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HOST RESPONSE AND INFLAMMATION

The Ubiquitin-Modifying Enzyme A20 Terminates C-Type Lectin Receptor Signals and Is a Suppressor of Host Defense against Systemic Fungal Infection

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ABSTRACT C-type lectin receptors (CLRs) play key roles in antifungal defense. CLR-induced NF-κB is central to CLR functions in immunity, and thus, molecules that control the amplitude of CLR-induced NF-κB could profoundly influence host defense against fungal pathogens. However, little is known about the mechanisms that negatively regulate CLR-induced NF-κB, and molecules which act on the CLR family broadly and which directly regulate acute CLR-signaling cascades remain unidentified. Here, we identify the ubiquitin-editing enzyme A20 as a negative regulator of acute NF-κB activation downstream of multiple CLR pathways. Absence of A20 suppression results in exaggerated CLR responses in cells which are A20 deficient and also cells which are A20 haplosufficient, including multiple primary immune cells. Loss of a single allele of A20 results in enhanced defense against systemic *Candida albicans* infection and prolonged host survival. Thus, A20 restricts CLR-induced innate immune responses *in vivo* and is a suppressor of host defense against systemic fungal infection.

KEYWORDS A20, C-type lectin receptors, NF-κB, TRAF6, cytokines, dendritic cells, fungal immunity, innate immunity, ubiquitination

The C-type lectin receptor (CLR) family of pattern recognition receptors recognizes a diversity of microbial ligands to mediate host defense against fungal and mycobacterial infections (1–3). Within this large family of receptors, signals induced by Dectin-1, Dectin-2, and Mincle are the most well-studied, and they are most abundantly expressed by innate immune cells, including neutrophils, macrophages, and dendritic cells (4–6). In these immune cells, Dectin-1, Dectin-2, and Mincle use a Syk- and CARD9-dependent signaling pathway to activate NF-κB and induce downstream cellular activation and immune responses (3, 7–10). Amid the many different cellular responses induced by these pathways, which include phagocytosis, reactive oxygen species, and antigen presentation, NF-κB activation plays a critical role in cytokine expression (e.g., interleukin-1β [IL-1β], IL-6, tumor necrosis factor alpha [TNF-α], IL-12, and IL-23) and is essential for immunity to pathogen challenge (10–13).

Given the prominent roles played by CLR-induced NF-κB, molecular regulators that control the amplitude of NF-κB activation could have a profound influence on host defense against fungal infection. However, little is known about the mechanisms that negatively regulate CLR-induced responses, and the identity of such negative regula-

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tors have only recently begun to be explored. The expression of a few negative regulators is induced in response to CLR stimulation, such as specific microRNA (miRNA) species and CD23 (also known as Fc epsilon RII) (14–16). These inducible regulators act to control secondary responses to a stimulus encountered subsequent to an initial ligand encounter. Acute regulation of initial CLR ligand-induced NF-κB is another means of negative regulation, and thus far, only one molecule has been identified in this category. A recent study identified Cbl-b as a negative regulator of signals transduced by Dectin-2 and also the Dectin-2-binding receptor macrophage C-type lectin (MCL, also called Dectin-3, or *Clec4d*) (17). Mechanistically, Cbl-b downregulates Dectin-2 and MCL signals by inducing endocytosis and degrading these receptors. While restricting the cell surface expression of CLRs has a clear effect on their availability for ligand binding, this mechanism of suppression does not appear to act on Dectin-1 (17), and thus, the identity of molecules with a broad function to suppress CLR-induced NF-κB and which have a direct influence on the acute signaling cascade remain unidentified.

The ubiquitin-modifying enzyme A20 (gene name *Tnfaip3*) is a negative regulator of NF-κB activation downstream of multiple innate and inflammatory receptors, including Toll-like receptors (TLRs), IL-1R, TNF receptor [TNF-R], IL-17R, and NOD2 (18–22). In these pathways, A20 regulates the ubiquitination of key signaling molecules (e.g., TRAF6 and RIP1) to control the amplitude and duration of NF-κB activation (reviewed in reference 23). A20's expression is upregulated by NF-κB, and in this way, A20's module of suppression is 2-fold, as follows: (i) to restrict acute NF-κB signaling that occurs immediately upon receptor-ligand binding and (ii) to restrict secondary cellular responses to stimulus encountered subsequent to an initial ligand exposure. A20's influential role as a negative regulator of NF-κB is well demonstrated by human genetic studies, which link A20 to multiple autoimmune, malignant, and inflammatory diseases (23, 24). Given these genetic links to human disease, the main investigative focus has been on the mechanisms by which the loss of A20, or reduced expression of A20, gives rise to these disease phenotypes (24–26). In contrast, little is known about A20's functions in host defense against pathogens. In this arena, we discovered that during early-stage *Candida albicans* infection, a subset of $A20^{high}$ tissue-resident macrophages degrade intracellular A20 protein, which otherwise restricted their capacity to secrete chemoattractants (27). That A20 restricted early-stage antifungal responses by this macrophage subset suggests that A20 could act broadly to suppress host defense against fungal pathogens. However, whether A20's mode of suppression in fungal immunity is active in multiple cell types and whether A20 suppresses CLR signals directly are unknown. Here, we identify A20 as a potent negative regulator of acute NF-κB signals triggered by multiple CLR pathways and further show that A20's mode of suppression on CLR responses restricts cytokine production by many primary cell types. As a consequence, A20 suppresses host defense against systemic fungal infection.

RESULTS

A20 is upregulated upon Dectin-1 stimulation and restricts Dectin-1-induced cytokine responses. To assess the potential role of A20 as a negative regulator of CLR signals, we first determined whether A20 protein expression was upregulated in response to curdlan, a purified β-1,3-glucan that is a potent and specific ligand for Dectin-1. Bone marrow-derived dendritic cells (BMDCs) from granulocyte-macrophage colony-stimulating factor (GMCSF) cultures were chosen for initial studies since they have robust responses to Dectin-1 ligands (28) and because we have previously shown that A20 is a critical suppressor of DCs *in vivo* (29, 30). The A20 protein was readily detectable in unstimulated BMDCs, and its expression was highly upregulated following Dectin-1 stimulation (Fig. 1a). These data parallel the dramatic induction of A20 seen after TNF or TLR stimulation (18, 31), pathways in which A20 suppression plays a major role. That A20 is also significantly upregulated after CLR stimulation raises the possibility that A20 may suppress CLR responses.

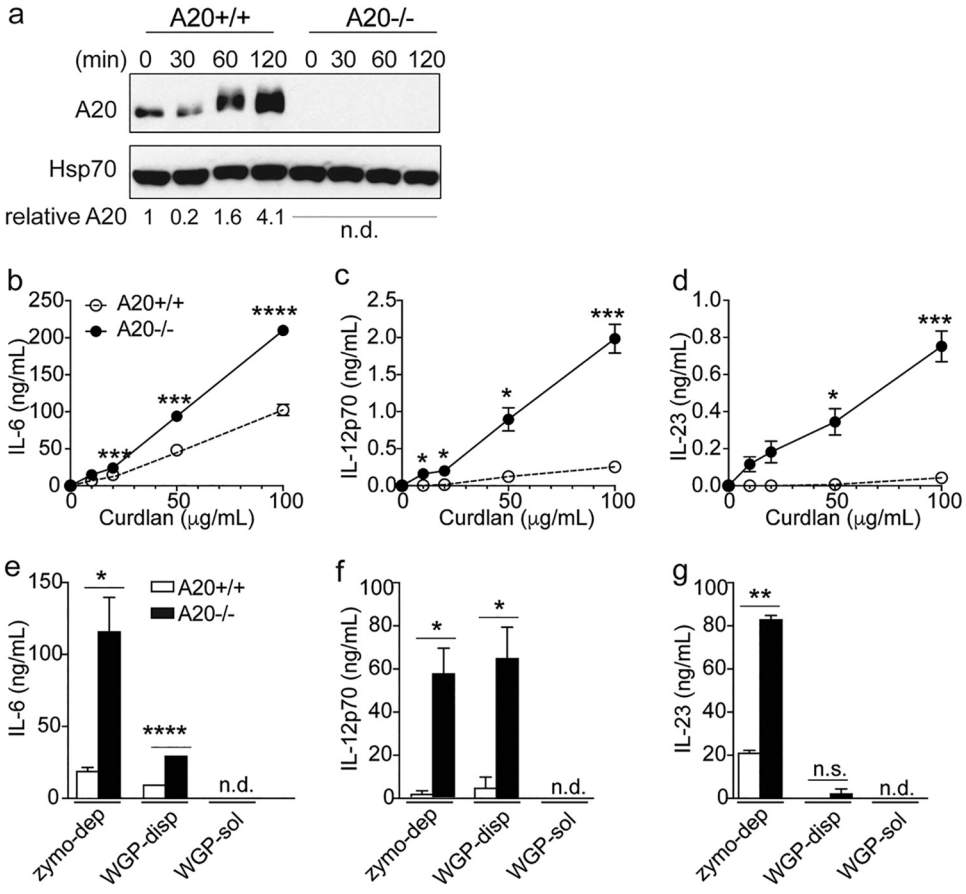

FIG 1 A20 is upregulated in response to Dectin-1 ligands and is required to restrict downstream cytokine production. (a) Wild-type and A20-deficient BMDCs were stimulated with curdlan (100 μg/ml) for the indicated times, and cell lysates were probed by Western blot for A20 or Hsp70 (loading control). Numbers below the blot indicate the A20:Hsp70 ratio, relative to time zero. (b to d) ELISA quantification of IL-6 (b) IL-12p70 (p35/p40) (c), and IL-23 (p19/p40) (d) in culture supernatant of WT or A20-deficient BMDCs stimulated for 7 h with the indicated concentration of curdlan. (e to g) ELISA quantification of IL-6 (e) IL-12p70 (f), and IL-23 (g) in culture supernatant of WT or A20-deficient BMDCs stimulated for 7 h with Dectin-1 ligands zymosan-depleted (zymo-dep, 100 μg/ml) or particulate/dispersible whole glucan particles (WGP-disp, 100 μg/ml). In tandem, cells were stimulated with the Dectin-1 antagonist whole glucan particles in soluble form (WGP-soluble, 100 μg/ml). Error bars represent mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (unpaired Student's *t* test). Results are representative of at least 3 independent experiments.

We next assessed the relative contribution of A20 to Dectin-1-induced cytokine responses. Surface expression of the Dectin-1 receptor was equivalent between wild-type ($A20^{+/+}$) and A20-deficient ($A20^{-/-}$) BMDCs (data not shown), and we stimulated them with curdlan to assess Dectin-1-induced cytokine production. At curdlan concentrations of 10 to 100 μg/ml, the production of IL-6, IL-12p70, and IL-23 was 2 to 50-fold higher in A20-deficient BMDCs than in the wild type (WT), indicating that A20 restricted curdlan-induced cytokine responses (Fig. 1b to d). To determine whether A20 restricted cytokine production to Dectin-1 ligands broadly, we tested the following two additional Dectin-1 ligands: (i) alkaline-treated zymosan (zymo-dep), which is depleted of a TLR2 agonist; and 2) whole glucan particles in particulate/dispersible form (WGP-disp). Similar to curdlan-induced responses, zymo-dep and WGP-disp triggered 3- to 38-fold higher cytokine production by A20-deficient BMDCs (Fig. 1e to g). Importantly, neither WT nor A20-deficient BMDCs elaborated cytokines in response to soluble whole glucan particles (WGP-sol), which efficiently bind Dectin-1 but are incapable of activating the receptor (32). These data indicate that the exaggerated cytokine responses of A20-deficient BMDCs were specific to bona fide Dectin-1 ligands and were not a result of nonspecific activation of Dectin-1 or aberrant activation of other pattern recognition

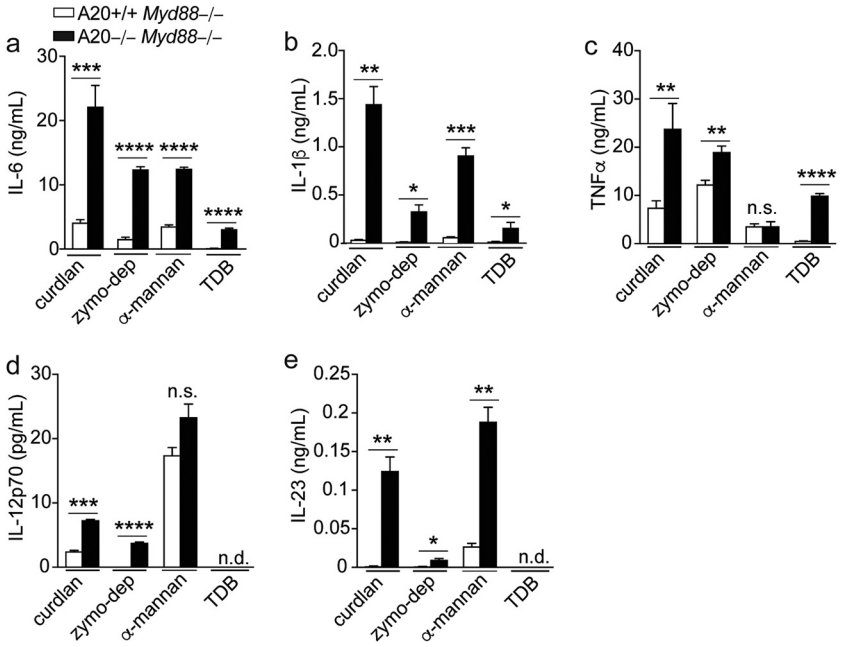

FIG 2 A20 restricts cytokine production downstream of multiple C-type lectin receptors. Myd88-deficient BMDCs, either A20 sufficient or deficient, were stimulated for 7 h with ligands to different C-type lectin receptors: curdlan (100 μg/ml), zymosan-depleted (zymo-dep; 100 μg/ml), α-mannan (300 μg/ml), or trehalose-6,6-dibehenate (TDB; 44 μg/ml). IL-6 (a), IL-1β (b), TNF-α (c), IL-12p70 (d), and IL-23 (e) in culture supernatant were quantified by ELISA. Error bars represent mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (unpaired Student's *t* test). Results are representative of at least 3 independent experiments.

receptors. Taken together, our data identify A20 as a negative regulator of cytokine responses to multiple Dectin-1 ligands.

A20 restricts cytokine responses downstream of Dectin-1, Dectin-2, and Mincle pathways. Given the known diversity among CLR signaling modules, it was important to ascertain whether A20 was also a negative regulator of Dectin-2 and Mincle which, unlike Dectin-1, signal through the Fc receptor common γ-chain (8, 12). We tested this by stimulating BMDCs with α-mannan and trehalose-6,6-dibehenate (TDB), respective ligands for Dectin-2 and Mincle receptors (4, 6). Although all ligands tested were of the highest purity possible, for these tests, we used BMDCs that were deficient in *Myd88* (derived from *Myd88*$^{-/-}$ mice [33]) to formally exclude the possible influence of TLR ligand contaminants.

Consistent with our findings on MyD88-sufficient BMDCs, IL-6 production by A20$^{-/-}$*Myd88*$^{-/-}$ BMDCs was significantly increased in response to curdlan and zymo-dep, thus excluding the possible influence of TLR ligands in our analyses (Fig. 2a). We also analyzed IL-1β and TNF-α, which we had not tested previously, and found that these cytokines were robustly produced by A20$^{-/-}$*Myd88*$^{-/-}$ BMDCs in response to curdlan and zymo-dep, with IL-1β and TNF-α production 3- to 40-fold higher than that of control *Myd88*$^{-/-}$ BMDCs (Fig. 2b and c). Importantly, A20$^{-/-}$*Myd88*$^{-/-}$ BMDCs also produced heightened amounts of IL-6 and IL-1β in response to α-mannan and TDB (increased 3- to 38-fold), indicating that A20 was also a negative regulator of Dectin-2 and Mincle cytokine responses (Fig. 2a and b). Among the inflammatory cytokines, TNF-α responses to α-mannan was the only outlier, as α-mannan-induced TNF-α production was not significantly different between A20-deficient and -sufficient *Myd88*$^{-/-}$ BMDCs (Fig. 2c). This outcome was not because FcRγ-dependent CLR-induced TNF-α was generally independent of A20 regulation since FcRγ-dependent TNF-α production in response to TDB was increased >10-fold in A20$^{-/-}$*Myd88*$^{-/-}$ BMDCs.

Among cytokines relevant to T cell stimulation, we again found cytokine-specific effects for the impact of A20 on Dectin-1 and Dectin-2 responses. Thus, while A20 restricted IL-23 production in response to both Dectin-1 and Dectin-2 ligands, IL-12p70 was exaggerated only in response to Dectin-1 ligands (Fig. 2d and e). Neither A20-sufficient or -deficient BMDCs produced IL-12p70 in response to TDB, which is consistent with the known action of Mincle signals to block the production of the IL-12p35 subunit (34). We did not detect IL-23 in response to TDB in any of our assays, possibly suggesting a reduced potency of our source of TDB ligand or differences in serum used for cell culture, compared with that of other reports. Despite the nuances for specific cytokines, taken together, our data indicate that A20 restricts cytokine production downstream of multiple CLRs, both FcRγ-dependent and -independent.

A20 terminates C-type lectin receptor signals that activate NF-κB and JNK. To investigate the mechanism by which A20 restricted cytokine responses to CLR ligands, we focused on Dectin-1 signals since these signaling cascades are conserved for Dectin-2 and Mincle. Autophosphorylation of spleen tyrosine kinase (Syk) is one of the first receptor proximal signaling events (5, 12, 35, 36), and in WT BMDCs, Syk-phosphorylation was markedly increased within 5 min of curdlan stimulation. Curdlan-induced Syk-phosphorylation was not exaggerated in A20-deficient BMDCs and actually appeared reduced (Fig. 3a) (the p-Syk:Syk ratio was increased 1.4-fold compared with 2.4-fold in WT). That A20 deficiency did not enhance Syk-phosphorylation was confirmed using two different antibodies, each recognizing two different phosphorylated tyrosine residues (Fig. 3a; see Fig. S1 in the supplemental material). We investigated signaling events downstream of Syk and found Iκbα phosphorylation was markedly increased in A20-deficient BMDCs. Thus, the WT response to curdlan induced a peak of phosphorylated Iκbα (p-Iκbα) 60 minutes poststimulation, which diminished thereafter (Fig. 3b). In contrast, phosphorylated Iκbα persisted well after 60 minutes in A20-deficient BMDCs, even up to 2 h. Correspondingly, the abundance of total Iκbα was markedly diminished in curdlan-stimulated A20-deficient BMDCs, resulting in a far greater p-Iκbα:Iκbα ratio at all time points, indicative of dramatically increased signals that lead to the activation of NF-κB. These results strongly suggest that A20 negatively regulates both the strength and duration of CLR signals that activate NF-κB.

Among CLR-induced MAP kinases (37–39) the loss of A20 modestly increased phosphorylated c-Jun N-terminal kinase (JNK), but we did not observe any significant changes to extracellular signal -regulated kinase (ERK) or p38 signals (Fig. 3c; see Fig. S2 in the supplemental material). These results indicate that A20 potently restricts Dectin-1 signals that activate NF-κB and, to a lesser extent, JNK. Accordingly, hyperactivation of these signaling cascades resulted in dramatically enhanced mRNA transcription of all cytokine genes tested (*Il1b*, *Il6*, *Tnfα*, *Il12p35*, *Il12p40*, and *Il23a*) (see Fig. S3 in the supplemental material), consistent with the increased abundance of these cytokines in the supernatant of CLR ligand-stimulated BMDCs (Fig. 1 and 2).

A20 restricts TRAF6 ubiquitination in C-type lectin receptor signaling. CLR signals require CARD9, Bcl10, and MALT1 to activate NF-κB. Although less well studied, TRAF6 is also an essential signaling component, and interestingly, TRAF6 is polyubiquitinated in the cellular response to *Candida albicans* (38). TRAF6 ubiquitination is a well-established regulatory module for multiple innate immune receptor pathways, including TLRs, and in TLR pathways, A20 regulates TRAF6 ubiquitination (18). We thus hypothesized that A20 was a negative regulator of TRAF6 ubiquitination in CLR pathways. We used curdlan to test our hypothesis since *Candida* sp. stimulates both CLRs and TLRs. We used TRAF6 antibodies to immunoprecipitate (IP) TRAF6 from curdlan-stimulated BMDCs and probed the IP by Western blot for ubiquitinated proteins. TRAF6 polyubiquitination is molecularly heterogenous and typically appears as a "smear" when detected via Western blot (18, 19, 40). Ubiquitinated proteins were detected in the TRAF6 IP from WT BMDCs, the abundance of which peaked 30 minutes after curdlan stimulation and declined thereafter (Fig. 4). These results suggest that TRAF6 is indeed polyubiquitinated in Dectin-1 signals, which is consistent with TRAF6's

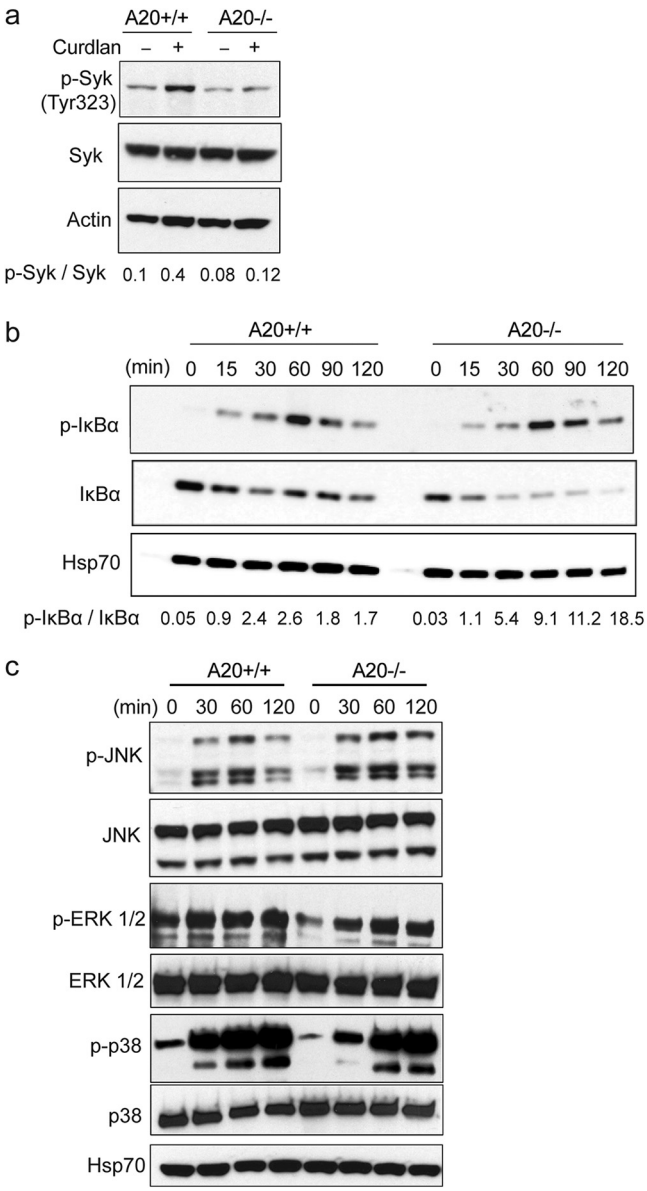

FIG 3 A20 restricts Dectin-1 signals that activate NF-κB. Wild-type and A20-deficient BMDCs were stimulated with curdlan (100 μg/ml) or left untreated. (a) Cell lysates were probed by Western blot for the abundance of phosphorylated Syk (p-Syk; Tyr323) and total Syk after 5 minutes of curdlan stimulation. Actin was used as loading control. (b) Cell lysates from curdlan-stimulated BMDCs were prepared at the indicated time and analyzed for the abundance of phosphorylated Iκbα (p-Iκbα) and total Iκbα. Hsp70 was used as loading control. (c) Curdlan-stimulated cell lysates were probed for the abundance of phosphorylated JNK (p-JNK), phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38), as well as the unmodified forms of these proteins. Hsp70 was used as a loading control. The ratio of phosphorylated to unmodified forms of these proteins is provided in Fig. S2. Results in panels a to c are representative of at least 3 independent experiments.

dynamics in response to *Candida* sp. (38). Ubiquitinated proteins were also abundant in the TRAF6 IP from A20-deficient BMDCs, and compared with the WT, ubiquitinated proteins were of increased abundance 30 minutes poststimulation and were sustained at high levels, even up to 2 hours (Fig. 4). Based on these results, we conclude that A20 regulates TRAF6 ubiquitination and perhaps also TRAF6-associated molecules in the CARD9/Bcl10/MALT1 complex to restrict and terminate NF-κB activation in CLR signals.

Heterozygous expression of A20 results in exaggerated responses to C-type lectin receptor responses in multiple primary cell types. We next set out to determine whether A20-mediated suppression of CLR signals was important in primary

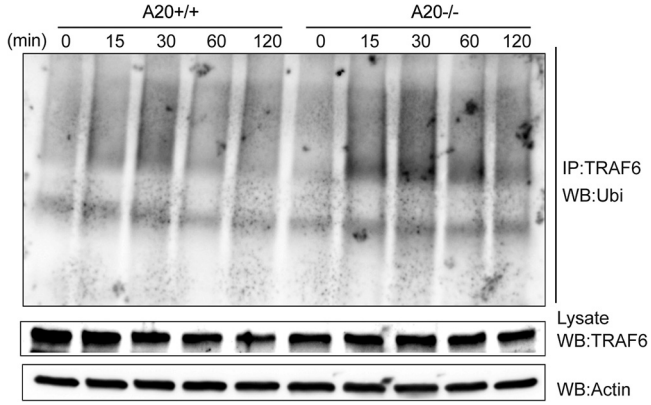

FIG 4 A20 restricts TRAF6 ubiquitination in Dectin-1 signals. Wild-type and A20-deficient BMDCs were stimulated with curdlan (100 μg/ml) for the indicated times. Cell lysates prepared using nondenaturing conditions were subjected to TRAF6 immunoprecipitation (IP) and the IP analyzed by Western blot (WB) for the abundance of ubiquitinated proteins. The abundances of TRAF6 and actin in whole-cell lysates were used as loading controls. Results are representative of at least 3 independent experiments.

cells. A20-heterozygous mice ($A20^{+/-}$) were appropriate for these tests since they express reduced levels of A20 protein; yet, unlike A20-deficient mice (19, 41), they are healthy without obvious signs of inflammation (25; unpublished observations). Heterozygous expression of A20 in BMDCs resulted in exaggerated responses to Dectin-1 ligands, thus supporting our rationale to analyze primary cells from $A20^{+/-}$ mice (see Fig. S4 in the supplemental material).

To exclude the influence of MyD88 signals on baseline phenotypes, we used primary cells from $A20^{+/-}$ mice that were also deficient in *Myd88* ($A20^{+/-}$*Myd88*$^{-/-}$ mice). Multiple types of innate immune cells naturally reside in the peritoneal cavity, and we leveraged this cellular heterogeneity to determine the requirements for A20 in each of these cell subsets (gating strategy shown in Fig. S5 in the supplemental material). In the absence of stimulation, baseline cytokine production was similar between all peritoneal cell subsets from $A20^{+/+}$*Myd88*$^{-/-}$ and $A20^{+/-}$*Myd88*$^{-/-}$ cells (Fig. S5). Stimulation with the Dectin-1 agonist curdlan induced IL-6 and TNF-α production in multiple cell types from A20-sufficient mice, including F4/80^{high} and F4/80^{low} macrophages, small peritoneal macrophages, and dendritic cells (Fig. 5a). Compared with control *Myd88*$^{-/-}$ cells, cytokine production was significantly more robust in $A20^{+/-}$*Myd88*$^{-/-}$ cells, with the percentage of cells producing either IL-6 or TNF-α increased 2- to 3-fold for all cell types tested (Fig. 5b to d). Additionally, in all subsets of peritoneal macrophages, responders producing both IL-6 and TNF-α (IL-6$^+$TNF$^+$) were significantly increased in $A20^{+/-}$*Myd88*$^{-/-}$ mice (Fig. 5e). Heterozygous expression of A20 also resulted in exaggerated IL-6 and IL-6$^+$TNF$^+$ responses of F4/80^{high} and F4/80^{low} macrophages to α-mannan, thus highlighting key roles for A20 to restrict both Dectin-1 and Dectin-2 responses of these two macrophage subsets (Fig. 5f to h). Dectin-2 responses of $A20^{+/-}$*Myd88*$^{-/-}$ small peritoneal macrophages and dendritic cells trended toward increased cytokine production, but these responses did not reach significance. We also analyzed neutrophils, which we purified from bone marrow. Reactive oxygen species, degranulation, and cytokine responses of $A20^{+/-}$*Myd88*$^{-/-}$ neutrophils were similar to controls, suggesting that heterozygous expression of A20 had a negligible impact on CLR responses of neutrophils (see Fig. S6 in the supplemental material). Taken together, our data show that in select subsets of primary cells, A20's mode of suppression on CLR responses is compromised by the heterozygous expression of A20.

A20 is a suppressor of immunity to systemic *Candida albicans* infection. We next tested the impact of A20's suppression on host defense against the fungal pathogen *Candida albicans*. Since fungal defense against *Candida* sp. involves both CLRs (6, 42, 43) and MyD88 signals (44–48), we used *Myd88*$^{-/-}$ mice to highlight CLR-mediated immunity. The loss of A20 indeed exaggerated *Candida* sp.-induced IL-6

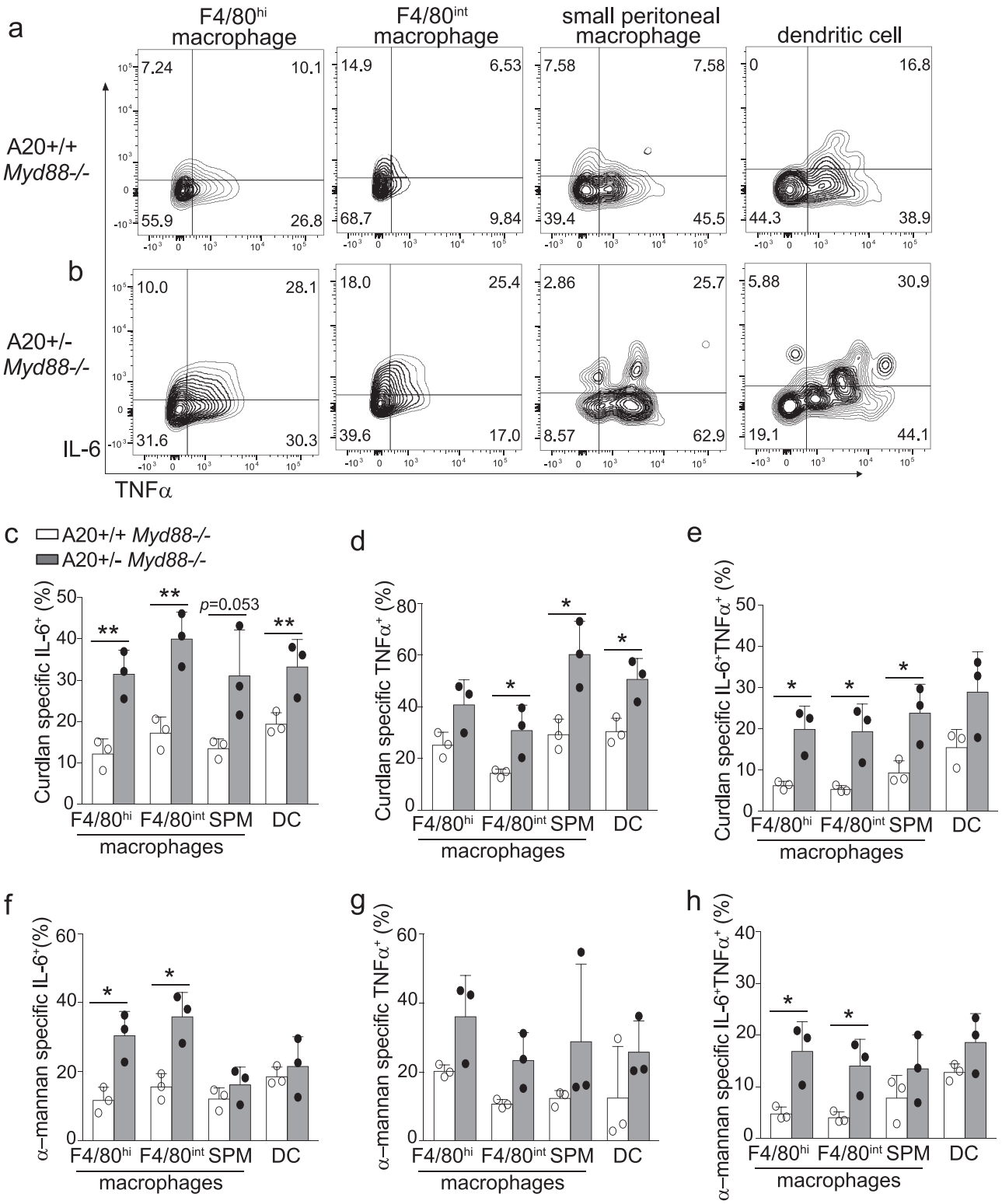

FIG 5 In multiple cell types, the heterozygous expression of A20 leads to exaggerated CLR-induced cytokine responses. Cells from the peritoneal cavity of *Myd88*$^{-/-}$ mice, either A20 heterozygous or wild-type, were stimulated for 4 hours with Dectin-1 ligand curdlan (25 μg/ml) or Dectin-2 ligand α-mannan (300 μg/ml) and analyzed by flow cytometry. Representative flow plots of IL-6 and TNF-α production from curdlan-stimulated wild type (a) or A20 heterozygous (b) *Myd88*$^{-/-}$ peritoneal cells of the indicated subset. The percentage of each cell type producing IL-6 (c), TNF-α (d), or both cytokines (e) in response to curdlan stimulation. Each dot represents one mouse. For each mouse, cytokine production in unstimulated cultures was used to determine the curdlan specific response. (f to h) Cytokine production in response to α-mannan was analyzed as in panels c to e. Error bars represent mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$ (unpaired Student's *t* test). Results are representative of 3 independent experiments, each including 2 to 3 mice of each genotype.

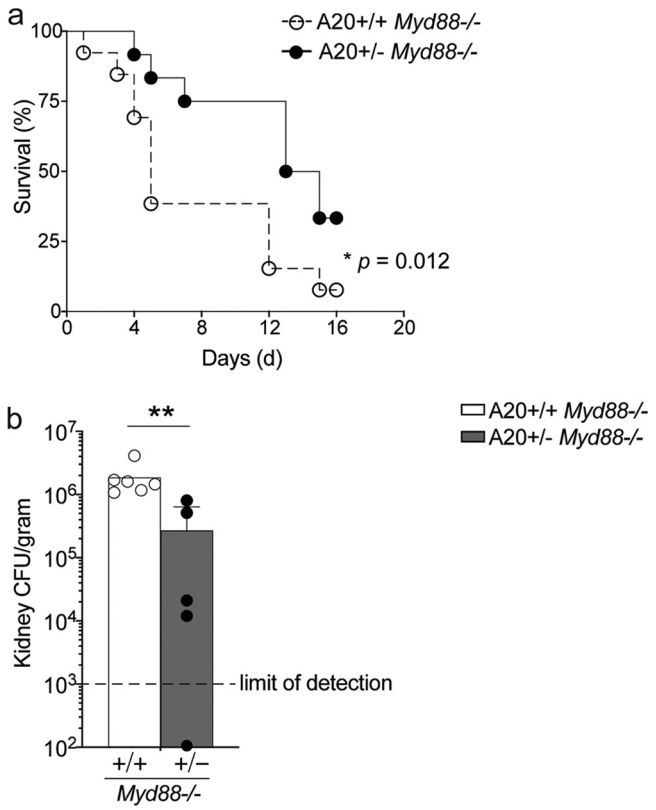

FIG 6 Heterozygous expression of A20 restricts *Candida albicans* growth *in vivo* and is protective against systemic *Candida albicans* infection. *Myd88*$^{-/-}$ mice, either A20 heterozygous or wild type, were infected intravenously with 2×10^6 *C. albicans* spores. (a) Survival was evaluated over a period of 16 days. Data are combined from two separate experiments, each with at least 6 mice of each genotype. Log-rank test; *, $P < 0.012$. (b) Separate cohorts of mice were analyzed for the abundance of *C. albicans* CFUs in kidneys on day 3 postinfection. Each dot represents one mouse. Error bars represent mean ± SD. Samples where CFUs were below the limit of detection were excluded from statistical analysis. **, $P < 0.01$ (unpaired Student's *t* test). Results are representative of 2 independent experiments, each including at least 5 mice of each genotype.

and IL-6$^+$TNF$^+$ responses of MyD88-deficient BMDCs (see Fig. S7 in the supplemental material), and we thus used A20$^{+/-}$*Myd88*$^{-/-}$ mice to test the impact on systemic infection with *C. albicans*. A20$^{+/+}$*Myd88*$^{-/-}$ mice exhibited severe mortality due to *Candida* infection, with >50% of mice succumbing to the infection within 5 days (Fig. 6a). In comparison, the survival of A20$^{+/-}$*Myd88*$^{-/-}$ mice at this same time point was significantly improved, with a mortality rate of only 20%. Improved survival at these early time points correlated with reduced fungal load in kidneys, the organ where *Candida* sp. typically undergoes its most robust expansion (Fig. 6b). Kidney failure is thought to be the underlying cause of death due to systemic *Candida* infection, and considering that relief of A20 suppression resulted in decreased fungal burden in the kidney at early time points, it is worth noting that the survival of A20$^{+/-}$*Myd88*$^{-/-}$ mice was improved even at late stages of infection, with 50% of mice surviving even until day 12, a time point at which most A20-sufficient mice had already succumbed to the infection. These results demonstrate the potent influence of A20 on CLR responses *in vivo* and identify A20 as a suppressor of host defense to systemic fungal infection.

DISCUSSION

CLRs, including Dectin-1, Dectin-2, and Mincle, have well-established roles in host defense against fungal and mycobacterial infections (1–6). In the current study, we identify A20 as a potent negative regulator of acute ligand-induced signals triggered by CLRs, both FcRγ dependent and independent. Furthermore, we show that A20 regulates CLR responses in several different types of innate immune cells. Ultimately, A20's

regulation of these pathways has a suppressive effect on antifungal immunity. Our results establish A20 as a broad regulator of several different CLR responses and suggest that modulation of A20's mode of suppression could improve the outcomes of fungal and mycobacterial infection.

A20 is a negative regulator of NF-κB that suppresses multiple signaling pathways in innate and adaptive cells, as well as pathways triggered in nonhematopoietic cells (18–22, 49–51). Like immune cells, nonhematopoietic cells also respond to CLR ligands (52), raising the possibility that A20's mode of suppression could play important roles in regulating the CLR responses of many different cell types. Mechanistically, A20's role in restricting CLR-induced NF-κB acts downstream of Syk since the absence of A20 did not increase CLR-induced Syk phosphorylation. In fact, acute Syk phosphorylation was reduced in the absence of A20, although the explanation underpinning this outcome is currently unclear. Given the robust levels of p-Iκbα in A20-deficient BMDCs, it would appear that exaggeration of the p-Iκbα component of the CLR signaling cascade is more than sufficient to compensate for the reduced levels of acute Syk-phosphorylation. A20 restricted CLR-induced NF-κB by controlling the amplitude and duration of TRAF6 ubiquitination, which was significantly amplified in the absence of A20. In addition to TRAF6 (38), CARD9 has recently been shown to be ubiquitinated in response to CLR ligands (53), and our tests here do not exclude the possibility that A20 regulates both TRAF6 and CARD9 ubiquitination. However, CLR signals do indeed induce direct ubiquitination of TRAF6 (38), and since TRAF6 is a well-established target of A20 when activated in TLR pathways (18, 19), evidence suggests that TRAF6 is also a direct target for A20 suppression in CLR pathways. Whether or not A20 has multiple targets in the CARD9/Bcl10/MALT1 complex, it is clear that the absence of A20 regulation results in robust NF-κB activation and hyperresponsiveness to CLR ligands.

It is important to note that relief of A20 suppression did not interfere with the mode of action of known antagonists to CLR signaling, which is an important consideration for the strategic design of approaches that modulate A20 during vaccination or immunity. Thus, the Dectin-1 antagonist WGP-soluble, which binds Dectin-1 but does not trigger signals (32), did not induce cytokine responses in A20-deficient BMDCs. Similarly, Mincle signals that interfere with IL-12p70 production (34) were intact in A20-deficient BMDCs. Thus, our data suggest that relief of A20 suppression does not increase the "ligand potential" for CLRs, nor redirect CLR signals to exclude the influence of antagonists. Rather, relief from A20 suppression amplifies the normal responses downstream of bona fide CLR ligands.

Using primary cells which were haplosufficient for A20, we showed that A20's mode of suppression on CLR responses is active in many primary cell types. Individuals with A20 haploinsufficiency (a result of loss-of-function mutations in *Tnfaip3*) develop autoinflammatory disease, and our findings here suggest that dysregulated CLR responses may contribute to these disease conditions (24). In the context of pathogen immunity, different cell types express different baseline levels of A20, and it is thus interesting to note that specific macrophage subsets with naturally high A20 expression must first reduce A20 protein levels by autophagy in order to secrete chemoattractants in response to fungal pathogens (27). High levels of macrophage-expressed A20 is also linked to an increased rate of expansion of mycobacterium within infected macrophages, suggesting that decreased A20 expression improves immunity to mycobacterial infection (54). These results, together with our results showing that decreased A20 expression improves antifungal immunity, suggest that in settings where CLRs play a major role, A20 is a suppressor of host-protective responses to pathogen infection. That decreased expression of A20 exaggerates the cellular response to CLR ligands has implications beyond pathogen immunity since A20's ability to restrict CLRs likely also impacts autoimmune, inflammatory, and malignant diseases which are linked to reduced A20 function (25, 26). Indeed, there is a growing body of literature supporting a role for CLRs in autoimmunity, allergy, inflammation, and malignancy, and even obesity (55–61). Given the potent influence of A20 on CLR responses, future

studies are warranted to investigate whether A20 expression is linked to the protection or development of these conditions, as they are driven in a CLR-dependent fashion.

Finally, our findings that A20 restricts responses to FcRγ-dependent CLRs raises the possibility that A20 also suppresses other FcRγ-dependent responses, including those mediated by IgG-opsonized particles. In this way, A20's mode of suppression could be intimately linked to the action of the almost unlimited diversity of IgG antibodies, in which the FcRγ-mediated component plays a major role in pathogen and vaccine-induced immunity. In summary, our discovery here that A20 regulates FcRγ-dependent and -independent CLRs expands our knowledge of A20's axis of influence over immunity, health, and disease.

MATERIALS AND METHODS

Mice. A20-heterozygous mice (generated from A20-floxed mice described in reference 29) were bred together to generate fetal livers of the following genotypes: A20$^{+/+}$, A20$^{+/-}$, A20$^{-/-}$, A20$^{+/+}$*Myd88*$^{-/-}$, or A20$^{+/-}$*Myd88*$^{-/-}$. *Myd88*$^{-/-}$ mice were purchased from The Jackson Laboratory (Jax stock number 009088) (33). For experiments with A20$^{+/+}$*Myd88*$^{-/-}$ and A20$^{+/-}$*Myd88*$^{-/-}$ mice (neither of which develop inflammation) or primary cells from these mice, cohoused littermates were used directly as a source of peritoneal cavity cells and for *Candida albicans* infection. The routine screening of animals in maximum barrier facilities was used to confirm exclusion of several pathogens, including *Helicobacter* sp., *Pasteurella* sp., and murine norovirus. Animals were used according to protocols approved by Duke University Institutional Animal Care and Use Committee.

Bone marrow-derived DCs. All bone marrow was harvested from fetal liver hematopoietic chimera. Fetal livers were collected at day 14 of gestation, and fetal liver cells were used to reconstitute CD45.1 C57BL/6 mice that were irradiated with 2 doses of 600 cGy (X-Rad 320), with 3-h rest between doses. Bone marrow from chimera mice was harvested at 4 weeks after reconstitution and treated with CD45.1 antibody:bead conjugates (Stem Cell) to deplete residual host cells. The resulting bone marrow was cultured in complete RPMI 1640 and 10% fetal calf serum, supplemented with GMCSF supernatant to generate BMDCs. Fresh medium was replaced every other day. BMDCs were harvested on day 7 of culture and enriched using CD11c-positive enrichment kits (Stem Cell) before stimulation.

C-type lectin receptor ligand stimulation of BMDCs and primary cells. CLR ligands curdlan, WGP-dispersible, WGP-soluble, zymosan, zymosan-depleted (zymo-dep), α-mannan, and trehalose-6,6-dibehenate (TDB) were purchased from Invivogen and resuspended or reconstituted following the manufacturer's protocol. BMDCs were plated at a density of 1×10^6 cells/ml for stimulation with CLR ligands. For ELISA analysis, cells were incubated with CLR ligands for 7 h. Cytokines in culture supernatants were analyzed by ELISA kits purchased from BioLegend. Peritoneal cavity cells harvested by peritoneal washing were plated at a density of 1.5×10^6/ml in 24-well dishes and incubated for 4 hours with or without CLR ligands in media containing 10% fetal calf serum and 1$\times$ Golgiplug (BD Biosciences). Cells were then stained with a live/dead fixable reagent (Thermo), and the following antibodies from BioLegend were used: CD11b, Ly6C, IA/E, CD88, CD24, and F4/80. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and then stained with antibodies to IL-6 and TNF-α (BioLegend). Neutrophils isolated from bone marrow were enriched by negative selection (Stem Cell) and were routinely at $>$80% Ly6G$^+$. Enriched neutrophils were plated at a density of 2×10^5/well in 96-well U-well dishes and stimulated with 0.25 μg/ml zymo-dep. All neutrophil assays were performed as previously described (62). Cytokine production after a 4-h stimulation was analyzed as described above. Separate cultures were evaluated for degranulation, which was measured by the upregulated cell surface expression of CD11b at 30 min poststimulation. To assess the production of reactive oxygen species, neutrophils were cultured in Hanks balance salt solution (HBSS), 20 mM HEPES, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ and preloaded with 3 μM dichlorodihydrofluorescein diacetate (DCFDA; Abcam) prior to stimulation. DCFDA fluorescence was assessed at 30 minutes poststimulation. For all assays, mock-treated cells were analyzed in parallel. This background response was subtracted or divided, as appropriate, from the response to stimulus, and data were reported as the zymo-dep-specific response. Flow cytometry was performed on a BD Canto instrument and analyzed with FlowJo software.

Western blot and immunoprecipitation. For Western blot, cells were lysed in 2$\times$ Laemmli buffer (Bio-Rad) supplemented with 1$\times$ proteinase inhibitor cocktail (Roche), 50 mM sodium fluoride, and 200 μM sodium orthovanadate. Lysates were boiled at 95°C for 10 min and separated in a 4% to 20% SDS-PAGE gel (Bio-Rad). Semidry transfer was performed onto a nitrocellulose membrane. The following antibodies were used for Western blot: anti-IκBα (rabbit polyclonal; Cell Signaling), anti-phospho-IκBα (5A5), anti-Syk (D3Z1E), anti-phospho-Syk (Y323, rabbit polyclonal; Cell Signaling), anti-phospho-Syk (Y352, rabbit polyclonal; Cell Signaling), anti-TRAF6 (H274), anti-ubiquitin (P4D1, horseradish peroxidase [HRP] conjugated), anti-A20 (A12), anti-p38 MAPK (D13E1), anti-phospho-p38 mitogen-activated protein kinase [MAPK] (D3F9), anti-ERK1/2 (137F5), anti-phospho-ERK1/2 (D13.14.4E), anti-JNK (56G8), anti-phospho-JNK (rabbit polyclonal; Cell Signaling), anti-actin (I-19), anti-rabbit IgG-HRP (L27A9), anti-mouse IgG-HRP (horse polyclonal; Cell Signaling), and anti-rabbit IgG-HRP (goat polyclonal; Cell Signaling). Bands were visualized using the SuperSignal West Pico kit (Thermo) on autoradiography film. Membranes were stripped in Restore Western stripping buffer (Thermo). For immunoprecipitation of TRAF6, cells were lysed in buffer containing 1% NP-40, 50 mM Tris-HCl, 150 mM NaCl, 20 mM *N*-ethylmaleimide, 50 mM sodium fluoride, 200 μM sodium orthovanadate, 2 mM EDTA, and 1$\times$ proteinase inhibitor cocktail (Roche). Lysates were sonicated twice for 5 s. IP was carried out overnight with protein G magnetic beads

(Thermo). Beads were washed 3 times with lysis buffer containing 1% sodium deoxycholate, 20 mM *N*-ethylmaleimide, 1% NP-40, 100 mM TrisHCl, and 1× proteinase inhibitor cocktail (Roche). At each wash, beads were incubated with lysis buffer with constant rotation for 2 min. IP products were eluted in 3× SDS buffer and boiled at 95°C for 10 min. Elutes were separated on a 4% to 20% SDS-PAGE gel (Bio-Rad) and transferred overnight in 1× Towbin buffer supplemented with 15% methanol. Bands were visualized using the SuperSignal West Femto kit (Thermo) on the ChemiDoc imaging system (Bio-Rad).

Quantitative PCR. CLR ligand-stimulated BMDCs were lysed in TRIzol (Thermo) and RNA prepared according to the manufacturer's instructions. cDNA was prepared using QuantiTect reverse transcription kit (Qiagen). A total of 30 ng cDNA was used for each qPCR using the following TaqMan probe sets (Invitrogen): *il6* (assay identifier, Mm00446190_m1), *tnfa* (Mm00443258_m1), *il23a* (Mm00518984_m1), *il12a* (Mm00434165_m1), *il12b* (Mm00434174_m1), and *il1b* (Mm00434228_m1). qPCRs were performed on a Roche Lightcycler.

***Candida albicans* infection and stimulation *in vitro*.** *Candida albicans* (ATCC 18804) infection was performed as described previously (27). Briefly sex-matched, cohoused littermate A20$^{+/+}$*Myd88*$^{-/-}$ and A20$^{+/-}$*Myd88*$^{-/-}$ mice of age 6 to 8 weeks were infected by intravenous (i.v.) injection of 2×10^5 *C. albicans* spores. Body weights, survival, and humane endpoints were evaluated every 24 h, as approved by the mouse protocol. *Candida* CFUs were counted from tissue lysates prepared from kidneys of live mice. Lysates were first treated with H_2O to lyse host cells (*C. albicans* does not lyse in this H_2O treatment) and then incubated overnight on yeast extract-peptone-dextrose (YPD) agar plates. *C. albicans* used to test *in vitro* responses was first heat killed (95°C, 1 h) and incubated at a ratio of 5:1 with BMDCs. Cytokine production was analyzed by flow cytometry, as described above. Mock-treated cells were analyzed in tandem to subtract background responses. Data are reported as the *C. albicans*-specific cytokine response.

Statistical analysis. Student's *t* test (GraphPad Prism) was used for statistical analysis. Survival was analyzed by the log-rank test (GraphPad Prism).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

SUPPLEMENTAL FILE 2, PDF file, 3.4 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 7, PDF file, 0.7 MB.

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