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Cord Factor and Peptidoglycan Recapitulate the Th17-Promoting Adjuvant Activity of Mycobacteria through Mincle/CARD9 Signaling and the Inflammasome

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Although adjuvants are critical vaccine components, their modes of action are poorly understood. In this study, we investigated the mechanisms by which the heat-killed mycobacteria in CFA promote Th17 CD4+ T cell responses. We found that IL-17 secretion by CD4+ T cells following CFA immunization requires MyD88 and IL-1 receptor signaling, through measurement of Ag-specific responses after adoptive transfer of OTII cells. We confirmed that MyD88-dependent signaling controls Th17 differentiation rather than simply production of IL-17. Additional experiments showed that CFA-induced Th17 differentiation involves IL-1β processing by the inflammasome, as mice lacking caspase-1, ASC, or NLRP3 exhibit partially defective responses after immunization. Biochemical fractionation studies further revealed that peptidoglycan is the major component of heat-killed mycobacteria responsible for inflammasome activation. By assayng Il1b transcripts in the injection site skin of CFA-immunized mice, we found that signaling through the adaptor molecule caspase activation and recruitment domain 9 (CARD9) plays a major role in triggering pro–IL-1β expression. Moreover, we demonstrated that recognition of the mycobacterial glycolipid trehalose dimycolate (cord factor) by the C-type lectin receptor mincle partially explains this CARD9 requirement. Importantly, purified peptidoglycan and cord factor administered in mineral oil synergized to recapitulate the Th17-promoting activity of CFA, and, as expected, this response was diminished in caspase-1– and CARD9-deficient mice. Taken together, these findings suggest a general strategy for the rational design of Th17-skewing adjuvants by combining agonists of the CARD9 pathway with inflammasome activators.

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The choice of adjuvants is often a critical factor in the success of vaccines, but the number of adjuvants available for clinical use is very limited. Effective adjuvants are known to act on the innate immune system to not only increase the magnitude of vaccine-induced immune responses, but also to direct the appropriate class of effector response (1). However, the innate immune pathways that must be targeted to elicit particular types of adaptive immunity are poorly understood, thus hampering the development of rationally designed adjuvants.

Historically, mycobacteria and their components have been the basis of numerous adjuvants and immunotherapies. For instance, bacillus Calmette-Guérin instillation is widely used to treat superficial bladder cancer, and this attenuated mycobacterial strain has been employed as an adjuvant in experimental vaccines against Leishmania spp. and other pathogens (2). However, the best-known use of mycobacteria for stimulating the immune system is in CFA, a water-in-oil emulsion containing heat-killed Mycobacterium tuberculosis strain H37Ra or M. butyricum. Although not appropriate for human use because of its adverse inflammatory side effects, CFA has for decades been the adjuvant of choice for inducing strong humoral and cellular immune responses in experimental animals (3). Moreover, administering self-Ags (e.g., myelin basic protein) in CFA breaks self-tolerance and results in autoimmunity (1, 3). Previous studies have shown that CFA

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Abbreviations used in this article: AG, arabinogalactan; alum, aluminum hydroxide; BMDM, bone marrow–derived dendritic cell; BDMM, bone marrow–derived macrophage; CARD9, caspase activation and recruitment domain 9; DP, double-producing; LN, lymph node; mAGP, mycolyl-arabinogalactan-peptidoglycan; MDP, muramyl dipeptide; NIAID, National Institute of Allergy and Infectious Diseases; NOD, nucleotide-binding oligomerization domain; PGN, peptidoglycan; RORγt, retinoic acid-related orphan receptor γt; TDM, trehalose dimycolate; WT, wild-type.
promotes strong Th1 and Th17 CD4+ T cell responses, and this is a likely explanation for the potency of the adjuvant in inducing experimental autoimmune disease (3, 4).

Although Th1 and Th17 responses cause autoimmunity under certain circumstances, they are also protective against many pathogens, and adjuvants triggering their differentiation could be useful tools for vaccination. Whereas Th1 responses have long been appreciated for their role in cell-mediated immunity against intracellular pathogens, Th17 responses are now known to be protective against fungi and some extracellular bacteria (5). This is clearly evident in Job’s syndrome patients, who mount defective Th17 responses as a result of mutations in the STATS gene and are highly susceptible to Candida albicans and other fungal infections (6). Moreover, it has recently been shown that Th17 and Th1 cells can cooperate in host defense against two major intracellular pathogens, M. tuberculosis and Francisella tularensis (7, 8).

T cell subset differentiation is largely directed by the innate immune system. Recognition of pathogen-associated molecular patterns and danger signals by germline-encoded innate immune receptors leads to cellular activation and production of T cell–polarizing cytokines (3). However, in the case of complex microbial stimuli, it is not clear how activation of particular combinations of pattern recognition receptors causes innate immune cells to promote CD4+ T cell differentiation into specific subsets. For instance, the mycobacteria in CFA are known to activate several TLRs, the intracellular nucleotide-binding oligomerization domain (NOD) 1 and NOD2 receptors, and multiple C-type lectin receptors (9–16), but the respective contributions of these innate immune pathways in triggering Th17 differentiation in response to CFA immunization are poorly understood.

In the current study, we have undertaken a systematic investigation of innate immune receptors activated by CFA to understand their respective roles in promoting Th17 polarization. We demonstrate a major role for IL-1β/IL-1R signaling in driving CFA-induced Th17 responses. Moreover, in investigating the mechanisms involved in IL-1β production in response to CFA, we have elucidated important roles for caspase/caspase activation and receptor-activation domain 9 (CARD9)–dependent signaling and the inflammasome, a molecular complex that proteolytically activates pro–IL-1β and pro–IL-18 (17). Finally, we have assigned functions for the mycobacterial cord factor and peptidoglycan (PGN) components of CFA in triggering IL-1β induction and processing respectively. Together, these findings elucidate a major pathway for the generation of IL-17–producing CD4+ T cells in response to mycobacterial products, which could be used in the design of novel Th17-promoting adjuvants.

Materials and Methods

Mice

C57BL/6 mice were purchased from Tacomin Farms. Il1r1−/− mice were purchased from The Jackson Laboratory, CD45.1 congenic Rag1−/− OTII mice and Il1r1−/− mice backcrossed to B6 for 10 generations, were obtained from S. Akira (Osaka University, Osaka, Japan). Il10−/− mice, backcrossed to B6 for 10 generations, were originally obtained from G. Nunez (University of Colorado, of Virginia, VA). E. Lien (University of Massachusetts, Worcester, MA). Nod1−/− Nod2−/− mice (18), backcrossed to B6 for 10 generations, were originally obtained from G. Nunez (University of Michigan, Ann Arbor, MD). Il1a−/− and Il1b−/− mice were generated by Y. Ikawara (University of Tokyo, Tokyo, Japan) and generously provided by T. Merkel (Food and Drug Administration, Bethesda, MD). C3d−/− mice (19) were generated by X. Lin (MD Anderson, Houston, TX). Clec4e−/− (mice 20), backcrossed at least seven generations to B6, were kindly provided by S. Yamasaki (Kyushu University, Kyushu, Japan). Pycard−/− mice were generated by Millenium Pharmaceuticals (Cambridge, MA). Casp1−/− mice were generously supplied by R. Flavell (Yale University, New Haven, CT) and subsequently back-crossed to B6 until 10 generations. Nlrp3−/− and Nlrc4−/− mice were kindly provided by V. Dixit (Genentech, San Francisco, CA). All animals were maintained in an AALAC-accredited animal facility at the NIAID, National Institutes of Health (Bethesda, MD). Mice were used according to animal study proposals approved by the NIAID Animal Care and Use Committee.

Bone marrow from P2x7−/− mice was kindly provided by F. Tani (Imperial College, London, U.K.).

Reagents

CFA, IFA, ATP, brefeldin A, cytocalasin D, propyl gallate, butylated hydroxyanisole, NaCl, and KCl were purchased from Sigma-Aldrich. Trehalose dimycolate (TDM) purified from M. tuberculosis was from Sigma-Aldrich or Enzo Life Sciences. Immunostaining of species and Rag1−/− mice were kindly provided by F. Tam (Imperial College, London, U.K.).

Immunizations

For IFA and CFA immunizations, mice were injected s.c. at four sites along the back (100 μl/site) with a total of 100 μg OVA protein or OVA323-339 peptide. CFA emulsions contained 0.5 mg/ml heat-killed M. tuberculosis H37Ra. For IFA supplementation experiments, TDM was dissolved in IFA emulsion at 5% prior to preparing emulsions with OVA323-339 peptide. TDM was used at a concentration of 0.125 mg/ml in these emulsions. H37Ra PGN was suspended in IFA and used in emulsions at a concentration of 0.5 mg/ml.

Adaptive transfer and ex vivo T cell restimulation

To measure endogenous polyclonal CD4+ T cell responses, draining (inguinal, axillary, and brachial) lymph nodes (LNs) were harvested 14 d after CFA/OVA immunization. LNs were dissociated through 100-μm cell strainers, and LN cells were plated at 1 × 106 cells per well in a flat-bottom 96-well plate. The cells were incubated with 100 μg/ml OVA protein for 1.5 h at 37°C. Brefeldin A (5 μg/ml) was then added, and the cells were incubated for an additional 4 to 6 h at 37°C. For adaptive transfer experiments, mice received 5 × 106 CD45.1 congenic Rag1−/− OTII cells i.v. and were then immunized with CFA/OVA323-339 peptide the next day. Draining LNs were harvested 10 d later, and cells were restimulated with 25 μg/ml OVA323-339 as above.

Abs and flow cytometry

Abs against the following molecules were purchased from eBioscience, BioLegend, or BD Biosciences: CD4 (RCM-4), CD45.1 (A20), CD45.2 (104), CD44 (IM7), IFN-γ (XMG1.2), IL-17 (eBio17B7), Foxp3 (FJK-16s), and retinoic acid-related orphan receptor γ (RORγt; B2D). Blue fireable live/dead cell stain and streptavidin-Qdot605 were from Molecular Probes/Invitrogen. All samples were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Tissue harvest and quantitative PCR

Skin was harvested from the injection site of CFA/OVA-immunized mice at various times after immunization. Subcutaneous fat was removed, and the skin was homogenized in TRIzol (Invitrogen). Total RNA was extracted according to the manufacturer’s protocol, and cDNA was reverse transcribed with Superscript III reverse transcriptase and random primers (Invitrogen). Quantitative PCR was performed on an ABI Prism 7900 HT Sequence Detection System using Power SYBR Green Master Mix (Applied Biosystems) and analyzed using the manufacturer’s software (Tree Star).

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2 INNATE IMMUNE PATHWAYS IN CFA-INDUCED Th17 DIFFERENTIATION

Macrophage and dendritic cell stimulation and immunoblotting

Bone marrow–derived macrophages (BMDM) were prepared by growing bone marrow cells in 30% L929 cell–conditioned medium for 6–8 d. Bone
marmor–derived dendritic cells (BMDC) were prepared as previously described (21). BMDM and BMDC were then harvested, primed where indicated for 3 to 4 h with 20 ng/ml LPS, and then stimulated for 1 h with 5 mM ATP or overnight with heat-killed H37Ra (500 μg/ml), alumn (400 μg/ml), or flagellin (1 μg/ml) in OptiMEM serum-free media. DOTAP liposomal transfection reagent (Roche) was used according to the manufacturer’s instructions to deliver flagellin intracellularly. For inhibition of potassium efflux, KCl or NaCl were added to the OptiMEM medium. For experiments with cytochalasin D or reactive oxygen species scavengers, the inhibitors were present both during LPS priming and during the subsequent inflammasome stimulation step. For stimulation with heat-killed H37Ra fractions, all fractions except for polar and nonpolar lipids were used at a concentration of 250 μg/ml. Lipids were used at 50 μg/ml because of toxicity at higher concentrations.

After stimulation, supernatants were harvested, and cells were lysed with cell lysis buffer (Cell Signaling Technology) supplemented with 2 mM PMSF (Sigma-Aldrich) and a protease inhibitor mixture (EMD Chemicals). Supernatants were precipitated with 1 volume methanol and 0.25 volumes chloroform, and the protein pellets were resuspended in reducing sample buffer (Pierce/Thermo Fisher Scientific). The samples were then separated on 15% polyacrylamide gels and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare). IL-1β was detected with a goat anti–IL-1β Ab (AF-401; R&D Systems), and caspase-1 was detected with rabbit anti-mouse caspase-1 (sc-514; Santa Cruz Biotechnology). For loading controls, equal amounts of cell lysates were separated on 12% polyacrylamide gels and blotted as above or with mouse anti-GAPDH Ab (sc-32233; Santa Cruz Biotechnology).

Polar and nonpolar lipid extraction from heat-killed H37Ra

Polar and nonpolar lipids were extracted as described (22). Briefly, freeze-dried M. tuberculosis H37Ra cells were treated with 22 ml methanolic solutions (20 ml 0.3% NaCl and 20 ml CH3OH) and 22 ml petroleum ether for 2 h. The suspension was centrifuged, and the upper layer containing nonpolar lipids was separated. An additional 22 ml petroleum ether was added, mixed, and centrifuged as described above. The two upper petroleum ether fractions were combined and dried. For polar lipids, 26 ml CHCl3/CH3OH/0.3% NaCl (9:10:3, v/v/v) was added to the lower aqueous phase and stirred for 1 h. The mixture was filtered, and the filter cake was re-extracted twice with 8.5 ml CHCl3/CH3OH/0.3% NaCl (5:10:4, v/v/v). The delipidated cells were retained for further extraction and purification of lipoglycans as described below. Equal amounts of CHCl3 and 0.3% NaCl (14.5 ml each) were added to the combined filtrates and stirred for 1 h. The mixture was allowed to settle, and the lower layer containing the polar lipids recovered and dried. The polar and nonpolar lipid extracts were examined by two dimensional thin-layer chromatography on aluminum-backed plates of silica gel 60 F 254 (Merck 5554; Merck) using solvent systems A–E with the appropriate staining solution to detect the presence of lipids, glycolipids, or phospholipids as described (22).

Isolation and extraction of lipoglycans

Lipoglycans were extracted from the above delipidated cells as previously described (23). Briefly, cells were broken by sonication (MSE Soniprep 150; 12 μm amplitude, 60 s on, 90 s off for 10 cycles, on ice) and the cell debris refluxed five times with 50% C2H5OH at 68˚C, for 12-h intervals. The crude insoluble material from the above lipoglycan extraction was methyl esterified, recentrifuged at 27,000 × g, and the pellet (AGP) and soluble mycolic acid methyl esters recovered in the diethyl ether supernatant. The extraction process using diethyl ether was repeated three times. The combined ether fractions were evaporated to dryness to provide the mycolic acid methyl esters. The AGP complex was hydrolyzed with 0.2 M H2SO4 at 85˚C for 30 min and neutralized with BaCO3. The supernatant, which contained the solubilized arabinogalactan (AG), was recovered after centrifugation at 27,000 × g for 30 min and dialyzed (m.w. cutoff 3.500). The residual pellet was the PGN fraction. The supernatant was then made up to 80% cold ethanol and left at −20˚C overnight to precipitate polysaccharides. The pellet was then recovered by centrifugation, at 27,000 × g for 30 min, and freeze-dried to afford AG, which was stored at −20˚C. The recovered PGN fraction was finally extensively washed with water, freeze-dried, and stored at −20˚C.

Statistical analysis

The statistical significance of differences between groups was analyzed via unpaired Student t test using GraphPad Prism software version 5.0c (GraphPad). The p values are shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

MyD88 signaling is required for CFA-induced Th17 polarization

MyD88 signaling has previously been shown to play an important role in the adjuvant activity of CFA (26). To specifically investigate the requirement for this adaptor molecule in CFA-induced Th17 responses, we immunized wild-type (WT) and Myd88−/− mice s.c. with OVA in CFA and measured OVA-specific IL-17 and IFN-γ production by CD4+ T cells in the draining LNs 2 wk later. As expected, we found that CFA immunization induced robust differentiation of IL-17 and IFN-γ single-producing CD4+ T cells, as well as a population of IL-17/IFN-γ double-producing (DP) cells (Fig. 1A). However, in Myd88−/− mice, the Th17 and IL-17/IFN-γ DP responses were almost completely abrogated, although there was still a residual IFN-γ response.

To test whether there was a specific impairment in IL-17 production or a more general defect in Th17 differentiation in Myd88−/− mice, we adoptively transferred CD45.1 congenic Rag1−/−/OTII cells into CD45.2 congenic WT or Myd88−/− mice and then immunized the mice with OVA323−339 peptide in CFA. We found that Myd88−/− recipients contained markedly reduced frequencies of IL-17–producing and IL-17/IFN-γ DP OTII T cells compared with WT recipients (Supplemental Fig. 1A, 1B). Importantly, this correlated with a major loss in expression of the Th17 master regulator RORγt in the OTII cells isolated from Myd88−/− recipients, suggesting that MyD88 is required for Th17 differentiation, rather than simply secretion of IL-17 (Supplemental Fig. 1C, 1D).

The requirement for MyD88 in Th17 responses reflects a role for IL-1β/IL-1R signaling rather than TLRs or IL-18R

MyD88 is required for signaling via most TLRs as well as IL-1R family members. To understand the requirement for MyD88 in CFA-induced CD4+ T cell polarization, we thus investigated the role of TLRs, IL-1R, and IL-18R in the response to CFA. The myocardiasa in CFA contain the TLR2 agonists 19 kDa lipo-protein and arabinose-capped lipoarabinomannan, the TLR2/LR2 activating phosphatidylinositol, and unmethylated CpG DNA (recognized by TLR9) (13). Therefore, we examined the response of Tlr2−/− Tlr9−/− and Tlr2−/− Tlr9−/− mice to immunization. OVA-specific IL-17 production by LN CD4+ T cells was not significantly reduced in these mice (Fig. 1B), suggesting that recognition of CFA by these TLRs is dispensable for adjuvant-induced CD4+ Th17 differentiation.

The mycobacteria in CFA contain the TLR2 agonists 19 kDa lipo-protein and arabinose-capped lipoarabinomannan, the TLR2/LR2 activating phosphatidylinositol, and unmethylated CpG DNA (recognized by TLR9) (13). Therefore, we examined the response of Tlr2−/− Tlr9−/− and Tlr2−/− Tlr9−/− mice to immunization. OVA-specific IL-17 production by LN CD4+ T cells was not significantly reduced in these mice (Fig. 1B), suggesting that recognition of CFA by these TLRs is dispensable for adjuvant-induced CD4+ Th17 differentiation.
**FIGURE 1.** MyD88-dependent IL-1β/IL-1R signaling is critical for CFA-induced Th17 polarization. (A) Draining LN cells were harvested from WT or Myd88−/− mice 14 d after CFA/OVA immunization and intracellular cytokine staining for IL-17 and IFN-γ was performed after ex vivo OVA restimulation. Plots are gated on CD4+CD44hiFoxp3− cells. (B-D) The indicated mice were immunized and draining LN responses were measured as in (A). Frequencies of cytokine-producing cells within the CD4+CD44hiFoxp3− population are shown. Data are representative of at least three independent experiments (A, B) or are pooled from three to four experiments (C, D). Each point represents data from an individual mouse. *p < 0.05, **p < 0.01, ***p < 0.001.

II1a−/− mice, compared with a marked reduction in II1b−/− mice (Fig. 1D), suggesting that IL-1β is the primary IL-1 cytokine required for CFA-induced Th17 responses. Taken together, these findings argue that the role of MyD88 in CFA-triggered Th17 and IL-17/IFN-γ DP cell differentiation is primarily downstream of IL-1β/IL-1R signaling.

**Activation of the inflammasome by M. tuberculosis PGN contributes to CFA-induced Th17 differentiation**

IL-1β is initially produced as an immature cytokine that must be proteolytically processed to its bioactive form. The cleavage of pro–IL-1β to its mature form is usually thought to be accomplished by the caspase-1 inflammasome (17), so we asked whether activation of this complex contributed to CFA-induced Th17 responses. To address this question, we immunized Casp1−/−, Pycard (Asc)−/−, Nlrp3−/−, and Nlrc4−/− mice with CFA/OVA. We observed a substantial defect in Th17 responses in Casp1−/− and Asc−/− mice, but only a slight reduction in Nlrp3−/− mice and unimpaired responses in Nlrc4−/− animals (Fig. 2A, 2B).

To further analyze the inflammasome’s role in the response to CFA, we stimulated BMDM in vitro and assayed their activation of caspase-1 and ability to secrete mature IL-1β. We found that stimulation of LPS-primed BMDM with heat-killed *M. tuberculosis* H37Ra was sufficient to induce caspase-1 cleavage and IL-1β secretion and that this required NLRP3 as well as phagocytosis of the *M. tuberculosis* potassium efflux, and reactive oxygen species (Fig. 2C, 2D, Supplemental Fig. 2). NLRP3-dependent IL-1β production and caspase-1 cleavage in response to heat-killed H37Ra were also observed in the absence of LPS priming, indicating that H37Ra itself is sufficient for inducing pro–IL-1β and components of the inflammasome (data not shown). Similar results were obtained with BMDC (data not shown).

Infection of macrophages with live *M. tuberculosis* is known to activate the NLRP3 inflammasome, and this is thought to occur in response to the mycobacterial ESX-1 secretion system (27). Because heat-killed mycobacteria do not secrete proteins, we investigated alternative pathways by which mycobacterial products could activate the inflammasome. To do so, we biochemically fractionated heat-killed H37Ra, successively extracting polar and nonpolar lipids, lipoglycans, mycolic acid methyl esters, and arabinogalactan, as described previously (22–25). We tested each of these soluble fractions as well as the insoluble fractions remaining after each step. We found that none of the soluble fractions triggered the inflammasome, whereas all of the insoluble fractions, including PGN alone, contained this activity (Fig. 2E, 2F). These data implicate PGN as the primary stimulus for inflammasome activation by heat-killed H37Ra.

Previous studies using other bacteria (e.g., *Salmonella*) have shown that PGN can activate the inflammasome, and this activity has generally been ascribed to the ubiquitous PGN subunit MDP acting via the receptor NOD2 (28–30). However, we found that macrophages from *Nod1−/−*/*Nod2−/−* mice displayed normal caspase-1 cleavage and IL-1β secretion in response to H37Ra PGN (Supplemental Fig. 3), indicating that mycobacterial PGN can activate the inflammasome independently of these NOD receptors.

The signaling adaptor CARD9 is required for pro–IL-1β production and Th17 differentiation in response to CFA

Having shown that CFA-induced Th17 differentiation involves the inflammasome, we next investigated how the pro–IL-1β substrate for the inflammasome was being produced in this model. Transcription of the II1b gene is induced by signals such as TLRs that cause translocation of NF-kB to the nucleus (17). To determine whether II1b induction in response to CFA involves TLR signaling, we immunized WT and Myd88−/−/Trif−/− mice (which lack all TLR signaling) with CFA/OVA. We then harvested injection site skin from these mice at various time points and measured
mRNA levels of Il1b and other proinflammatory cytokines. We found that although Il1b induction was slightly delayed in Mydd88−/− Trif−/− mice, total transcript levels were not reduced compared with WT mice (Fig. 3A), arguing that TLR signaling is largely dispensable for pro–IL-1β production in this system.

Bacterial PGN subunits can induce innate cytokine production via the cytosolic receptors Nod1 and Nod2 (31, 32), and mycobacteria have also been shown to trigger several C-type lectins that signal via the adaptor CARD9 (9–12, 16, 33). To determine whether either of these pathways contribute to pro–IL-1β induction in response to CFA, we immunized Nod1−/− Nod2−/− or Card9−/− mice and measured Il1b transcripts in the injection site skin. We found that whereas postimmunization Il1b mRNA levels were comparable in the skin of Nod1−/− Nod2−/− and WT mice (Fig. 3B), they were greatly reduced in Card9−/− mice (Fig. 3C). These data suggest that if Card9−/− mice affect CD4+ T cell responses, we measured Il1b induction after CFA immunization, although the response was less impaired than in the Card9−/− mice (Fig. 3C). This is of particular interest because recent studies have identified TDM as one of its ligands (9, 10). The latter is of particular interest because recent studies have identified TDM as one of its ligands (9, 10). The latter is of particular interest because recent studies have identified TDM as one of its ligands (9, 10). The latter is of particular interest because recent studies have identified TDM as one of its ligands (9, 10).

To determine whether the defects in proinflammatory cytokine induction in the Card9−/− mice affect CD4+ T cell responses, we adoptively transferred CD45.1 congenic Rag1−/− OTII cells into CD45.2 congenic WT or Card9−/− mice and then immunized the mice with CFA/OVA (323–339). We found that the IL-17 and IL-17/ IFN-γ DP OTII responses were nearly absent in the Card9−/− recipients, as was the RORγt+ population (Fig. 3D, 3E), demonstrating that CFA-induced Th17 differentiation requires CARD9-dependent signaling.

**FIGURE 2.** Activation of the inflammasome by mycobacterial PGN contributes to CFA-induced Th17 polarization. (A and B) The indicated mice were immunized with CFA/OVA, and draining LN cells were harvested 14 d after CFA/OVA immunization. IL-17 and IFN-γ production were analyzed by ICS after OVA restimulation and frequencies of cytokine-producing cells within the CD4+ CD44hi Foxp3− population are shown. (C) LPS-primed BMDM were stimulated with heat-killed M. tuberculosis H37Ra (20, 100, or 500 μg/ml), alum, or ATP. Supernatants and cell lysates were harvested and immunoblotted for IL-1β, caspase-1, and GAPDH. (D) LPS-primed BMDM generated from WT, Nlrp3−/−, or Nlrc4−/− mice were stimulated with heat-killed H37Ra or flagellin and supernatants and cell lysates were immunoblotted for IL-1β and caspase-1. (E) Polar lipids, nonpolar lipids, lipoglycans, mycolic acid methyl esters (MAMES), and AG were sequentially extracted from the cell wall of heat-killed H37Ra, as described in the Materials and Methods, and used to stimulate LPS-primed BMDM. Supernatants and cell lysates were then immunoblotted as above. (F) The insoluble H37Ra fractions remaining after each extraction described in (E) were used to stimulate LPS-primed BMDM and supernatants and cell lysates were immunoblotted as above. Data are pooled from three experiments (A) or representative of at least three independent experiments (B–F). CL, cell lysate; SN, supernatant. *p < 0.05, **p < 0.01, ***p < 0.001.

Recognition of mycobacterial cord factor by mincle is a major CARD9-dependent signal for CFA-induced IL-1β production and Th17 polarization

Mycobacteria are known to trigger several CARD9-dependent C-type lectin receptors, including dectin-1, dectin-2, and mincle (9–12). The latter is of particular interest because recent studies have identified TDM as one of its ligands (9, 10). TDM, also known as cord factor, is a mycobacterial glycolipid with potent adjuvant activity that was discovered while investigating the cording phenomenon observed during infection with virulent mycobacteria (16, 34, 35). To determine whether TDM could account for the CARD9-dependent Il1b induction observed after CFA injection, we compared Il1b mRNA expression in the skin of mice immunized with IFA (mineral oil and surfactant without mycobacteria), IFA supplemented with TDM, or CFA itself. IFA was a poor inducer of Il1b, but the addition of TDM resulted in robust Il1b upregulation comparable to that observed with CFA. Importantly, this response to TDM was ablated in Card9−/− mice (Fig. 4A).

Having shown that TDM in IFA stimulates Il1b expression, we asked whether recognition of TDM by mincle is required for CFA-induced pro–IL-1β production as well as Th17 polarization. We found that Clec4e−/− (Mincle−/−) mice exhibited reduced cutaneous Il1b induction after CFA immunization, although the response was less impaired than in the Card9−/− mice (Fig. 4B). Moreover, OTII cells transferred into Mincle−/− mice were defective in their differentiation into IL-17–producing cells and in their expression of RORγt (Fig. 4C, 4D). Nevertheless, the Th17 response was not as greatly reduced in Mincle−/− as in Card9−/− mice, thus implicating additional CARD9-dependent receptors in CFA-induced Th17 polarization.
IFA supplemented with TDM and PGN recapitulates the Th17-polarizing effects of CFA

The above experiments demonstrated major roles for mincle/CARD9-dependent recognition of TDM in driving pro–IL-1β production and for inflammasome-mediated recognition of PGN in triggering IL-1β maturation. Having found that IL-1β/IL-1R signaling as well as the CARD9 and inflammasome pathways are necessary for optimal Th17 responses after CFA immunization, we therefore asked whether the combination of TDM and PGN could recapitulate the Th17-polarizing capacity of the mycobacterial component of CFA by inducing production of IL-1β as well as other Th17-polarizing cytokines.

FIGURE 3. The signaling adaptor CARD9 is required for pro–IL-1β induction and Th17 differentiation in response to CFA. (A–C) The indicated mice were immunized with CFA/OVA, and injection site skin was excised at the time points shown. RNA was isolated, and transcript levels of the indicated genes were measured by quantitative PCR. Fold induction over mRNA levels in unimmunized skin is shown. Data are mean ± SEM. (D) CD45.1 congenic Rag1−/− OTII cells were transferred into CD45.2 congenic WT or Card9−/− mice prior to immunization with CFA/OVA323–339. Draining LNs were harvested 10 d after immunization and restimulated with OVA323–339. IL-17 and IFN-γ production by the OTII cells was analyzed by ICS. (E) RORγt expression in the draining LN OTII cells described in (D) was analyzed by ICS. Data are representative of two independent experiments (A) or pooled from two to four experiments (B–E). *p < 0.05, **p < 0.01, ***p < 0.001.

IFA supplemented with TDM and PGN signaling as the CARD9 and inflammasome pathways are necessary for optimal Th17 responses after CFA immunization, we therefore asked whether the combination of TDM and PGN could recapitulate the Th17-polarizing capacity of the mycobacterial component of CFA by inducing production of IL-1β as well as other Th17-polarizing cytokines.

FIGURE 4. Recognition of mycobacterial cord factor by mincle is upstream of the CARD9 requirement in CFA-induced pro–IL-1β production and Th17 polarization. (A) WT or Card9−/− mice were immunized with OVA in PBS, IFA, IFA supplemented with cord factor (TDM), or CFA. Injection site skin was excised 12 h postimmunization, RNA was isolated, and Il1b transcript levels were measured by quantitative PCR. Fold induction over mRNA levels in unimmunized skin is shown. Data are mean ± SEM. (B) WT, Card9−/−, or Mincle−/− (Clec4e−/−) mice were immunized with CFA/OVA, and RNA was isolated from injection site skin 12 h after immunization. Levels of indicated transcripts were measured by quantitative PCR, and fold induction over mRNA levels in unimmunized skin is shown. (C) CD45.1 congenic Rag1−/− OTII cells were transferred into CD45.2 congenic WT, Card9−/−, or Mincle−/− mice prior to immunization with CFA/OVA323–339. The draining LNs were harvested 10 d after immunization. IL-17 and IFN-γ production by OTII cells in the draining LNs was analyzed by ICS after restimulation with OVA323–339. (D) RORγt expression in the draining LN OTII cells described in (C) was analyzed by intracellular cytokine staining. Data are pooled from three to six experiments (A–D). *p < 0.05, **p < 0.01, ***p < 0.001.
In contrast to CFA, IFA is incapable of inducing Th17 responses even though it promotes CD44 T cell priming (Fig. 5A, 5B), thereby demonstrating the importance of the mycobacteria in CD44 T cell polarization. To test the immunostimulatory effects of TDM and PGN, we added these components to IFA either individually or in combination. We then prepared emulsions with OVA323–339 and immunized mice that had received adoptively transferred CD45.1 Rag1−/− OTII cells. IFA containing TDM or PGN alone induced little or no Th17 response, but IFA supplemented with both TDM and PGN induced robust Th17 differentiation comparable to that induced by CFA itself (Fig. 5C, Supplemental Fig. 4). Importantly, as expected, the IL-17 response to the IFA+TDM+PGN adjuvant was dependent on both CARD9 and caspase-1 (Fig. 5D).

Discussion

In this manuscript, we have deconstructed the key innate immune recognition events required for the induction of Th17 differentiation by the mycobacterial component of CFA in vivo. We identify a two-step mechanism by which dual recognition of mycobacterial cord factor and PGN synergistically drives production of IL-1β and other cytokines necessary for Th17 polarization. Our findings suggest a general strategy for the rational design of Th17-skewing adjuvants.

The role of MyD88 in CFA-induced CD44 T cell responses was initially thought to reflect the involvement of TLRs in recognition of the mycobacterial component of the adjuvant (26). However, we found that deficiencies in TLRs implicated in the response to mycobacteria failed to have a major impact on Th17 differentiation following CFA immunization. Instead, the requirement for MyD88 in Th17 skewing could largely be explained by the role of IL-1β/IL-1R signaling, although a significant role for IL-1α was also observed. These findings are consistent with previous reports demonstrating a major function for IL-1R signaling in driving CFA-induced Th17 polarization, especially in the context of autoimmune disease models such as experimental autoimmune encephalomyelitis and uveitis (36–39).

Previous studies have shown that there is a T cell–intrinsic role for IL-1R/MyD88-dependent signaling in driving Th17 differentiation (36, 40). Interestingly, however, we found that MyD88–sufficient OTII cells transferred into Myd88−/− recipients are unable to differentiate into Th17 cells after CFA immunization, suggesting that MyD88-dependent signaling in the non–T cell compartment also plays a critical role in inducing Th17 differentiation. Therefore, MyD88–dependent signaling may act in both the T cell and non–T cell compartments to drive Th17 differentiation.

After identifying IL-1β as a key mediator of the CFA-induced Th17 response, we investigated the innate immune pathways that drive production of this cytokine after immunization. We found that the Th17-skewing effects of CFA were partially dependent on the inflammasome. In agreement with previous studies, in vitro stimulation with heat-killed M. tuberculosis–induced caspase-1 cleavage and mature IL-1β production by macrophages in an Nlrp3-dependent manner (41). However, Nlpr3−/− mice displayed only a mild reduction in Th17 differentiation in response to CFA compared with Casp1−/− and Asc−/− mice, suggesting the involvement of other inflammasome sensors in vivo. In addition, the Th17 defect in Casp1−/− mice was not as profound as that in Il1b−/− mice, implicating inflammasome-independent mechanisms.
of IL-1β maturation acting in vivo. Multiple enzymes other than caspase-1 have been shown to be capable of cleaving pro–IL-1β, including several neutrophil serine proteases and caspase-8 (42, 43), and these alternative pathways for IL-1β maturation could explain the incomplete inflammasome requirement observed in vivo in our studies.

The results of our fractionation experiments implicate PGN as the primary trigger for the inflammasome in heat-killed M. tuberculosis. Interestingly, experiments performed decades ago identified the PGN subunit MDP as the minimal mycobacterial component that recapitulated the adjuvant activity of CFA when added to IFA (44). More recently, MDP has also been reported to activate the inflammasome in a NOD2-dependent manner (28–30). However, we found that, in contrast to MDP, polymeric PGN isolated from heat-killed M. tuberculosis was able to induce inflammasome activation and IL-1β maturation in Nod1−/−Nod2−/− macrophages. Although mycobacterial diaminopimelic acid–containing PGN is distinct from Staphylococcus aureus lysine-type PGN, the latter has also recently been reported to activate the inflammasome independently of NOD2 (45). Therefore, in principle, PGN need not be processed into subunits recognized by NOD receptors to activate the inflammasome.

In addition to finding a role for the inflammasome in CFA-induced IL-1β cleavage and Th17 differentiation, we analyzed the signals involved in the induction of pro–IL-1β in response to CFA immunization. Although TLRs are thought to provide a major stimulus for NF-kB–dependent pro–IL-1β induction (46), we found that upregulation of Il1b message in response to CFA was intact in Myd88−/−Trif−/− mice. In contrast, we observed that CARD9-dependent signaling plays a major role in inducing pro–IL-1β production in CFA-immunized mice and that mircine is an important upstream receptor that triggers this pathway. However, although CFA-induced Th17 differentiation was strongly impaired in the Card9−/− mice, it was only partially reduced in the Mincle−/− mice, suggesting the involvement of additional CARD9-dependent receptors in the recognition of CFA. Dectin-1 and dextrin-2 have each been shown to recognize mycobacteria and are therefore logical candidates (11, 12). Moreover, the dextrin-1 agonist curdulan was previously shown to augment Th17 differentiation in vivo via a CARD9-dependent pathway, although the role of IL-1 signaling was not investigated in this model (47).

Confirmation of the ability of mycobacterial cord factor and PGN to drive Th17 responses was obtained in experiments in which these components were admixed into IFA, and CARD9- and caspase-1–dependent Th17 polarization comparable to that observed with CFA was achieved. Importantly, either component alone did not trigger a substantial Th17 response, likely because cord factor and PGN act synergistically by providing the two signals necessary for mature IL-1β production. Recent studies investigating the basis of vaccine-induced protection to fungal and some bacterial infections have revealed a key role for the Th17 response (7, 48). For this reason, chemically defined agents that promote the differentiation of Th17 cells are likely to become important tools in the development of vaccines against these pathogens. Although we did not attempt to optimize our mycobacterial adjuvant (e.g., by changing the ratio of the two mycobacterial ligands or varying the nature of the oil component) to enhance the efficacy or safety of the emulsion, our findings establish the principle of combining a CARD9-dependent pro–IL-1β inducer with an inflammasome activator as a strategy for developing Th17-skewing immunostimulants. This rationale is not necessarily limited to mycobacterial products and could conceivably be applied to the formulation of adjuvants with components derived from other sources.

Note added in proof. During the revision of our manuscript, Lang and colleagues published a report (49) showing that the cord factor analog trehalose dibenenate (TDB) incorporated into cationic liposomes can induce Th17 responses in an IL-1R– and ASC-dependent manner.

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Disclosures

The authors have no financial conflicts of interest.

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