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C-Type Lectin Receptor MCL Facilitates Mincle Expression and Signaling through Complex Formation

Yasunobu Miyake,* Masatsugu Oh-hora,* and Sho Yamasaki*,†

C-type lectin receptors expressed in APCs are recently defined pattern recognition receptors that play a crucial role in immune responses against pathogen-associated molecular patterns. Among pathogen-associated molecular patterns, cord factor (trehalose-6,6′-dimycolate [TDM]) is the most potent immunostimulatory component of the mycobacterial cell wall. Two C-type lectin receptors, macrophage-inducible C-type lectin (Mincle) and macrophage C-type lectin (MCL), are required for immune responses against TDM. Previous studies indicate that MCL is required for TDM-induced Mincle expression. However, the mechanism by which MCL induces Mincle expression has not been fully understood. In this study, we demonstrate that MCL interacts with Mincle to promote its surface expression. After LPS or zymosan stimulation, MCL-deficient bone marrow–derived dendritic cells (BMDCs) had a lower level of Mincle protein expression, although mRNA expression was comparable with wild-type BMDCs. Meanwhile, BMDCs from MCL transgenic mice showed an enhanced level of Mincle expression on the cell surface. MCL was associated with Mincle through the stalk region and this region was necessary and sufficient for the enhancement of Mincle expression. This interaction appeared to be mediated by the hydrophobic repeat of MCL, as substitution of four hydrophobic residues within the stalk region with serine (MCL^{4S}) abolished the function to enhance the surface expression of Mincle. MCL^{4S} mutant failed to restore the defective TDM responses in MCL-deficient BMDCs. These results suggest that MCL positively regulates Mincle expression through protein–protein interaction via its stalk region, thereby magnifying Mincle-mediated signaling. ***The Journal of Immunology*****, 2015, 194: 5366–5374.**

Mycobacterium tuberculosis is a deleterious pathogen that causes the life-threatening infectious disease, tuberculosis. A wide variety of pattern recognition receptors (PRRs) elicit an anti-mycobacterial response upon sensing *M. tuberculosis* (1). Among these PRRs, macrophage-inducible C-type lectin (Mincle; also called Clec4e) recognizes mycobacterial glycolipid, trehalose-6,6′-dimycolate (TDM; also called cord factor), and activates innate and acquired immune responses, including proinflammatory cytokine production, Th1/Th17 responses, and granulomagenesis (2, 3). These TDM responses are completely abrogated in Mincle-deficient mice, indicating that Mincle is an essential receptor for TDM responses (2, 3). Importantly, Mincle is involved in the host immune responses against mycobacterial infection (4–6).

Macrophage C-type lectin (MCL; also called Clec4d) is a type II transmembrane C-type lectin receptor (CLR) expressed in myeloid cells that shares a high sequence similarity with Mincle (7, 8). MCL has been proposed to be an activating receptor (9). We and other groups have reported that MCL also plays a crucial role in TDM responses (6, 10, 11). MCL deficiency results in impaired TDM-induced Mincle expression and, consequently, MCL-deficient mice show a severe defect in TDM responses. Recently, MCL has been reported to trigger Mincle expression through NF-κB activation during stimulation with TDM (11). However, the regulation of Mincle expression by MCL has not been fully understood.

Mincle expression is also induced by other pathogen-associated molecular patterns and cytokines (10, 12, 13). Mycobacteria contain a wide variety of immunostimulatory components other than Mincle ligand (14). Mincle induction upon *M. tuberculosis* infection or LPS stimulation was impaired in MCL-deficient mice (6, 10, 12, 13). It is therefore possible that MCL may also have additional functions on Mincle beyond transcriptional regulation.

Several CLRs form homo-/hetero-oligomers to increase their avidity for multivalent ligands (15). MCL and Dectin-2 form a heteromeric complex to enhance their ability to recognize fungus (16). MCL is also reported to associate with Mincle, and the coexpression of MCL with Mincle increases the expression of Mincle (17). However, the physiological significance of this interaction has not been clear.

In this study, we examined the molecular mechanisms underlying MCL–Mincle complex formation. We found that MCL–Mincle interaction via the stalk region of MCL enhances protein expression of Mincle, resulting in the augmentation of TDM-induced responses.

Materials and Methods

Reagents

Anti-Mincle (1B6 and 4A9) and anti-MCL (1K2-5) were established as described previously (6, 18). Anti-actin (4970) and anti-Flag (1E6) were obtained from Cell Signaling Technology and Wako Pure Chemical Industries, respectively. TDM (T3034), zymosan (Z4250), and LPS (L4516) were purchased from Sigma-Aldrich. ELISA kits for MIP-2 (DY452) and TNF (558534) were from R&D Systems and BD Biosciences.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; CLR, C-type lectin receptor; CRD, carbohydrate recognition domain; HEK, human embryonic kidney; MCL, macrophage C-type lectin; MDchimera, macrophage C-type lectin–Dectin-2 chimera; Mincle, macrophage-inducible C-type lectin; PRR, pattern recognition receptor; TDM, trehalose-6,6′-dimycolate; Tg, transgenic; WT, wild-type.

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Mice

Mincle$^{-/-}$ mice were generated previously (19) and backcrossed at least 10 generations with C57BL/6 mice. FcRγ$^{-/-}$ mice on a C57BL/6 background were provided by T. Saito (RIKEN Institute, Saitama, Japan) (20). MCL$^{-/-}$ mice were established previously (6) and backcrossed at least five generations with C57BL/6 mice. MCL transgenic (Tg) mice and Mincle Tg mice were generated on a C57BL/6 background. In brief, murine MCL cDNA containing Flag tag at the C terminus or murine Mincle cDNA was inserted into pCAGGS vector (21). Tg mouse lines were produced by injecting the linearized vector into C57BL/6 fertilized eggs. Among several lines, we used line no. 10-4 for MCL Tg mice and line no. 9-2 for Mincle Tg in this study. C57BL/6 mice were obtained from CLEA Japan. All mice were maintained in a filtered-air laminar-flow enclosure and given standard laboratory food and water ad libitum. Animal protocols were approved by the Committee of Ethics on Animal Experiments, Faculty of Medical Sciences, Kyushu University.

Cells

Bone marrow–derived dendritic cells (BMDCs) were generated in RPMI 1640 medium supplemented with 10% FBS and GM-CSF containing conditioned medium. For cell stimulation, TDM dissolved in chloroform/MeOH (2:1) were diluted in isopropanol and added to the plates, followed by evaporation of the solvents in the laminar-flow cabinet. LPS or zymosan was dissolved in RPMI 1640 medium and added to the culture medium. 2B4 cells expressing Mincle together with FcRγ were described elsewhere (6, 18).

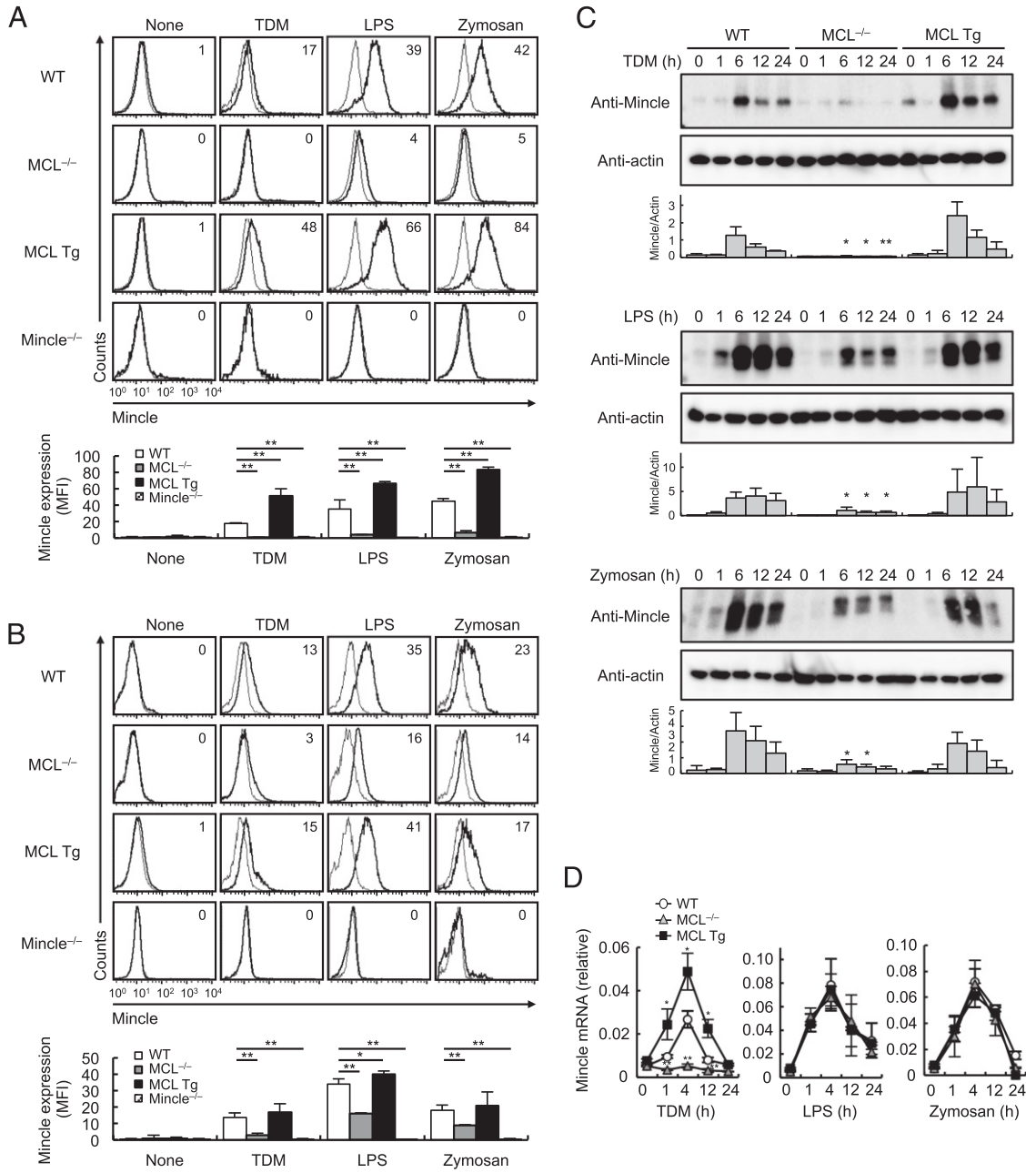

FIGURE 1. Mincle expression is posttranscriptionally controlled by MCL. (**A**) Surface expression of Mincle. BMDCs from WT, MCL$^{-/-}$, MCL Tg, and Mincle$^{-/-}$ mice were stimulated with plate-coated TDM (1 μg/well), LPS (50 ng/ml), or zymosan (100 μg/ml) for 15 h. Cells were then stained with anti-Mincle 1B6 (thick line) or isotype control (thin line) and analyzed by flow cytometry. Numbers in histograms indicate mean fluorescence intensity (MFI) of anti-Mincle staining. (**B**) Intracellular expression of Mincle. BMDCs were stimulated with plate-coated TDM (1 μg/well), LPS (50 ng/ml), or zymosan (100 μg/ml) for 15 h and permeabilized and fixed. Cells were then stained with anti-Mincle 1B6 (thick line) or isotype control (thin line) and analyzed by flow cytometry. Numbers in histograms indicate MFI of anti-Mincle staining. (**C**) Immunoblotting of Mincle. BMDCs were stimulated with TDM, LPS, or zymosan for indicated times. Cell lysates were blotted with anti-Mincle and anti-actin. (**D**) mRNA expression of Mincle. Quantitative PCR was performed with mRNA from BMDCs after stimulation. Data are presented as mean ± SD of triplicate experiments (D) and are representative of two (B and D) or three (A and C) separate experiments. *$p < 0.05$, **$p < 0.01$ versus WT.

Immunoprecipitation

Cells were lysed with lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 12.5 μg/ml chymostatin, 50 μg/ml leupeptin, 25 μg/ml pepstatin, and 1 mM PMSF). For immunoprecipitation analysis, 0.5 μg anti-Flag and protein A–Sepharose (GE Healthcare) were added to the lysates and rotated for 2 h at 4°C and then beads were washed with the lysis buffer. Bound proteins were eluted by boiling with sample buffer.

Quantitative PCR

Total RNA was isolated by Sepasol-RNA I Super G (Nacalai Tesque). Real-time PCR was performed by using Thunderbird SYBR qPCR mix (Toyobo) and ABI Prism 7000 (Applied Biosystems). The primer sets for murine Mincle were 5′-CAGTGGCAATGGGTGGATGATAC-3′ and 5′-AGTCCCTTATGGTGGCACAGTC-3′, and for murine MCL were 5′-CCGAGAGGAGCCACAGCC-3′ and 5′-TCATGCCAGGTCTGGTTGTCA-3′.

Construction of MCL chimeras and mutants

The receptor chimeras and mutants were generated using overlapping extension PCR and cloned into pMX-IRES-hCD8 retroviral vector containing Flag tag at the C terminus. Murine MCL (mMCL)^{4S} mutant was generated by substituting four hydrophobic residues (Leu^{57}, Tyr^{60}, Val^{64}, Ile^{67}) into hydrophilic serine residues by PCR with the following primers: 5′-CCCACACGAGATCAACGTGCAGCCGAG-3′ and 5′-CTCGTGTGGGAGTCTGACGATTTCAC-3′. mMCL^{WAA} was generated by the mutagenesis of calcium-binding motif, WND sequence (Trp^{193}, Asn^{194}, Asp^{195}) into WAA sequence by using the following primer: 5′-TCTGGGCTGCCTTTCCTTGTCAC-3′ and 5′-GAAAGGCAGCCCAGACCCATTTTTC-3′. Mutated sites are underlined.

Lentivirus-mediated gene transfer

MCL and its mutant genes were introduced into expression vector (CSII-CMV-MCS-IRES-Bsd). Human embryonic kidney (HEK)293T cells were transfected with expression vector together with packaging vectors (pCMV-VSV-G-RSV-Rev and pCAG-HIVgp). Culture supernatant was collected at 48–72 h after transfection. Virus was concentrated by the ultracentrifugation at 50,000 × *g* for 2 h at 20°C. For transduction, MCL-deficient BMDCs were incubated with lentivirus (multiplicity of infection of 10–100) with 8 μg/ml Polybrene. After 16 h, the medium was replaced with fresh culture medium. Lentivirus-infected cells were selected by 10 μg/ml blasticidin S for 3 d.

Statistical analysis

An unpaired two-tailed Student *t* test was used for all statistical analyses.

Results

MCL positively regulates Mincle expression on the cell surface

Mincle is a stress-inducible gene and its expression is upregulated upon cell stimulation (12). We and other groups have demonstrated that TDM-induced Mincle expression is severely impaired in MCL-deficient mice (6, 10, 11). Mincle was also induced by

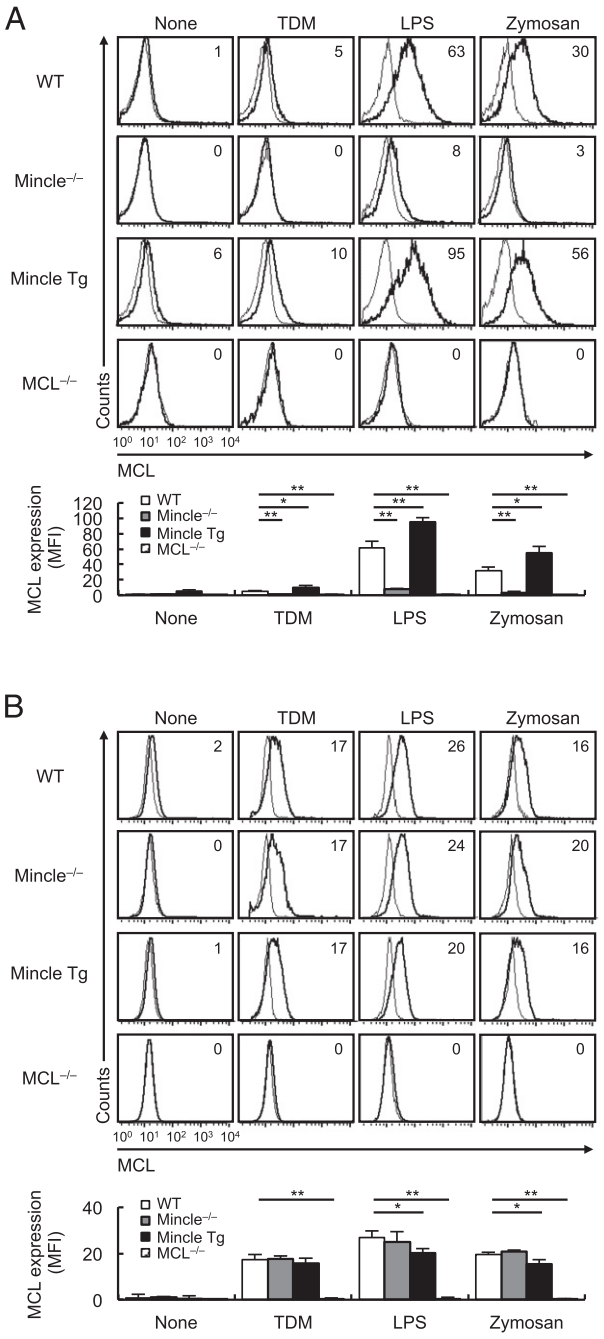

FIGURE 2. Surface expression of MCL requires Mincle. (**A**) Surface expression of MCL. BMDCs from WT, Mincle$^{-/-}$, Mincle Tg, and MCL$^{-/-}$ mice were stimulated with plate-coated TDM (1 μg/well), LPS (50 ng/ml), or zymosan (100 μg/ml) for 15 h. Cells were then stained with anti-MCL (thick line) or isotype control (thin line) and analyzed by flow cytometry. Numbers in histograms indicate mean fluorescence intensity (MFI) of anti-MCL staining. (**B**) Intracellular expression of MCL. BMDCs were stimulated for 15 h and permeabilized and fixed. Cells were then stained with anti-MCL (thick line) or isotype control (thin line) and analyzed by flow cytometry. Numbers in histograms indicate MFI of anti-MCL staining. Data are presented as mean ± SD of triplicate experiments and are representative of three separate experiments. *$p < 0.05$, **$p < 0.01$.

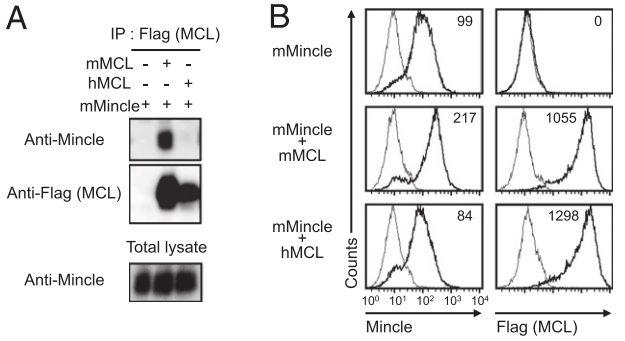

FIGURE 3. mMCL interacts with mMincle and enhances the surface expression. (**A**) Association of MCL and Mincle. HEK293T cells were transfected with Flag-tagged mMCL or hMCL together with mMincle and murine FcRγ. Lysates were immunoprecipitated (IP) with anti-Flag and blotted with anti-Mincle and anti-Flag. Total lysates were also blotted with anti-Mincle. (**B**) mMCL but not hMCL enhances mMincle expression on the cell surface. Flag-tagged mMCL or hMCL was transfected into cells stably expressing mMincle and murine FcRγ. Cells were stained with anti-Mincle, anti-Flag (thick line), or isotype control (thin line) and analyzed by flow cytometry. Numbers in histograms indicate mean fluorescence intensity. Data are representative of three separate experiments.

TLR-dependent pathogen-associated molecular patterns, such as LPS or zymosan, in wild-type (WT) BMDCs and bone marrow–derived macrophages, whereas its inducible expression on the cell surface was compromised in MCL-deficient mice (Fig. 1A, Supplemental Fig. 1A, *upper panels*). To further confirm the role of MCL in Mincle expression, we generated MCL Tg mice (Supplemental Fig. 1B). Mincle expression was significantly enhanced in MCL Tg mice (Fig. 1A, Supplemental Fig. 1A, *upper*

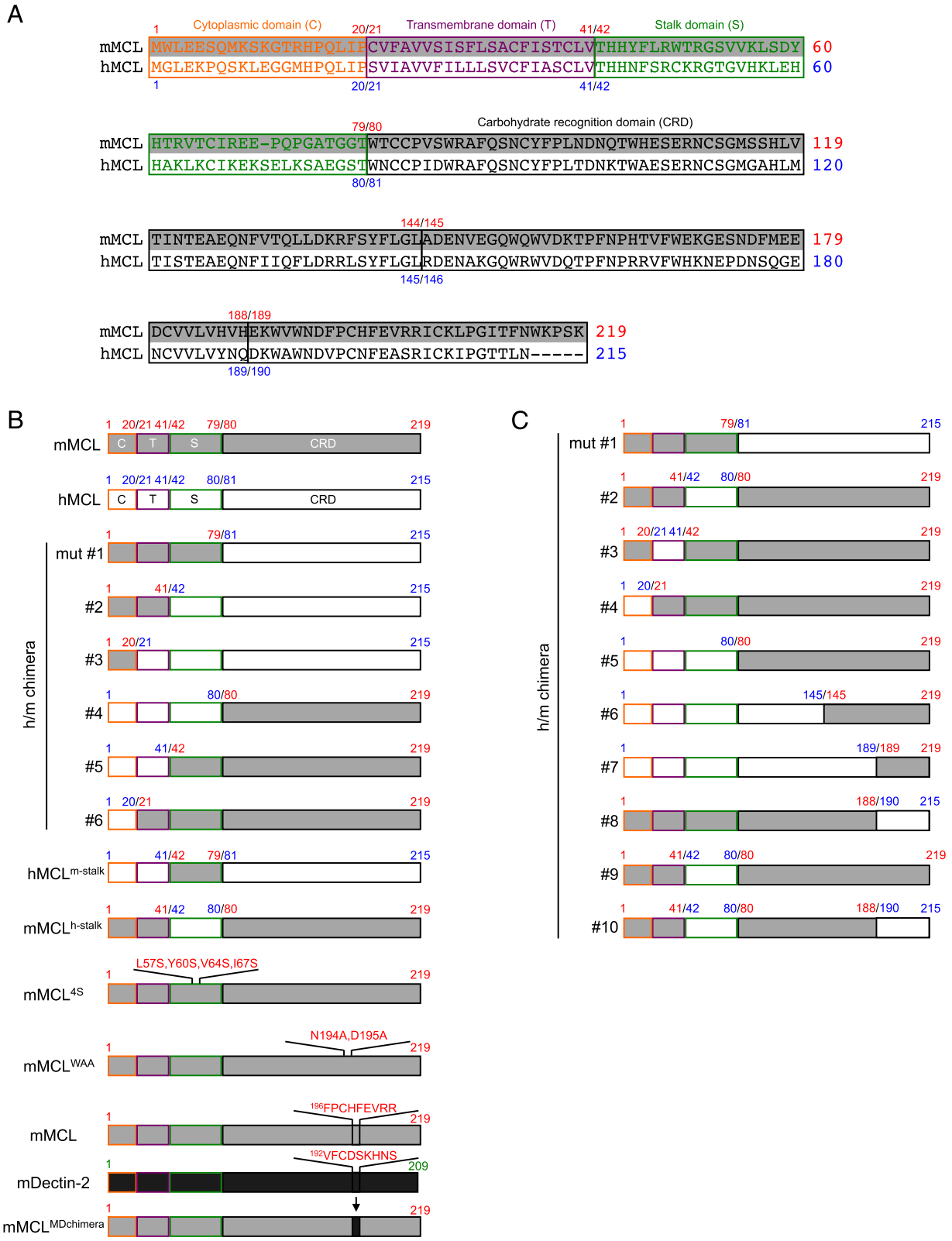

FIGURE 4. Schematic structure of MCL mutant proteins. (**A**) Sequence alignment of mMCL and hMCL. Red and blue numbers indicate the number of amino acid residues of mMCL and hMCL, respectively. (**B**) Schematic structure of mMCL, hMCL, human/murine (h/m) chimeras, and MCL mutants used in Fig. 5. Gray and white boxes represent the domain of mMCL and hMCL, respectively. C, cytoplasmic domain; S, stalk domain; T, transmembrane domain. (**C**) Schematic structure of h/m chimeras used in Fig. 6.

panels). Intracellular staining and Western blot analyses revealed that the total protein expression of Mincle was significantly reduced in MCL-deficient BMDCs and bone marrow–derived macrophages regardless of stimulants (Fig. 1B, 1C, Supplemental Fig. 1A, *lower panels*). TDM-induced Mincle mRNA expression was well correlated with the protein expression in a MCL-dependent manner (Fig. 1D, *left panel*). In contrast, LPS- or zymosan-induced Mincle mRNA expression did not alter in BMDCs from WT, MCL-deficient, and MCL Tg mice (Fig. 1D, *middle* and *right panels*). These results suggest that MCL may positively regulate Mincle expression at the protein level.

Mincle is indispensable for the surface expression of MCL

We next analyzed the surface expression of MCL in BMDCs. Although MCL mRNA is abundantly expressed in the resting state and is slightly upregulated upon stimulation (6, 10), surface expression of MCL was only detected after cell stimulation (Fig. 2A). This induction was significantly reduced in Mincle-deficient BMDCs and conversely increased in Mincle Tg BMDCs (Fig. 2A), suggesting that Mincle facilitates MCL expression on the cell surface. In contrast, intracellular expression of MCL protein was not dependent on Mincle (Fig. 2B). Taken together, Mincle and MCL mutually enhance their expressions on the cell surface.

MCL enhances Mincle expression on the cell surface through its stalk region

A previous report demonstrated that rat Mincle forms a complex with rat MCL (17). We found that mMCL, but not human MCL (hMCL), coprecipitated with murine Mincle (mMincle) in HEK293T cells (Fig. 3A). The transfection of mMCL into cells stably expressing mMincle significantly enhanced the surface expression of mMincle (Fig. 3B, *middle panels*), whereas hMCL had no effect on mMincle expression (Fig. 3B, *bottom panels*). We next tried to identify the region within mMCL responsible for the enhancement of mMincle expression. MCL consists of four domains: cytoplasmic (C), transmembrane (T), stalk (S), and carbohydrate recognition domain (CRD). We generated a series of murine/human chimeric proteins that replace the domain of mMCL with the corresponding domain of hMCL (Fig. 4) and transfected them into mMincle-expressing cells. The chimeric proteins carrying the stalk region of mMCL potently increased the surface expression of mMincle (Fig. 5A, mutant nos. 1, 5, and 6), whereas chimeras harboring the hMCL stalk region failed to enhance the expression of mMincle (Fig. 5A, mutant nos. 2–4). Finally, we found that the stalk region of mMCL is necessary and sufficient for the enhancement of mMincle expression on the cell surface (Fig. 5B).

Several C-type lectins form multimers via hydrophobic interaction of the stalk region (22–24). We found that mMCL has four hydrophobic residues (Leu^{57}, Tyr^{60}, Val^{64}, Ile^{67}) in the stalk region and satisfied criteria for a typical heptad repeat. Substitution of these residues with serine was found to abolish the enhancement of mMincle expression (Fig. 5C, $mMCL^{4S}$).

Because MCL regulates Mincle expression upon LPS or zymosan stimulation (Fig. 1A), this function is likely to be independent of its ligand binding. To address this possibility, we generated two MCL mutants in which functional residues were mutated in the CRD region. One is $mMCL^{WAA}$ in which the conserved calcium-binding WND sequence was mutated into the WAA sequence. The other is the MCL–Dectin-2 chimera ($mMCL^{MDchimera}$) in which the amino acid stretch mediating TDM recognition (25) was replaced with the corresponding portion of Dectin-2, which does not recognize TDM. These two CRD mutants still retained the ability to enhance mMincle expression (Fig. 5C).

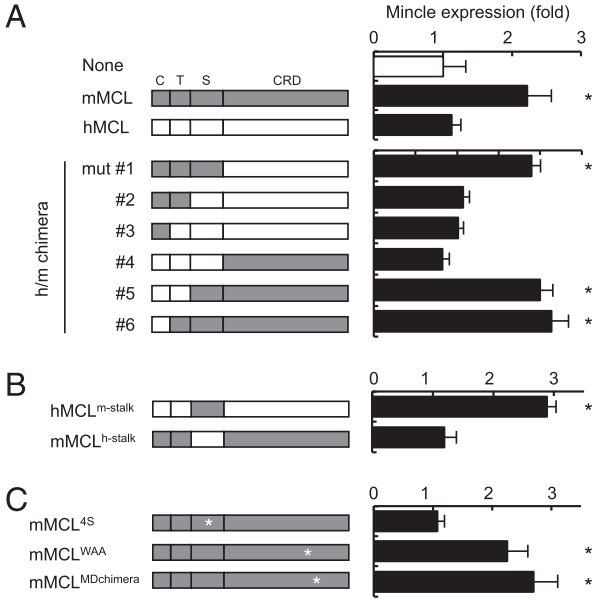

FIGURE 5. Stalk region of MCL is crucial for the enhancement of Mincle expression on the cell surface. (**A**) Stalk region is crucial for Mincle regulation. mMCL, hMCL, or chimeric proteins of mMCL and hMCL (h/m chimera) were transfected into cells stably expressing mMincle and mFcRγ and surface expression of mMincle was determined by flow cytometry. Enhancement of mMincle expression was calculated as fold induction of mean fluorescence intensity (ratio to the mMincle expression of non-transfected cells). (**B**) Stalk region is necessary and sufficient for Mincle regulation. Two MCL mutants that were swapped each stalk region of hMCL and mMCL were transfected into cells stably expressing mMincle and surface expression of mMincle was determined. (**C**) Hydrophobic residues in the stalk region but not the calcium-binding site of mMCL are critical for mMincle regulation. $mMCL^{4S}$ mutant was generated by substituting four hydrophobic residues (Leu^{57}, Tyr^{60}, Val^{64}, Ile^{67}) in the stalk region into hydrophilic serine residues. Calcium binding WND sequence in the CRD was mutated into the WAA sequence ($mMCL^{WAA}$). $mMCL^{MDchimera}$ was generated by replacing the hydrophobic residues of mMCL (196–204 aa) with the corresponding residues of murine Dectin-2 (192–200 aa). The white asterisk indicates the position of mutation. Data are presented as mean ± SD of triplicate experiments (A–C) and are representative of three separate experiments. *$p < 0.05$.

Collectively, the stalk region, rather than the CRD, of mMCL is essential for the regulation of mMincle expression. Importantly, we confirmed comparable expression of mMCL mutants (Supplemental Fig. 2).

Role of stalk region of MCL in MCL–Mincle interaction

We next examined the physical interaction of MCL with Mincle using transfected cells. Although the stalk region of mMCL was important for supporting Mincle expression, $mMCL^{4S}$ and $mMCL^{h\text{-}stalk}$ still coprecipitated with mMincle (Fig. 6A). The CRD of mMCL largely explained this mMCL–mMincle interaction in lysates (Fig. 6B, mMCL versus mutant no. 1), however non-CRD portions still contributed (Fig. 6B, hMCL versus mutant no. 1). Within the CRD, the C-terminal end (189–219 aa) mediated this strong interaction with mMincle in aqueous solution (Fig. 6C, hMCL versus mutant no. 7). Because the C-terminal end of mMCL was dispensable for the positive regulation of mMincle expression (Fig. 6D), we evaluated the interaction of mMCL with mMincle by replacing the C-terminal end of mMCL with that of hMCL (Fig. 6E). mMCL lacking its C-terminal end still bound to mMincle significantly (Fig. 6E, mutant no. 8), whereas this interaction was completely abrogated by the additional substitution of the stalk region (Fig. 6E, mutant no. 10). These results suggest that the stalk region of mMCL is involved in the interaction with mMincle.

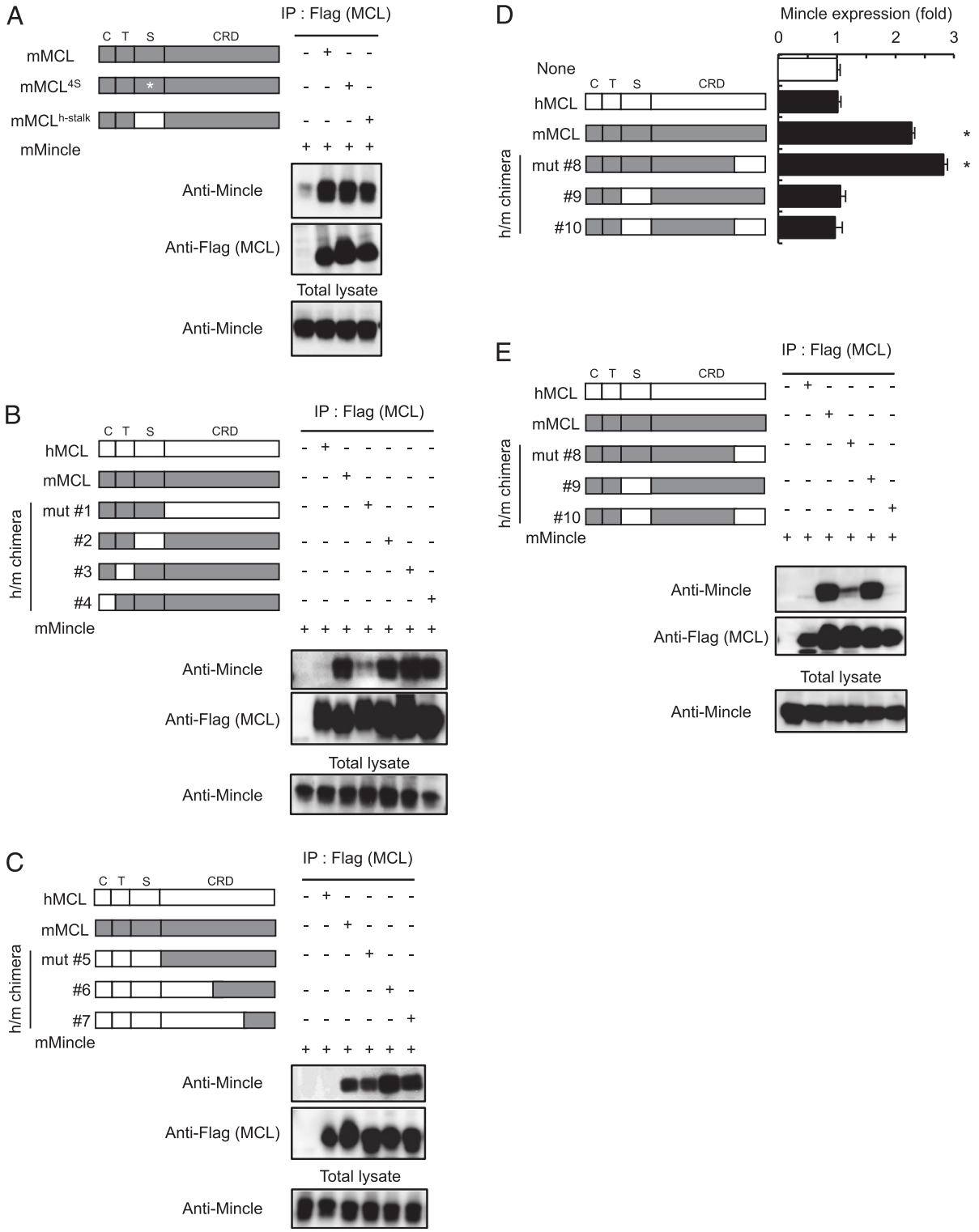

FIGURE 6. MCL binds to Mincle via the C-terminal region of the CRD and the stalk region. (**A**) A mutation of the mMCL stalk region is capable of binding to mMincle. HEK293T cells were transfected with Flag-tagged mMCL or mMCL mutants ($mMCL^{4S}$ and $mMCL^{h\text{-}stalk}$) together with mMincle and mFcRγ. Lysates were immunoprecipitated with anti-Flag and blotted with anti-Mincle and anti-Flag. Total lysates were also blotted with anti-Mincle. C, cytoplasmic domain; S, stalk region; T, transmembrane domain. Asterisk indicates the position of mutation. (**B**) CRD of mMCL mediates interaction with mMincle. Human/murine (h/m) chimeras were generated by replacing each domain of hMCL into that of mMCL. (**C**) Association of mMincle with mMCL via its C-terminal end. The h/m chimeras were generated by replacing the part of the CRD of hMCL into that of mMCL. (**D**) The C-terminal end of mMCL is dispensable for mMincle regulation. The h/m chimeras were generated by replacing the C terminus region of mMCL into that of hMCL or the stalk domain of mMCL into that of hMCL or both of them. Flag-tagged mMCL, hMCL, or h/m chimeras were transfected into cells stably expressing mMincle and mFcRγ. Surface expression of mMincle was determined by flow cytometry. (**E**) Stalk region of mMCL is responsible for the interaction with mMincle together with the C-terminal end. HEK293T cells were transfected with Flag-tagged mMCL, hMCL, or mMCL mutants together with mMincle and mFcRγ. Lysates were immunoprecipitated with anti-Flag and blotted with anti-Mincle and anti-Flag. Total lysates were also blotted with anti-Mincle. Data are presented as mean ± SD for triplicate experiments (D) and representative of two separate experiments. $*p < 0.05$.

Regulation of Mincle expression by MCL contributes to TDM responses

Finally, we investigated the physiological consequences caused by MCL mutants by reconstituting MCL-deficient BMDCs. WT MCL and MCL^{WAA} mutant, but not MCL^{4S} mutant, restored the defective surface expression of Mincle upon stimulation by LPS or TDM (Fig. 7A). The total protein expression of Mincle was correlated with the surface expression of Mincle (Fig. 7B). Thus, the stalk region of MCL also controls Mincle expression in the context of BMDCs.

We next investigated DC activation by evaluating the secretion of proinflammatory cytokines and chemokines such as TNF and MIP-2. TDM-induced production of these cytokines and chemokines was impaired in MCL-deficient BMDCs and enhanced in MCL-overexpressing BMDCs (Fig. 7C). This trend was correlated with the surface expression of Mincle (Fig. 1A). These results suggest that the level of Mincle expression faithfully reflects the potencies of DC activation in response to TDM. Consistently, the MCL^{4S} mutant failed to induce cytokine production upon TDM stimulation in MCL-deficient BMDCs (Fig. 7D). In sharp contrast, MCL^{WAA} and MCL^{MDchimera} mutants restored TDM-induced cytokine production markedly as did WT (Fig. 7D, Supplemental Fig. 3).

These results suggest that MCL can augment TDM response by modulating Mincle expression independent of the CRD region.

Discussion

In this study, we show that MCL positively regulates both Mincle protein expression and its signaling. This suggests that MCL potentially contributes to immunity against all pathogens that are recognized by Mincle, such as fungi and bacteria as well as mycobacteria (3, 14, 19, 26), although we cannot exclude Mincle-independent MCL function. In line with this idea, MCL-deficient mice show high susceptibility to infection by *Klebsiella pneumonia*, *Candida albicans*, and *Mycobacterium bovis* bacillus Calmette–Guérin (6, 16, 27), all of which are reported to be poorly eliminated in Mincle-deficient mice (5, 26, 28). As Mincle also recognizes dead cells (18) as well as microorganisms, MCL may also participate in inflammatory responses against damaged tissue.

The precise mechanism through which MCL controls Mincle expression on the cell surface is still an intriguing issue that needs to be clarified. Several CLRs bind to pathogens and are internalized into lysosomes for killing, degradation, or Ag presentation (29). Dectin-1 is internalized following interaction with its ligand β-glucan, which leads to the attenuation of inflammatory responses (30). Interestingly,

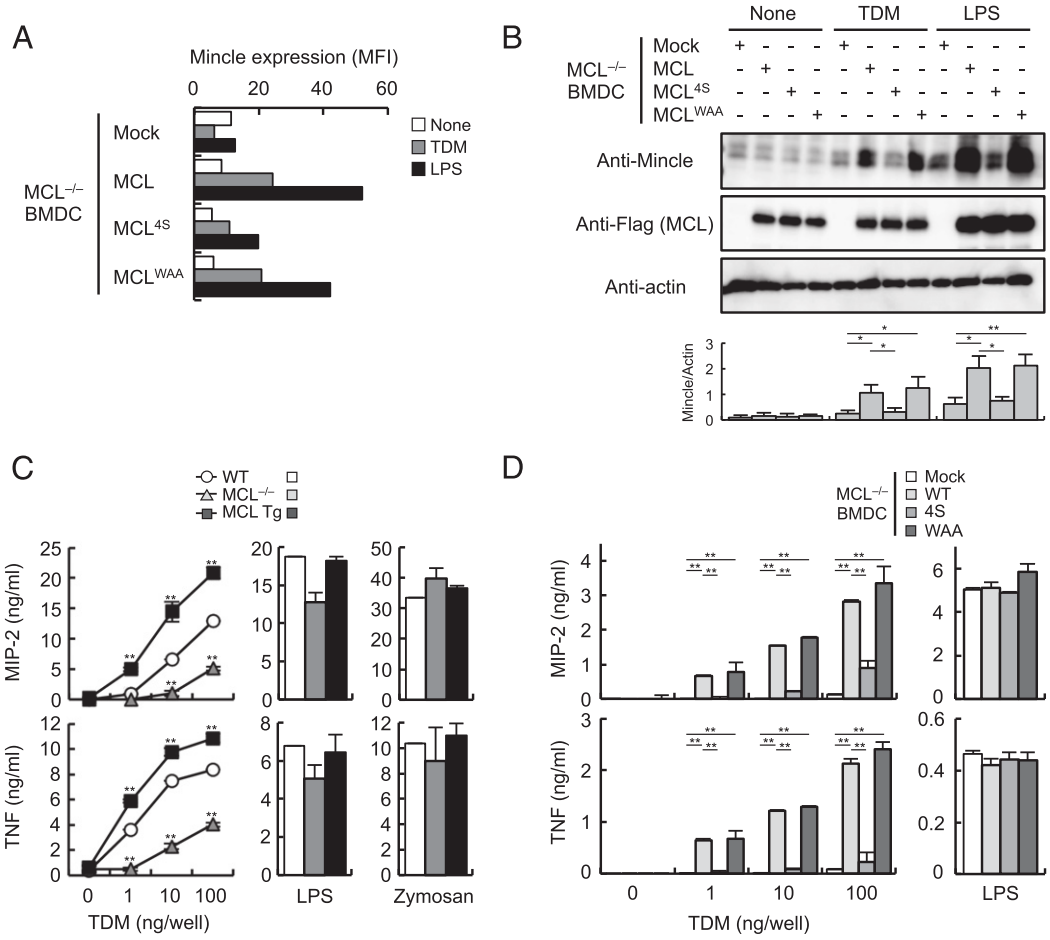

FIGURE 7. MCL contributes to the efficient TDM responses through the regulation of Mincle expression but not through the direct recognition of TDM. (**A** and **B**) Restoration of Mincle expression in MCL$^{-/-}$ BMDCs by exogenous MCL. BMDCs from MCL$^{-/-}$ mice were transduced with Flag-tagged WT MCL, MCL^{4S}, or MCL^{WAA} through lentivirus vector, followed by the stimulation with plate-coated TDM or LPS for 14 h. (A) Surface expression of Mincle. (B) Immunoblotting of anti-Mincle, anti-Flag, and anti-actin. $*p < 0.05$, $**p < 0.01$. (**C**) TDM-induced cytokine production. BMDCs from WT, MCL$^{-/-}$, and MCL Tg mice were stimulated with plate-coated TDM, LPS (50 ng/ml), or zymosan (100 μg/ml) for 24 h. LPS and zymosan were used as a positive control. Concentrations of MIP-2 and TNF were determined by ELISA. $**p < 0.01$ versus WT. (**D**) Restoration of TDM-induced cytokine production in MCL$^{-/-}$ BMDCs by exogenous MCL. BMDCs from MCL$^{-/-}$ mice were transduced with WT MCL, MCL^{4S}, or MCL^{WAA}, followed by the stimulation with plate-coated TDM or LPS (50 ng/ml) for 24 h. Concentrations of MIP-2 and TNF were determined by ELISA. Data are presented as mean ± SD of triplicate experiments (C and D) and are representative of two (A, B, and D) or three (C) separate experiments. $**p < 0.01$.

tetraspanin CD37 stabilizes Dectin-1 through direct interaction to inhibit its internalization (31). MCL might stabilize Mincle on the cell surface through a similar mechanism, although this issue warrants further investigation.

Mycobacterial species, one of the major targets of Mincle, reside in the intracellular organelles of myeloid cells. It is still unclear how Mincle expression on the plasma membrane contributes to host immune responses against these intracellular bacteria. However, after stimulation, the amount of total Mincle protein, as assessed by intracellular staining and immunoblot, was also markedly upregulated in the presence of MCL. One possibility is that MCL might stabilize Mincle in the membrane of intracellular organelles as well as the plasma membrane, which enables the host to recognize both intracellular and extracellular pathogens. Alternatively, but not exclusively, increased levels of surface Mincle may capture and internalize extracellular pathogens (26). In this respect, cell surface Mincle may serve as a "reservoir" for Mincle protein within whole cells.

Lobato-Pascual et al. (17) demonstrated that rat MCL and rat Mincle form heterodimers in HEK293T cells. We confirmed MCL–Mincle interaction in mice and humans (Fig. 3A, Supplemental Fig. 4). Thus, the heteromeric complexes between MCL and Mincle appear to be a conserved phenomenon among species, such as mice, rats, and humans. However, it was recently reported that human MCL was not coprecipitated with human Mincle in RAW264.7 cells (11). Although different Abs were used in these studies, the reason for these apparent discrepancies is currently unclear.

Although the terminal CRD region of mMCL was involved in "strong" interaction with mMincle in coimmunoprecipitation experiments, mMCL mutant lacking this region still interacted with mMincle through the stalk region (Fig. 6E). Furthermore, the deletion mutant of the C-terminal end of CRD ($mMCL^{\Delta C\text{-}CRD}$) still bound to Mincle and enhanced Mincle expression on the cell surface (Supplemental Fig. 3A, 3B), further suggesting that the intact CRD is not essential for MCL expression on the cell surface with Mincle. Indeed, $mMCL^{\Delta C\text{-}CRD}$ could restore TDM responses in MCL-deficient BMDCs (Supplemental Fig. 3). Likewise, the corresponding region of hMCL was dispensable for interacting with human Mincle (Supplemental Fig. 4A). Interestingly, guinea pig MCL lacks the C-terminal region of CRD due to stop codon insertion, despite the fact that Mincle acts as a functional TDM receptor in guinea pigs (13). Taken together, the stalk region, rather than terminal CRD region, of MCL is likely to be essential for the functional interaction of MCL and Mincle.

It was reported that MCL forms a heteromeric complex with Dectin-2, which facilitates the efficient recognition of and immune responses against *C. albicans* (16). We also confirmed the interaction of MCL with Dectin-2 in HEK293T cells; however, Dectin-2 expression was not altered in BMDCs from MCL-deficient or MCL Tg mice (data not shown). MCL may play distinct roles dependent on its partners.

Our study uncovers a novel role of CLRs in regulating protein expression of other CLRs through direct interaction. The potent adjuvanticity of TDM can be explained by the synergistic contribution of two CLRs, MCL and Mincle. Additionally, MCL may be able to use Mincle-coupled FcRγ to respond efficiently to its specific ligands, although it has not yet been identified. It is also tempting to speculate that heteromeric complex formation among other CLRs might allow the host to diversify PRR repertoire against a broad range of pathogens.

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Disclosures

The authors have no financial conflicts of interest.

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