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C-Type Lectin Receptors Differentially Induce Th17 Cells and Vaccine Immunity to the Endemic Mycosis of North America

Huafeng Wang,*,1 Vanessa LeBert,*,1 Chiung Yu Hung,‡ Kevin Galles,*, Shinobu Saijo,‡ Xin Lin,§ Garry T. Cole,‡ Bruce S. Klein,*‡,†,‖ and Marcel Wüthrich*†

Vaccine immunity to the endemic mycoses of North America requires Th17 cells, but the pattern recognition receptors and signaling pathways that drive these protective responses have not been defined. We show that C-type lectin receptors exert divergent contributions to the development of antifungal Th17 cells and vaccine resistance against Blastomyces dermatitidis, Histoplasma capsulatum, and Coccidioides posadasii. Acquired immunity to B. dermatitidis requires Dectin-2, whereas vaccination against H. capsulatum and C. posadasii infection depends on innate sensing by Dectin-1 and Dectin-2, but not Mincle. Tracking Ag-specific T cells in vivo established that the Card9 signaling pathway acts indispensably and exclusively on differentiation of Th17 cells, while leaving intact their activation, proliferation, survival, and migration. Whereas Card9 signaling is essential, C-type lectin receptors offer distinct and divergent contributions to vaccine immunity against these endemic fungal pathogens. Our work provides new insight into innate immune mechanisms that drive vaccine immunity and Th17 cells. The Journal of Immunology, 2014, 192: 1107–1119.

Despite several million new systemic fungal infections annually worldwide, there are no commercial vaccines available. We and others have engineered vaccines that protect against experimental infection with the dimorphic fungal pathogens Blastomyces dermatitidis (1), Histoplasma capsulatum (2), and Coccidioides posadasii (3). Our studies showed that vaccine-induced immunity is chiefly mediated by CD4+ T cells (4). Despite the crucial roles of Th1 cells in protective immunity against fungal infection (3, 5, 6) and the controversial roles of Th17 cells in some other infection models (7–13), in our vaccination model Th1 immunity is dispensable, whereas fungus-specific Th17 cells are necessary and sufficient for vaccine-induced protection against these three pathogenic fungi that cause the major endemic mycoses of North America (14). Thus, engaging Th17 cells could be a promising strategy to develop effective fungal vaccines. However, the mechanisms underlying the vaccine-induced Th17 immunity are still largely unknown and need to be determined to develop rationale strategies for antifungal vaccines.

Fungi-specific T cell responses are initiated through the recognition of pathogen-associated molecular patterns by pattern recognition receptors (PRRs) on innate immune cells. Among the best-characterized PRRs that recognize fungi are the ITAM-coupled receptors Dectin-1, Dectin-2, and Mincle. They are C-type lectin receptors (CLRs), which are predominantly expressed in myeloid cells (15, 16). There is accumulating evidence that stimulation of the most-studied CLR, Dectin-1, by β-glucans induces Th17 differentiation of naive CD4+ T cells (17). Recently, Viriyakosol et al. (18) showed that Dectin-1−/− mice infected with Coccidioides immitis have lower levels of Th17 cytokines in their lungs. Patients who are homozygous for a single polymorphism of Dectin-1 are susceptible to mucocutaneous Candida albicans infections (19, 20) and invasive aspergillosis (21, 22) owing to defective IL-17 production. We have found, however, that Dectin-1 is unexpectedly dispensable in the development of vaccine-induced Th17 cell responses and resistance to B. dermatitidis (14). It is unknown whether Dectin-1 is required for the development of vaccine-induced Th17 cells and resistance to H. capsulatum and C. posadasii infection.

In contrast to Dectin-1, few reports describe the role of Dectin-2 in driving Th17 responses. In mice, Dectin-2 is required for the differentiation of Th17 cells induced by C. albicans infection (23). In human dendritic cells (DCs), Dectin-2 activation by Candida results in the selective activation of the NF-κB subunit c-Rel and the production of IL-1β and IL-23 p19, which skews CD4+ T cell responses toward a Th17 profile (24). Although Mincle has been reported to induce Th1/Th17 immunity in response to the mycobacterial cell wall glycolipid trehalose 6,6′-dimycolate (TDM) and its synthetic analog trehalose-6,6′-dibehenate (TDB) (25), to our knowledge its role in driving anti-fungal Th17 responses has not been investigated.

Although Dectin-1 recognizes fungi via β-1,3-glucan exposed on the cell wall and recruits Syk directly through its hemITAM motif (26), Dectin-2 and Mincle recognize mannose-like structures (23, 27–29) and need to pair with the ITAM-bearing adaptor FcRy to
activate the Syk-Card9 pathway (30–32). In mice, Card9 signaling induces DC maturation, the production of proinflammatory cytokines, and the induction of Th17 responses (17). In humans, a Card9 mutation results in susceptibility to chronic mucocutaneous candidiasis (33). Notably, C. albicans—induced Th17 responses are dependent on Card9 (17), and greatly reduced numbers of Th17 cells are associated with patients carrying homozygous Card9 mutations (33), indicating that Card9 is essential for antifungal Th17 responses. However, it is unclear at what stage the Card9 signaling pathway governs Th17 cell development in vivo. To dissect whether Card9 affects the priming, expansion, differentiation, contraction, and migration of vaccine-induced Th17 cells, we exploited an adoptive transfer system that we have previously established using TCR transgenic 1807 cells that recognize a shared Ag from B. dermatitidis, H. capsulatum, and C. posadasii (34). On adoptive transfer into recipient mice, 1807 cells become activated, proliferate, and expand in the draining lymph node (LN); 1807 cells differentiate into cytokine-producing effector T cells after trafficking to the site of vaccination and the lung upon challenge and confer resistance against the three dimorphic fungi (14, 34, 35). Thus, the autologous adoptive transfer system offers a powerful tool to dissect normal or defective development of vaccine-induced Ag-specific T cells responsive to multiple dimorphic fungi.

Although Card9 and CLRs have been implicated in mediating innate resistance to primary fungal infection and priming of Th17 cells, their role in vaccine-induced resistance to fungi and the effect on the sequential stages of T cell development has not been investigated. In this study, we demonstrate that the adaptor Card9 is indispensable for the acquisition of vaccine immunity and the development of Th17 cells against all three systemic dimorphic fungi of North America, but the upstream CLRs play distinctly different roles for each pathogen. We also pinpoint at what stage of the immune response Card9 controls Th17 cell development and show that this adaptor promotes the differentiation of antifungal Th17 cells, but does not influence downstream stages of T cell expansion, activation, contraction, or migration to the lung upon challenge. With B. dermatitidis, the upstream PRR Dectin-2, but neither Mince nor Dectin-1, recognizes yeast and induces ITAM signaling that initiates the development of Th17 cells and vaccine immunity to fungal infection. Conversely, for the related dimorphic fungi H. capsulatum and C. posadasii, while Card9 is required for the induction of vaccine immunity, both Dectin-1 and Dectin-2, but not Mince, participate in the recognition and induction of protective Th17 cell responses. Our data offer insight into the mechanisms that underpin acquisition of vaccine immunity to pathogenic fungi.

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. Care was taken to minimize animal suffering. The work was done with the approval of the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison.

Mouse strains

Inbred wild type C57BL/6 and congenic B6.PLT-Thy1.1/Cy (stock #000406) mice carrying the Thy 1.1 allele were obtained from Jackson Laboratories (Bar Harbor, ME). B6.129P2-FcrlgmutN2 NL12 mice (model no. 583) that lack FcγR were purchased from Taconic. B. dermatitidis specific TCR transgenic (Tg) 1807 mice were generated in our laboratory and were backcrossed to congenic Thy1.1 mice as described elsewhere (35). Card9−/−, Dectin-2−/−, Mince−/−, and Syk−/− mice were a generous gift from Dr. Victor L. J. Tybulewicz. All mice were 7–8 wk old at the time of experiments. Mice were housed and cared for according to guidelines of the University of Wisconsin Animal Care Committee, who approved all aspects of this work.

Fungi and growth conditions

B. dermatitidis strains used were ATCC 26199, a wild type virulent strain, and the isogenic, attenuated mutant lacking BAD1, designated strain #55 (39). Isolates of B. dermatitidis were maintained as yeast on Middlebrook TH1 agar with oleic acid-albumin complex (Sigma, St. Louis, MO) at 39°C. H. capsulatum strain G217B (provided by George S. Deepe, University of Cincinnati, Cincinnati, OH) was maintained on brain-heart infusion slants supplemented with 5% sheep blood at 37°C. C. posadasii isolate C735 is a virulent, human clinical strain that was used to generate a live, attenuated vaccine strain (ΔT) by deleting the chitinases 2, 3, and α-arabinofuranosyl-dehydroxynase genes as described previously (3). The saprobic phase of both the parental and mutant strains were grown on glucose yeast extract medium (1% glucose, 0.5% yeast extract, 1.5% agar) at 30°C for 3–4 wk to generate a confluent layer of arthroconidia (spores) on the agar surface. Formalin-killed endospore-spherical spores of C. posadasii were generated as described (40).

Vaccinations

Mice were vaccinated s.c. at two sites: dorsally and at the base of the tail in mice. B. dermatitidis and C. posadasii attenuated BAD1−/− yeast, designated strain #55, were injected as live or heat-killed cells using a dose range of 10⁶–10⁷ yeast per mouse. For vaccination with H. capsulatum G217B, 10⁶ heat-killed or 107 live yeast per mouse was used. For vaccination with C. posadasii, mice were immunized with 5.0 × 10⁶ viable spores of the ΔT strain (3). Formalin-killed spherical spores (FKS) of C. posadasii were used at 10⁶ per mouse for vaccination. Resistance experiments included one (B. dermatitidis and H. capsulatum) or two (C. posadasii) booster vaccinations of the same vaccine dose and site 2 weeks apart.

Experimental infection

Mice were infected intratracheally with 2 × 10³ or 2 × 10⁴ wild type 26199 B. dermatitidis yeast as described previously (14). At day 4 after infection, coinciding with the peak of T cell influx (14), the mice were sacrificed and lung T cells were analyzed by FACS analysis. Extent of lung infection was examined 2 wk after infection and determined by plating lung homogenates on glucose yeast extract plates containing 50 µg/ml chloramphenicol.

Adaptive transfer of transgenic 1807 T-cells and surface staining

Single-cell suspensions of 10⁶ magnetic bead purified CD4⁺ cells from 1807 Tg Thy1.1 mice were injected i.v. into Thy1.2 C57BL/6 recipients. When indicated, transgenic 1807 cells were labeled with CFSE (Molecular Probes) before adoptive transfer. After vaccination or infection, single-cell suspensions from draining lymph nodes (brachial and inguinal) and lung cells of recipient mice were stained with mAbs directed against the following surface markers: CD4, CD8, Thy1.1, CD44, CD62L, and B220 (as a dump marker to exclude events that are stained nonspecifically). mAbs were obtained from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA), and cytomtery data were gathered with an LSRII Flow Cytometer (BD Biosciences, San Jose). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). 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.

Intracellular cytokine staining

Effector T cells from the site of vaccination and lung were obtained as described previously (14). An aliquot of isolated cells was stained for surface CD4 and Thy 1.1 to determine the percentage of transferred 1807 cells. The numbers of 1807 cells in the lung was derived by multiplying the percentage of cells by the total number of cells per organ isolated. The rest
of the cells were stimulated with anti-CD3 and anti-CD28 mAb in the presence of Golgi-Stop (BD Pharmingen). After 4–6 h of stimulation, cells were stained for surface markers, fixed, and permeabilized using the in Cytofix/Cytoperm kit (BD Pharmingen) and stained with anticytokine Abs as described (14).

Generation of bone marrow dendritic cells

Bone marrow–derived dendritic cells (BMDCs) were obtained from the femurs and tibias of individual mice. Each bone was flushed with 10 ml of RPMI 1640 containing 10% FBS plus penicillin-streptomycin (HyClone), 2-ME, and 20 ng/ml of rGM-CSF. The culture media were refreshed every 3 d, and BMDCs were harvested after 10 d in vitro coculture assays.

Ex vivo and in vitro coculture for cytokine protein measurement

Ex vivo cell culture supernatants were generated using the bronchial and inguinal draining lymph nodes harvested from mice 10 d after vaccination, washed, and resuspended in complete RPMI 1640 containing 10 µg/ml yeast cell wall membrane Ag (CWM) (1), and plated in 96-well plates at a concentration of 5 × 10^5 cells/well. Supernatants were collected from ex vivo cocultures after three days of incubation at 37°C and 5% CO₂ (14). In vitro cell culture supernatants were generated using BMDCs plated in complete RPMI 1640 at a concentration of 10⁵ cells/well for 18 h. The dissolved tissue was washed with ice-cold PBS containing 5 mM EDTA and 1% BSA and strained again. Filtered cells were resuspended in complete media for stimulation.

Isolation of effector cells at the site of vaccination

Mice were vaccinated once s.c. with 10⁷ heat-killed strain #55 yeast at a single dorsal site. On day 10 after vaccination, inflamed s.c. tissue was excised from the site of vaccination, placed in ice-cold collagenase buffer and minced into fine pieces. To digest the tissue, 5 ml of dissociation buffer containing 5 mM EDTA and 1% BSA and strained again. Filtered cells were resuspended in complete media for stimulation.

RT-PCR analysis for the detection of Card9–associated gene transcripts

BMDCs were raised from 15 individual Card9⁻/⁻ or wild type mice and 3 × 10⁵ nonadherent DCs from each individual mouse were cocultured with 3 × 10⁵ B. dermatitidis yeast vaccine or medium alone in a 24-well plate for 24 h. Total RNA from the BMDC cultures described above was purified using the Qiagen RNeasy kit (catalog #74106) according to the manufacturer’s protocol. Genomic DNA was removed using Turbo DNase (Ambion, catalog no. AM2238). Following DNase treatment, the RNA was cleaned up using Qiagen RNeasy columns according to the manufacturer’s protocol and by performing additional washes with buffer RPE and 80% ethanol to remove contaminants. The n-fold change of gene expression for yeast stimulation versus no stimulation was calculated using the comparative C₇ method (41).

Generation of Fc fusion proteins

The Dectin-2-Fc expression vector was constructed by cell surface staining and FACS analysis using an anti-mouse FcR chain (44) and the parent BWZ cells (45), were a generous gift from Dr. Caetano Reis e Sousa (London Research Institute, London, U.K.). To construct Mincle-expressing reporter cells, the full-length cDNA of mouse Mincle was subcloned from the pFF1HIBS-mMincle plasmid into a pFB-IRES-EGFP retrovector. BMDCs were retrovirally transduced with pFB-mMincle-IRES-EGFP, selected based on EGFP expression, and verified by cell surface staining and FACS analysis using an anti-mouse Mincle mAb (MBL). To generate B3Z cells coexpressing Mincle and the adopter FcRγ, Mincle-expressing reporter cells were retrovirally transduced with pMX-IP-FcRγ-chain (wild type) or pMX-IP-FcRγm (mutant) (31). FcRγ expression was verified with Western blotting using an anti-FcRγ Ab (Upstate).

For B3Z/BWZ cell stimulation, 10⁵ B3Z/BWZ cells/well in a 96-well plate were incubated for 18 h with heat-killed fungal cells. lacZ activity was measured in total cell lysates using CPG (Roche) as a substrate. OD 560 was measured using OD 620 as a reference. For the B3Z/BWZ cell binding assay, 2 × 10⁵ B3Z/BWZ cells were prestained with CFSE (Molecular Probes) and incubated for 6 h with 10⁶ B. dermatitidis, 2 × 10⁵ H. capsulatum, or 2 × 10⁵ C. posadasi prestained with LIVE/DEAD Violet staining (Invitrogen). Bound fungal cells were quantified with flow cytometry.

Statistical analysis

Differences in the number and percentage of activated, proliferating or cytokine-producing T cells, and in the number of CFUs were analyzed using the Wilcoxon rank test for nonparametric data or the t test when data were normally distributed (46). Factorial ANOVA analysis using the SAF software was used to compare the vaccine-induced reduction in lung CFUs between different strains of mice; p < 0.05 is considered statistically significant.

Results

Vaccine-induced immunity against systemic dimorphic fungi requires the adaptor Card9

We previously reported that vaccine-induced Th17 cells were necessary and sufficient to protect mice against the three major systemic mycoses in North America (14). Because Card9 has been reported to be required for the development of Th17 cells in responses to primary C. albicans infection (17), we investigated whether Card9⁻/⁻ mice can acquire vaccine-induced immunity against infection with the systemic dimorphic fungi. Card9⁻/⁻ mice were unable to control the live, virulence-attenuated #55 vaccine strain of B. dermatitidis and succumbed to widespread dissemination and infiltration of the lungs by the yeast (Fig. 1A). To circumvent susceptibility of Card9⁻/⁻ mice to the vaccine strain, we immunized them with heat-killed yeast and tested their ability to resist a lethal pulmonary infection with wild type yeast.

Vaccinated Card9⁻/⁻ mice had a burden of lung infection similar to the wild type mice.
to unvaccinated littersmates, which was more than 5 logs higher than in vaccinated wild type controls, indicating the adaptor Card9 is essential for the acquisition of immunity to B. dermatitidis infection (Fig. 1B). Interestingly, unvaccinated Card9−/− mice were more susceptible to primary infection than unvaccinated wild type controls, suggesting that Card9 also contributes to innate resistance. To determine whether Card9 is required for the development of Th17 cells to the lung upon challenge. Vaccinated Card9−/− mice recruited reduced numbers and frequencies of IL-17– and IFN-γ–producing Ag-specific 1807 cells to the lungs compared with vaccinated wild type controls (Fig. 1C–D). Thus, the adaptor Card9 is required for the development of antifungal Th17 responses after vaccination with B. dermatitidis.

To test whether Card9 is necessary for the acquisition of vaccine-induced immunity and the development of Th17 cell responses to other systemic dimorphic fungi, we vaccinated mice with H. capsulatum and C. posadasii. Card9−/− mice vaccinated with live G217B H. capsulatum yeast as described (14) contained the vaccine in the s.c. tissue and did not succumb to dissemination (data not shown), but they failed to acquire resistance to pulmonary challenge (Fig. 1B) and recruited reduced numbers and frequencies of IL-17– and IFN-γ–producing 1807 cells to the lungs (Fig. 1C–D). Card9−/− mice succumbed to dissemination when vaccinated s.c. with live C. posadasii conidia from wild type strain C735 (C.-Y. Hung, M. del Pilar Jimenez-Alzate, A. Gonzalez, M. Wüthrich, B.S. Klein, and G.T. Cole, submitted for publication).

To determine whether Th17 cells develop in the absence of Card9,
knockout mice were instead vaccinated with either FKS or live conidia of an attenuated ΔT vaccine strain. This strain lacks the chitinases 2, 3, and β-arabinotol-2-dehydrogenase genes (3) and fails to endosporulate and disseminate in s.c. vaccinated Card9<sup>−/−</sup> mice. Card9<sup>−/−</sup> mice vaccinated with FKS failed to acquire resistance to challenge (Fig. 1B) and recruited lower numbers and frequencies of IL-17– and IFN-γ–producing 1807 cells to the lungs upon challenge compared with wild type controls (Fig. 1C–D). Vaccination with the attenuated ΔT vaccine strain yielded similar results (data not shown). In contrast to primary infection with <i>B. dermatitidis</i> and <i>H. capsulatum</i>, unvaccinated Card9<sup>−/−</sup> mice were not more susceptible than wild type controls to infection with <i>C. posadasii</i>. Thus, the Card9 signaling pathway is required for the development of vaccine-induced resistance and antifungal Th17 responses to the three major systemic dimorphic fungi.

**Expansion and activation of Ag-specific cells does not require Card9**

Receptor signaling pathways can affect T cell development at multiple stages. In the setting of LCMV infection, Tc1 cells require intrinsic TLR/Myd88 signals not for activation, expansion, or differentiation, but to survive apoptosis after these events (47). Because Card9 is required for the development of vaccine-induced anti-fungal Th17 cells, we investigated the stage of the immune response at which Card9 governs T cell priming. To do so, we adoptively transferred magnetic bead-purified CD4<sup>+</sup>, CFSE-labeled naive T cells from 1807 mice into Card9<sup>−/−</sup> and wild type recipients prior to vaccination with <i>B. dermatitidis</i> and analyzed T cell development at serial time points thereafter. At day 7 after vaccination, mice lacking Card9 showed no deficit in the proliferation and activation, but had a small reduction in expansion of Ag-specific 1807 cells (Fig. 2A–C), indicating that Card9 minimally affects the early stages of vaccine-induced T cell development. At subsequent time points (days 14 and 35 after vaccination), activated 1807 cells underwent similar contraction. Upon recall at day 4 after infection, the frequency and number of activated CD4<sup>+</sup> 1807 cells were comparable in the skin-draining lymph node (sdlN) and in the lung of Card9<sup>−/−</sup> and wild type recipient mice (Fig. 2B–C). These data indicate that the adaptor Card9 is not required for the activation, proliferation, and contraction of Ag-specific 1807 cells in the sdlN. Although the expansion of 1807 cells was minimally affected by the absence of Card9, this did not affect the number of 1807 cells in the sdlN and the lung at day 4 after challenge.

**Th17 cell differentiation requires Card9**

Because Card9<sup>−/−</sup> mice failed to recruit IL-17– and IFN-γ–producing 1807 cells to the lung (Fig. 1C–D), we investigated whether Card9 governs the differentiation of effector T cells during the vaccine-priming phase. We analyzed T cell differentiation by three independent approaches. First, we harvested activated T cells at the peak of expansion from the sdlN and stimulated them ex vivo with cell wall membrane (CW/M) Ag. T cells from Card9<sup>−/−</sup> mice produced ~8-fold less IL-17 protein in response to fungal Ag than did wild type T cells (Fig. 3A). Similarly, IFN-γ protein levels were reduced by at least 30-fold compared with wild type controls. Although Card9 is primarily responsible for receptor signaling in myeloid but not lymphoid cells, we sought to exclude formally a T cell–intrinsic role of Card9 for the differentiation of Th17 cells. Thus, in a second approach we cocultured BMDCs from Card9<sup>−/−</sup> and wild type mice together with vaccine yeast and naive wild type 1807 cells in vitro. 1807 cells cocultured with Card9<sup>−/−</sup> DCs produced ~7-fold less IL-17 than did cocultures with wild type DCs (Fig. 3B). Third, to investigate Th17 cell differentiation in vivo, we determined the frequency of cytokine-producing wild type 1807 cells at the site of vaccination. The frequency of IL-17– and IFN-γ–producing

**FIGURE 2.** Card9 is dispensable for T cell expansion and activation. (A) Wild type and Card9<sup>−/−</sup> mice received an adoptive transfer of 10<sup>6</sup> CD4<sup>+</sup>-purified, CFSE-labeled, naive 1807 Tg cells and were vaccinated with 10<sup>6</sup> HK <i>B. dermatitidis</i> yeast or not. Transferred 1807 cells were harvested from the sdlN and proliferation was assessed by enumerating the frequency of CFSE<sup>low</sup> cells (mean ± SEM; <i>n</i> = 4–6 mice/group; representative of three experiments) at day 7 after vaccination. (B) At serial times after vaccination, the frequency of activated (CD44<sup>hi</sup>) 1807 Tg cells was determined in the sdlN and in the lung at day four after infection. Data are the mean ± SEM (<i>n</i> = 4–6 mice/group); representative of five experiments. (C) The number of activated (CD44<sup>hi</sup>) 1807 Tg cells (mean ± SEM; <i>n</i> = 4–6 mice/group) and are representative of five experiments. *<i>p</i> < 0.05 versus vaccinated wild type controls.
1807 cells recruited to the s.c. tissue was reduced in Card9<sup>−/−</sup> versus wild type mice (Fig. 3C). Thus, extrinsic Card9 signaling is required for the differentiation of Th17 and Th1 cells during the priming phase of vaccine-induced immunity.

**Card9-dependent genes induced by vaccine yeast**

Upon vaccination, APCs become activated and generate a milieu that fosters the generation of Th17 and Th1 cells. To investigate genes that are induced upon yeast exposure, we cocultured the *B. dermatitidis* vaccine with BMDCs from 15 individual wild type and Card9<sup>−/−</sup> mice, harvested their RNA, and analyzed gene expression by microarray. Among the upregulated genes, some were canonical genes that are known to induce the differentiation of Th17 cells (data not shown). To verify differential gene expression, we used T hybridoma cells expressing an NFAT-lacZ reporter of ITAM signaling (31, 44). In response to vaccine yeast stimulation, lacZ activity was increased in T hybridoma cells coexpressing Dectin-2 and wild type yeast, but not in reporter cells expressing either Dectin-2 or FcR<sub>y</sub> (Supplemental Fig. 1A). Fluorescent microscopy showed punctate staining of Fc fusion proteins around the cell wall (Fig. 4B). The carbohydrate recognition domain of Dectin-2 and Mincle requires calcium for binding, so we added 5 mM EDTA to the scaffolding conditions. Staining of the yeast was abrogated by the addition of 5 mM EDTA (data not shown), indicating that the binding of the soluble CLRs to the yeast occurred in a C-type lectin-specific manner.

To investigate whether vaccine yeast are recognized by cell surface-bound CLRs and induce downstream receptor signaling, we used T hybridoma cells expressing an NFAT-lacZ β-galactosidase reporter of ITAM signaling (31, 44). In response to vaccine yeast stimulation, lacZ activity was increased in T hybridoma cells coexpressing Dectin-2 and FcR<sub>y</sub>, but not in reporter cells expressing either Dectin-2 or FcR<sub>y</sub> alone (Fig. 5A). Because surface expression of Dectin-2 requires coexpression of FcR<sub>y</sub> (31), reporter cells expressing Dectin-2 and FcR<sub>y</sub> together bound vaccine yeast (Fig. 5B), whereas reporter cells expressing Dectin-2 or FcR<sub>y</sub> alone bound yeast poorly (Supplemental Fig. 1A). As expected, reporter cells coexpressing an ITAM signaling-defective mutant of FcR<sub>y</sub> chain (31) and Dectin-2 failed to increase reporter activity (Fig. 5A), indicating that ITAM signaling mediates the reporter cell activation. However, this reporter cell line bound vaccine yeast as efficiently as T hybridoma cells coexpressing Dectin-2 and wild type FcR<sub>y</sub> (Supplemental Fig. 1A), indicating that the FcR<sub>y</sub> mutations did not affect the ability of Dectin-2 to mediate binding of vaccine yeast. In contrast to Dectin-2, reporter cells expressing Dectin-1 or Mincle did not increase lacZ activity (Fig. 5A) and...
bound vaccine yeast poorly (Fig. 5B; Supplemental Fig. 1B–C), although the respective soluble CLRs bound the yeast. Thus, Dectin-2, but not Dectin-1 or Mincle, recognized B. dermatitidis vaccine yeast and triggered downstream ITAM signaling through FcRγ.

**Dectin-1 and Dectin-2 recognize multiple dimorphic fungi**

The three CLRs differentially recognize B. dermatitidis; therefore, we investigated how they recognize H. capsulatum and C. posadasii. We incubated the latter two fungi with Fc fusion proteins and analyzed them by FACS. All three soluble CLR fusion proteins stained H. capsulatum yeast to various degrees (Fig. 4A). Soluble Dectin-1–Fc stained the yeast strongly, Dectin-2–Fc stained intermediately and Mincle–Fc stained the least. All three soluble CLR fusion proteins stained C. posadasii efficiently (Fig. 4A). Fluorescent microscopy showed punctate staining of Fc fusion proteins on the cell wall of H. capsulatum (Fig. 4B), whereas a more evenly distributed staining pattern was observed on the surface of C. posadasii (Fig. 4B). Dectin-1– and Dectin-2–expressing T hybridoma cells showed increased lacZ reporter activity when stimulated with H. capsulatum or C. posadasii (Fig. 5A), whereas no increase in reporter activity was detected with Mincle-expressing reporter cells. Dectin-1– and Dectin-2–expressing T hybridoma cells consistently bound H. capsulatum and C. posadasii, whereas Mincle-expressing and control reporter cells failed to do so (Fig. 5B; Supplemental Fig. 1). Because all three dimorphic fungi failed to increase reporter activity of Mincle-expressing T hybridoma cells, we used TDB, a known ligand for Mincle and synthetic analog of the mycobacterial cord factor, as a positive control reporting cells (data not shown). Thus, Dectin-1 and Dectin-2, but not Mincle, recognized and responded to both H. capsulatum and C. posadasii.

Th17 cell differentiation and vaccine immunity to B. dermatitidis requires Dectin2 and FcRγ

Because Dectin-2 recognizes and binds B. dermatitidis vaccine yeast and triggers functional ITAM signaling, we tested to determine whether Dectin-2 and its downstream signaling components are necessary to prime Th17 cells and acquire vaccine-induced resistance. We studied Th17 cell differentiation using several approaches. First, we adoptively transferred naive magnetic bead purified CD4+ 1807 cells into Dectin-2−/−, FcRγ−/−, and Mincle−/− mice before vaccination, harvested the primed T cells from the sdLN at the peak of expansion, and stimulated them with CW/M Ag in vitro as described above. T cells primed in Dectin-2 and FcRγ-deficient mice produced significantly less IL-17 and IFN-γ than did T cells from wild type or Mincle−/− mice (Fig. 6A). In this assay, both endogenous and adoptively transferred CD4+ T cells contributed to cytokine production and a T cell intrinsic contribution cannot be evaluated. Thus, second, to exclude a T cell intrinsic role of either Dectin-2 or FcRγ, we studied T cell differentiation of naive wild type 1807 cells in vitro. When cocultured with BMDCs from Dectin-2 and FcRγ-deficient mice, 1807 cells produced significantly less IL-17 compared with wild type recipient mice (Fig. 6C). Fourth, we studied Th17 cell differentiation using several approaches. First, we adoptively transferred naive magnetic bead purified CD4+ 1807 cells into Dectin-2−/−, FcRγ−/−, and Mincle−/− mice before vaccination, harvested the primed T cells from the sdLN at the peak of expansion, and stimulated them with CW/M Ag in vitro as described above. T cells primed in Dectin-2 and FcRγ-deficient mice produced significantly less IL-17 and IFN-γ than did T cells from wild type or Mincle−/− mice (Fig. 6A). In this assay, both endogenous and adoptively transferred CD4+ T cells contributed to cytokine production and a T cell intrinsic contribution cannot be evaluated. Thus, second, to exclude a T cell intrinsic role of either Dectin-2 or FcRγ, we studied T cell differentiation of naive wild type 1807 cells in vitro. When cocultured with BMDCs from Dectin-2 and FcRγ-deficient mice, 1807 cells produced significantly less IL-17 compared with wild type recipient mice (Fig. 6C). Fourth, we studied cultivation of wild type 1807 cells in vivo at the site of vaccination. The frequency of 1807 cells producing IL-17 and IFN-γ was significantly reduced in Dectin-2−/− and FcRγ−/− compared with wild type recipient mice (Fig. 6C). Fourth, we determined the number and frequencies of IL-17− and IFN-γ− producing 1807 cells that migrated to the lung upon recall. Both the numbers and frequencies of Ag-specific Th17 and Th1 1807...
cells that migrated to the lungs were significantly reduced in vaccinated Dectin-2−/− mice, compared with wild type controls that were vaccinated and then challenged with H. capsulatum (Fig. 7A). Fewer IFN-γ–producing T cells also were found in the lungs of vaccinated and challenged Dectin-2−/− mice compared with wild type mice. Histoplasma vaccinated Dectin-2−/− mice likewise reduced lung CFU significantly less after challenge than did vaccinated wild type or Mincle−/− mice (Fig. 7B). Although Dectin-1−/− mice were nearly 10-fold less resistant than wild type mice, this difference did not achieve statistical significance.

In the Coccidioides model, the development of Th17 (and Th1) cells were impaired in C. posadasii vaccinated and challenged Dectin-2−/− mice, as compared with wild type and Mincle−/− recipients. The acquisition of vaccine immunity likewise was blunted in these mice, as determined by the reduction of lung and spleen CFU (Fig. 7C+D). Thus, the Dectin-2/FcRγ/Syk/Card9 signaling pathway is required for the optimal development of vaccine-induced Th17 cells and acquired resistance to multiple systemic dimorphic fungi. In addition, whereas Dectin-1 is required for the acquisition of vaccine-induced Th17 cells and resistance to H. capsulatum, this CLR is dispensable for eliciting these responses to C. posadasii (data not shown and paper in preparation).

**FIGURE 5.** Differential recognition of three dimorphic fungi by surface bound Dectin-1, Dectin-2, and Mincle receptors. (A) BWZ cells and a subline expressing Dectin-1-CD3ζ (Dectin-1), as well as B3Z cells expressing Dectin-2, Mincle, FcRγ-chain, Dectin-2 + FcRγ, Dectin-2 + FcRγm (a signaling-defective FcRγ-chain), Mincle + FcRγ, or Mincle + FcRγm were stimulated with the indicated numbers of heat-killed B. dermatitidis (left column), H. capsulatum (middle column), or C. posadasii (right column). After 18 h, lacZ activity was measured using a colorimetric assay and expressed as OD 560/620 values. Data are the mean ± SD of duplicate wells. (B) Reporter cells expressing Dectin-1-CD3ζ, Dectin-2 + FcRγ, or Mincle + FcRγ were prestained with CFSE and incubated for 6 h with heat-killed fungal cells prestained with LIVE/DEAD Violet staining. The numbers indicate the frequencies of B3Z/BWZ cells bound by fungi as analyzed by FACS. Data are representative of two independent experiments.
Discussion

The development of effective vaccines requires a fundamental understanding of how protective immune responses are induced. In this study, we identify the signaling adaptors and PRRs that govern the acquisition of vaccine immunity to the three major systemic mycosis of North America by differentiating naive fungus-specific T cells into protective Th17 cells. Although Card9 has been implicated in host protection to primary infection and the development of Th17 cells (17), we establish that it has a requisite role in the induction of vaccine immunity, and we uncover the
and wild type conidia of controls. s.c. delivery of live attenuated vaccine yeast of spherules of \textit{H. capsulatum} live \textit{B. dermatitidis} yeast, live vaccinated with 10^7 HK \textit{H. capsulatum} G217B yeast and challenged with 10^6 FKS. Data are the mean ± SEM (n = 4–6 mice/group); representative of two experiments. *p < 0.05 versus vaccinated wild type controls. (B) Wild type, Dectin-2^-/-, FcRγ^-/-, and Mincle^-/- mice were vaccinated with 10^7 HK \textit{H. capsulatum} G217B yeast (or not as a control) and challenged with 10^5 \textit{H. capsulatum} yeast 2 wk after booster vaccination. Lung CFU were determined 13 d postinfection. Data are the mean ± SEM (n = 8–12 mice/group); representative of two experiments. Numbers indicate the n-fold change in lung CFU versus unvaccinated controls. *p < 0.05 versus vaccine-induced reduction in CFU in wild type mice. (C) Same as (A), but mice were vaccinated with 10^6 FKS of \textit{C. posadasii}. Two wk after the boost, the mice were challenged with 10^6 FKS. Data are the mean ± SEM (n = 4–6 mice/group); representative of two experiments. *p < 0.05 versus vaccinated wild type controls. (D) Wild type, Dectin-2^-/-, FcRγ^-/-, and Mincle^-/- mice were vaccinated with 10^6 FKS or 5 × 10^5 viable spores of the ΔT strain (or not as a control) and challenged with 50–80 \textit{C. posadasii} spores 4 wk later. Lung and spleen CFU were determined 14 d postinfection. Data are the averages ± SEM of two independent experiments (n = 8–12 mice/group/experiment); representative of two experiments. Numbers indicate the n-fold change in lung CFU versus unvaccinated controls. *p < 0.05 versus vaccine-induced reduction in lung CFU in wild type mice.

we found that Card9 is essential for the acquisition of vaccine-induced resistance to infection with the dimorphic fungi \textit{B. dermatitidis}, \textit{H. capsulatum}, and \textit{C. posadasii}. Card9^-/- mice failed to control s.c. delivery of live attenuated vaccine yeast of \textit{B. dermatitidis} and wild type conidia of \textit{C. posadasii}, but survived vaccination with live \textit{H. capsulatum} yeast. In response to vaccination with heat killed \textit{B. dermatitidis} yeast, live \textit{H. capsulatum} yeast or formalin-fixed spherules of \textit{C. posadasii}, Card9^-/- mice failed to acquire vaccine resistance. These results indicate that the Card9 signaling pathway is essential and can be harnessed for the induction of vaccine-induced protection against dimorphic fungal infection.

Because the generation of Th17 cells directly correlates with vaccine-induced resistance to the systemic dimorphic fungi (14), we determined the number of IL-17–producing CD4^+ T cells in the lungs of vaccinated mice upon recall after challenge. We also enumerated the number of IFN-γ–producing CD4^+ T cells because they can contribute to vaccine-induced resistance (4), although Th1 cells are dispensable, unlike Th17 cells (14). We observed that the numbers of Th17 and Th1 cells in all three vaccine models were significantly reduced in vaccinated Card9^-/- versus wild type mice. Using our recently developed adoptive transfer system with TCR transgenic 1807 cells, which recognize a shared and protective Ag among the systemic dimorphic fungi (34), we demonstrated that although Card9 is required for the differentiation of naïve T cells into Th17, the adaptor is largely dispensable for the activation, expansion, proliferation, and survival during the contraction phase, and for recruitment to the lung upon recall. Moreover, Card9 not only affected the differentiation of Th17 cells; it likewise affected Th1 cells. Although the role of the CLR-Card9 signaling pathway on the development of effector Th17 and Th1 cells has not been studied in vivo, the TLR-Myd88 pathway...
pathway has been shown to exert an incremental impact on the recognition of A. fumigatus–specific CD4+ T cells during pulmonary infection (48). Myd88-mediated signals were dispensable for the recruitment and expansion of Ag-specific T cells to the mediastinal lymph nodes (MLN) and trafficking to the lung after priming. However, Myd88 signaling was required to enhance T-bet induction and differentiation of Th1 cells in the MLN. Further maturation of MLN-primed T cells into fully differentiated IFN-γ-producing Th1 cells occurred upon arrival of the cells in the airways and was TLR and Myd88 independent.

To discern how Card9 induces T helper differentiation, we performed a microarray analysis and verified differential gene expression by real-time RT-PCR. Among those differentially expressed were genes known to induce Th17 and Th1 cells, such as IL-6, IL-23, TGF-β, IL-1β, and IL-12 p35. Thus, Card9 governs the expression of cytokines that are instrumental in driving differentiation of Th17 and Th1 cells. Using the microarray data, we also found noncanonical genes that have not been linked previously to Th17 differentiation (V. LeBert, H. Wang and M. Wüthrich, unpublished data). We intend to study the role of novel candidates in elucidating how Card9 governs the expression of the Th17 and Th1 priming cytokines.

To investigate which PRRs upstream of Card9 are required to induce the differentiation of Th17 cells and vaccine-induced immunity, we first tested whether soluble fusion proteins consisting of the extracellular domain of Dectin-1, Dectin-2, and Mincle and human Fc can stain fungal yeast and spherules. Soluble Dectin-1 and Dectin-2 have been used previously to stain H. capsulatum and H. capsulatum, such as IL-6, IL-23, TGF-β, IL-1β, and IL-12 p35. Thus, Card9 governs the expression of cytokines that are instrumental in driving differentiation of Th17 and Th1 cells. Using the microarray data, we also found noncanonical genes that have not been linked previously to Th17 differentiation (V. LeBert, H. Wang and M. Wüthrich, unpublished data). We intend to study the role of novel candidates in elucidating how Card9 governs the expression of the Th17 and Th1 priming cytokines.

To investigate which PRRs upstream of Card9 are required to induce the differentiation of Th17 cells and vaccine-induced immunity, we first tested whether soluble fusion proteins consisting of the extracellular domain of Dectin-1, Dectin-2, and Mincle and human Fc can stain fungal yeast and spherules. Soluble Dectin-1 and Dectin-2 have been used previously to stain A. fumigatus (42), Fonsecaea pedrosii (49), C. albicans (27, 30), and C. immitis (18). Here, all three soluble Fc chimeras were able to bind to the three dimorphic fungi; however, the binding efficiency between soluble receptors and fungal species varied presumably based on respective ligand expression and size of the organisms. Second, to explore whether cell surface–bound CLRs can recognize and bind the fungal vaccine strains we cocultured T cell hybridoma cells that were engineered to express a β-galactosidase NFT-lacZ reporter of ITAM signaling (43). By using this nonmyeloid reporter system, we were able to distinguish between signaling events triggered by putative CLR ligands versus other ligands. To our surprise, some of the hybridoma cell–expressed CLRs showed a recognition pattern different from soluble CLR chimera. For example, soluble Dectin-1 stained B. dermatitidis vaccine yeast, but surface expressed receptor did not recognize the vaccine yeast. Similarly, soluble Mincle stained all three dimorphic fungi, but Mincle expressing reporter cells did not become activated and failed to bind the three fungi. On the other hand, soluble and surface-expressed Dectin-2 yielded consistent results for each of the fungi tested. Although soluble CLR chimera gave valid binding results, they were less reliable than surface expressed CLRs in forecasting the in vivo role of CLRs in vaccine resistance.

One potential explanation for the discrepant findings between the two assays could be that only surface-exposed fungal ligands can be recognized by cell surface–expressed receptors, but not ligands that are embedded deeper in the cell wall; however, the latter might still be accessible by soluble CLRs. Thus, cell surface–expressed receptors on the T hybridoma reporter cells helped to predict the importance of the receptor for the priming of T helper responses in vivo. Indeed, the failure of cell surface–expressed Dectin-1 to recognize B. dermatitidis is consistent with our previous data showing that Dectin-1 is dispensable for the induction of Th17 and Th1 cells and vaccine immunity (14). Similarly, the phenotypes of Dectin-2 and Mincle in the vaccination model with B. dermatitidis showed good fidelity with the results of the NFT reporter cells. Dectin-2−/− mice showed a severe defect in differentiation of fungus-specific Th17 cells and vaccine immunity, indicating that Dectin-2 is indispensable for the acquisition of adaptive immunity to B. dermatitidis infection. The inability of T hybridoma–expressed Mincle to trigger NFAT-reporter activity or bind to the three dimorphic fungi correlated with the dispensability of this CLR in the vaccine models.

Similar to Card9, the upstream receptor Dectin-2 affected the development of both B. dermatitidis–specific Th17 and Th1 but not Th2 cells during vaccination. Therefore, Dectin-2 and Card9 deficiency did not shift the balance of Th cell differentiation as has been reported for Th17 responses that depended on Dectin-1 during a pulmonary infection of mice with A. fumigatus (50). Dectin-1 deficiency disproportionally increased Th1 responses and decreased Th17 differentiation. The authors showed that Dectin-1 mediated signals alter CD4 T cell responses to fungal infection by reducing the production of IFN-γ and IL-12p40 in innate cells, thereby decreasing T-bet expression in responding CD4 T cells and enhancing Th17 responses (50).

Despite the fact that cell surface–expressed Dectin-1 and Dectin-2 recognized and bound H. capsulatum yeast and C. posadasii spherules, the effect on Th17 cell priming and the acquisition of vaccine immunity was modest, albeit statistically significant. Our data are compatible with the interpretation that these two CLRs are redundant or cooperate in the innate recognition of these two dimorphic fungi. Receptor collaboration has been reported for Dectin-1 and multiple TLRs (51), and for the recognition of Malassezia by Dectin-2 and Mincle through distinct ligands (29). The fact that, H. capsula-tatum and C. posadasii vaccinated FcRγ−/− mice showed stronger phenotypes in acquiring vaccine-induced immunity and Th17 cells compared with Dectin-2−/− mice is compatible with the notion of receptor cooperation. Alternatively, other FcRγ coupled CLR alone or together with Dectin-2 could play a prominent role in innate fungal recognition as well. A recently identified CLR, MCL, is a potential candidate, and mediates TDM-induced acquired immune responses (52); however, its role in vaccine immunity to fungi has not been studied.

A prior study showed that β-glucan on H. capsulatum yeast was shielded by α-glucan and not recognized by Dectin-1 (53), whereas we observed that Dectin-1 expressing reporter cells bound to H. capsulatum, which triggered lacZ reporter activity in vitro. Consequently, in vivo, the induction of Th17 cells, the acquisition of vaccine-induced resistance to H. capsulatum infection, and resistance to primary infection were all blunted in Dectin-1−/− versus wild type control mice. The differing results between the two studies may be due to strain specific differences in the cell wall composition. We used the North American, RFLP class 2 and chemotype I strain G217B (ATCC 26032) that mostly lacks α-1,3-glucan on the yeast surface. In contrast, Rappeleye et al. (53) used the Central and South American, RFLP class 3 and chemotype II yeast strain G186AR (ATCC 26029), which expresses surface α-1,3-glucan that shields β-glucan from detection by Dectin-1. In a recent report (54), liquid grown G217B yeast bound to Dectin-1–expressing 3T3 fibroblasts when yeast were harvested during the exponential phase, but not during the stationary phase (54). In our study, we grew G217B yeast for 3 to 7 d on slants and heat-killed them to perform overnight incubation in the lacZ reporter assay. Surface staining of yeast with Dectin-1–fused protein was comparable in live versus heat-killed cells, indicating that heat inactivation did not increase exposure of β-glucan. However, we have seen large differences in the gross appearance of the yeast cell wall by electron microscopy (H. Wang and M. Wüthrich, unpublished data) when comparing liquid versus agar grown yeast, which could account for the different Dectin-1 binding phenotypes. Nonetheless, our data suggest that β-glucan is not shielded in H. capsu-
latum strain G217B yeast in vivo, and it is recognized by Dectin-1 during primary and adaptive immunity to recruit and prime Th17 cells, respectively. Dectin-1 induces two independent signaling pathways—one through Syk and one through Raf-1—to control adaptive immunity (55, 56), but we have not determined whether the FcRy/Card9 and Dectin-1 phenotypes in the vaccination model with H. capsulatum are functionally linked or unrelated/Raf-1 dependent.

The fungal ligands of most CLR s remain largely unclear. It is likely that differences in the cell wall composition and structure between the endemic fungi account for the differential recognition by Dectin-1, Dectin-2, and Mincle. In addition, individual CLR can recognize several distinct ligands within one fungal species and among different fungi and microorganisms. For example, Mincle recognizes TDM from mycobacteria, Corynebacteria, and Nocardia (57), its synthetic analog TDB (25) and two structurally different glycolipids 44-1 and 44-2 from Malassezia (29). Similarly, Dectin-2 recognizes the terminal mannose of N-linked glycan (27) and β-linked 1-2-mannosyl residues from Malassezia (29). Thus, it is conceivable that Dectin-2 recognizes ligands in the systemic fungi that are structurally distinct from those that have been described above.

CLR s have been of burgeoning interest as targets to enhance vaccine efficacy (56, 58). A promising strategy is to use carbohydrate ligands of CLR s to drive vaccine responses (59, 60). For example, the particulate β-glucan curdlan, a well-defined ligand for Dectin-1, has been reported to be a potent adjuvant that primes anti-CD4 Th17 responses (61). Moreover, porous β-glucan particles consisting primarily of β-1,3,1,6-glucans and allow for high Ag loading have been exploited as a receptor-targeted vaccine delivery system (62). Thus, the glucan particle-based vaccine yields an attenuated Blastomyces dermatitidis strain that induces host resistance (63). In contrast, Mincle recognizes TDM from mycobacteria, Corynebacteria, and Nocardia (57), its synthetic analog TDB (25) and two structurally different glycolipids 44-1 and 44-2 from Malassezia (29). Similarly, Dectin-2 recognizes the terminal mannose of N-linked glycan (27) and β-linked 1-2-mannosyl residues from Malassezia (29). Thus, it is conceivable that Dectin-2 recognizes ligands in the systemic fungi that are structurally distinct from those that have been described above.

References


