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Caspase-8 Modulates Dectin-1 and Complement Receptor 3–Driven IL-1β Production in Response to β-Glucans and the Fungal Pathogen, Candida albicans


Inflammasomes are central mediators of host defense to a wide range of microbial pathogens. The nucleotide-binding domain and leucine-rich repeat containing family (NLR), pyrin domain–containing 3 (NLRP3) inflammasome plays a key role in triggering caspase-1–dependent IL-1β maturation and resistance to fungal dissemination in Candida albicans infection. β-Glucans are major components of fungal cell walls that trigger IL-1β secretion in both murine and human immune cells. In this study, we sought to determine the contribution of β-glucans to C. albicans–induced inflammasome responses in mouse dendritic cells. We show that the NLRP3–apoptosis-associated speck-like protein containing caspase recruitment domain protein–caspase-1 inflammasome is absolutely critical for IL-1β production in response to β-glucans. Interestingly, we also found that both complement receptor 3 (CR3) and dectin-1 play a crucial role in coordinating β-glucan–induced IL-1β processing as well as a cell death response. In addition to the essential role of caspase-1, we identify an important role for the proapoptotic protease caspase-8 in promoting β-glucan–induced cell death and NLRP3 inflammasome–dependent IL-1β maturation. A strong requirement for CR3 and caspase-8 was also found for NLRP3–dependent IL-1β production in response to heat-killed C. albicans. Taken together, these results define the importance of dectin-1, CR3, and caspase-8, in addition to the canonical NLRP3 inflammasome, in mediating β-glucan– and C. albicans–induced innate responses in dendritic cells. Collectively, these findings establish a novel link between β-glucan recognition receptors and the inflammatory proteases caspase-8 and caspase-1 in coordinating cytokine secretion and cell death in response to immunostimulatory fungal components. The Journal of Immunology, 2014, 193: 2519–2530.

Normally part of the human commensal flora, certain fungal species can become opportunistic pathogens when antibiotic treatment, immune suppressants, or pathogenic microorganisms perturb normal homeostatic balance. Candida spp., especially Candida albicans, can lead to invasive, mucosal, and systemic infections (1). The host innate immune system maintains a tolerant interaction with commensal microbial flora and in healthy individuals is normally able to protect the host against invasive fungal infection. Understanding how these immune responses are elicited and controlled has important therapeutic implications for the treatment of fungal disease in immune-compromised conditions. In this context, the proinflammatory cytokines IL-1β and IL-18 have been identified as integral components of antifungal immune defenses. Mouse models of candidiasis and human ex vivo studies have uncovered a critical role for IL-1β and IL-18 in protecting the host against fungal dissemination via their ability to trigger T cell–mediated production of IL-17 and IFN-γ, respectively (2–9).

In most cell types, secretion of mature IL-1β (Uniprot: P10749) requires at least two signals: synthesis of the precursor pro–IL-1β (and sometimes nucleotide-binding domain and leucine-rich repeat containing family [NLR], pyrin domain–containing 3 [NLRP3]) through NF-kB activation (signal 1) and the subsequent activation of caspase-1 through the formation of an inflammasome (signal 2). This inflammasome complex is responsible for cleaving pro–IL-1β and triggering the release of mature IL-1β. Inflammasomes are

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Abbreviations used in this article: AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing caspase recruitment domain; BMDC, bone marrow–derived DC; BMDCM, BM-derived macrophage; CARD, caspase recruitment domain; CM-curdlan, carbonyl-methylated-curdlan; CR3, complement receptor 3 (Mac-1); curdlan (I), curdlan (InvivoGen); DC, dendritic cell; EHEC, enterohemorrhagic Escherichia coli; FasL, Fas ligand; HK, heat-killed; LDH, lactate dehydrogenase; MEF, mouse embryonic fibroblast; NLR, nucleotide-binding domain and leucine-rich repeat containing family; NLRC4, NLRD, CARD domain-containing 4; NLRP3, nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain–containing 3; PAMP, pathogen-associated molecular pattern; PYHIN, PYRIN and HIN domain–containing protein; WGP, whole glucan particle.
assembled and activated in the cytosol in response to both microbial and nonmicrobial danger signals. NLRs as well as DNA binding PYRIN and HIN domain–containing (PYHIN) proteins are capable of forming caspase-1-activating inflammasomes. Apoptosis-associated speck–like protein containing caspase recruitment domain (CARD) (ASC) (UniProt: Q9EPB4), an adaptor protein that comprises a PYRIN and a CARD, bridges the homotypic interaction between NLR/PYHIN proteins and CARD-containing caspase-1 (UniProt: P29452). Seminal studies have established the importance of NLR and PYHIN proteins such as NLRP3 (UniProt: Q8R48), NLR, CARD containing 4 (NLRC4) (UniProt: Q3UP24), and absent in melanoma 2 (AIM2) (UniProt: Q9V1J1) in the maturation of the IL-1 family of proinflammatory cytokines in response to pathogenic and sterile assaults (reviewed in Ref. 10). We and others have shown important roles for NLRP3, NLRC4, and NLRP10 (UniProt: Q8CCN1) in different aspects of antifungal immune responses (2, 11–13).

Our interest in understanding the molecular mechanisms underlying inflammasome activation during infection with *C. albicans* was particularly focused on defining the contribution of cell wall polysaccharide structures, which constitute 90% of the yeast cell wall and are the primary mechanism by which the innate system senses fungal infection (14, 15). One major component of fungal cell walls are the β(1,3) and β(1,6) glucans that are important for its structural framework, along with mannans, proteins, and chitin (14). β-Glucans are highly immunostimulatory and also have been considered effective as immune supplements and as well as possible vaccine adjuvants. Detection of β(1,3) glucan and Abs to β(1,3) glucan in the plasma are considered biomarkers of candidiasis, and many diagnostic tests that exploit this finding are under clinical development (16–18). Studies on inflammasome responses to β-glucans are key to advancing our understanding of host fungal sensing pathways and their mode of action as immunomodulators.

In this study, we investigated the molecular and mechanistic details of inflammasome responses to *C. albicans* by using β-glucans and heat-killed (HK) *C. albicans* in conjunction with live *C. albicans*. Although previous studies have yielded contrasting observations regarding the role of NLRP3 in β-glucan–triggered inflammasome responses (19–21), we found the canonical NLRP3-ASC-caspase-1 inflammasome to be essential in mediating IL-1β production by both β-glucans and *C. albicans* (live or HK) in mouse dendritic cells (DC). The receptors linking β-glucan to inflammasome activation were dectin-1 (UniProt: Q6QLQ4) and complement receptor 3 (CR3), another receptor implicated in β-glucan sensing. We also uncovered an essential role for CR3 in mediating IL-1β production to HK and to a lesser extent, to live *C. albicans*. Moreover, Dectin-1 and CR3 appear to also function non-redundantly in a less-well characterized cellular death response to β-glucans and HK *C. albicans*.

A growing body of literature points to the existence of diverse and complex molecular platforms for inflammasomes including other caspases such as caspase-11 and -8 (19, 22–25). Gringhuis et al. (19) showed that IL-1β triggered by β-glucan and some HK strains of *C. albicans* was caspase-8 (UniProt: O989110) dependent but independent of caspase-1 in human cells. Furthermore, recent studies show that caspase-8 localizes to ASC specks (26). Caspase-8 also has been found to be required for inflammasome responses during *Salmonella* infection or upon treatment of cells with proapoptotic chemotherapeutic drugs and in Fas signaling (22, 26, 27). Casp8-/- mice are embryonic lethal because of RIP3-dependent necrosis (28). This lethality is rescued by the genetic deletion of RIP3 (UniProt: Q9QZL0) (29, 30). Using the viable Casp8+/-Rip3+/- double knockout and the Rip3+/- single knockouts as a control, we present evidence for caspase-8 involvement in β-glucan–triggered IL-1β production as well as cell death in mouse DC. Comparing the molecular requirements of inflammasome response between β-glucan and HK *C. albicans* (which has exposed β-glucans) with that of live *C. albicans* (which has most of its β-glucans masked by mannans), we identify a differential requirement for CR3, dectin-1, and caspase-8 in IL-1β and cell death responses.

Our findings suggest a β-glucan–specific dectin-1/CR3/caspase-8 pathway that synergizes with the canonical NLRP3-ASC-caspase-1 pathway for optimal inflammasome responses. This study gives rise to new insights into the differences in host immune sensing of fungal molecular patterns such as β-glucans in contrast to live *C. albicans* infection.

### Materials and Methods

#### β-Glucans

Curdlan was purchased from Sigma-Aldrich, InvivoGen, or Wako Chemicals. Carboxy-methylated curdlan also was from Wako Chemicals. Curdlan was reconstituted in sterile PBS at 10 mg/ml and used at 100 μg/ml to stimulate bone marrow–derived DC (BMDC) or 1 mg/ml for BM-derived macrophages (BMMDM), unless otherwise indicated. Fungal whole glucan particle (WGP) agonist or antagonist (WGP dispersible or WGP soluble) was purchased from InvivoGen. WGP (dispersible/soluble) was used at 100 μg/ml, unless otherwise indicated.

#### Mice

Mice were maintained and bred at the University of Massachusetts Medical School in accordance with the Institutional Animal Care and Use Committee. Casp8+/-Rip3+/- mice were provided by Dr. D. Green (St. Jude Children’s Research Hospital, Memphis, TN), and in some cases, Casp8+/-Rip3+/- femurs were from Dr. W.J. Kaiser and Dr. E.S. Mocarski (Emory University School of Medicine, Atlanta, GA). Card9+/- femurs were from Dr. R. Xavier (Massachusetts General Hospital, Boston, MA) (31), and Itgam+/- femurs were from Dr. T.N. Mayadas (Brigham and Women’s Hospital, Boston, MA) (32). Casp1+/- and casp1+/- mice were provided by Dr. V. Dixit (Genentech, South San Francisco, CA) (24), and Clec7a+/- (Dectin-1+/-) mice were from Dr. G. Brown (University of Aberdeen, King’s College, Aberdeen, U.K.) (33). Nlrp3+/- and Asc+/- mice were from Millennium Pharmaceuticals. Casp8+/-Rip3+/- mice were on a mixed C57BL/6-129 background and were intercrossed to generate Casp8+/-Rip3+/- and Casp8+/-Rip3+/- mice, which were used as littermate controls in all of our experiments. All other mice were on a C57BL/6 background.

*C. albicans* and enterohemorrhagic Escherichia coli culture conditions

*C. albicans* UC820 (ATCC MYA-3573) strain, obtained from Dr. M. Netea (Nijmegen Institute for Infection, Inflammation, and Immunity, Nijmegen, the Netherlands), was used in all experiments (34). *C. albicans* was maintained as glycerol stocks and cultured in the yeast phase in Sabouraud Dextrose broth at 30°C, 250 rpm. Overnight cultures were reinoculated 1:20 in Sabouraud Dextrose broth for 4 h to obtain yeast forms. Live log-phase yeast cultures was washed twice and resuspended in PBS, counted, and used directly or HK (95°C for 30 min), and used for BMDC stimulation. For differentiation into hyphal forms, overnight cultures were reinoculated in serum-free, antibiotic-free RPMI 1640 medium, with 10% heat-inactivated FBS for 4 h at 37°C, 250 rpm. Hyphal were washed twice and resuspended in PBS, counted, and used. Enterohemorrhagic Escherichia coli (EHEC, strain 0157:H7, EDL 933) was maintained as glycerol stocks and cultured overnight in Luria–Bertani medium at 37°C, 250 rpm and used in stationary phase for experiments as described previously (25).

#### BMDC and BMDM culture

For BMDC, progenitor cells were cultured in DMEM with 10% heat-inactivated serum, Pen-strep and 20% L929 supernatant as the source of MCSF. Medium was changed on days 3, 6, and 8, and cells were used for experiments between days 8 and 10. For DC, bone marrow progenitor cells were counted and plated at 8 × 10⁶ cells/25 cm² dish in RPMI 1640 medium with 10% heat-inactivated serum, Pen-strep, 20 ng/ml recombinant GM-CSF, and 50 μM 2-ME and cultured for 9–10 d as described previously (35).
For ELISAs, BMDC or BMDM were plated in 96-well plates at 1 x 10^5 cells/ml. Cells were unprimed or primed with Pam2CSK4 at 100 ng/ml for 3 h and stimulated with curdlin, WGP agonist, or EHEC for 6 or 24 h. Medium was replaced with fresh gentamicin containing medium for EHEC-infected cells after 1 h of infection. For controls, cells were stimulated with canonical inflammasome activators such as pAdET or silica for 6 h or nigericin or ATP for 1 h after priming. For experiments with the WGP antagonist, unprimed or primed cells were pretreated with 100 ng/ml antagonists for 1 h before stimulation with β-glucan ligands. For C. albicans infection, cells were infected with HK or live log-phase cultures at 1:1, 10:1 for 6 h postpriming. Cytokine levels were measured in triplicate, and data are representative of three independent experiments.

For Western blot analysis, cells were plated at 2 x 10^5 cells/well in 12-well plates in serum-free, antibiotic-free medium, primed, and stimulated as described above. Supernatant proteins were precipitated with methanol and chloroform. Cells were lysed with 1% Nonidet P-40 lysis buffer, and Bradford assay was performed to normalize the amount of protein. Samples were run on 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed with the Abs to caspase-1 p10 (s) of C. albicans were fixed with 4% paraformaldehyde and stained with 1:250 dilution of the mouse monoclonal β-glucan Ab and washed, followed by staining with CF488A goat anti mouse secondary Ab. The stained preparations were then visualized by confocal microscopy for glucan staining.

Confocal microscopy
Silica, WGP agonist, and live or HK C. albicans were fixed with 4% paraformaldehyde and stained with 1:250 dilution of the mouse monoclonal β-glucan Ab and washed, followed by staining with CF488A goat anti mouse secondary Ab. The stained preparations were then visualized by confocal microscopy for glucan staining.

Serum opsonization and C3 staining
Blood was harvested from C57BL/6 mice by cardiac puncture and serum isolated by centrifugation. Live and HK C. albicans were left untreated or opsonized with serum (20%) and incubated at 37˚C for 30 min. The cells were then spun down, washed twice with PBS, and resuspended in RPMI 1640 medium. Pam2CSK4-primed BMDC were then stimulated with opsonized/unopsonized live or HK C. albicans for 6 h. C3 deposition was verified by fixing these cells with 1% paraformaldehyde and staining them with goat anti-mouse C3 FITC Ab or isotype control and analyzing them by flow cytometer.

ELISA
The amount of IL-1β present in cell culture supernatants was measured by ELISA (BD Biosciences or eBioscience), according to the manufacturer’s instructions.

Quantitative RT-PCR
RNA was extracted from cells using RNeasy kit (Qiagen). iScript Select cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA from 1 µg total RNA from each sample. Quantitative RT-PCR for pro–IL-1β and β-actin was performed using iQ SYBR green supermix (Bio-Rad). Primers used for pro–IL-1β were 5′-TCCCCAGCCCTTTGGTGA-3′ (forward), 5′-TTAGAACCAAATGTGGCCGTG-3′ (reverse), and β-actin were 5′-TTGGAATGACATTGTTACCAA-3′ (forward), 5′-TGGCATAAGGCTCTTACCGGA-3′ (reverse). Levels of pro–IL-1β mRNA were normalized to that of β-actin.

Caspase-8 activity and cell death assay
Caspase-8 activity in cell lysates was assayed using Caspase-Glo 8 Assay kit (Promega). Cell viability was assessed using Cell-Titer Glo kit (Promega), according to the manufacturer’s instructions.

Statistics
Data were analyzed by two-way ANOVA, followed by Bonferroni test using Prism software. A p value < 0.05 was considered significant. For cytokine measurements, experiments were performed in triplicate from one mouse, and the graphs depicted are representative of at least two and, in most cases, three independent experiments. The triplicates of the representative experiment shown were analyzed using two-way ANOVA. The significance of comparisons was marked by an asterisk (p < 0.05), and the unmarked groups in the same graph are not. In the case of experiments with Casp8p43Rip3−/−, Casp8p88Rip3−/−, and Casp8p68Rip3−/− DC, the groups Casp8p43Rip3−/− and Casp8p88Rip3−/− were compared to assess significance.

Results
NLRP3-ASC-caspase-1 inflammasome is essential for C. albicans- and β-glucan-induced IL-1β
We and others have previously demonstrated that C. albicans–induced IL-1β is mediated by the NLRP3-ASC-caspase-1 inflammasome (2, 11). In this study, we first examined whether our strain of live C. albicans triggers IL-1β production in BMDC. As shown in Fig. 1A, we observed that both live yeast as well as hyphal forms induce IL-1β. In particular, the yeast form is highly immunostimulatory (as seen with both the multiplicity of infection), has high colonizing and disseminating potential, and can transition into the hyphal form during infection. Using the yeast form of C. albicans, we set out to characterize the mechanism of inflammasome activation and the immunostimulatory component (s) of C. albicans responsible for this activity. Interestingly, HK C. albicans triggers levels of IL-1β production similar to that seen with live fungi, and this response is also completely dependent on the NLRP3-ASC-caspase-1 inflammasome in mouse DC (Fig. 1B, 1C). β-Glucan, which is part of fungal cell walls, is a highly immune-stimulatory pathogen-associated molecular pattern (PAMP) that is exposed in HK fungi (36). Hence, we sought to understand the underlying mechanism of inflammasome activation by β-glucans. The composition of microbial glucans may vary depending on the length, m.w., frequency of branching, and the type of glycosidic linkage. Two representative β-glucans used in this study are 1) curdlan, a β(1,3) glucan derived from the bacterium Alcaligenes faecalis; and 2) dispersible whole fungal glucan particles derived from the cell walls of the baker’s yeast Saccharomyces cerevisiae (WGP agonist). Curdlans as well as WGP are high m.w., insoluble particles. Glucan particles derived from S. cerevisiae (WGP agonist) as well as C. albicans contain a linear 1,3-linked glucan backbone with occasional 1,6 branches. Although the cell wall of S. cerevisiae and C. albicans are considered qualitatively similar (37, 38), they exhibit quantitative differences in the wall components (39). Previous studies showed that the alkali-insoluble glucan of C. albicans contains ∼43–53% β(1,6) linkages, 32–39% β(1,3) linkages and is highly branched. In sharp contrast, the alkali-insoluble glucan of S. cerevisiae are primarily composed of linear β(1,3) glucose with ∼3% β(1,6) linkages (39, 40). However, curdlan from A. faecalis is just linear 1,3 glucan with no branching. Curdlan and WGP agonist are considered to be good models for studying the innate immune responses to fungal β(1,3) glucans. We observed that curdlan as well as WGP agonist induced IL-1β production in DC in the absence or presence of Pam2CSK4 (TLR2) priming (Fig. 1D). Pam2CSK4 priming greatly boosted this response. All remaining experiments in this study were performed in Pam2CSK4-primed DC, unless otherwise indicated. Furthermore, we tested increasing doses of curdlan and WGP agonist and determined 100 µg/ml to be optimal for inducing IL-1β production in Pam2CSK4-primed BMDC (Fig. 1E).

To determine the role of the NLRP3-ASC-caspase-1 inflammasome in β-glucan–induced IL-1β production, Pam2CSK4-primed wild-type (WT), Nlrp3−/−, Asc−/−, and Casp1−/− BMDC were stimulated with curdlan and WGP agonist. IL-1β secretion was completely abrogated in Nlrp3−/−, Asc−/−, and Casp1−/− DC (Fig. 1F, 1G). IL-1β processing detected by immunoblotting of BMDC culture supernatants was severely impaired in the absence of caspase-1 (Fig. 1H). Of note, our previous studies used the Casp1−/− mice that carried the Casp11 passenger mutation associated with the 129 mouse strain (24, 41, 42). Caspase-11 is essential for inflammasome activation by Gram-negative bacteria (24, 25). To carefully examine the role of caspase-1, the experiments in Fig. 1 used Casp1−/− mice expressing caspase-11.
performed on results in (B) and (C). The statistical analysis was performed on results in (G) and (H).

Sensing and phagocytosis of particulate β-glucan triggers IL-1β production

To ensure that the responses elicited by the curdlan preparations were specifically because of β-glucans, cells were pretreated with a well-established antagonist, soluble β-glucan (WGP antagonist), to competitively bind and block signaling through host receptors. WGP antagonist–treated cells were then stimulated with curdlan from two independent sources (InvivoGen or Wako Chemicals), and IL-1β production was assayed. Curdlan is capable of triggering the synthesis (signal 1) and maturation (signal 2) of pro–IL-1β in DC, as observed in the experiments with unprimed cells shown in Fig. 1D. In the unprimed condition, WGP antagonist treatment led to an attenuated curdlan-induced IL-1β response (Fig. 2A). Because the WGP antagonist could be blocking either of the two signals, the effect of WGP antagonist specifically on signal 2 was addressed by priming the cells with a non–β-glucan ligand, Pam3CSK4, to induce signal 1. WGP antagonist also attenuated IL-1β production in the primed BMDCs specifically in response to curdlan but not pdAdT (an activator of the AIM2 inflammasome) (Fig. 2B), indicating that WGP antagonist inhibits β-glucan–induced inflammasome activation and IL-1β release (signal 2). Taken together, these results highlight that receptor mediated sensing of β-glucan is important for inflammasome activation.

Considerable research is devoted to water-soluble chemical derivatives of β-glucans to improve the ease of their use in clinical applications. One such water-soluble derivative of curdlan, carboxy-methylated–curdlan (or CM-curdlan), was tested for its stimulatory activity of CM-curdlan also has been observed in vivo, where preinjection of curdlan but not CM-curdlan protects mice from lethal E. coli infection (43). The weak ability of CM-curdlan to trigger IL-1β production indicates that the particulate properties of β-glucans are important for triggering inflammasome activation. In line with that idea, chitosan, a deacetylated derivative of chitin, induces higher IL-1β in macrophages in particulate form compared with its soluble counterpart (44).

Because the particulate form of curdlan was more immunostimulatory and DC are highly phagocytic, we determined whether inflammasome responses to β-glucans require their uptake. Cells were pretreated with cytochalasin D, an inhibitor of actin rear-
significant, as described in (Wako Chemicals) were used at increasing concentrations (WGP antagonist (pdAdT or silica for 6 h, nigericin for 1 h in the presence of the soluble was performed on results in (Supplemental Fig. 1A). Phagocytosis is essential for this process similar to that of the particulate stimulus silica (Supplemental Fig. 1A). Of note, when we measured lactate dehydrogenase (LDH) in the supernatants, minimal to no increase was observed in β-glucan–stimulated cells, unlike nigericin-treated cells (data not shown), suggesting that either the LDH assay was not as sensitive as the ATP-based assay or that LDH may not be released in the cell death process observed in this study.

**Dectin-1 and CARD9 signaling are important for β-glucan–triggered IL-1β processing and release**

The C-type lectin receptor family of proteins is considered the primary recognition and phagocytic receptors for fungal cell wall components, and dectin-1, in particular, plays an important role in anti-fungal immunity (46, 47). To identify innate immune receptors that couple β-glucan recognition to inflammasome activation, we tested the role of dectin-1 in IL-1β maturation and secretion. Curdlan (InvivoGen) [curdlan (I)–induced IL-1β production was markedly decreased in Pam2CSK4–primed Clec7a−/− (dectin-1-deficient) BMDC (Fig. 3A). Western blotting of precipitated supernatants from curdlan (I)–stimulated cells reflected this decrease in the amount of processed IL-1β in Clec7a−/− BMDC (Fig. 3B). Curdlan induced caspase-1 processing also was markedly reduced in Clec7a−/− BMDC, indicating that dectin-1 signaling is important for β-glucan–induced caspase-1 activation (Fig. 3B). CARD9 is an adaptor molecule that mediates NF-κB activation by many ITAM-associated receptors including dectin-1 and is an indispensable component of antifungal innate immune responses in myeloid cells (48). Consistent with a role for dectin-1 in inflammasome activation by curdlan, IL-1β production by Pam2CSK4–primed Card9−/− BMDC also was decreased in response to curdlan (I) (Fig. 3C). Card9−/− BMDC exhibited reduced IL-1β processing in response to curdlan (Fig. 3D), indicating that the dectin-1–CARD9 signaling axis is critical for curdlan (I)–induced IL-1β maturation. Dectin-1 deficiency also protected cells from β-glucan–induced cell death (Supplemental Fig. 1B), indicating that dectin-1 not only triggers IL-1β processing but also cell death.

**FIGURE 3.** Dectin-1 and CARD9 signaling are important for β-glucan–triggered IL-1β production and release. Pam2CSK4–primed BMDC from C57BL/6, Clec7a−/− or Card9−/− mice were stimulated with curdlan (I), EHEC or silica for 6 h. IL-1β released in the supernatants was measured by ELISA (A and C). Cells were stimulated as indicated, and precipitated supernatants and lysates were probed for IL-1β or caspase-1 p10 (B and D). Statistical analysis was performed on results in (A) and (C) with *p < 0.05 considered significant, as described in Materials and Methods.
Another receptor that has been implicated in β-glucan recognition is CR3, an integrin composed of CD11b/CD18 (Uniprot: P05555/P11835). In addition to binding the complement component iC3b, CR3 has a distinct lectin site for binding β-glucan (49–51). Similar to other leucocyte integrins, upon receiving intracellular signals, CR3 undergoes conformational changes required for it to optimally bind its ligand. Recently, it was shown that dectin-1 activates inside-out signaling of CR3 that in turn promotes CR3 binding of C. albicans and subsequent neutrophil antifungal effector functions (52). The role of CR3 in inflammasome activation generally and particularly in response to β-glucans or C. albicans has not been investigated to date. To test the role of CR3 in this pathway, Pam3CSK4-primed Itgam−/− (CR3-deficient) BMDC were stimulated with curdlan (I) and IL-1β release was measured. CR3-deficient BMDC (Itgam−/− BMDC) displayed severely impaired IL-1β production in response to curdlan (Fig. 4A).

Curdlan-induced IL-1β processing also was drastically reduced in the Itgam−/− BMDC while that induced by silica was unaffected (Fig. 4B). Remarkably, curdlan-triggered caspase-1 processing also was impaired in CR3-deficient cells, suggesting that CR3 is important for inflammasome-mediated caspase-1 activation in response to β-glucans. The role of CR3 observed in caspase-1 activation and IL-1β production appears to be complement independent, because all of these experiments were performed in serum-free medium. Interestingly, CR3 deficiency also protected DC from β-glucan–triggered cell death (Supplemental Fig. 1C). Taken together, these results indicate an essential role for CR3 in innate immune responses to β-glucans.

**Caspase-8 promotes β-glucan–induced IL-1β production**

We have shown that dectin-1 is required not only for β-glucan–induced IL-1β maturation but also for DC death (Fig. 3A, Supplemental Fig. 1B). A recent study reported that dectin-1–mediated caspase-8 activation is essential for β-glucan–induced IL-1β responses in human DC (19). We also have linked caspase-8 deficiency to Fas ligand (FasL)–induced IL-1β production (53–55), yet this remains controversial (56, 57). Pro–IL-1β is a transcriptional target of NF-κB, and hence, we examined the impact of caspase-8 deficiency on pro–IL-1β synthesis in response to β-glucans. In the absence of Pam3CSK4 priming, pro–IL-1β mRNA levels were similar in WT, Rip3−/−, and Casp8−/−/− BMDC stimulated with curdlan (Supplemental Fig. 3A), suggesting that caspase-8 deficiency does not affect curdlan-induced signal 1. Curdlan has been found to synergize with TLR2 and TLR4 agonists for TNF induction (58), and hence, we also investigated the role of caspase-8 in TLR2- and TLR4-induced pro–IL-1β synthesis. When cells were primed with the TLR2 agonist Pam3CSK4, prior to curdlan (I) stimulation (similar to the experimental conditions used in most of this study), pro–IL-1β protein levels in the Casp8−/−/− BMDC were comparable to that in WT or Rip3−/− cells (Supplemental Fig. 3B), as is also seen in Fig. 5C and Supplemental Fig. 2D. These results indicate that caspase-8 does not play a role in β-glucan or lipopeptide triggered signal 1 (NF-κB–mediated pro–IL-1β induction) and confirm the effect of caspase-8 on signal 2 (inflammasome-mediated pro–IL-1β processing). However, LPS (a TLR4 agonist)–induced pro–IL-1β was markedly decreased in Casp8−/−/− BMDC compared with WT or Rip3−/− cells (Supplemental Fig. 3C). Consistent with a defect in TLR4 induction) and LPS-induced TNF, another well-characterized NF-κB target gene, also was decreased in Casp8−/−/−/− BMDC (Supplemental Fig. 3D). Other recent studies published also have demonstrated the critical role of caspase-8 in the proinflammatory cytokine (TNF, IL-6, and IL-1β) production.

**FIGURE 4.** CR3 is critical for β-glucan–induced inflammasome activation and IL-1β production. Pam3CSK4-primed BMDC from C57Bl/6, Itgam−/− mice were stimulated with curdlan (I) for 6 h or ATP for 1 h. IL-1β released in the supernatants was measured by ELISA (A). Cells were stimulated as indicated, and precipitated supernatants and lysates were probed for IL-1β or caspase-1 p20 (B). Statistical analysis was performed on results in (A) with *p < 0.05 considered significant, as described in Materials and Methods.
Having identified the innate immune pathways involved in the execution of IL-1β and cell death responses to β-glucan, we sought to explore the relevance of these signaling pathways during infection with C. albicans. C57BL/6, Casp8<sup>−/−</sup> Rip3<sup>−/−</sup>, and littermate Rip3<sup>−/−</sup> BMDC were infected with C. albicans at 1:1 or 10:1. To facilitate higher β-glucan presentation by C. albicans, we also used HK fungus. As expected, IL-1β production by Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> cells was significantly decreased in response to HK C. albicans (Fig. 6A). However, live log-phase C. albicans, did not require caspase-8 for IL-1β production (Fig. 6B). Consistent with the IL-1β phenotype, HK C. albicans–triggered cell death was protected to an extent in Casp8<sup>−/−</sup> Rip3<sup>−/−</sup> cells, whereas the response to live C. albicans was unaffected (Fig. 6C, 6D). Because HK C. albicans required caspase-8 for IL-1β production similar to curdlan and WGP agonist, we checked whether the β-glucans exposure is higher in HK C. albicans compared with that of the live fungus. A monoclonal β(1,3) glucan–specific Ab, which robustly stained WGP agonist but not the negative control, silica was used (Supplemental Fig. 4A, 4B). We observed relatively higher staining of β-glucan in HK C. albicans compared with that of its live counterpart (Supplemental Fig. 4C, 4D). These results link the requirement of caspase-8 to the β-glucans exposed by specific stimuli. To further validate that the exposed β-glucans are the main immunostimulatory components of HK C. albicans, we used the WGP antagonist to test whether HK C. albicans–induced responses are more β-glucan dependent. Cells treated with HK C. albicans, but not live C. albicans exhibited reduced IL-1β and protection from cell death in the presence of the antagonist (Fig. 6E, 6F).

Although β-glucans and HK C. albicans show an additional requirement for caspase-8, the canonical NLRP3 inflammasome was essential for all for these stimuli (Fig. 1). To understand the position of caspase-8 in these pathways, we monitored caspase-8 activity in cells exposed to HK C. albicans and assessed the same in Asc<sup>−/−</sup> BMDC, which are deficient in inflammasome function (Supplemental Fig. 3F). This rules out any possible role of caspase-8 on β-glucan–induced IL-1β indirectly through its role in TLR4 signaling or LPS contaminants in curdlan. Importantly, the use of Pam<sub>2</sub>CysK<sub>4</sub> priming throughout our study provided an ideal tool to avoid any potential impairment of pro–IL-1β that could result from LPS priming.

Differential requirement of caspase-8, dectin-1, and CR3 for HK versus live C. albicans-induced IL-1β production and cell death

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During the course of infection in humans and mice, C. albicans is often opsonized by serum and Abs, and the myeloid cells may encounter the opsonized fungus. Hence, we tested whether dectin-1 or CR3 was required for C. albicans–induced IL-1β in our system. No significant reduction in IL-1β production was noticed in Clec7a−/− BMDC treated with HK or live C. albicans (Fig. 7A). In contrast, HK C. albicans showed a near complete requirement for CR3, whereas the live fungus exhibited reduced IL-1β production (Fig. 7B), suggesting that CR3 may in fact be the dominant receptor involved in C. albicans–induced IL-1β. Interestingly, Clec7a−/− BMDC were protected from cell death triggered by HK, but not live C. albicans–induced cell death (Fig. 7C). Similarly, cell death triggered by HK, but not live C. albicans, was CR3 dependent (Fig. 7D). Thus, both CR3 and dectin-1 appear to be involved in the cell death response to HK fungi.

During the course of infection in humans and mice, C. albicans is often opsonized by serum and Abs, and the myeloid cells may encounter the opsonized fungus. Hence, we assessed the IL-1β production and cell death observed with opsonized live and HK yeasts. As shown in Supplemental Fig. 4G, IL-1β production and cell death responses of BMDC when exposed to HK but not live C. albicans required CR3 for IL-1β production, whereas the live fungus exhibited reduced IL-1β production (Fig. 7B), suggesting that CR3 may in fact be the dominant receptor involved in C. albicans–induced IL-1β. Interestingly, Clec7a−/− BMDC were protected from cell death triggered by HK, but not live C. albicans–induced cell death (Fig. 7C). Similarly, cell death triggered by HK, but not live C. albicans, was CR3 dependent (Fig. 7D). Thus, both CR3 and dectin-1 appear to be involved in the cell death response to HK fungi.

Discussion

The data presented in this study demonstrate that the canonical NLRP3 inflammasome (NLRP3, ASC, and caspase-1), dectin-1, CARD9, CR3, and caspase-8 all contribute to β-glucan–induced inflammasome responses (Fig. 8). These molecular requirements are highly specific to β-glucans, because dectin-1, CARD9, CR3, and caspase-8 were dispensable for IL-1β induced by canonical inflammasome triggers such as silica or pdAdT. Strikingly, our data implicates CR3 in addition to dectin-1 as a critical sensor coupling β-glucan recognition to inflammasome activation. Strikingly, dectin-1 and CR3 were both essential, and appeared to function in a nonredundant manner in the IL-1β and cell death response to β-glucans. We used an ATP-based luminescence assay to measure the metabolic activity of DC as a surrogate for cell viability. Intracellular ATP can fluctuate in response to other physiological and mitochondrial perturbations and, hence, an alternate interpretation of this assay, in the case of β-glucans in particular, is that ATP levels are decreased because of reduced metabolic activity, rather than cell death. Dectin-1 has previously been shown to activate the integrin CR3 through inside-out signaling to recognize C. albicans PAMPs (52), indicating a potential functional connection between these two receptors. Thus, dectin-1...
and CR3 could be acting sequentially for sensing and signaling in response to β-glucans.

C. albicans–induced IL-1β and cell death profiles showed some similarities and some differences from that of β-glucans (Fig. 8). Live or HK C. albicans triggered IL-1β production didn’t require dectin-1. Instead, CR3 appeared to the critical receptor for C. albicans–induced IL-1β production, with HK and live fungus showing a complete or partial requirement, respectively. Both dectin-1 and CR3 were important for HK C. albicans–induced cell death. We observed higher staining of β-glucan in HK C. albicans and the recognition of β-glucan is important for HK C. albicans–triggered IL-1β and cell death, in contrast to live C. albicans. This is consistent with many published studies that have indicated that β-glucan is masked within the cell wall of live fungus (64), which might impede dectin-1/CR-3 recognition of live C. albicans. The differential requirement of these receptors for HK and live C. albicans may represent their independent and redundant function or alternatively the involvement of receptors for other PAMPs, such as chitin and mannan, in triggering inflammasome activation. In vivo studies suggest that β-glucan that is masked by live C. albicans, especially in the yeast form, gets exposed later during the course of infection in organs such as the kidneys (64). Varied degree of β-glucan unmasking that occurs with disseminated infection, might elicit a qualitatively different cytokine response. In line with our findings with unopsonized yeast, a previous study has reported that CR3 drives caspase-8 activation and neutrophil apoptosis in response to phagocytosis of serum-opsonized yeast (65). Because serum opsonized live and HK C. albicans yeast also trigger IL-1β production, it will be very interesting to examine the role of caspase-8 and CR3 in this response in future.

Our findings present striking similarities and differences with that of Gringhuis et al. (19), where IL-1β maturation in human DC, triggered by curdlan and some strains of HK and live C. albicans was dependent on dectin-1, ASC, and caspase-8, but independent of NLPR3, caspase-1, and phagocytosis. Our studies in mouse DC, using genetic models, similarly show key roles for dectin-1 and caspase-8, but also a critical requirement for NLPR3, ASC, and caspase-1 for optimal IL-1β maturation following β-glucan stimulation. Other studies also have linked the NLPR3 inflammasome to β-glucan–induced IL-1β production in mouse and human macrophages and DC (20, 21). In contrast to β-glucans, dectin-1 and caspase-8 were not required for live C. albicans–induced IL-1β production in mouse DC.

How caspase-8 is engaged by β-glucan–sensing pathways is currently unclear. Caspase-8 deficiency did not have a profound impact on curdlan-induced caspase-1 processing, suggesting that NLPR3 inflammasome assembly and caspase-1 activation are intact in Casp8−/− Rip3−/− cells. This is consistent with a recent study that linked caspase-8 to Salmonella-induced pro–IL-1β synthesis and processing in a manner that is independent of caspase-1 (26). Furthermore, caspase-8–mediated cell death in response to HK C. albicans is not dependent on NLPR3 or ASC, even though all of these molecules are important for IL-1β production. These results position caspase-8 on a parallel β-glucan–activated pathway that is independent of inflammasome assembly but converges with the assembled NLPR3 inflammasome complex to promote IL-1β maturation (Fig. 8).

Future studies will dissect the mode of action of caspase-8 to determine whether caspase-8 promotes the activation and processing of IL-1β through direct or indirect mechanisms. Interestingly, recombinant caspase-8 has been shown to cleave pro–IL-1β at the same site as caspase-1, in an in vitro system (23), suggesting that caspase-8 may directly cleave pro–IL-1β. Caspase-1 and caspase-8 have CARD and DED, respectively, which are related protein–protein interaction domains involved in a number of distinct homotypic interactions. Thus, one intriguing possibility would be direct interaction between these two caspases. It also would be interesting if caspase-8 regulates caspase-1 through activating cellular inhibitors of apoptosis proteins, cIAPs, and K63 ubiquitination (66). In our own studies, we have shown that the
Drosophila caspase-8-like factor DREDD triggers IAP-dependent K63-ubiquitination (67) and perhaps some aspects of these pathways are conserved. Overall, the role of caspase-8 in bridging β-glucan sensing by dectin-1 and CR3 and potentiating cell death and NLRP3 inflammasome–dependent IL-1β processing is reminiscent of the role of caspase-11 in gram-negative bacterial infection, where caspase-11 integrates LPS sensing with cell death and NLRP3-dependent IL-1β maturation (25).

Extrinsic cell death pathways show significant overlap with innate immune signaling mechanisms and share molecular components including RIP1 and cIAPs. Ripoptosomes are dynamic cytosolic multiprotein complexes, which determine cell fate along the survival/apoptosis/necrosis axis (68, 69). Recent studies have elucidated their role in regulation of IL-1β processing in addition to their well-characterized roles in cell death. Two recent reports indicated that RIP3 triggers NLRP3-ASC–dependent caspase-1 activation and IL-1β processing when caspase-8 is deficient or when cIAPs are depleted (70, 71). Surprisingly, IL-1β production observed in these scenarios required just a priming signal by TLRs. This suggests a tight quality control of the ripoptosome complex where removal of caspase-8 or cIAP proteins skews the ripoptosome to feed into the NLRP3-ASC-caspase-1 pathway for IL-1β processing. Although any potential role for RIP3 in caspase-8 deficient conditions is not evident in our assays, which use RIP3 and caspase-8 double deficient cells, we noticed that Rsp3-/- DC exhibit IL-1β and cell death responses comparable to WT, in response to all stimuli examined (Figs. 5A, 6A–D, Supplemental Fig. 2A, 2E).

Our efforts to characterize the activation of the enzyme caspase-8 by HK C. albicans revealed that induction of caspase-8 activity, albeit detectable, was very weak as compared with that elicited by FasL vesicles (Supplemental Fig. 4E). However, consistent with our data, caspase-8 activation has been observed when dectin-1 is activated by HK C. albicans or CR3 phagocytosis opsonized yeast in other cell types (19, 65). Catalytic functions of caspase-8 are regulated by a non-catalytic homolog and partner, cFLIP, which is also involved in NLRP3 and AIM2 inflammasome responses (72). Recent evidence that hemizygotic cFLIP deficient cells exhibit higher caspase-8–mediated IL-1β production specifically in response to HK C. albicans supports the important role of caspase-8 activity in the β-glucan signaling pathway (72).

Currently, no antifungal vaccines are available for humans, but β-glucan conjugates have shown promise as vaccines in rodent experimental models (73). Laminarin, a β-glucan derived from the alga, Laminaria digitata conjugated with a diphtheria toxoid conferred protection to mice against systemic C. albicans and A. fumigatus infection that was attributed to β-glucan specific Abs (74). β-Glucan, as a glucose polymer, finds applications in many therapeutic and adjuvant preparations and is considered to have anticancer potential (75, 76). Moreover, β-glucans also act as potent adjuvants and delivery systems for Ags and in eliciting Th1 and Th17 responses against protein Ags (77, 78). It would be exciting to determine whether the anticancer and/or adjuvant properties of β-glucans results from engagement of caspase-8 and/or caspase-1 pathways.

Altogether, the findings presented here uncover new roles for CR3, dectin-1 as well as caspase-8 in coordinating cell death and inflammasome responses to β-glucans. These studies significantly enhance our understanding of β-glucan elicited inflammatory responses; provide improved understanding of anti-fungal immune responses and mechanistic understanding of the immune-modulating properties of β-glucans.

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Disclosures

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