was less pronounced upon CpG stimulation (Fig. 7H). Furthermore, release of NO into the supernatant was reduced by 75% from Hif1α-deficient macrophages stimulated with TDB or TDM, whereas NO production induced by CpG or LPS was only moderately reduced (Fig. 7I). In clear contrast with NO production, Hif1α deficiency did not impair G-CSF mRNA expression and
secretion (Fig. 7J and Supplemental Fig. 3C) and expression of Cxcl10 and Cxcl11 (Fig. 7K). Together, these data show that HIF1α is a C/EBPβ-dependent Mincle-inducible transcription factor controlling the expression of Nos2, but not of Csf3, Cxcl10, and Cxcl11 in macrophages (Fig. 8).

Discussion

In this article, we have investigated the requirements for Mincle expression, signaling, and target gene induction in macrophages in response to the mycobacterial cord factor. We demonstrate that the TLR-MYD88 pathway is not required for TDM-induced kinase activation and gene expression, but primes and enhances the response to cord factor stimulation through marked upregulation of the Mincle receptor. The transcription factor C/EBPβ is a central regulator of the response to TDM, because it mediates TLR priming and positive feedback through inducible Mincle expression and it governs inflammatory target genes and inducible transcription factors. One C/EBPβ-dependent transcriptional regulator identified in this study is HIF1α, which is essential for the production of NO, whereas other Mincle-dependent responses are intact.

A requirement for Mincle and FcRγ-SYK–dependent signaling in the response to the mycobacterial cord factor has been previously shown by use of genetically deficient phagocytes and mice (7, 11, 12, 14, 15), and by pharmacological inhibitors of the SYK kinase (7, 12). In this article, to our knowledge, we provide the first biochemical demonstration of SYK phosphorylation after stimulation with TDB/TDM, which was not observed after TLR9 ligation with CpG DNA consistent with the genetic data (7). The ERK1/2 MAPK was also activated dependent on the presence of Mincle/FcRγ, but was similarly triggered by CpG stimulation. We did not address in this article which other kinases are activated and contribute to TDB/TDM-triggered Mincle signaling. Strasser et al. (27) recently showed that protein kinase Cδ links SYK-activation by CLR (including Mincle and Dectin-1) to the CARD9–BCL10–MALT1 complex. To obtain a more comprehensive picture of kinase signaling downstream of CLR, a global phosphoproteomic analysis, as recently performed by us for TLR4-dependent macrophage activation (22), should be informative and help to define kinases common and unique to Mincle- and TLR-driven signaling pathways.

Consistent with the initial description of Mincle as TLR4-inducible gene in macrophages (49), we observed strong induction of Mincle mRNA expression by LPS and also the TLR9 ligand CpG. We show in this study that Mincle protein levels are increased accordingly and Mincle displays an increased m.w.
because of N-glycosylation as predicted by Matsumoto et al. (49).
In addition to TLR ligands, the Mincle ligands TDB and TDM themselves triggered a strong induction of Mincle mRNA and protein, matching recent findings of Mincle induction by TDM in alveolar macrophages (15) and in neutrophils (11). Receptor up-regulation was functionally important as we observed enhanced responsiveness of TLR-primed macrophages to TDB/TDM. We conclude that TLR priming synchronizes the response to TDB/TDM by Mincle induction that allows for efficient binding to ligand and initiation of signaling through FcRγ-SYK activation. In contrast, when resting macrophages with low Mincle expression encounter TDB/TDM, a weak response is slowly amplified by a positive feedback loop of Mincle upregulation (Fig. 8). An alternative mechanism for generation of sufficiently high Mincle expression levels has recently been provided by Yamasaki’s group (53) with the identification of Mcl (encoded by the Clec4d gene) as a second receptor for TDB/TDM. Although Mcl has a much lower affinity to the glycolipids, its constitutive expression in resting macrophages appears to be required for Mincle upregulation. Our results confirm the impaired induction of Mincle and G-CSF mRNA in Mcl-deficient macrophages. However, Mcl<sup>2/2</sup> macrophages can respond to TDB/TDM if Mincle expression is increased through TLR priming (Fig. 3). Thus, although the reported formation of Mcl-Mincle heterodimers may alter the scope and efficiency of ligand recognition (19, 54), it is not essential for TDB/TDM-induced macrophage activation.

Our results regarding the enhanced responsiveness of TLR-primed macrophages shed some light on the controversial involvement of the TLR adapter protein MYD88 in the response to TDM. Inflammatory responses to application of TDM-coated beads in vivo were MYD88 dependent (but TLR2/4 independent) (3); furthermore, Bowdish et al. (9) reported that the scavenger receptor MARCO binds TDM and delivers the cord factor to TLR2/4, thereby initiating MYD88-dependent macrophage activation. In contrast, the work of several other laboratories, including ours reported in this article, suggests that the TDB/TDM response is

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**FIGURE 7.** TDB stimulation strongly induces Hif1α expression, and Hif1α deficiency results in decreased NO production. (A) wt and Mincle<sup>−/−</sup> BMMs were prestimulated with LPS overnight and then for 2–24 h with TDB or CpG. qRT-PCR for Hif1α was performed. (B) BMMs were stimulated as described for (A) for 8 and 24 h. Cells were lysed in PEG lysis buffer for WB analysis. Band intensities of Hif1α were standardized to GRB2 and untreated WT BMMs (bottom panel). (C) Hif1α expression was determined after 24-h stimulation with TDB, Crd (400 μg/ml), or LPS by qRT-PCR. (D) Knockout efficiency in Tie2 Cre<sup>+/−</sup> Hif1fl<sup>ox/lox</sup> macrophages was checked by WB analysis. Cells were treated as described for (B) (24 h). (E) Mincle protein levels in TDB (5 μg/ml) or CpG-stimulated macrophages were assessed after 24 h; lane loading as in (D). (F) BMMs were prestimulated with LPS overnight, and SYK and ERK phosphorylation and total protein levels were assessed after 30-min stimulation with TDB or CpG; lane loading as in (D). (G) Cells were treated as described for (H), and Egr1 expression was analyzed by WB after 2.5 and 4 h; lane loading as in (D). (H) BMMs from Tie2 Cre<sup>−/−</sup>, Hif1α<sup>Δ</sup> mice were stimulated with TDB or CpG, and Nos2 expression levels were assessed after 24 h. (I) For Griess assay, Tie2 Cre<sup>−/−</sup> BMMs were stimulated in the presence of IFN-γ as indicated with TDB or TDM (both 5, 1 μg/ml), CpG, or LPS for 48 h. Nitrates in supernatants of Hif1α<sup>Δ</sup> cells (104.2 μM) were set to 100%. (J) BMMs from Tie2 Cre<sup>−/−</sup>, Hif1α<sup>Δ</sup> mice were stimulated with TDB or CpG for 8 and 24 h, and Csf3 expression levels were analyzed by qRT-PCR. (K) Expression of Cxcl10 and Cxcl11 was determined 8 and 24 h after stimulation by qRT-PCR. (A–G and K) Representative of at least three independent experiments, mean and SD. Fold changes in qRT-PCR experiments were calculated using unstimulated WT or Cebpb<sup>fl</sup> BMMs as calibrators. (H–J) Data show mean and SEM of three independent experiments in percent, with TDB-stimulated heterozygous cells set to 100%. *p < 0.05, **p < 0.01.
regulates the expression of TDB. The importance of IL-1R signaling (13). Similar findings of IL-18, and IL-33 for adjuvanticity of TDB, and identified an adjuvanticity have been reported by Sher’s group (56) for CFA. IL-1–dependent signal amplification via MYD88 could also contribute to Mincle-dependent responses in vitro. In this context, differential triggering of the inflammasome by bead-coated versus plate-bound glycolipids may explain some of the differences observed in different studies.

The transcriptional networks involved in reprogramming gene expression in response to TLR stimuli (24, 25, 57) have been studied much more intensively than those related to CLR such as Dectin-1, Dectin-2, and Mincle. In this study, we have obtained new insight into the mechanism of macrophage transcriptional responses to Mincle ligation. Using transcriptome data sets as the starting point, we first identified several transcription factors whose expression was upregulated preferentially by Mincle ligation and selected Egr family members, Cebpb and Hif1a, for more detailed analyses. The Egrs were rapidly induced, direct targets of Mincle triggering, as shown by CHX-blockade experiments. Several reports have indicated that Egr family members are induced by Dectin-1 ligands in macrophages and DCs (34, 58) or neutrophils (59) in a calcineurin/NF-AT–dependent manner, which is likely also the case after Mincle stimulation. Although there are a number of reports on genes regulated by EGR family members in innate immune cells (60–62), the direct targets of EGRs in macrophages and DCs, especially in response to CLR activation, are currently unknown. We used expression of Egrs in this study primarily as a readout for early Mincle-dependent gene expression.

In contrast, the other two transcription factors in the focus of this study, Hif1a and Cebpb, were upregulated by TDB with slower kinetics, suggesting that they are indirect target genes. A central role of C/EBPβ in the TLR-induced upregulation of Mincle expression was described earlier (49). Our data extend this finding by showing that also the TDB-induced Mincle induction requires C/EBPβ. Impaired TDB/TDM-induced gene expression could therefore be because of a lack of feed-forward signal by Mincle upregulation in the absence of C/EBPβ, or may indicate that C/EBPβ controls Csf3 and Nos2 expression downstream of Mincle. To dissect these possibilities, first, we rescued Mincle expression in Cebpb−/− DCs by retroviral complementation, and second, we stimulated macrophages through Dectin-1 (whose expression is not influenced by Cebpb deficiency) as a means to determine the role of C/EBPβ in SYK-CARD9–dependent gene expression. Both approaches yielded a similar pattern of Cebpb dependence: expression of the immediate response gene Egr1 was not affected, Nos2 expression was partially dependent with a strong effect on NO levels in the supernatant, and the production of G-CSF mRNA and protein was completely abrogated (Fig. 8). Although, to the best of our knowledge, the role of C/EBPβ in gene expression downstream of CLR is not influenced by Cebpb deficiency, it has a dual function in the response to macrophages and DCs to the cord factor, in that it first drives inducible Mincle expression in response to a TLR- or CLR-dependent priming, synergy, or feed-forward amplification loop, and in addition controls the expression of a subset of Mincle-target genes, most strikingly of G-CSF. Of note, mice deficient in Cebpb are highly susceptible to infection with M. tuberculosis, show a selective impairment of G-CSF expression in the lungs, and can be partially protected by injection of rG-CSF (64).

HIF1α was identified in this study as TDB-induced, Cebpb-dependent Mincle target gene. To our knowledge, induction of
HIF1α mRNA or protein by CLR signaling has not been reported previously. HIF1α was not uniquely upregulated by the Mincle ligand TDB but also by TLR9 triggering, in agreement with the literature on TLR-induced HIF1α expression and stabilization (65–67). TLR-triggered Hif1a mRNA induction requires NF-kB signaling (68), and the NF-kB family member RelA is recruited to the Hif1a promoter in response to LPS (69). However, the kinetics of Hif1a mRNA induction by TDB was quite slow, suggesting that, in addition to NF-kB, other transcriptional regulators are involved. Consistent with this assumption, translational blockade with CHX showed that Hif1a is a secondary target gene of Mincle ligation. Our observation that Cebpb-deficient macrophages fail to upregulate Hif1a in response to TDB and Curdlan, but also to LPS, establishes a novel connection between these transcription factors in the control of macrophage gene expression and suggests that C/EBPβ synergizes with NF-kB at the Hif1a promoter. Our experiments using conditional knockout macrophages show that HIF1α is selectively required for TDB-induced Nos2 expression and NO production, but not for upregulation of Egr1, Mincle, or Csf3. A critical role for HIF1α in Nos2 expression has been established previously in macrophages stimulated with LPS (65), lipoteichoic acid (70), or infected with Group A Streptococci (GAS) (71). Hif1a-deficient macrophages have a defect in the intracellular killing of GAS and P. aeruginosa, rendering mice with myeloid HIF1α deficiency more susceptible to GAS infection (71). Antimycobacterial immunity in mice critically depends on NO production through Nos2 upregulation in infected macrophages (72). Our finding that the mycobacterial glycolipid TDM induces NO production to a large degree via the action of HIF1α indicates a potentially important role for this transcription factor in host defense against tuberculosis.

Taken together (see the model in Fig. 8), we show in this article that responsiveness of macrophages to the cord factor depends on Mincle expression levels, which can be strongly induced by TLR activation requiring FcRγ-Syk-Card9-dependent innate immune activation. J. Exp. Med. 206: 89–97.


