Cutting Edge: Mincle Is Essential for Recognition and Adjuvanticity of the Mycobacterial Cord Factor and its Synthetic Analog Trehalose-Dibehenate

Hanne Schoenen, Barbara Bodendorfer, Kelly Hitchens, Silvia Manzanero, Kerstin Werninghaus, Falk Nimmerjahn, Else Marie Agger, Steffen Stenger, Peter Andersen, Jürgen Ruland, Gordon D. Brown, Christine Wells and Roland Lang

*J Immunol* 2010; 184:2756-2760; Prepublished online 17 February 2010; doi: 10.4049/jimmunol.0904013
http://www.jimmunol.org/content/184/6/2756

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/02/15/jimmunol.0904013.DC1

**References**

This article cites 26 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/184/6/2756.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Mincle Is Essential for Recognition and Adjuvanticity of the Mycobacterial Cord Factor and its Synthetic Analog Trehalose-Dibehenate

Hanne Schoenen,* Barbara Bodendorfer,* Kelly Hitchens,† Silvia Manzanero,‡ Kerstin Werninghaus,* Falk Nimmerjahn,‡ Else Marie Agger,‡ Steffen Stenger,‡ Peter Andersen,‡ Jürgen Ruland,‖ Gordon D. Brown,‡ Christine Wells,† and Roland Lang*

The mycobacterial cord factor trehalose-6,6-dimycolate (TDM) and its synthetic analog trehalose-6,6-dibehenate (TDB) are potent adjuvants for Th1/Th17 vaccination that activate Syk-Card9 signaling in APCs. In this study, we have further investigated the molecular mechanism of innate immune activation by TDM and TDB. The Syk-coupling adapter protein FcRy was essential for macrophage activation and Th17 adjuvanticity. The FcRy-associated C-type lectin receptor Mincle was expressed in macrophages and upregulated by TDM and TDB. Recombinant Mincle-Fc fusion protein specifically bound to the glycolipids. Genetic ablation of Mincle abolished TDM/TDB-induced macrophage activation and induction of T cell immune responses to a tuberculosis subunit vaccine. Macrophages lacking Mincle or FcRy were impaired in the inflammatory response to Mycobacterium bovis bacillus Calmette-Guérin. These results establish that Mincle is a key receptor for the mycobacterial cord factor and controls the Th1/Th17 adjuvanticity of TDM and TDB. The Journal of Immunology, 2010, 184: 2756–2760.

The development of recombinant subunit vaccines has been hampered by the lack of safe and effective adjuvants for human use. For many years, research into adjuvants was neglected and only recently has been invigorated by the discovery of pattern recognition receptors (PRR) for microbial ligands (so-called pathogen-associated molecular patterns) on APCs. Modern, molecularly defined adjuvants may be used to induce appropriate types of immune responses through engagement of specific receptors and pathways.

*Institute of Clinical Microbiology, Immunology and Hygiene and †Division of Rheumatology and Immunology, Medical Department, Friedrich-Alexander-Universität Erlangen-Nürnberg and University Clinics of Erlangen, Erlangen; ‡Institute of Medical Microbiology, University Hospital Ulm, Ulm; †Department of Hematology, Technical University Munich, Munich, Germany; †Griffith University, Queensland, Australia; ‡Adjuvant Research, Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark; and †Section of Immunology and Infection, Division of Applied Medicine, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom

Received for publication December 16, 2009. Accepted for publication January 20, 2010.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB796, TP B6 to R.L.; SFB576 to J.R.; SFB 643 and FOR832 to F.N.) and the European Union (FP6 TBVAC to E.M.A., P.A., and R.L., Contract Number LSHP-CT-2005-503367), a Max-Eder-Programm grant from Deutsche Krebshilfe (to J.R.), the Wellcome Trust (to G.D.B.), and The National Health and Medical Research Council, Australia (455947, 597452, and 481945 to C.W.).

Address correspondence and reprint requests to Dr. Roland Lang, University Clinics Erlangen, Wasserturmstrasse 3-5, Erlangen 91054, Germany. E-mail address: roland.lang@uk-erlangen.de.

The online version of this article contains supplemental material.

Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; BMM, bone marrow-derived macrophage; CLEC, C-type lectin receptor; DDA, dimethyldioctadecylammonium; PRR, pattern recognition receptor; qRT-PCR, quantitative RT-PCR; TDB, trehalose-6,6-dibehenate; TDM, trehalose-6,6-dimycolate.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00
which is also essential for adjuvanticity in vivo. We excluded Dectin-1 as an essential receptor for TDB and TDM, but obtained evidence in vitro that the adapter protein FcR may couple a receptor protein to Syk-Card9 signaling (4).

In this study, we show that the FcR-coupled CLEC Mincle binds the glycolipids TDB and TDM, is essential for activation of macrophages and adjuvant activity, and plays a non-redundant role in the macrophage response to intact mycobacteria.

Materials and Methods

Reagents, mice, and immunizations
TDB, TDM, CpG, and dimethyl dioctadecylammonium (DDA) liposomes have been described (4). Myd88<sup>/−/−</sup> and Fcεr1<sup>/−/−</sup> (referred to as FcR<sub>g</sub><sup>/−/−</sup> mice in this paper) were used with permission from Drs. Akira and Takai, respectively (16, 17). Card9<sup>/−/−</sup> and Mincle<sup>/−/−</sup> mice have been described (2, 18). Animal experiments were approved by the Regierung von Oberbayern. Mice were immunized s.c. with Ag85B-ESAT-6 (H1) mixed with adjuvants as described (4).

Bacteria

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) glycerol stocks were prepared at a density of 1.7×10<sup>9/ml</sup>. *Escherichia coli* (DH5α) and *Staphylococcus aureus* ATCC R5923 were cultured on blood agar, picked, and adjusted to a density of 10<sup>8/ml</sup> before freezing. All bacteria were diluted in complete DMEM before use for macrophage stimulation at the indicated multiplicity of infection.

Generation of macrophages from bone marrow and stimulation with glycolipids was done as described (4). The glycolipids TDB and TDM were coated directly to cell culture plates following the procedure described in Ref. 19.

Measurement of nitrites, cytokines, and mRNA expression was done as described (4).

Recombinant CLECs and glycolipid-binding ELISA
TDB, TDM, and curdlan were coated onto 96-well cell culture plates at 40, 10, and 2.5 µg/ml. After washing and blocking with 3% BSA in HBSS, supernatant of Mincle-Fc–transfected HEK-293 cells was added at a dilution of 1:4 in blocking buffer. Following incubation at 4°C overnight, plates were washed three times, bound protein detected by incubation with anti-human Fc Ab conjugated to peroxidase, washed again, and developed with ELISA substrate.

Results and Discussion

FcγR-dependent activation of macrophages by TDB and the cord factor

Previously, we demonstrated that the glycolipid adjuvants TDM and TDB activate the Syk-Card9-Bcl10-Malt1 pathway in APCs (4). In addition, TDM/TDB-induced NO production is independent of Dectin-1, but requires FcRγ (4). To corroborate and extend these data, we tested whether the expression of G-CSF, IL-1β, and arginase-1 requires FcRγ (Supplemental Fig. 1). As expected, TLR9– as well as Dectin-1–triggered induction was not affected by the deletion of FcRγ, whereas TDB and TDM failed to induce G-CSF and IL-1β expression in FcRγ<sup>/−/−</sup> bone marrow-derived macrophage (BMM). FcRγ also associates with the activating Fcγ receptors [FcγRI (CD64), FcγRIII (CD16), and FcγRIV] that can also bind non-Ig ligands (20). However, although NO production was completely abrogated in the absence of...
FCR, none of the Fcγ receptor-deficient BMM genotypes showed a substantial impairment in NO release (Supplemental Fig. 2). Thus, FcRγ is essential for transcriptional responses to TDM/TDB, but Fcγ receptors are not involved.

**FcRγ-dependent Th17 adjuvant activity of TDB**

The adjuvanticity of TDB/TDM is abrogated in Card9−/− mice, including the locoregional inflammatory reaction and the induction of Th1/Th17 biased Ag-specific T cells (4). As FcRγ deletion closely mimicked the phenotype of Card9−/− in BMM, we next asked whether FcRγ is also essential for the induction of cellular immunity to subunit vaccination with the M. tuberculosis fusion protein Ag85B-ESAT-6 (H1) by the adjuvant TDB in vivo. The moderate footpad swelling post-injection of H1 in liposomes alone was not affected by the absence of FcRγ. In contrast, the large increase in footpad thickness in the TDB-containing adjuvant group was almost entirely abrogated in FcRγ−/− mice (Fig. 1A). The induction of IFN-γ-producing T cells was reduced to the levels obtained with liposomes alone in FcRγ−/− (Fig. 1B), and the strong induction of IL-17–producing H1-specific T cells by the adjuvant TDB was completely FcRγ-dependent (Fig. 1C). The similarity between the phenotype of FcRγ−/− and Card9−/− mice indicates that FcRγ is critical for the Syk-Card9–dependent activation of APCs. The lack of residual adjuvant activity of TDB in FcRγ-deficient mice suggested that other adapter molecules or receptors that directly recruit Syk are most likely not involved.

**Mincle expression, regulation, and binding to TDB and cord factor**

In our search for a receptor for the TDM and TDB, we focused on the family of CLEC because we had excluded Fcy receptors (Supplemental Fig. 2). We reasoned that the putative receptor should be expressed in macrophages and mined our microarray datasets from BMM (GSE10530 and GSE10532 in Gene Expression Omnibus, www.ncbi.nlm.nih.gov/sites/entrez?db=geo; see Ref. 4) for expression of CLEC family members (Supplemental Fig. 3). Among the expressed CLECs, Dectin-1 had already been ruled out as a receptor (4); Clec5a associates with Dap12 (10), and the adapter protein coupling Clec4d/Clecfs8 to Syk is unknown. In contrast, Mincle and Dectin-2 stood out because they are FcRγ-associated receptors and were expressed and upregulated in BMM by TDB. In a second microarray dataset, all TDB regulation of CLEC receptors was Card9-dependent (Supplemental Fig. 4). Validating expression of Mincle mRNA by quantitative RT-PCR (qRT-PCR), we observed a complete dependence of the TDM/TDB-induced increase on FcRγ (Fig. 2A). Mincle and Dectin-2 are most likely not involved.

In our search for a receptor for the TDM and TDB, we focused on the family of CLEC because we had excluded Fcγ receptors (Supplemental Fig. 2). We reasoned that the putative receptor should be expressed in macrophages and mined our microarray datasets from BMM (GSE10530 and GSE10532 in Gene Expression Omnibus, www.ncbi.nlm.nih.gov/sites/entrez?db=geo; see Ref. 4) for expression of CLEC family members (Supplemental Fig. 3). Among the expressed CLECs, Dectin-1 had already been ruled out as a receptor (4); Clec5a associates with Dap12 (10), and the adapter protein coupling Clec4d/Clecfs8 to Syk is unknown. In contrast, Mincle and Dectin-2 stood out because they are Fcγ-associated receptors and were expressed and upregulated in BMM by TDB. In a second microarray dataset, all TDB regulation of CLEC receptors was Card9-dependent (Supplemental Fig. 4). Validating expression of Mincle mRNA by quantitative RT-PCR (qRT-PCR), we observed a complete dependence of the TDM/TDB-induced increase on Fcγ and Card9, but not on the TLR adapter protein Myd88 (Fig. 2A). Mincle can interact with ligands of diverse chemical nature (9, 18, 21). We therefore investigated whether it directly binds to TDM and TDB. A Mincle-Fc fusion protein specifically and dose-dependently bound to TDM and TDB, but not to curdlan (Fig. 2B). These data demonstrate that the mycobacteria-derived glycolipids TDM and TDB are newly recognized ligands of Mincle.
Mincle is required for macrophage activation and Th1/Th17 induction by TDM and TDB

Mincle−/− mice have been generated independently by two groups (18, 21). We first studied Mincle−/− macrophages in vitro and observed a complete abrogation of the TDM/ TDB-induced expression of G-CSF and IL-6; in contrast, the response to curdlan or CpG was unaltered (Fig. 3A, Supplemental Fig. 5). Likewise, the production of NO by IFN-γ–primed BMM specifically required Mincle when the cells were stimulated with TDM or TDB (Fig. 3B). The role of Mincle in generating the adjuvant effect of TDB in vivo was studied again using the H1 subunit vaccination protocol. The cellularity in the draining lymph node was significantly reduced in Mincle−/− mice when TDB was the adjuvant, but not when CpG was applied (Fig. 3C). The functional analysis of the T cell response to H1 revealed a significantly impaired Th1 response for TDB but not CpG or liposomes alone (Fig. 3D). Robust IL-17 production postimmunization was only observed when TDB was included as adjuvant and strictly depended on Mincle (Fig. 3E). Thus, the deletion of any component of the Mincle-FcR-Card9 signaling pathway destroyed the capacity of TDB to drive Th1 and Th17 immune responses to protein Ag in vivo. These data identify Mincle as an innate PRR that is targeted by TDM and TDB and controls the generation of cellular immunity known to be linked to protection against mycobacterial infection (4).

Mincle-FcRγ–dependent signaling in recognition of mycobacteria

The cord factor is a virulence factor of pathogenic mycobacteria that is sufficient to cause granuloma formation and tissue damage (22). Given the abundance of TDM in the mycobacterial cell wall, binding to Mincle may occur when macrophages encounter mycobacteria. Indeed, Mincle−/− BMM showed a significant reduction in the expression of G-CSF and IL-6 poststimulation with M. bovis BCG that was not observed for E. coli or S. aureus (Fig. 4). Deletion of FcRγ caused a similar effect on the response to BCG, suggesting that Mincle-FcRγ–dependent recognition of TDM synergizes with Myd88-dependent activation by multiple mycobacterial TLR ligands (1) for full induction of gene expression in macrophages with mycobacteria (Supplemental Fig. 6).

Concluding remarks

The data presented in this study show that Mincle is a pivotal receptor for the mycobacterial cord factor, entirely in agreement with the findings of Ishikawa et al. (23) published during the review process of this paper. It should be pointed out that additional receptors may bind TDM independently or in cooperation with Mincle. Candidates include other CLEC proteins, such as Dectin-2, which also associates with FcRγ, is expressed in macrophages, and binds to M. tuberculosis (24). The scavenger receptor MARCO interacts with TDM, yet lacks an intracellular domain for signal initiation (25). In contrast, Mincle can directly trigger Syk-Card9 signaling via its association with FcRγ. The impaired macrophage response to mycobacteria in the absence of Mincle raises the question about the function of Mincle and the subsequent Syk-Card9–dependent response for the control of mycobacteria in vivo; cord factor recognition by Mincle may be beneficial for the host by increasing cytokines and antimicrobial effector molecules, but a downregulatory role (e.g., through G-CSF and IL-10) also seems possible (26).

Our data reveal the molecular mechanism underlying the Th1/Th17 adjuvanticity of the glycolipids TDM and TDB. The identification of Mincle as the TDB receptor provides the target structure for this promising synthetic glycolipid adjuvant that has entered clinical studies for tuberculosis subunit vaccination. These results also suggest new possibilities of modulating vaccination responses (e.g., by targeting Mincle with specific Abs coupled to Ag) as a dual function receptor for Ag delivery and APC activation.

Acknowledgments

The technical assistance of Katrin Jozefowski is appreciated. We thank Dr. Christian Bogdan for critical review of the manuscript and Manfred Kirsch, Melissa Wojak, and Verena Laux for help with mice.

Disclosures

P.A. is a coinventor of patents relating to cationic liposomes as vaccine adjuvants.

References


Supplemental Figure 1
FeRγ-dependent macrophage activation

BMM of FeRγ1−/− and wt mice were stimulated as indicated for 24 hours. TDB (5 µg/ml), TDM (5 µg/ml) and Curdlan (500 µg/ml) were immobilized on cell culture plastic before addition of the cells, CpG (1 µM) was added in solution. Changes in expression of G-CSF, Arginase-1 and IL-1β were measured by qRT-PCR using the Roche Universal Probe Library system. Fold changes were calculated with the ΔΔCT method using the untreated wt sample as calibrator. Mean and SD of four replicates of one representative experiment of at least three performed.
**Supplemental Figure 2:**

**Intact NO production in Fc gamma receptor knockout macrophages stimulated with TDB or TDM.**

BMM of the indicated genotypes were stimulated as indicated in the presence of IFNγ (20 ng/ml) for 48 h. Supernatants were analyzed by Griess assay for nitrite levels. Mean and SD of triplicates wells from one experiment of two giving similar results.
Supplemental Figure 3:
CLEC expression in BMM

Microarray data mining for changes in CLEC expression in BMM stimulated with CpG ODN or TDB (Gene Expression Omnibus dataset GSE 10532). Probesets detecting CLEC mRNAs with a signal intensity of >20 were selected, z-score normalized and subjected to hierarchical clustering. Yellow indicates relative upregulation, blue relative downregulation across all experimental conditions. Alternative gene names are indicated for TDB-regulated CLEC.

Supplemental Figure 4:
Regulated expression of CLEC genes in wt and Card9-/- macrophages after stimulation with CpG, TDB and Curdlan.

Data are from Gene Expression Omnibus dataset GSE 10530, IFNγ-primed macrophages stimulated for 48 h. Heatmap of z-score normalized expression data from CLEC mRNA detecting probesets showing a signal of >40 in at least one of eight microarray samples. Original microarray data can be accessed at http://www.ncbi.nlm.nih.gov/sites/entrez?db=geo
Supplemental Figure 5:
Mincle-/- macrophages fail to produce cytokines after stimulation with TDB or TDM.

BMM were stimulated in 96-well plates for 48 h with TDB/TDM (0.015 – 4 µg/ml) or CpG (1 µM). Supernatants were analysed by ELISA for G-CSF and IL-6 production. Mean and SD of triplicate samples from a representative experiment of at least three performed.
Supplemental Figure 6: Contribution of Mincle-FcRγ-signaling to recognition of mycobacteria.

BMM were stimulated with live *Mycobacterium bovis* BCG for 24 h at the indicated MOI, followed by preparation of RNA from the cell lysate. Expression of IL-6 (left panels) and G-CSF (right panels) was analysed by qRT-PCR. Shown are mean and SD of quadruplicate samples from a representative of at least two experiments.

(A) MyD88-/-, (B) FcRγ-/-, (C) Mincle-/- BMM.