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HOST RESPONSE AND INFLAMMATION

Toll-Like Receptor 2 and Mincle Cooperatively Sense Corynebacterial Cell Wall Glycolipids

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ABSTRACT Nontoxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* cause invasive disease in humans and animals. Host sensing of corynebacteria is largely uncharacterized, albeit the recognition of lipoglycans by Toll-like receptor 2 (TLR2) appears to be important for macrophage activation by corynebacteria. The members of the order *Corynebacterineae* (e.g., mycobacteria, nocardia, and rhodococci) share a glycolipid-rich cell wall dominated by mycolic acids (termed corynomycolic acids in corynebacteria). The mycolic acid-containing cord factor of mycobacteria, trehalose dimycolate, activates the C-type lectin receptor (CLR) Mincle. Here, we show that glycolipid extracts from the cell walls of several pathogenic and nonpathogenic *Corynebacterium* strains directly bound to recombinant Mincle *in vitro*. Macrophages deficient in Mincle or its adapter protein Fc receptor gamma chain (FcRγ) produced severely reduced amounts of granulocyte colony-stimulating factor (G-CSF) and of nitric oxide (NO) upon challenge with corynebacterial glycolipids. Consistently, cell wall extracts of a particular *C. diphtheriae* strain (DSM43989) lacking mycolic acid esters neither bound Mincle nor activated macrophages. Furthermore, TLR2 but not TLR4 was critical for sensing of cell wall extracts and whole corynebacteria. The upregulation of Mincle expression upon encountering corynebacteria required TLR2. Thus, macrophage activation by the corynebacterial cell wall relies on TLR2-driven robust Mincle expression and the cooperative action of both receptors.

KEYWORDS C-type lectin receptor, Mincle, Toll-like receptor, macrophage, *Corynebacterium diphtheriae*, cell wall lipids, mycolate, mycolic acid, corynomycolate

Diphtheria caused by toxin-producing *Corynebacterium diphtheriae* is a severe, life-threatening infection, which has become rare in Europe due to the efficacy and coverage of toxoid immunization but still causes a considerable burden of disease globally. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* can also harbor the *tox* gene encoding diphtheria toxin (1) and in addition may secrete the exotoxin phospholipase D, a virulence factor involved in caseous lymphadenitis of sheep and goats (2, 3). Rates of infections with nontoxigenic strains of *C. diphtheriae*, including bloodstream infections and endocarditis, appear to be increasing in Europe (4, 5). Of the 90 species that comprise the genus *Corynebacterium*, many inhabit the human skin or mucosa as commensals (6), with *C. striatum*, *C. tuberculostearicum*, *C. amycolatum*, and *C. jeikeium* being typical examples of skin inhabitants, whereas *C. urealyticum* and *C. glucuronolyticum* are frequently found in the urogenital tract. All

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these *Corynebacterium* spp. can cause invasive disease in patients with compromised skin or mucosal barrier function (e.g., patients suffering from burn wounds or carrying intravascular devices) or in immunocompromised patients (e.g., diabetics and cancer patients) (7–13).

While it is clear that protection against toxin-producing strains of *C. diphtheriae* relies on the presence of neutralizing antibodies, not much is known about the immune response against nontoxigenic *Corynebacterium* spp. in patients. A few studies in the mouse model have started to investigate tissue tropism and inflammatory responses (14), revealing strain-dependent arthritogenic potential independent of toxin and phospholipase D production (15, 16).

Taxonomically, *Corynebacterium* spp. belong to the suborder *Corynebacterineae* of the order *Actinomycetales*, which includes *Mycobacterium* spp., *Nocardia* spp., *Rhodococcus* spp., and *Tsukamurella* spp. All *Corynebacterineae* are characterized by a lipid-rich cell wall with an outer membrane layer dominated by long-chain α-alkyl β-hydroxy fatty acids called mycolic acids (termed corynomycolic acids in *Corynebacterium*) covalently linked to arabinogalactan and additionally to trehalose as trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (17). Among the *Corynebacterineae*, *Corynebacterium* spp. have the shortest chain length, with corynomycolic acids comprising a total carbon length of 22 to 38 carbons, whereas *Mycobacterium* spp. harbor mycolic acids with a total carbon length of 60 to 90 carbons (17). TDM from pathogenic mycobacteria is known as cord factor, based on its role in the clumping of mycobacteria during *in vitro* culture, and has been recognized for decades for its role in inducing the inflammatory response, granuloma formation, and adjuvant activity for T cell responses during mycobacterial infection (18–20). Recently, the direct recognition of TDM by the C-type lectin receptor (CLR) Mincle was shown to trigger macrophage activation through the adapter protein Fc receptor gamma chain (FcRγ), the kinase Syk, and the Card9-Bcl10-Malt1 complex (21–23). Mincle, the related CLR Mcl, and the components of this signaling pathway are essential for granuloma formation and adjuvant effects in response to TDM in mice (20, 22, 24–26) and mediate the response of human macrophages to TDM and the synthetic glycolipid trehalose dibehenate (TDB) (27).

However, the mechanisms of innate immune recognition of corynebacteria are not well studied. Early work from the 1960s to the 1980s in the last century described surface lipids of *C. pseudotuberculosis* as virulence factors with toxic activity toward macrophages and correlated the lipid content to abscess formation *in vivo* (28, 29). Toll-like receptors (TLRs) mediate *C. diphtheriae*-driven macrophage activation in a TLR2-dependent yet TLR4-independent manner (30). Mishra et al. demonstrated that lipomannan (LM) and lipoarabinomannan (LAM) of *C. glutamicum* interact with TLR2 (31). In addition, TLR2-activating lipoglycans of *C. glutamicum* include glycosylated diacylglycerol-anchored lipids (32). A stimulatory effect of purified trehalose dicorynomycolate (TDCM) from *C. glutamicum* on macrophages was shown previously, without characterization of the receptors involved (33). Recently, synthetic corynomycolates were demonstrated to be recognized through mouse and human Mincle by using a reporter cell system (34). However, the role for Mincle and other CLRs in the recognition of corynebacteria by innate immune cells has not been tested.

Here, we used cell wall preparations of specific isolates of *C. diphtheriae*, *C. ulcerans*, and *C. glutamicum* to comparatively address the roles of TLR2 and Mincle receptors in macrophage activation by corynebacteria. Mincle directly bound glycolipid cell wall extracts and was required for cytokine and nitric oxide (NO) production in response to lipid extracts but not to whole bacteria. Consistently, one isolate of *C. diphtheriae* lacking cell wall mycolates was deficient in Mincle binding and macrophage activation in response to its glycolipid extracts. TLR2 was essential for macrophage activation by corynebacteria and upregulated Mincle expression, suggesting a synergistic recognition of corynebacterial glycolipids via a feed-forward loop of TLR2-dependent Mincle induction.

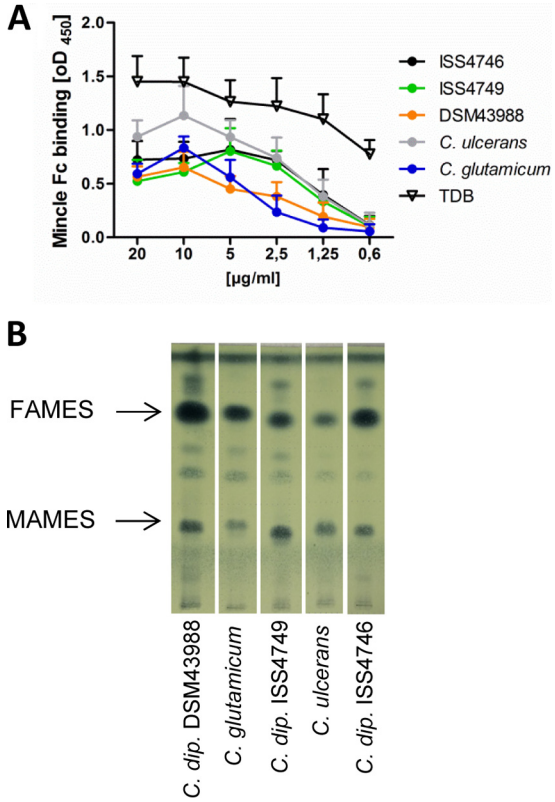

FIG 1 Cell wall extracts of corynebacteria bind the Mincle-Fc fusion protein *in vitro*. (A) The binding affinity of the Mincle-Fc fusion protein for different concentrations of corynebacterial glycolipid extracts was analyzed by using a Mincle-Fc binding assay. Data are depicted as mean values and standard deviations from 3 experiments performed in duplicates ($n = 6$). (B) TLC of fatty acid and mycolic acid methyl esters stained with MPA. *C. dip.*, *C. diphtheriae*.

RESULTS

Mincle-Fc binds to cell wall extracts of corynebacteria. To test for the presence of Mincle ligands in the cell wall of corynebacteria, chloroform-methanol extracts were prepared and used in an enzyme-linked immunosorbent assay (ELISA)-based Mincle-Fc binding assay (35, 36). Plate-bound lipid extracts of several strains of *C. diphtheriae*, *C. ulcerans*, and *C. glutamicum* dose-dependently bound to Mincle-Fc (Fig. 1A). Binding activity was detectable starting at a concentration of around 1 μg/ml of the extract. Corynebacterial glycolipids bound specifically to Mincle-Fc, as no signal was obtained by using Clec9a-Fc or the Fc protein alone (see Fig. S1 in the supplemental material). The synthetic Mincle ligand TDB, which was used as a positive control, yielded detectable signals at lower concentrations and reached somewhat higher signals at saturation. Thin-layer chromatography (TLC) and staining of fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) from cell wall lipid extracts with molybdophosphoric acid (MPA) gave overall similar results for the different corynebacterial strains, with prominent signals representing FAMES and MAMES (Fig. 1B). Together, the data from Mincle-Fc binding assay clearly demonstrated the presence of ligands for this CLR among the complex mixture of corynebacterial cell wall glycolipids.

Mincle-FcRγ signaling is required for the response to corynebacterial cell wall glycolipids. To assess the response of innate immune cells to corynebacteria and their cell wall, bone marrow-derived macrophages (BMM) were stimulated with titrated amounts of plate-bound glycolipid extracts or heat-inactivated corynebacteria, followed by measurement of granulocyte colony-stimulating factor (G-CSF) release as a robust readout of Mincle-dependent BMM activation (22, 37) (Fig. 2A) and nitrites as an indicator of inducible nitric oxide synthase (iNOS) activity (Fig. 2B). As positive controls,

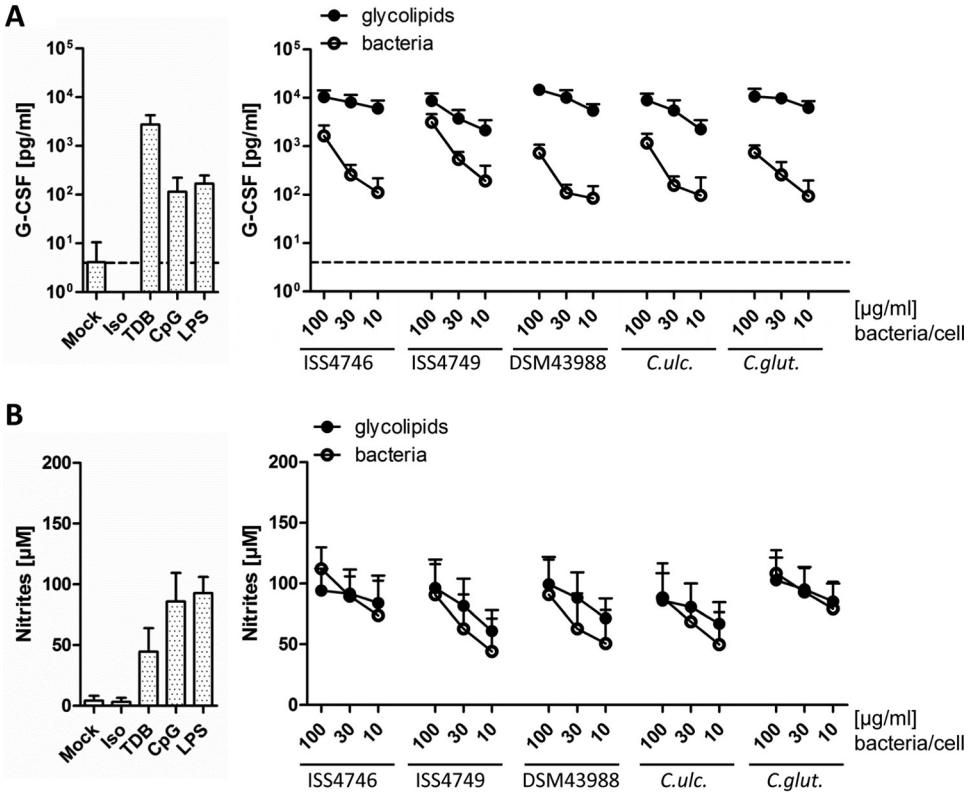

FIG 2 Corynebacterial glycolipids induce G-CSF and NO production in macrophages in a concentration-dependent manner. BMM from C57BL/6 mice were stimulated with different concentrations of glycolipids (100, 30, or 10 μg/ml) or heat-killed bacteria (100, 30, or 10 bacteria per cell), followed by determination of G-CSF (A) and nitrite (B) levels in cell culture supernatants via an ELISA and a Griess assay, respectively. The detection limit for G-CSF was 4 pg/ml (dotted line). For comparison, unstimulated cells or TDB-, CpG-, or LPS-stimulated cells were used as controls. Data are represented as means and standard deviations of results from 6 independent experiments performed in duplicates ($n = 12$). Iso, isopropanol; *C. ulc.*, *C. ulcerans*; *C. glut.*, *C. glutamicum*.

ligands for TLR4 and TLR9 (lipopolysaccharide [LPS] and CpG oligodeoxynucleotide [ODN], respectively) and for Mincle (TDB) were included. While all synthetic ligands triggered both G-CSF from resting BMM and NO release from gamma interferon (IFN-γ)-cotreated BMM, TDB more potently induced G-CSF, and the TLR ligands induced higher levels of NO. G-CSF production was induced very robustly and dose dependently by the glycolipid extracts of corynebacteria independent of the species tested. Whole corynebacteria also triggered G-CSF production, yet even at a high bacterium-to-cell ratio of 100, the levels were not as high as those with relatively small amounts of glycolipids. In contrast, NO production from BMM cotreated with IFN-γ was induced to similar levels by cell wall extracts and whole corynebacteria.

We next asked whether the recognition of cell wall glycolipids by Mincle and signaling via its adapter protein FcRγ were required for the macrophage response to corynebacteria. As expected, BMM lacking Mincle or FcRγ did not produce G-CSF or NO upon stimulation with TDB but responded normally to CpG or LPS (Fig. 3A and D). The release of G-CSF after stimulation with glycolipid extracts was strongly attenuated (20-fold reduction on average) but not completely abrogated in the absence of Mincle or FcRγ (Fig. 3B). In contrast, the comparably weak induction of G-CSF by whole corynebacteria was only partially Mincle-FcRγ dependent (Fig. 3C). NO release from IFN-γ-cotreated BMM was significantly reduced in the absence of Mincle or FcRγ after glycolipid stimulation (Fig. 3E) but not when whole corynebacteria were used (Fig. 3F). Mincle-FcRγ deficiency especially impaired the G-CSF response to glycolipid extracts over a broad range of concentrations (Fig. 3G), whereas for NO production, a shift in the dose response to glycolipids was apparent (Fig. 3H). Results for *C. diphtheriae* strain

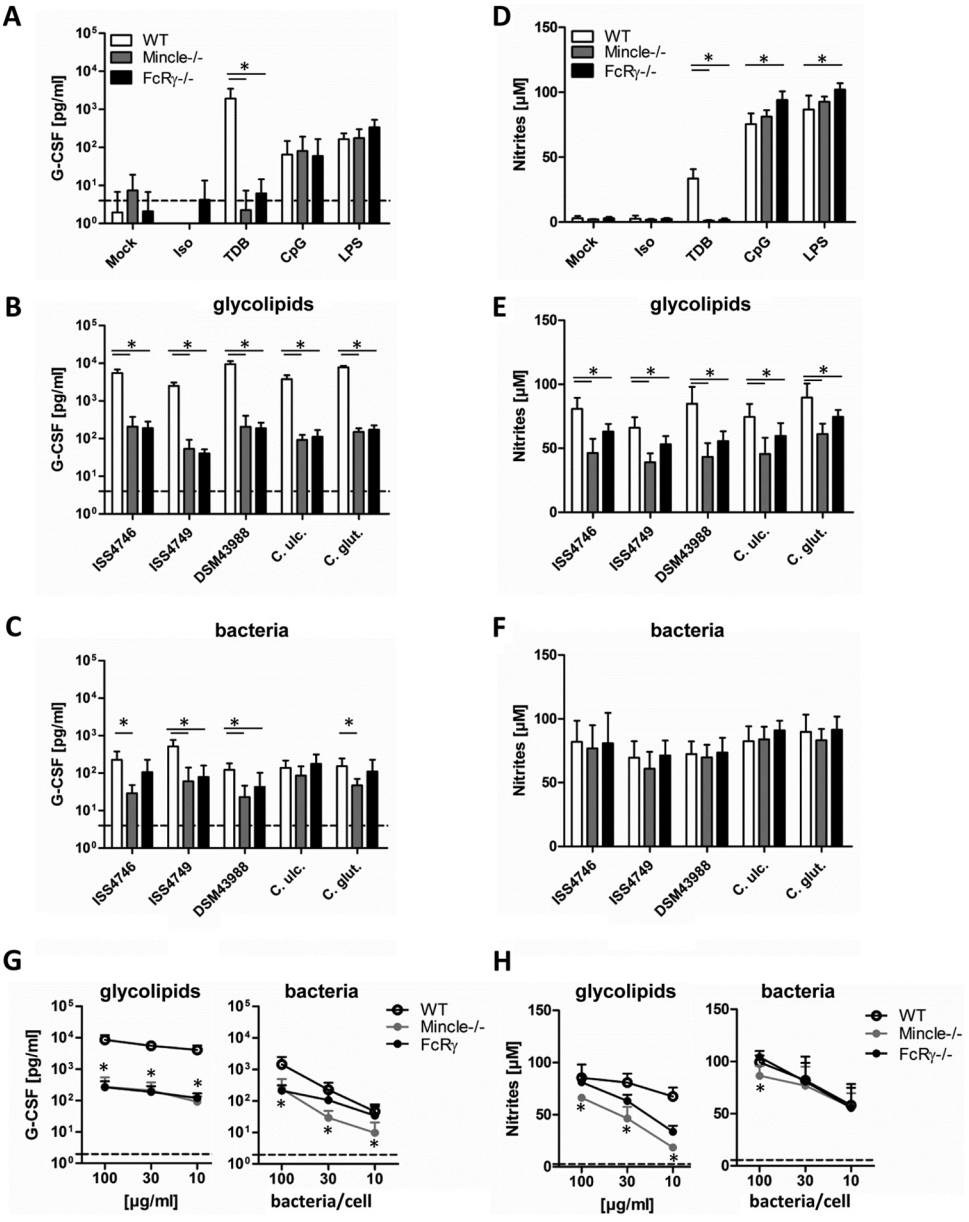

FIG 3 Mincle/FcRγ deficiency impairs G-CSF and NO production in macrophages stimulated with corynebacteria or their cell wall glycolipids. (A to C) BMM from Mincle- or FcRγ-deficient mice and their respective wild-type controls were stimulated with different glycolipid extracts (30 μg/ml) or the corresponding heat-killed bacteria (30 bacteria per cell) as well as controls for 48 h, as indicated. G-CSF cytokine levels in cell culture supernatants were determined by using an ELISA. The detection limit was 4 pg/ml (dotted line). (D to F) NO production in cell culture supernatants was determined by using a Griess assay. BMM were cotreated with 10 ng/ml IFN-γ. (G and H) BMM were stimulated with different concentrations of glycolipids (100, 30, or 10 μg/ml) or heat-killed bacteria (100, 30, or 10 bacteria per cell). Data shown were obtained with glycolipids from *C. diphtheriae* ISS4746; extracts from the other corynebacterial strains/species showed similar dose-response curves (not shown for clarity). G-CSF and NO production was analyzed as described above. Dotted lines represent G-CSF or nitrite levels from unstimulated BMM. Note that data points for some Mincle$^{-/-}$ and FcRγ$^{-/-}$ BMM overlap in panel G. Data are depicted as means and standard deviations of results from 3 independent experiments performed in duplicates ($n = 6$). In panels B to H, * indicates a P value of <0.05 for the indicated comparison between genotypes.

ISS4746 are shown in Fig. 3G and H as a representative example, since a similar pattern was observed for *C. diphtheriae* strains ISS4749 and DSM43988, *C. ulcerans*, and *C. glutamicum*.

Absence of corynomycolic acid-derived glycolipids, lack of Mincle binding, and macrophage activation in the cell wall of *C. diphtheriae* strain DSM43989. Our present studies with DSM43989, a *tox*$^{+}$ *C. diphtheriae* isolate (identical to ATCC 13812,

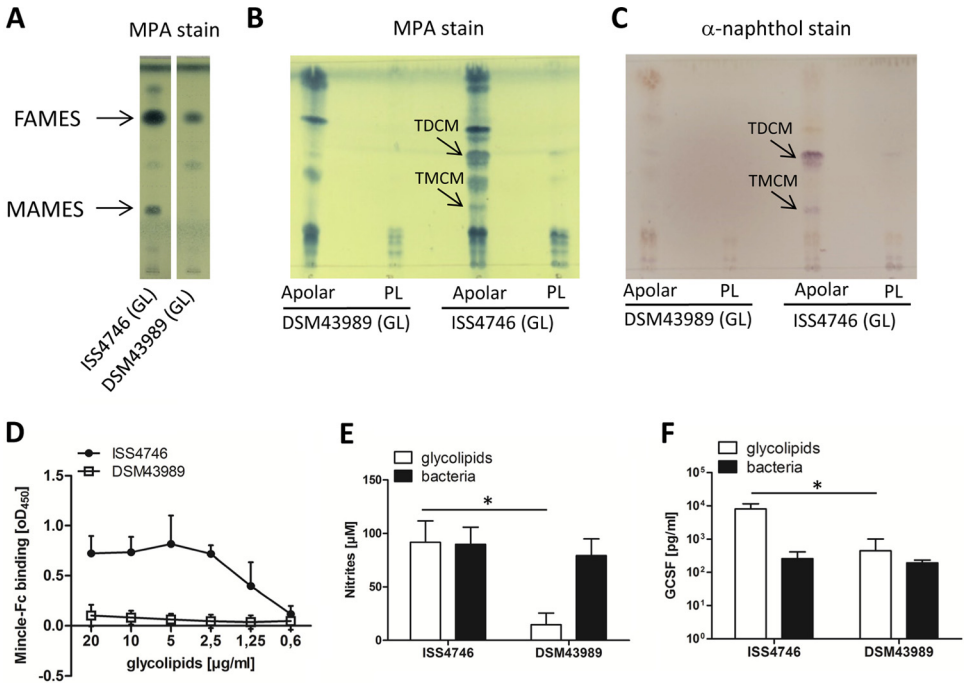

FIG 4 Cell wall extracts of *C. diphtheriae* DSM43989 fail to activate macrophages and do not bind Mincle-Fc. (A) TLC of different lipid extracts stained by using MPA. (B and C) Apolar compounds and phospholipids (PL) of glycolipid (GL) extracts were purified and also detected by TLC using MPA (B) or α-naphthol (C). (D) The binding affinity of the Mincle-Fc fusion protein for different concentrations of *C. diphtheriae* ISS4746 and DSM43989 glycolipid extracts was analyzed by using a Mincle-Fc binding assay. Shown are means and standard deviations of results from three independent experiments performed in duplicates ($n = 6$). (E and F) Bone marrow-derived murine macrophages from C57BL/6 mice were stimulated with lipid extracts (30 μg/ml) or the corresponding heat-killed bacteria (30 bacteria per cell) from *C. diphtheriae* ISS4746 or DSM43989 in the presence (E) or absence (F) of 10 ng/ml IFN-γ. NO production (E) and G-CSF levels (F) were measured in the supernatant 48 h after stimulation. Data are depicted as means and standard deviations of results from 6 independent experiments performed in duplicates ($n = 12$).

a PW8 strain used for toxoid production [38]), revealed a lack of corynomycolic acids in this strain (L. Ott, E. Hacker, T. Kunert, I. A. Kline, P. Etschel, R. Lang, V. Wiesmann, T. Wittenberg, A. C. Varela, A. Bhatt, V. Sangal, and A. Burkovski, submitted for publication) (Fig. 4A). This strain, which lacks TMM and TDM, provided us with an opportunity to probe the role of corynomycolic acid-derived glycolipids in Mincle signaling. Indeed, analysis of fractions from whole lipids revealed the absence of TDCM and trehalose monocorynomycolate (TMCM) in the apolar fraction of DSM43989 cells (Fig. 4B and C). Whole-lipid extracts from this isolate did not bind the Mincle-Fc protein even at high concentrations (Fig. 4D), consistent with the notion that the MAMES and TDCM/TMCM visible by TLC analysis represent the major Mincle ligands of the corynebacterial cell wall. The capacity of the cell wall glycolipids of DSM43989 to stimulate NO release and G-CSF production from BMM was strongly attenuated, whereas heat-killed whole DSM43989 cells activated macrophages as potently as did cells of a representative MAMES-positive *C. diphtheriae* strain (Fig. 4E and F).

TLR2 is essential for macrophage activation by whole corynebacteria and cell wall glycolipids. BMM from *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$;*Tlr4*$^{-/-}$ mice were employed to test the role of these TLRs in macrophage activation by corynebacteria and their cell wall glycolipids (Fig. 5). Control stimulations with ligands for Mincle, TLR4, and TLR9 showed no effect on TDB and CpG responses, as expected, yet strongly reduced responses to LPS in *Tlr4*$^{-/-}$ and no response in *Tlr2*$^{-/-}$;*Tlr4*$^{-/-}$ BMM (Fig. 5A and D). The residual production of NO in *Tlr4*$^{-/-}$ BMM might be attributable to potential contaminating TLR2 ligands in the LPS preparation used or contributive LPS recognition through TLR2 (39). *Tlr4*$^{-/-}$ macrophage responsiveness to the glycolipid extract as well as to whole heat-inactivated corynebacteria, in terms of both G-CSF production (Fig. 5B and C) as well as NO release (Fig. 5E and F), equaled that of wild-type (WT) controls. In contrast,

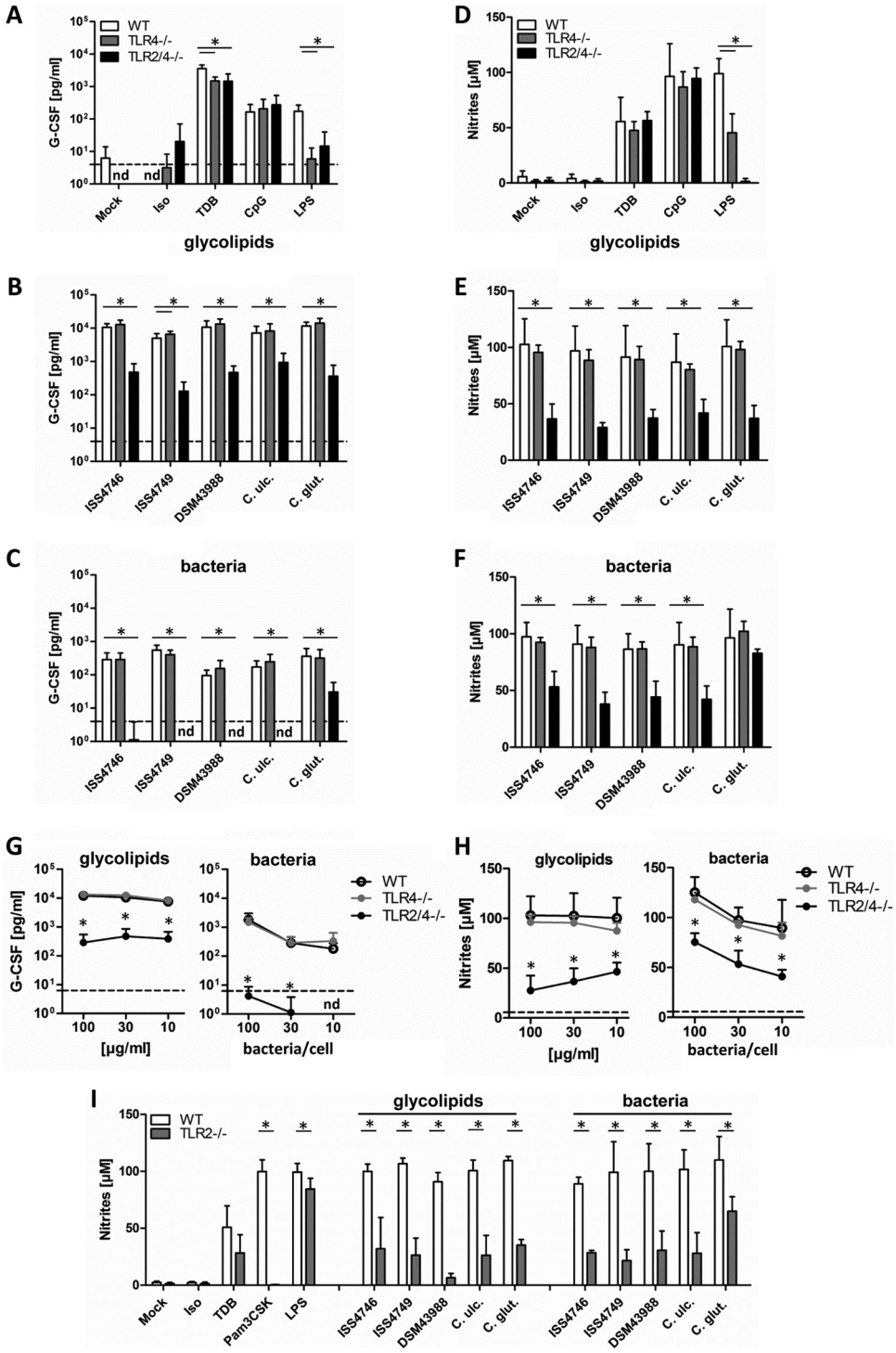

FIG 5 TLR2 deficiency impairs G-CSF and NO production in macrophages stimulated with corynebacteria or their cell wall glycolipids. (A to C) BMM from TLR4-deficient or TLR2/4-double-deficient mice and their respective wild-type controls were stimulated with different glycolipid extracts (30 μg/ml) or the corresponding heat-killed bacteria (30 bacteria per cell) as well as controls for 48 h, as indicated. G-CSF cytokine levels in cell culture supernatants were determined by using an ELISA. The detection limit was 4 pg/ml (dotted line). nd, not detectable. (D to F) NO production in cell culture supernatants was determined by using a Griess assay. BMM were cotreated with 10 ng/ml IFN-γ. BMM were stimulated with different concentrations of glycolipids (100, 30, or 10 μg/ml) or heat-killed bacteria (100, 30, or 10 bacteria per cell). Data shown were obtained with glycolipids from *C. diphtheriae* ISS4746; extracts from the other corynebacterial strains/species showed similar dose-response curves (not shown for clarity). (G and H) G-CSF levels and NO production were analyzed as described above. Dotted lines represent G-CSF or nitrite levels from unstimulated BMM. Note that data points for WT and TLR4$^{-/-}$ BMM overlap in panel G. Data are depicted as means and standard deviations of results from 3 independent experiments performed in

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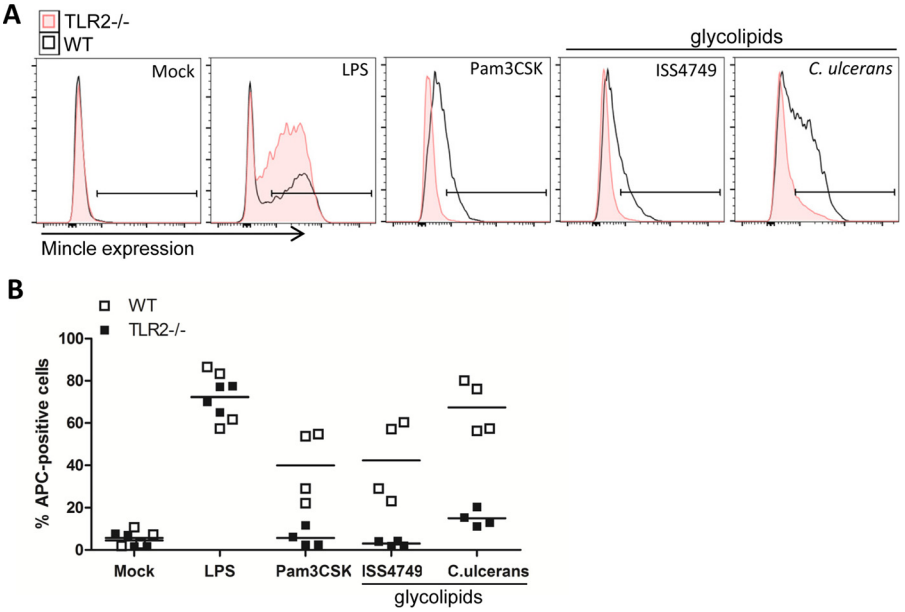

FIG 6 TLR2 deficiency leads to impaired Mincle upregulation on the cell surface. Shown are data from flow cytometric analysis of Mincle receptor expression on TLR2-deficient BMM and the respective wild-type controls. BMM were stimulated with 30 μg/ml cell wall extract of *C. diphtheriae* ISS4749 or *C. ulcerans* for 48 h. Unstimulated cells (mock) or BMM stimulated with 10 ng/ml LPS or 50 ng/ml Pam3CSK4 were used as controls. Panel A depicts representative histograms, indicating the gate used for the determination of Mincle-expressing macrophages shown in panel B. Data are depicted as single values from 2 independent experiments with biological duplicates ($n = 4$).

Tlr2$^{-/-}$;*Tlr4*$^{-/-}$ BMM secreted substantially less G-CSF in response to cell wall glycolipids and no detectable G-CSF after stimulation with whole corynebacteria (Fig. 5B and C). In addition, the levels of nitrites were significantly reduced in supernatants of double-deficient BMM (Fig. 5E and F), except for *C. glutamicum*. Dose-response experiments confirmed that *Tlr2*$^{-/-}$;*Tlr4*$^{-/-}$ but not *Tlr4*$^{-/-}$ BMM lack responsiveness to both cell wall extracts and whole corynebacteria (Fig. 5G and H). Results for *C. diphtheriae* strain ISS4746 are shown in Fig. 5G and H as a representative example, since a similar pattern was observed for *C. diphtheriae* strains ISS4749 and DSM43988, *C. ulcerans*, and *C. glutamicum*. Furthermore, the response to glycolipid extracts of DSM43989 was further reduced in *Tlr2*$^{-/-}$;*Tlr4*$^{-/-}$ BMM (see Fig. S2 in the supplemental material). Next, we applied *Tlr2*$^{-/-}$ BMM, which failed to respond to cell wall lipid extracts and whole corynebacteria (including *C. glutamicum*) (Fig. 5I), excluding an involvement of TLR4. Together, these results corroborate the previous implication of TLR2 as a receptor for corynebacterial cell wall lipoglycans (30, 31).

TLR2 is required for upregulation of Mincle expression on macrophages in response to corynebacteria. The expression of Mincle can be strongly induced by LPS in a C/EBPβ-dependent manner (40), which increases the sensitivity of macrophages to the Mincle ligand mycobacterial cord factor (37). We therefore asked whether TLR2-dependent stimulation by corynebacterial ligands may similarly upregulate Mincle expression and enhance the response to Mincle ligands in a feed-forward loop. Flow cytometry of resting BMM from WT and *Tlr2*$^{-/-}$ mice showed very low levels of cell surface Mincle protein and, as expected, strong and TLR2-independent upregulation after stimulation with LPS (Fig. 6A and B). Glycolipid extracts of *C. diphtheriae* strain ISS4749 and of *C. ulcerans* led to a more moderate but clearly detectable induction of

FIG 5 Legend (Continued)
duplicates ($n = 6$). (I) Wild-type and TLR2-deficient BMM were stimulated as described above for panels A to C, followed by NO measurement. Data are depicted as means and standard deviations of results from 3 independent experiments performed in duplicates ($n = 6$). In panels B, C, and E to I, * indicates a P value of <0.05.

Mincle receptor levels on WT, but not *Tlr2*$^{-/-}$, BMM (Fig. 6A and B). As expected, the widely used TLR2 ligand lipopeptide Pam3CSK4 upregulated Mincle in a TLR2-dependent manner (Fig. 6A and B). The inclusion of Mincle$^{-/-}$ BMM confirmed the specificity of staining with the 4A9 antibody in resting and stimulated BMM (see Fig. S3 in the supplemental material), consistent with previously reported results (41). Thus, TLR2 drives high-level Mincle expression in response to corynebacteria to optimize responsiveness to cell wall glycolipids of corynebacteria.

DISCUSSION

To the best of our knowledge, this is the first study investigating the role of a C-type lectin receptor in innate immune recognition of corynebacteria and to bring forward a nonredundant role for Mincle and its adapter protein FcRγ in macrophage activation by cell wall glycolipids of several pathogenic and commensal corynebacterial species. Our results suggest that corynomycolate esters of trehalose constitute the Mincle ligand(s), as the glycolipids from one *C. diphtheriae* strain lacking corynomycolic acids failed to bind Mincle and to activate macrophages. Our findings reveal the collaborative induction of inflammatory and antimicrobial gene expression by TLR2 and Mincle in corynebacterium-challenged macrophages involving the induction of Mincle expression. These results indicate that both TLR2 and Mincle-FcRγ may be important during human and veterinary infections with *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*.

The chemical nature of the Mincle ligand(s) from the corynebacterial cell wall preparations obtained by chloroform-methanol extraction in our study is unknown. However, the lack of stimulation by glycolipid extracts of *C. diphtheriae* strain DSM43989, which does not contain TMCM/TDCM (Fig. 4), suggested that mycolate-associated glycolipids confer Mincle binding and thereby activate macrophages. As reported in detail previously, the lack of cell wall mycolates in DSM43989 may be due to a deficiency in DIP0789, a putative enoyl coenzyme A (enoyl-CoA) hydratase (Ott et al., submitted). In a recent publication, van der Peet et al. reported the chemical synthesis of corynomycolic acids and tested trehalose and glucose mono- and diesters thereof for interactions with human and mouse Mincle using reporter cell lines (34). Notably, not only TDCM but also TMCM, and even glucose monocorynomycolate, triggered Mincle signaling and reporter gene activation. In contrast, when TDB was reduced toward a monosaccharide, namely, glucose behenate, it lost stimulatory activity (34). Similarly, testing of trehalose mono- and diesters of fatty acids of various lengths identified exclusively the diester forms as strong Mincle activators (36). Accordingly, a glucose monosaccharide is sufficient for binding to the carbohydrate recognition domain (CRD) provided that the lipid portion is of sufficient complexity (i.e., a corynomycolic acid and not a simple fatty acid). Conversely, the disaccharide trehalose binds with higher affinity to the CRD such that diesters of simple fatty acids can also trigger Mincle signaling.

Our finding that the robust production of G-CSF and of NO in response to cell wall extracts requires both TLR2 and Mincle suggests a collaboration of these pattern recognition receptors, which may occur at different levels (Fig. 7). First, we observed that the TLR2-driven induction of Mincle surface expression is operative and consistent with data from previous reports on TLR4 action (37, 40), MyD88-dependent upregulation in response to *Mycobacterium bovis* BCG (42), and TLR2 activity (43). This C/EBPβ-dependent feed-forward loop enhancing the response to Mincle ligands (37) most likely accounts for the TLR2-driven G-CSF production in response to cell wall glycolipid challenge, although we acknowledge that formal proof of this mechanism would require the demonstration that the overexpression of Mincle, perhaps in association with other CLRs like Mcl, circumvents TLR2 dependence. Second, the concurrent triggering of Mincle by trehalose corynomycolates and of TLR2 by ligands such as LM/LAM (31) and phosphatidylinositol mannosides (PIMs) (44), respectively, might quantitatively, qualitatively, and synergistically enhance signaling toward transcriptional and translational immune responses. While Mincle and TLR2 showed synergism

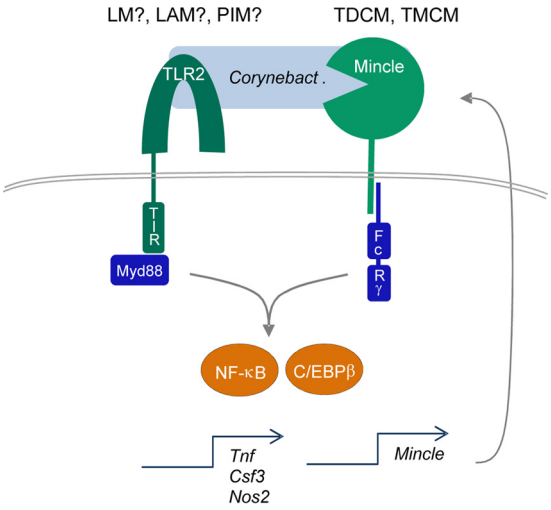

FIG 7 Synergistic induction of macrophage activation by the corynebacterial cell wall through TLR2 and Mincle signaling. The cell wall of corynebacteria contains TLR2 ligands, which activate macrophages through MyD88-dependent signaling. Lipomannan (LM) and Lipoarabinomannan (LAM) have been identified as TLR2 ligands from corynebacteria (31); PIMs from mycobacteria activate TLR2 (44) and are present in corynebacterial glycolipid extracts. Mincle binds to glycolipids containing mycolates (likely TDCM and TMCM), which activates FcRγ-Syk-dependent gene expression (*Csf3* encodes G-CSF, and *Nos2* encodes iNOS). Both pathways synergize at the level of NF-κB. In addition, TLR2- and C/EBPβ-dependent upregulation of Mincle mRNA and protein expression increases cell surface Mincle receptor expression and responsiveness to TDCM/TMCM in murine macrophages.

in the induction of G-CSF and NO, the dual triggering of both pathways may also have antagonistic effects on other responses and accordingly shape the output of the macrophage in terms of cytokines and antimicrobial mediators. Indeed, the Mincle ligand TDM inhibited interleukin-12p40 (IL-12p40) production induced by the TLR2 ligand Pam3CSK4 via IL-10 production in murine macrophages (45), and Wevers et al. observed that the ligation of Mincle downregulated IL-12p35 expression induced by TLR or Dectin-1 stimulation via phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB)-dependent MDM2-mediated degradation of IRF-1 in human dendritic cells (DC) (46). An interesting mechanism for the synergistic induction of the iNOS protein by TDM and Pam3CSK4 through increased translational efficiency dependent on hypusination of the elongation factor eukaryotic initiation factor 5 (eIF5) was recently described by Lee and colleagues (43). Thus, it will be interesting to investigate the differential regulation of a wider set of inflammatory and regulatory cytokines by Mincle and TLR2 pathways after macrophages encounter corynebacteria.

An important question arising is how the stimulation of the receptors Mincle and TLR2 on macrophages impacts the course of infections with corynebacterial species *in vivo*. *C. pseudotuberculosis* causes caseous lymphadenitis in sheep and goats, a chronic granulomatous infection with histopathological similarities to mycobacterial infections (47), and the amount of cell wall lipids in *C. pseudotuberculosis* strains has been correlated positively with the severity of abscess formation in a mouse model (29). Mice deficient in the Mincle-FcRγ and TLR2 pathways should be valuable to investigate the differential roles of glycolipid recognition in inflammation, the control of bacterial replication, and the differentiation of Th cell responses *in vivo*. As corynebacteria are abundant commensals on human skin and mucosal surfaces, Mincle- versus TLR2-dependent responses to their cell wall glycolipids by macrophages and DC may also contribute to protective and/or pathogenic immune responses. Therefore, it will be important to determine whether human myeloid cells respond similarly to corynebacterial cell wall components, as shown here for murine macrophages, in future experiments.

TABLE 1 *Corynebacterium* strains used

Strain	Description	Reference and/or source
C. diphtheriae ISS4746	*tox* negative, throat smear, *C. diphtheriae* subsp. *gravis*	16
C. diphtheriae ISS4749	*tox* negative, throat smear, *C. diphtheriae* subsp. *gravis*	16
C. diphtheriae DSM43988	Apathogenic isolate of throat smear, ATCC 11913	DSMZ
C. diphtheriae DSM43989	Pathogenic, unknown origin, ATCC 13812, tox^+	38, DSMZ
C. ulcerans 809	Patient isolate from fatal pulmonary infection	53
C. glutamicum ATCC 13032	Wild type	54

MATERIALS AND METHODS

Reagents. Synthetic TDB was purchased from Avanti Polar Lipids (Alabaster, AL, USA), solubilized in isopropanol at a concentration of 2.5 mg/ml. LPS of *Escherichia coli* serotype O55:B5 (Sigma-Aldrich, Deisenhofen, Germany) was taken up in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml. B-type CpG ODN 1826 was synthetized by TIB Molbiol (Berlin, Germany) and reconstituted to a stock concentration of 1 mM. Pam3CSK4 was obtained from EMC Microcollections GmbH (Tübingen, Germany). All TLR and CLR ligands were stored as stocks at −20°C.

Culture of corynebacteria. Bacterial strains used in this study are shown in Table 1. All *C. diphtheriae* and *C. ulcerans* strains were grown in Bacto heart infusion (HI) broth (Becton Dickinson and Company, France) at 37°C. *C. glutamicum* was grown in brain heart infusion (BHI) broth (Oxoid, Hampshire, England) at 37°C.

Preparation of cell wall glycolipids. For glycolipid extraction, bacterial cultures were grown to an optical density of 0.4 to 0.6 and harvested. Cells were incubated in chloroform-methanol (1:2) overnight, followed by filtration through the appropriate filter paper. Cells were incubated in chloroform-methanol (1:1) for 5 h and filtered through the same filter paper, followed by further overnight incubation in chloroform-methanol (2:1). Filtrates were collected in round-bottom flasks. The solvent was removed by rotary evaporation. Isolated glycolipids were dissolved in chloroform-methanol (2:1), sonicated, and stored at 4°C. To roughly correlate glycolipid extract amounts to the corresponding number of corynebacteria, we assumed that (i) bacterial growth stopped directly after transfer of the culture to ice and (ii) no glycolipid was lost during preparation. Calculating 5×10^8 bacteria per optical density at 600 nm (OD_{600}) unit, we estimate that 1 μg of glycolipid extracts corresponds to 1.45×10^7 CFU. Thus, a concentration of 10 μg/ml glycolipid extract in a well with 2×10^5 BMM would roughly correspond to a bacterium-to-cell ratio of 145.

Extraction and analytical thin-layer chromatography of total lipids and fatty acid and mycolic acid methyl esters. Total lipids were extracted from *Corynebacterium* spp. by using protocols described previously for *C. glutamicum* (48). The total lipids were further fractionated by chilled acetone precipitation to generate a predominantly phospholipid-rich, polar lipid pellet and a predominant apolar lipid-rich supernatant. Aliquots from all fractions were analyzed by TLC (silica gel 60 F254 plates) using chloroform-methanol-water (60:16:2) as the solvent system and MPA–α-naphthol as a stain to visualize lipids/glycolipids.

Methyl esters of fatty and mycolic acids were extracted by using protocols described previously for *C. glutamicum* (48). The bound mycolates were released from delipidated cells or free glycolipid extracts by using 2 ml of a 5% aqueous solution of tetrabutylammonium hydroxide and subsequent overnight incubation at 95°C. The cooled suspension was then mixed thoroughly with water (2 ml), CH_2Cl_2 (4 ml), and CH_3I (500 μl) for 30 min. The lower organic phase was recovered after centrifugation and washed three times with 4 ml of water. The organic extract was then dried and resuspended in 4 ml diethyl ether. Particulate, insoluble matter was removed by centrifugation; the resultant clear supernatant was dried and resuspended in 200 μl CH_2Cl_2. TLC of aliquots of the extract was performed by using silica gel 60 F254 plates developed in petroleum ether-acetone (95:5, vol/vol).

Mincle-Fc binding assay. Blocking and incubation steps were performed with Hanks' balanced salts solution (HBSS) plus 3% bovine serum albumin (BSA) in a working volume of 50 μl at room temperature. Washing steps were performed with HBSS only. Ninety-six-well cell culture plates were coated with glycolipids as previously described (22, 49) and blocked for 2 h. Murine Mincle-Fc and Clec9a-Fc fusion proteins or the Fc protein alone (35) was added and incubated at a final concentration of 200 ng/ml for 6 h, followed by at least 5 washing steps. Anti-human Fc horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson) was added at a final concentration of 1.6 μg/ml and incubated for 1 h, followed by at least 8 washing steps. Detection was performed by using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent (BD Bioscience).

Isolation and culture of mouse macrophages. *Clec4e*$^{-/-}$ mice were generated by the Consortium for Functional Glycomics and used with permission (50). *Fcerg1*$^{-/-}$ mice were described previously (51), and a breeding pair was kindly provided by Falk Nimmerjahn. C57BL/6, *Clec4e*$^{-/-}$, and *Fcerg1*$^{-/-}$ mice were bred at the Präklinische Experimentelle Tierzentrum of the Medical Faculty of the Friedrich Alexander University Erlangen-Nürnberg. *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *TLR2*$^{-/-}$;*Tlr4*$^{-/-}$ mice were bred at the animal facility of the University Hospital Essen. Bone marrow cells from femurs and tibiae were differentiated to macrophages by culture in complete Dulbecco's modified Eagle medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (FBS) (Biochrome), antibiotics, and 50 μM β-mercaptoethanol (complete DMEM [cDMEM]) plus 10% L929 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF), as previously described (52). On day 7, adherent macrophages were harvested by Accutase (Sigma, Deisenhofen, Germany) treatment, washed, and counted.

Cell stimulation. Cell wall extracts of corynebacteria (100 μg/ml, 30 μg/ml, or 10 μg/ml) as well as TDB (5 μg/ml; Polar Avanti) were used plate bound, as previously described (22, 49). For coating, cell wall extracts and TDB were dissolved in isopropanol. Isopropanol only was used as a negative control. LPS (100 ng/ml) and CpG (0.5 μM) were additionally used as positive controls. Whole bacteria were grown to an optical density (OD_{600}) of 0.4 to 0.6, harvested, and washed in PBS. Bacteria were inactivated in a water bath for 15 min at 70°C and resuspended in cDMEM before use. For stimulation, a bacterium-to-cell ratio of 100, 30, or 10 was used.

Cytokine ELISA. The cytokine concentration of murine G-CSF was analyzed by a sandwich ELISA (DuoSet ELISA; R&D Systems) using cell-free cell culture supernatants of cells stimulated as indicated.

Griess assay. NO production of BMM was assessed via a Griess assay. BMM were cotreated with IFN-γ.

Flow cytometry of Mincle surface receptor levels. A total of 2×10^5 bone marrow-derived macrophages of C57BL/6 and *Tlr2*$^{-/-}$ mice were stimulated with LPS (10 ng/ml), Pam3CSK4 (50 ng/ml), or different cell wall extracts of corynebacteria (30 μg/ml), as indicated. BMM were stimulated for 48 h in F-bottom 96-well cell culture plates. Receptor surface expression of Mincle was assessed by flow cytometry as described previously (41). Staining was performed by using anti-Mincle (clone 4A9; MBL) (24) as a primary antibody and anti-rat IgG1 conjugated to allophycocyanin (APC) as a secondary antibody (eBioscience, Frankfurt, Germany) at a final concentration of 1 μg/ml for anti-Mincle and anti-rat IgG-APC. Fc receptors were blocked by adding anti-mouse CD16/32 (clone 93; eBioscience) at a final concentration of 2.5 μg/ml before staining. Cells were stained with primary antibodies for 20 min at 4°C, washed, and then stained with secondary antibody for 20 min at 4°C. Flow cytometry data were acquired on a FACSCanto II instrument (BD), and analysis was carried out by using FlowJo (version 10).

Statistical analysis. Statistical analysis was performed by using GraphPad Prism (version 5). A two-tailed Mann-Whitney U test was applied as indicated for nonpaired testing between two groups. *P* values of <0.05 were considered significant and are indicated by asterisks in the figures.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00075-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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