AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity

The C-Type Lectin Receptor MCL Mediates Vaccine-Induced Immunity against Infection with *Blastomyces dermatitidis*

Huafeng Wang,[a] Mengyi Li,[a] Tassanee Lerksuthirat,[a] Bruce Klein,[a,b,c] Marcel Wüthrich[a]

Departments of Pediatrics,[a] Internal Medicine,[b] and Medical Microbiology and Immunology,[c] University of Wisconsin School of Medicine and Public Health, University of Wisconsin–Madison, Madison, Wisconsin, USA

C-type lectin receptors (CLRs) are essential in shaping the immune response to fungal pathogens. Vaccine-induced resistance requires Dectin-2 to promote differentiation of antifungal Th1 and Th17 cells. Since Dectin-2 and MCL heterodimerize and both CLRs use FcRγ as the signaling adaptor, we investigated the role of MCL in vaccine immunity to the fungal pathogen *Blastomyces dermatitidis*. MCL$^{-/-}$ mice showed impaired vaccine resistance against *B. dermatitidis* infection compared to that of wild-type animals. The lack of resistance correlated with the reduced recruitment of Th17 cells to the lung upon recall following experimental challenge and impaired interleukin-17 (IL-17) production by vaccine antigen-stimulated splenocytes *in vitro*. Soluble MCL fusion protein recognized and bound a water-soluble ligand from the cell wall of vaccine yeast, but the addition of soluble Dectin-2 fusion protein did not augment ligand recognition by MCL. Taken together, our data indicate that MCL regulates the development of vaccine-induced Th17 cells and protective immunity against lethal experimental infection with *B. dermatitidis*.

Vaccination is one of the greatest achievements in medicine and a powerful tool to protect humans against infectious diseases. The current armamentarium and efficacy of antifungal drugs is limited; thus, fungal vaccines are urgently needed (1). Today's vaccines against infectious diseases preferentially induce protective antibodies, driven by adjuvants such as alum, which has been used in a clinical trial of vaccination against *Candida albicans* with a recombinant fungal antigen (2). However, accumulating evidence suggests that antibodies contribute less to antifungal host defense than cellular immunity, which is required to resolve most fungal infections (3–5). Vaccine-induced resistance to fungi requires CD4$^+$ T cells that produce the proinflammatory cytokines interleukin-17 (IL-17; Th17 cells) and gamma interferon (IFN-γ; Th1 cells) (3, 4). While Th1 cells may be dispensable for vaccine-induced immunity against infection with systemic dimorphic fungi in murine models, Th17 cells generally are required for resistance against these infections (3). Hence, the identification of host pathogen recognition receptors (PRR) and signaling pathways that lead to the induction of vaccine-induced Th17 cell responses is critical for the rational design of antifungal vaccines.

C-type lectin receptors (CLRs) represent a large family of PRRs that share structurally homologous carbohydrate recognition domain(s) (CRD) (6, 7). CLRs expressed on antigen-presenting cells recognize carbohydrate structures on the fungal cell wall and tailor adaptive responses via the instruction of CD4$^+$ T helper cells (1, 8, 9). In a murine model of subcutaneous vaccination, we have previously uncovered an essential role of Dectin-2 in inducing antifungal immunity and CD4$^+$ T cell development (10). Using a reporter cell assay, we showed that Dectin-2 directly binds to vaccine yeast and triggers downstream NFAT signaling. Animals lacking Dectin-2 or its adaptor, FcRγ, fail to differentiate and recruit Th1/Th17 cells to the lung upon recall, and consequently the mice lack the ability to acquire vaccine-induced resistance.

MCL (also known as Dectin-3, CLECSF8, and CLEC4D) is a recently described Dectin-2 family member (11). It was originally cloned from macrophages (12) and later found to be expressed in other myeloid cell types, including monocytes and various subsets of dendritic cells (13, 14). Like Dectin-2, MCL is a type II transmembrane protein with a single extracellular CRD, and it associates with FcRγ to trigger intracellular signaling (15). Recent studies have shown that MCL recognizes mycobacterial cord factor TDM (trehalose-6,6′-dimycolate) (15, 16), a glycolipid ligand also recognized by another Dectin-2 family member, Mincle. MCL recognition of TDM induces Mincle expression and thus enhances host innate responses (15, 17, 18). Moreover, MCL is able to form a receptor complex with Mincle (19–21) to facilitate surface expression of the latter (19). Consequently, MCL is critically involved in TDM-induced experimental autoimmune encephalomyelitis (EAE) (15) and plays a nonredundant role in antimycobacterial innate immunity (17). MCL also has been shown to play a protective role in innate host defense against Gram-negative pneumonia (22). Aside from studies with *C. albicans* and *Fonsecaea pedrosoi* (13, 23), the role of MCL in antifungal immunity remains poorly defined. Interestingly, MCL was shown to form a heterodimer with Dectin-2 to synergistically induce NF-κB in response to *C. albicans* hyphae (24).

In view of the essential role of Dectin-2 and FcRγ in inducing protective immunity in our model of vaccine immunity and the facts that (i) MCL and Dectin-2 utilize FcRγ as their downstream signaling adaptor and (ii) MCL forms heterodimers with Dectin-2, we investigated whether MCL is instrumental in acquiring vaccine-induced immunity. Here, we report that MCL contributes to the acquisition of vaccine-induced resistance, promotes

Received 7 October 2015 **Returned for modification** 22 October 2015
Accepted 4 December 2015

Accepted manuscript posted online 14 December 2015

Citation Wang H, Li M, Lerksuthirat T, Klein B, Wüthrich M. 2016. The C-type lectin receptor MCL mediates vaccine-induced immunity against infection with *Blastomyces dermatitidis*. Infect Immun 84:635–642. doi:10.1128/IAI.01263-15.

Editor: G. S. Deepe, Jr., University of Cincinnati

Address correspondence to Marcel Wüthrich, mwuethri@wisc.edu.

the development of fungal antigen-specific Th17 cells, and recognizes a water-soluble ligand from the cell wall of vaccine yeast.

MATERIALS AND METHODS

Ethics statement. All animal procedures were performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (25). Care was taken to minimize animal suffering. The work was done with the approval of the IACUC of the University of Wisconsin–Madison.

Fungal growth conditions. *B. dermatitidis* strains used were ATCC 26199, a wild-type virulent strain, and the isogenic, attenuated mutant lacking BAD1, designated strain 55 (26). Isolates of *B. dermatitidis* were maintained as yeast on Middlebrook 7H10 agar with oleic acid-albumin complex (Sigma) at 39°C.

Mouse strains. Cryopreserved spermatozoa from C57BL/6-*Clec4dtm1.1Cfg*/Mmucd (Mcl-knockout) (stock 031935-UCD) mice were obtained from the Consortium of Functional Genomics and Mutant Mouse Resources & Research Centers (MMRRC) and used to fertilize wild-type C57BL6 eggs. The offspring were bred as brother/sister matings at our facility. *Blastomyces*-specific T-cell receptor (TCR) transgenic (Tg) 1807 mice were generated in our laboratory and were backcrossed to congenic Thy1.1$^+$ mice as described previously (27). Dectin-2$^{-/-}$ mice (28) were bred at our facility. All mice were 6 to 8 weeks of age at the time of experiments. Mice were housed and cared for according to guidelines of the University of Wisconsin Animal Care Committee, which approved all aspects of this work.

Vaccination and experimental infection. Virulence-attenuated yeast of *B. dermatitidis* strain 55 were injected as live cells using a dose range of 10^6 to 10^7 yeast per mouse. Mice were vaccinated subcutaneously (s.c.) at two sites, dorsally and at the base of the tail. Resistance experiments included one booster vaccination 2 weeks apart. Mice were infected intratracheally with 2×10^3 or 2×10^4 isogenic, wild-type *B. dermatitidis* 26199 yeast as described previously (3). At day 4 postinfection, the mice were sacrificed and lung T cells were analyzed by fluorescence-activated cell sorter (FACS) analysis. The burden of lung infection was determined by plating lung homogenates on brain heart infusion (Difco) agar followed by enumeration of CFU.

Adoptive transfer of transgenic 1807 T cells and intracellular cytokine staining. Single-cell suspensions of 10^6 magnetic bead-purified CD4$^+$ cells from TCR Tg 1807 (Thy1.1$^+$) mice were injected intravenously (i.v.) into Thy1.2$^+$ C57BL/6 recipients. After vaccination and challenge, effector T cells from the lung were isolated as described previously (3). To determine the percentage of transferred 1807 cells, an aliquot of isolated cells was stained with monoclonal antibodies (MAbs) directed against the following surface markers: CD4, CD8, Thy1.1, CD44, and B220 (BD Pharmingen). The number of 1807 CD4$^+$ T cells in a lung was calculated by multiplying the percentage of Thy1.1$^+$ CD4$^+$ cells by the number of viable cells as determined by trypan blue dye exclusion. The rest of the cells were stimulated with anti-CD3 and anti-CD28 MAbs in the presence of Golgi-Stop (BD Pharmingen). After 5 h of stimulation, cells were stained for surface markers, fixed, and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen), and then they were stained with anti-IFN-γ and -IL-17 MAbs (BD Pharmingen) as described previously (3). FACS data were gathered with an LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

***Ex vivo* coculture for cytokine protein measurement.** Splenocytes were harvested from mice at day 4 postinfection (34 days postvaccination), washed, resuspended in complete RPMI 1640 containing 10 μg/ml yeast cell wall membrane (CW/M) antigen (Ag) (29), and plated in 24-well plates at a concentration of 3×10^6 cells/well. Supernatants were collected from *ex vivo* cocultures after 5 days of incubation at 37°C and 5% CO_2. IFN-γ (R&D Systems) was used according to the manufacturer's specifications.

Generation of recombinant Fc fusion protein and binding assay. The generation of Dectin-2-Fc fusion protein has been described previously (10). To construct the MCL-Fc expression vector, the extracellular domain (amino acids 77 to 219) of MCL was amplified by PCR from mouse bone marrow dendritic cell cDNA and introduced in frame into the KpnI and EcoRI sites of pSecTag2(C)-mDectin-2-Fc (provided by Philip R. Taylor) to replace the Dectin-2 coding sequence. The pSecTag2(C)-mMCL-Fc vector was transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). Soluble MCL-Fc was expressed and purified as described previously (30). Human IgG1 Fc recombinant protein was purchased from Invitrogen.

For the enzyme-linked immunosorbent assay (ELISA)-based assay of CLR binding, *B. dermatitidis* cell wall extract (CWE) was prepared by moderate sonication of live or heat-killed (HK) strain 55 yeast for 5 min. Water-soluble fractions were collected and coated onto MaxiSorp ELISA plates (Nunc). Plate-coated CWE was incubated with 0.5 μg/ml Fc or 1 μg/ml Fc fusion proteins in 20 mM Tris-HCl, pH 7.6, 10 mM Ca^{2+}, 0.15 M NaCl, and 0.1% (wt/vol) Tween 20. Bound proteins were detected by using horseradish peroxidase (HRP)-conjugated anti-human IgG Ab (Jackson ImmunoResearch Laboratories). Absorbance was measured at 450 nm. Readings were measured against a blank of uncoated wells incubated with the appropriate Fc proteins.

For the binding of fusion proteins to *B. dermatitidis* yeast, Fc fragment alone or Fc fusion proteins were added at 10 μg/ml to 2×10^6 live, heat-killed, or UV-inactivated yeast in 100 μl binding buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Tween 20, pH 7.4) and incubated overnight at 4°C. The yeast cells were washed twice with binding buffer and incubated for an additional 30 min at room temperature with 1:100-diluted phycoerythrin (PE)-conjugated goat anti-human Fc Ab (Jackson ImmunoResearch Laboratories) in binding buffer. The cells then were washed three times with binding buffer before being fixed with 1% formaldehyde in binding buffer. Bound Fc proteins were quantified by flow cytometry.

CLR reporter cell construction and stimulation. Dectin-2- and MCL-expressing B3Z reporter cells and the control cell line expressing the FcRγ adaptor alone have been described previously (10). To generate a cell line coexpressing Dectin-2 and MCL, Dectin-2 reporter cells were retrovirally transduced with pMX-mMCL-Flag-IRES-hCD8 (23), purified using anti-hCD8 microbeads (Miltenyi Biotec), and verified by cell surface staining and FACS analysis using an anti-mouse MCL MAb (a gift from Sho Yamasaki) and an anti-Flag MAb (Sigma). For B3Z cell stimulation, 10^5 B3Z cells/well in a 96-well plate were incubated for 18 h with 5×10^5 heat-killed yeast from *B. dermatitidis* strain 55 or 2 μg plate-coated CWE. LacZ activity was measured in total cell lysates using CPRG (Roche) as a substrate. The optical density at 560 nm (OD_{560}) was measured using the OD_{620} as a reference.

Statistical analysis. Differences in the number and percentage of activated or cytokine-producing T cells were analyzed using the Wilcoxon rank test for nonparametric data or the *t* test (using GraphPad Prism) when data were normally distributed (31). Factorial analysis of variance (ANOVA) using SAF software was used to compare the vaccine-induced reduction in lung CFU between different strains of mice. $P < 0.05$ is considered statistically significant.

RESULTS

Vaccine-induced immunity against *Blastomyces dermatitidis* requires MCL. To test if MCL is required for vaccine-induced antifungal immunity, we immunized MCL$^{-/-}$ mice or wild-type littermates. Since MCL$^{-/-}$ mice have been backcrossed to C57BL6 mice only three times, we used wild-type littermates of the same genetic background as that of the controls. Unvaccinated MCL$^{-/-}$ mice had a burden of lung infection similar to that of unvaccinated wild-type controls, suggesting that MCL does not contribute to innate resistance (Fig. 1). Vaccination with live-attenuated yeast enhanced resistance to challenge in both wild-type mice and MCL$^{-/-}$ mice, but the level of resistance in MCL$^{-/-}$ mice was significantly diminished compared to that in wild-type mice (Fig. 1A and B). Thus, MCL is required for optimal vaccine-induced resistance against lethal experimental infection.

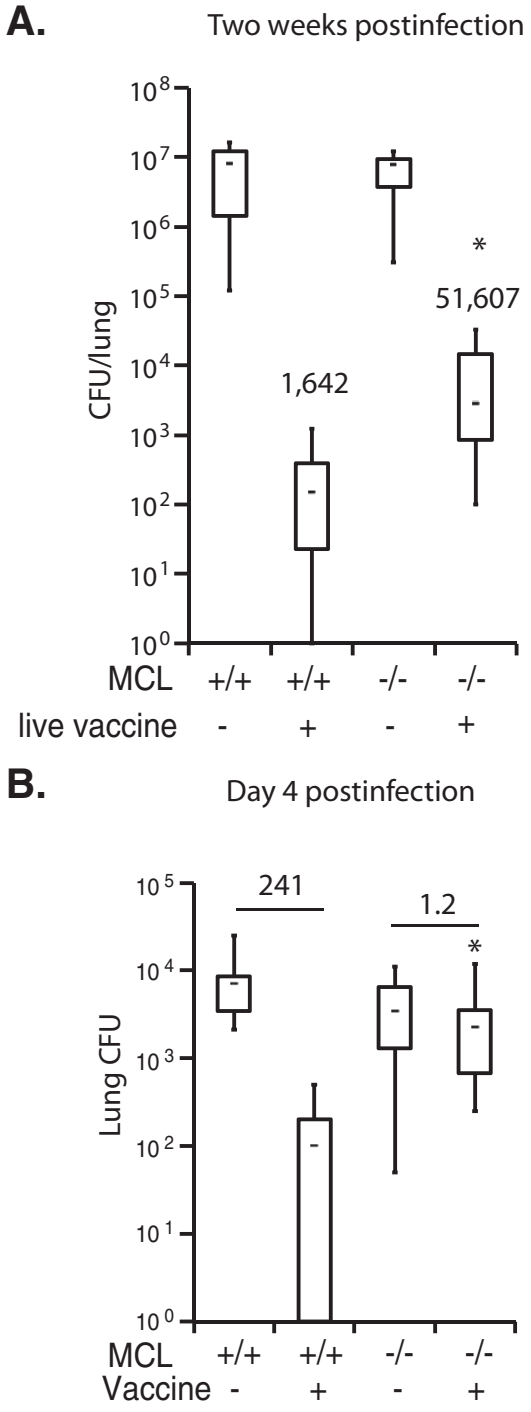

FIG 1 Vaccine-induced immunity against *B. dermatitidis* requires MCL signaling. MCL$^{-/-}$ mice and wild-type littermates were vaccinated with 10^6 live vaccine yeast twice over the course of 4 weeks. Two weeks after the boost, vaccinated and unvaccinated mice were challenged with 2×10^3 wild-type yeast. At 2 weeks postinfection (A), when unvaccinated mice were moribund, or 4 days postinfection (B), during the peak influx of antigen-specific CD4 T cells into the lung, lung CFU were assessed. Data are the means ± standard errors of the means (SEM) ($n = 8$ to 12 mice/group); data are from a single experiment representative of 3 independent experiments. *, $P < 0.05$ versus vaccine-induced reduction in lung CFU in wild-type mice. Numbers indicate the *n*-fold difference in lung CFU versus that of unvaccinated controls.

The recruitment of primed T cells is blunted in MCL$^{-/-}$ mice upon recall. CD4$^+$ T cells are the key players in mediating vaccine-induced resistance to fungi (4, 5, 32). Since MCL$^{-/-}$ mice showed impaired vaccine resistance, we sought to investigate whether the development and recruitment of CD4$^+$ T cells to the lung upon recall is impaired in the absence of MCL. To enumerate the number of fungus-specific CD4$^+$ T cells, we exploited TCR Tg 1807 cells that recognize calnexin (Cnx), an antigen shared by systemic dimorphic fungi (33, 34), and adoptively transferred naive 1807 cells into MCL$^{-/-}$ and wild-type mice prior to vaccination. At day 4 postinfection, the number of activated (CD44$^+$) 1807 cells that migrated to the lungs rose by >20-fold in vaccinated wild-type mice, whereas the respective number of cells rose by <10-fold in vaccinated MCL$^{-/-}$ mice (Fig. 2A and B). At day 4 postinfection, vaccinated MCL$^{-/-}$ mice likewise failed to significantly reduce lung CFU, whereas vaccinated wild-type mice reduced the lung burden by >2 logs versus unvaccinated controls (Fig. 1B). Thus, MCL is required for the optimal development and recruitment of antifungal T cells to the lungs, which coincided with the early reduction of lung CFU.

The numbers of lung Th17 cells are reduced in challenged MCL$^{-/-}$ mice. Vaccine-induced CD4$^+$ T cells mediate host resistance by producing the proinflammatory cytokines IFN-γ and IL-17, which recruit and activate phagocytes to kill the fungi (5). Consequently, we asked if MCL$^{-/-}$ mice have impaired Th1 and Th17 cell responses. First, we studied the recruitment of Ag-specific Th1/Th17 cells to the lung upon challenge. At day 4 postinfection, vaccinated MCL$^{-/-}$ mice recruited reduced numbers of IL-17-producing 1807 cells to the lungs compared to that of wild-type controls (Fig. 3A and B). Second, we harvested activated T cells from the spleen at day 4 postinfection and stimulated them *ex vivo* with cell wall membrane (CW/M) Ag. CD4$^+$ T cells from MCL$^{-/-}$ mice produced less IL-17 in response to stimulation than wild-type T cells. In contrast to these results, IFN-γ responses did not differ in MCL$^{-/-}$ versus wild-type mice. Thus, MCL contributes to the development of Th17 responses after vaccination.

MCL promotes T cell expansion and Th17 cell differentiation. We have previously shown that the downstream signaling adaptor Card9 regulates the development of Th17 cells primarily at the level of T cell differentiation and modestly during T cell expansion (10). The C-type lectin receptor Dectin-2 phenocopied the impairment of Th17 cell recruitment to the lung on recall (10). Thus, we sought to investigate at what stage MCL impacts the development of Th17 cells and compare its role to that of Dectin-2. We chose to analyze T-cell expansion, proliferation, and differentiation at the peak of the expansion phase at day 8 postvaccination. The number of activated (CD44$^+$) 1807 T cells was modestly reduced in the skin-draining lymph node (sdLN) and spleen of vaccinated MCL$^{-/-}$ versus MCL$^{+/+}$ mice and in the sdLN but not spleen of vaccinated Dectin-2$^{-/-}$ versus Dectin-2$^{+/+}$ mice (Fig. 4A). The proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled 1807 T cells was not impacted by the absence of MCL or Dectin-2 (data not shown). However, the differentiation of 1807 T cells into Th17 cells was sharply reduced in Dectin-2$^{-/-}$ versus Dectin-2$^{+/+}$ control mice and modestly reduced in MCL$^{-/-}$ versus MCL$^{+/+}$ mice (Fig. 4B and C). At day 8 postvaccination, differentiation of Th1 cells was not significantly reduced in the absence of Dectin-2 or MCL (Fig. 4C and data not shown). Thus, MCL promotes expansion and differentiation of

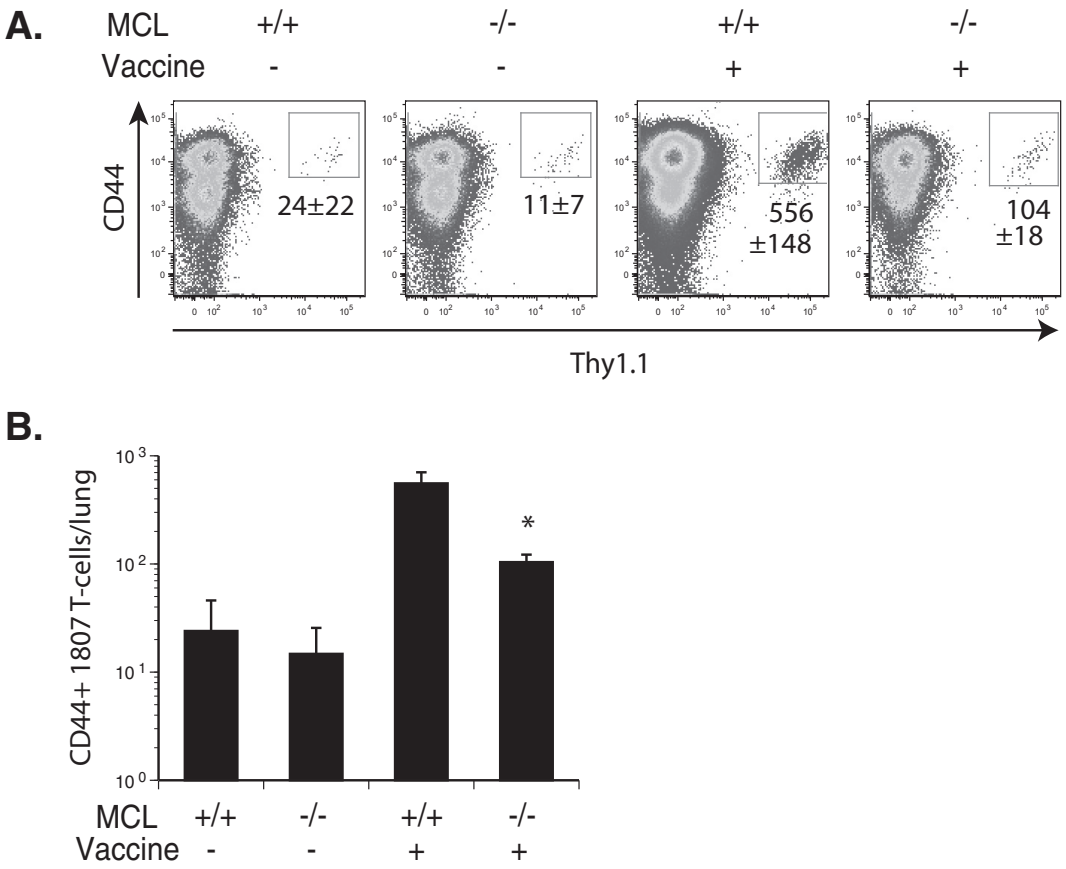

FIG 2 Number of activated T cells migrating to the lung on recall is reduced in MCL$^{-/-}$ mice. MCL$^{-/-}$ mice and wild-type littermates received an adoptive transfer of 10^6 CD4$^+$ purified, naive 1807 Tg cells and were vaccinated with 10^6 live vaccine yeast or left unvaccinated. At day 4 postinfection, the number of activated (CD44$^+$) transferred 1807 T cells was enumerated from the lung by flow cytometry. (A and B) Dot plots show concatenated samples of 4 to 6 mice/group. The numbers indicate the means ± SEM of activated (CD44^{hi}) 1807 Tg (Thy1.1$^+$) T cells. Data are expressed as means ± SEM (n = 4 to 6 mice/group). Data are means ± SEM (n = 4 to 6 mice/group) from single experiments representative of 3 independent experiments. *, $P < 0.05$ versus the number of 1807 T cells in vaccinated wild-type mice.

Th17 cells during the priming phase. Compared to MCL, Dectin-2 has a more powerful impact on the differentiation of Th17 cells.

Soluble MCL binds a water-soluble ligand from live and HK vaccine yeast. CLRs initiate immune responses by recognizing their cognate ligands and triggering intracellular signaling. Since our data indicate that MCL regulates vaccine-induced immunity, we hypothesized that MCL binds to ligands of vaccine yeast. To test this hypothesis, we exploited an established NFAT-*lacZ* reporter system (10) that reads out a LacZ signal when cell surface-expressed MCL is triggered by a cognate ligand that is present on the yeast cell wall. Live-attenuated yeast revealed an MCL phenotype during vaccine studies *in vivo*, but live yeast cannot be used in the *in vitro* reporter assay, since the replicating yeast and conditioned media from yeast cultures kill the reporter cells. MCL-expressing reporter cells showed no activation signal when stimulated with heat-killed vaccine yeast, whereas Dectin-2 and Dectin-2–MCL-coexpressing cells showed strong and comparable activation signals (Fig. 5A). These data suggest that the ligand for MCL does not augment Dectin-2 recognition and/or signaling, that the ligand is not on the yeast surface, or, alternatively, that the ligand for MCL is temperature sensitive and destroyed on heat-killed yeast.

To exclude the possibility that an MCL ligand is heat labile and to expose additional, embedded cell wall ligands to MCL in the reporter assay, we prepared water-soluble cell wall extract (CWE) from both live and HK vaccine yeast and tested them in the reporter assay. Whereas both extracts triggered Dectin-2 signaling in the reporter cells, neither of the extracts triggered MCL signaling in these cells (Fig. 5B). We next generated recombinant MCL protein fused to human IgG1 Fc to see whether soluble MCL receptor could bind to a ligand in water-soluble CWE from either live or HK vaccine yeast. An ELISA-based assay showed that soluble MCL-Fc fusion protein bound to plate-coated, water-washed ligands from live and HK yeast (Fig. 5C) and the yeast themselves (Fig. 5D), while Fc alone failed to do so. As a positive control, soluble Dectin-2-Fc fusion protein also bound strongly to the water-soluble ligands. Interestingly, the addition of MCL-Fc together with Dectin-2-Fc did not augment the binding of the latter to plate-coated ligand. These results indicate that MCL recognizes and binds to a water-soluble, heat-stable ligand from vaccine yeast, but neither the reporter cells nor the soluble form of the receptor reveal evidence for cooperativity of MCL with Dectin-2 in recognition of ligand(s) from *B. dermatitidis*.

DISCUSSION

We previously reported that innate sensing by Dectin-2 is required for the development of Th17 cells and acquired immunity

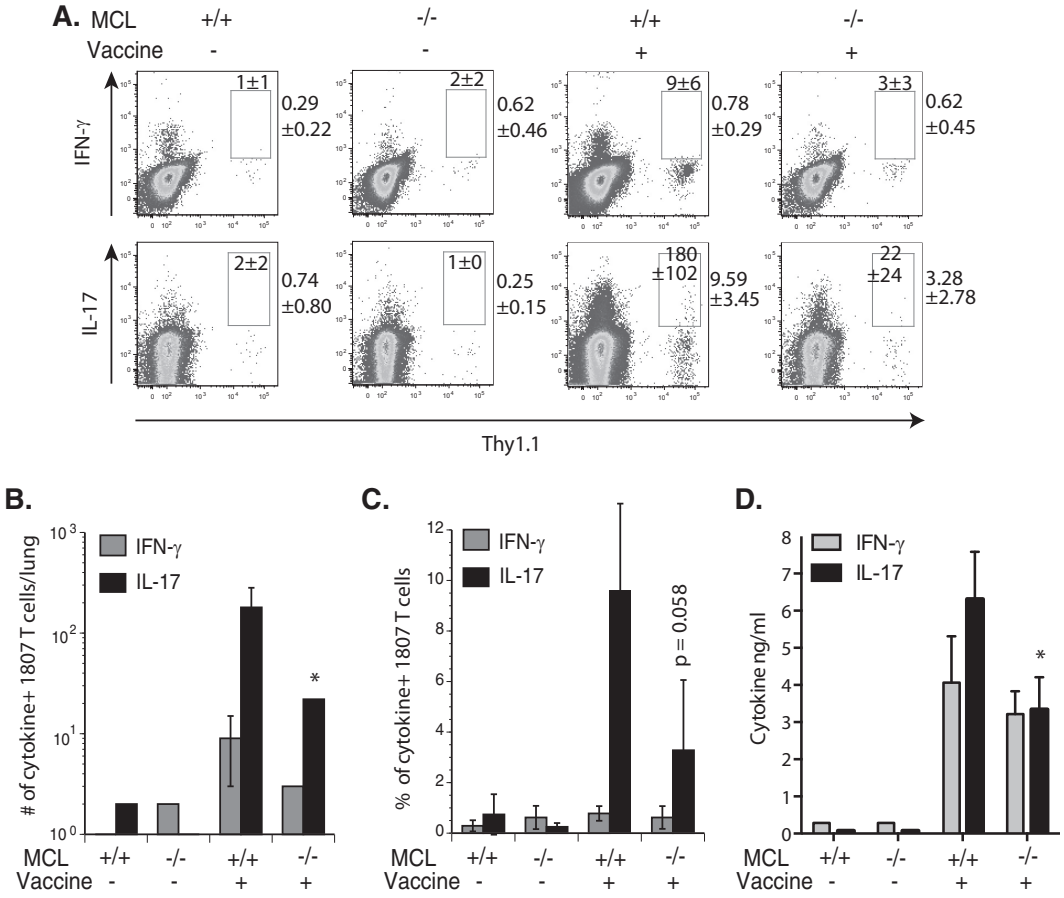

FIG 3 Number of IL-17-producing T cells migrating to the lung on recall is reduced in MCL$^{-/-}$ mice. MCL$^{-/-}$ mice and wild-type littermates received an adoptive transfer of 10^6 CD4$^+$ purified, naive 1807 Tg cells and were vaccinated with 10^6 live vaccine yeast cells or left unvaccinated. At day 4 postinfection, the number and frequency of cytokines producing 1807 T cells were enumerated from the lung by FACS. (A to C) Dot plots show concatenated samples of 4 to 6 mice/group. The numbers inside the dot plots indicate the mean number ± SEM of IFN-γ- and IL-17-producing 1807 Tg (Thy1.1$^+$) T cells. The numbers outside the dot plots indicate the mean frequencies ± SEM of IFN-γ- and IL-17-producing 1807 Tg (Thy1.1$^+$) T cells. Data are expressed as the means ± SEM (n = 4 to 6 mice/group). *, $P < 0.05$ versus cytokine production by T cells from wild-type mice. (D) At day 4 postinfection, the splenocytes were stimulated with CW/M Ag and IFN-γ and IL-17 production was determined by ELISA. Data are the means ± SEM (n = 4 to 6 mice/group) from single experiments representative of 3 independent experiments. *, $P < 0.05$ versus the number of lung CFU from vaccinated wild-type mice.

to *B. dermatitidis* (10). In a model of *C. albicans* infection, Dectin-2 signaling and downstream cytokine production in response to α-mannan on the fungal hyphae is augmented by the formation of heterodimers with MCL (24). Here, we sought to investigate whether MCL recognizes vaccine yeast and is required for T cell development and acquired resistance to *B. dermatitidis*. We also explored whether MCL acts together with Dectin-2 to augment receptor signaling, as in reports with *C. albicans*. Most strikingly, we found that MCL is required for the development of Th17 cells and optimal vaccine-induced protection against lethal pulmonary infection with *B. dermatitidis*. The absence of MCL was associated with striking decrements in Th17 development and sharply impaired resistance to infection. MCL regulated the development of Th17 cells during the priming phase by promoting T cell expansion and differentiation but not proliferation. These results are similar to the role of Dectin-2 and Card9 in inducing vaccine immunity and the development of Th17 cells (10) and add new insight into the requisite role for CLRs in vaccine-induced resistance to the fungi. However, a side-by-side analysis of the roles of Dectin-2 and MCL on T-cell priming indicated that Dectin-2 has a more profound impact on Th17 cell differentiation than MCL.

To understand how MCL recognizes vaccine yeast, we tested cell surface-bound and soluble forms of MCL receptor for signaling and binding in response to whole yeast or its extracted ligands. In contrast to Dectin-2-expressing cells, MCL-expressing reporter cells did not recognize and signal in response to HK vaccine yeast or to water-soluble ligands from either HK or live yeast. Likewise, coexpression of MCL on Dectin-2 reporter cells did not augment Dectin-2 signaling triggered by yeast or soluble ligands. In contrast to the reporter cells, soluble MCL receptor in the form of a fusion protein recognized a water-soluble ligand from both live and HK vaccine yeast and the yeast themselves. These data indicate that the soluble MCL receptor binds a heat-stable water-soluble ligand from the cell wall of vaccine yeast.

There are several possible explanations for the different functions of the cell surface-bound MCL receptor on reporter cells versus the soluble form of the MCL receptor. These possible explanations include the following: (i) a soluble receptor better accesses ligands buried deep in the yeast cell wall, which might not be accessible to a CLR that is more physically constrained while tethered on the surface of reporter cells; (ii) the relative affinity of soluble MCL versus cell surface-bound MCL is different and lower

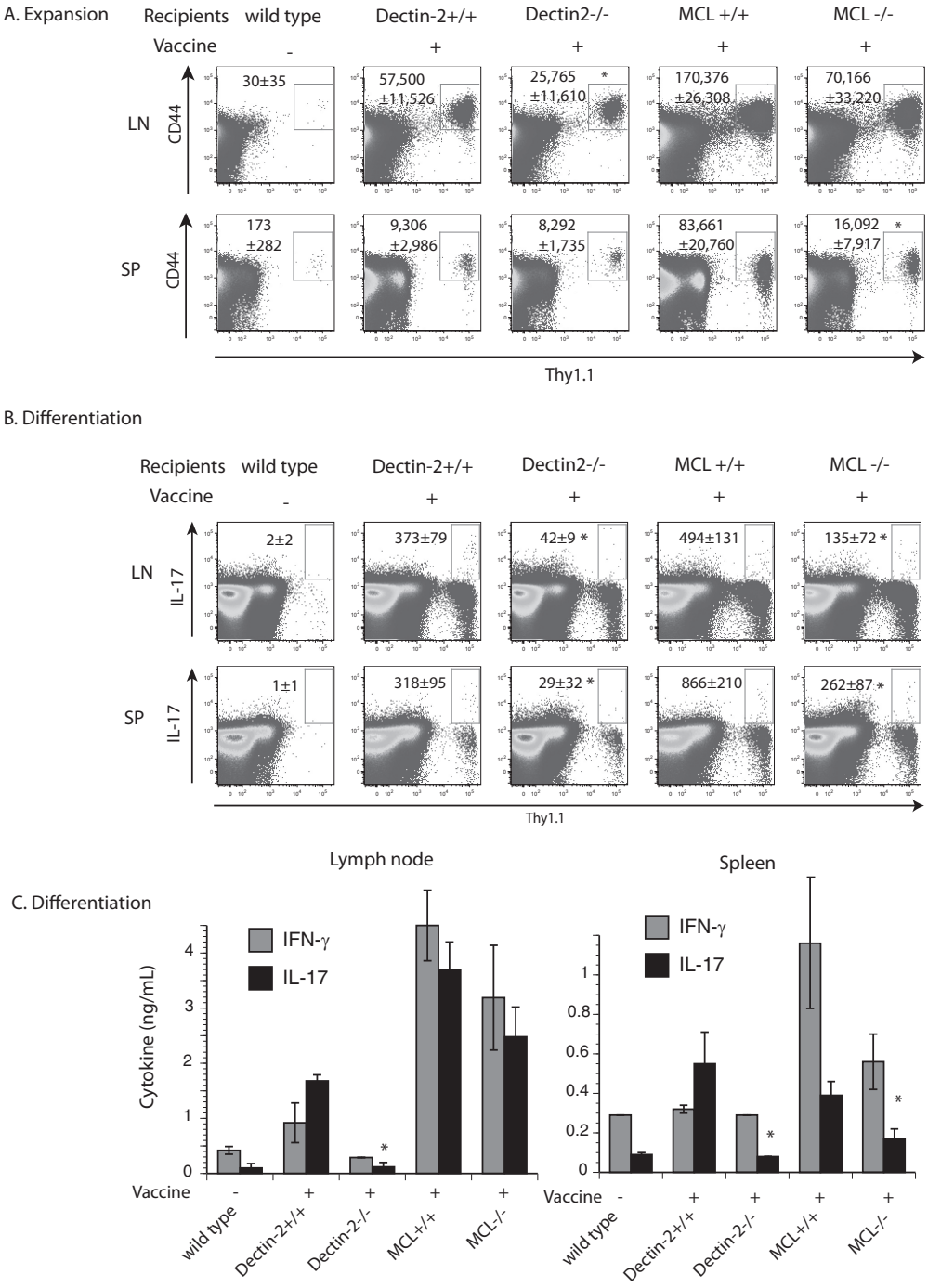

FIG 4 MCL and Dectin-2 regulate T-cell expansion and Th17 cell differentiation. C57BL6 wild-type mice (Dectin-$2^{+/+}$), Dectin-$2^{-/-}$ mice, and $MCL^{-/-}$ mice and wild-type littermates received an adoptive transfer of 10^6 $CD4^+$ purified, naive, CFSE-labeled 1807 Tg cells and were vaccinated with 10^6 live vaccine yeast or left unvaccinated. At day 8 postvaccination, the number of activated ($CD44^+$) (A) and cytokine-producing (B) 1807 T cells were enumerated from the lung by FACS. Dot plots show concatenated samples of 4 to 6 mice/group. The numbers inside the dot plots indicate the mean number ± SEM of activated ($CD44^+$) 1807 Tg ($Thy1.1^+$) T cells. Data are expressed as the means ± SEM ($n = 4$ to 6 mice/group). *, $P < 0.05$ versus activated 1807 T cells from wild-type mice. SP, spleen. (C) T cells from the sdLN and spleen were stimulated with CW/M antigen for 3 days, and cytokines were measured in the cell culture supernatant. *, $P < 0.05$ versus cytokines from wild-type control mice.

for MCL bound on the surface of reporter cells; (iii) the amount of MCL ligand present on vaccine yeast and in the water-soluble extract is too low to trigger MCL reporter cells but sufficient to generate a signal in the ELISA with soluble MCL; (iv) MCL receptor binding does not necessarily translate into receptor signaling; and (v) MCL signaling requires another coreceptor that is not present in the reporter cells.

We did not find evidence for receptor synergy as assessed by

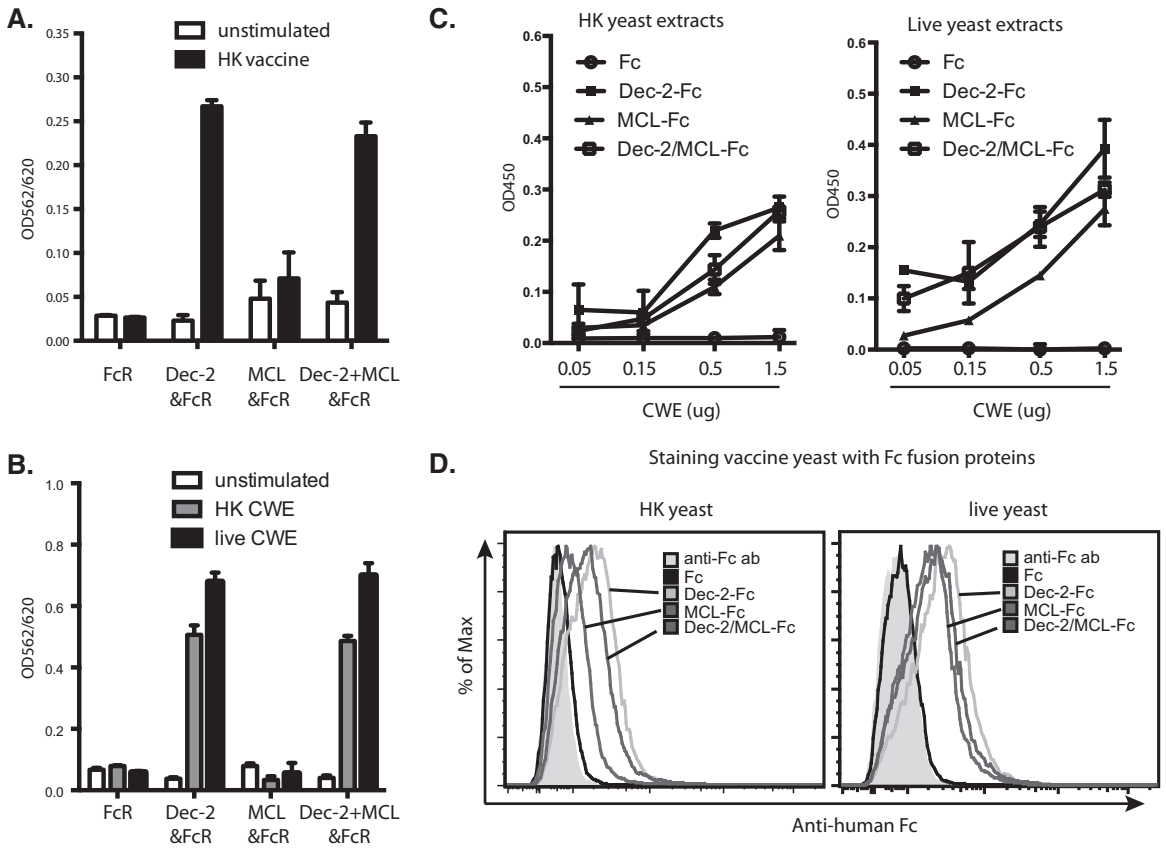

FIG 5 Recognition of *B. dermatitidis* ligands by Dectin-2 and MCL. (A and B) B3Z cells expressing FcRγ, Dectin-2 and FcRγ, MCL and FcRγ, or Dectin-2, MCL, and FcRγ were stimulated with heat-killed *B. dermatitidis* (A) or plate-coated cell wall extract (CWE) isolated from live and heat-killed *B. dermatitidis* (B). After 18 h, *lacZ* activity was measured using a colorimetric assay and expressed as OD_{560}/OD_{620} values. Data are the means ± standard deviations from duplicate wells. (C) Recombinant human IgG1 Fc, Dectin-2-Fc, MCL-Fc, and a mixture (1:1) of Dectin-2-Fc and MCL-Fc were incubated with the indicated amount of plate-coated cell wall extract isolated from live or heat-killed *B. dermatitidis*. Bound proteins were detected with anti-hIgG-HRP and expressed as OD_{450} values. (D) Fc protein staining of *B. dermatitis*. Heat-killed and live yeast cells were incubated with Fc fragment alone, Dectin-2-Fc, MCL-Fc, or a mixture of Dectin-2-Fc and MCL-Fc, as indicated by different colors, followed by staining with PE-conjugated anti-human Fc Ab and analysis by FACS. As a negative control, Ab-stained fungal cells without any Fc proteins are indicated by gray filled histograms.

augmented LacZ signal in B3Z T hybridoma cells that coexpressed MCL and Dectin-2. We purposely chose to express the CLRs in nonmyeloid B3Z T hybridoma cells that lack the myeloid signaling machinery and other CLRs and TLRs to let us individually test and identify receptors and adaptors required for vaccine yeast and ligand recognition. In contrast, Zhu et al. overexpressed MCL and Dectin-2 in RAW264.7 macrophages that also express other receptors and signal pathways when they reported synergistic sensing of *C. albicans* hyphae and NF-κB signaling (24). Thus, it is possible that other receptors expressed by the macrophage cell line could have formed a multireceptor complex with MCL and Dectin-2 to promote receptor synergy. However, our data do not exclude the possibility that the observed MCL phenotype is due to a synergistic interaction of MCL with other CLRs and receptors.

In our study, the absence of MCL led to the impaired development of protective Th17 cells and blunted vaccine resistance; however, MCL was dispensable for innate resistance during a primary lung infection with *B. dermatitidis*. This finding is contrary to what was described in an experimental model of pulmonary infection with *M. tuberculosis*. In that model, MCL was required for innate defense but dispensable for the development of acquired immunity (17). Moreover, the expression of MCL together with FcRγ was insufficient to mediate mycobacterial binding in transfected fibroblasts, implying that MCL must associate with other receptors to mediate its function (17). Thus, further investigation is needed to fully understand MCL sensing and signaling of microbial pathogen-associated molecular patterns.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 AI093553 (M.W.) and R01 AI040996 (B.K.).

FUNDING INFORMATION

HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Marcel Wüthrich under grant number AI093553. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Bruce Steven Klein under grant number AI040996.

The principal investigator for this work is Marcel Wüthrich.

REFERENCES

1. **Verma A, Wüthrich M, Deepe G, Klein B.** 2015. Adaptive immunity to fungi. Cold Spring Harb Perspect Med 5:a019612. http://dx.doi.org/10.1101/cshperspect.a019612.
2. **Schmidt CS, White CJ, Ibrahim AS, Filler SG, Fu Y, Yeaman MR, Edwards JE, Jr, Hennessey JP, Jr.** 2012. NDV-3, a recombinant alum-

adjuvanted vaccine for Candida and Staphylococcus aureus, is safe and immunogenic in healthy adults. Vaccine **30:**7594–7600. http://dx.doi.org/10.1016/j.vaccine.2012.10.038.

3. **Wüthrich M, Gern B, Hung CY, Ersland K, Rocco N, Pick-Jacobs J, Galles K, Filutowicz H, Warner T, Evans M, Cole G, Klein B.** 2011. Vaccine-induced protection against 3 systemic mycoses endemic to North America requires Th17 cells in mice. J Clin Investig **121:**554–568. http://dx.doi.org/10.1172/JCI43984.
4. **Lin L, Ibrahim AS, Xu X, Farber JM, Avanesian V, Baquir B, Fu Y, French SW, Edwards JE, Jr, Spellberg B.** 2009. Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice. PLoS Pathog **5:**e1000703. http://dx.doi.org/10.1371/journal.ppat.1000703.
5. **Wüthrich M, Filutowicz HI, Warner T, Klein BS.** 2002. Requisite elements in vaccine immunity to Blastomyces dermatitidis: plasticity uncovers vaccine potential in immune-deficient hosts. J Immunol **169:**6969–6976. http://dx.doi.org/10.4049/jimmunol.169.12.6969.
6. **Vautier S, MacCallum DM, Brown GD.** 2012. C-type lectin receptors and cytokines in fungal immunity. Cytokine **58:**89–99. http://dx.doi.org/10.1016/j.cyto.2011.08.031.
7. **Hardison SE, Brown GD.** 2012. C-type lectin receptors orchestrate antifungal immunity. Nat Immunol **13:**817–822. http://dx.doi.org/10.1038/ni.2369.
8. **Hoving JC, Wilson GJ, Brown GD.** 2014. Signalling C-type lectin receptors, microbial recognition and immunity. Cell Microbiol **16:**185–194. http://dx.doi.org/10.1111/cmi.12249.
9. **Wevers BA, Geijtenbeek TB, Gringhuis SI.** 2013. C-type lectin receptors orchestrate antifungal immunity. Future Microbiol **8:**839–854. http://dx.doi.org/10.2217/fmb.13.56.
10. **Wang H, Lebert V, Hung CY, Galles K, Saijo S, Lin X, Cole GT, Klein BS, Wüthrich M.** 2014. C-type lectin receptors differentially induce Th17 cells and vaccine immunity to the endemic mycosis of North America. J Immunol **192:**1107–1119. http://dx.doi.org/10.4049/jimmunol.1302314.
11. **Kerscher B, Willment JA, Brown GD.** 2013. The Dectin-2 family of C-type lectin-like receptors: an update. Int Immunol **25:**271–277. http://dx.doi.org/10.1093/intimm/dxt006.
12. **Balch SG, McKnight AJ, Seldin MF, Gordon S.** 1998. Cloning of a novel C-type lectin expressed by murine macrophages. J Biol Chem **273:**18656–18664. http://dx.doi.org/10.1074/jbc.273.29.18656.
13. **Graham LM, Gupta V, Schafer G, Reid DM, Kimberg M, Dennehy KM, Hornsell WG, Guler R, Campanero-Rhodes MA, Palma AS, Feizi T, Kim SK, Sobieszczuk P, Willment JA, Brown GD.** 2012. The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase. J Biol Chem **287:**25964–25974. http://dx.doi.org/10.1074/jbc.M112.384164.
14. **Arce I, Martinez-Munoz L, Roda-Navarro P, Fernandez-Ruiz E.** 2004. The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor. Eur J Immunol **34:**210–220. http://dx.doi.org/10.1002/eji.200324230.
15. **Miyake Y, Toyonaga K, Mori D, Kakuta S, Hoshino Y, Oyamada A, Yamada H, Ono K, Suyama M, Iwakura Y, Yoshikai Y, Yamasaki S.** 2013. C-type lectin MCL is an FcRgamma-coupled receptor that mediates the adjuvanticity of mycobacterial cord factor. Immunity **38:**1050–1062. http://dx.doi.org/10.1016/j.immuni.2013.03.010.
16. **Furukawa A, Kamishikiryo J, Mori D, Toyonaga K, Okabe Y, Toji A, Kanda R, Miyake Y, Ose T, Yamasaki S, Maenaka K.** 2013. Structural analysis for glycolipid recognition by the C-type lectins Mincle and MCL. Proc Natl Acad Sci U S A **110:**17438–17443. http://dx.doi.org/10.1073/pnas.1312649110.
17. **Wilson GJ, Marakalala MJ, Hoving JC, van Laarhoven A, Drummond RA, Kerscher B, Keeton R, van de Vosse E, Ottenhoff TH, Plantinga TS, Alisjahbana B, Govender D, Besra GS, Netea MG, Reid DM, Willment JA, Jacobs M, Yamasaki S, van Crevel R, Brown GD.** 2015. The C-type lectin receptor CLECSF8/CLEC4D is a key component of anti-mycobacterial immunity. Cell Host Microbe **17:**252–259. http://dx.doi.org/10.1016/j.chom.2015.01.004.
18. **Zhao XQ, Zhu LL, Chang Q, Jiang C, You Y, Luo T, Jia XM, Lin X.** 2014. C-type lectin receptor dectin-3 mediates trehalose 6,6′-dimycolate (TDM)-induced Mincle expression through CARD9/Bcl10/MALT1-dependent nuclear factor (NF)-kappaB activation. J Biol Chem **289:**30052–30062. http://dx.doi.org/10.1074/jbc.M114.588574.
19. **Miyake Y, Masatsugu OH, Yamasaki S.** 2015. C-type lectin receptor MCL facilitates mincle expression and signaling through complex formation. J Immunol **194:**5366–5374. http://dx.doi.org/10.4049/jimmunol.1402429.
20. **Yamasaki S.** 2013. Signaling while eating: MCL is coupled with Mincle. Eur J Immunol **43:**3156–3158. http://dx.doi.org/10.1002/eji.201344131.
21. **Lobato-Pascual A, Saether PC, Fossum S, Dissen E, Daws MR.** 2013. Mincle, the receptor for mycobacterial cord factor, forms a functional receptor complex with MCL and FcepsilonRI-gamma. Eur J Immunol **43:**3167–3174. http://dx.doi.org/10.1002/eji.201343752.
22. **Steichen AL, Binstock BJ, Mishra BB, Sharma J.** 2013. C-type lectin receptor Clec4d plays a protective role in resolution of Gram-negative pneumonia. J Leukoc Biol **94:**393–398. http://dx.doi.org/10.1189/jlb.1212622.
23. **Wuthrich M, Wang H, Li M, Lerksuthirat T, Hardison SE, Brown GD, Klein B.** 2015. Fonsecaea pedrosoi-induced Th17-cell differentiation in mice is fostered by Dectin-2 and suppressed by Mincle recognition. Eur J Immunol **45:**2542–2552. http://dx.doi.org/10.1002/eji.201545591.
24. **Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, Jia XM, Lin X.** 2013. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. Immunity **39:**324–334. http://dx.doi.org/10.1016/j.immuni.2013.05.017.
25. **National Research Council.** 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
26. **Brandhorst TT, Wüthrich M, Warner T, Klein B.** 1999. Targeted gene disruption reveals an adhesin indispensable for pathogenicity of Blastomyces dermatitidis. J Exp Med **189:**1207–1216. http://dx.doi.org/10.1084/jem.189.8.1207.
27. **Wüthrich M, Hung CY, Gern BH, Pick-Jacobs JC, Galles KJ, Filutowicz HI, Cole GT, Klein BS.** 2011. A TCR Transgenic Mouse Reactive with Multiple Systemic Dimorphic Fungi. J Immunol **187:**1421–1431. http://dx.doi.org/10.4049/jimmunol.1100921.
28. **Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y.** 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity **32:**681–691. http://dx.doi.org/10.1016/j.immuni.2010.05.001.
29. **Wüthrich M, Filutowicz HI, Klein BS.** 2000. Mutation of the WI-1 gene yields an attenuated Blastomyces dermatitidis strain that induces host resistance. J Clin Investig **106:**1381–1389. http://dx.doi.org/10.1172/JCI11037.
30. **McGreal EP, Rosas M, Brown GD, Zamze S, Wong SY, Gordon S, Martinez-Pomares L, Taylor PR.** 2006. The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. Glycobiology **16:**422–430. http://dx.doi.org/10.1093/glycob/cwj077.
31. **Fisher LD, van Belle G.** 1993. Biostatistics: a methodology for the health sciences, p 611–613. John Wiley & Sons, New York, NY.
32. **Romani L.** 2011. Immunity to fungal infections. Nat Rev Immunol **11:**275–288. http://dx.doi.org/10.1038/nri2939.
33. **Wüthrich M, Brandhorst TT, Sullivan TD, Filutowicz H, Sterkel A, Stewart D, Li M, Lerksuthirat T, LeBert V, Shen ZT, Ostroff G, Deepe GS, Jr, Hung CY, Cole G, Walter JA, Jenkins MK, Klein B.** 2015. Calnexin induces expansion of antigen-specific CD4(+) T cells that confer immunity to fungal ascomycetes via conserved epitopes. Cell Host Microbe **17:**452–465. http://dx.doi.org/10.1016/j.chom.2015.02.009.
34. **Wüthrich M, Ersland K, Sullivan T, Galles K, Klein BS.** 2012. Fungi subvert vaccine T cell priming at the respiratory mucosa by preventing chemokine-induced influx of inflammatory monocytes. Immunity **36:**680–692. http://dx.doi.org/10.1016/j.immuni.2012.02.015.