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Malassezia spp. induce inflammatory cytokines and activate NLRP3 inflammasomes in phagocytes

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Abstract

Malassezia spp. are common eukaryotic yeasts that colonize mammalian skin. Recently, we and others have observed that *Malassezia globosa* and *Malassezia restricta* can be found in the intestines in the context of certain diseases, including Crohn's disease and pancreatic cancer. In order to better understand the nature of innate inflammatory responses to these yeasts, we have evaluated inflammatory responses induced by *M. restricta* and *M. globosa* in mouse bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). While *Malassezia* yeasts induce proinflammatory cytokine production from both macrophages and dendritic cells, the levels of production from BMDC were more pronounced. Both *M. restricta* and *M. globosa* activated inflammatory cytokine production from BMDC in large part through Dectin2 and CARD9 signaling, although additional receptors appear to be involved in phagocytosis and activation of reactive oxygen production in response to the yeasts. Both *M. restricta* and *M. globosa* stimulate production of pro-IL-1β as well as activation of the NLRP3 inflammasome. NLRP3 inflammasome activation by *Malassezia* fungi requires SYK signaling, potassium efflux and actin rearrangement. Together, the data further our understanding of the coordinated involvement of multiple innate immune receptors in recognizing *Malassezia globosa* and *Malassezia restricta* and orchestrating phagocyte inflammatory and antimicrobial responses.

Summary sentence

Malassezia restricta and *Malassezia globosa* stimulate production of cytokines and chemokines from phagocytes and activate the NLRP3 inflammasome via multiple receptors and signaling pathways.

Keywords

phagocytosis; reactive oxygen species; IL-1β; yeast; innate immunity

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

INTRODUCTION

The gastrointestinal tract is a symbiotic environment established by the interactions between mammalian intestinal epithelial and immune cells, and the microbiota.[1,2] Intestinal microbes are essential for health of the gut and development of the immune system. Also, changes in the gut microbiota have been associated with diverse diseases including inflammatory bowel disease (IBD). While most studies have focused on defining normal and disease-associated bacterial members of the gut microbiota, there is an increasing appreciation that other microbes, including fungi, may also play important roles in health and disease.[3–5]

We initially reported finding diverse fungal species in fecal material of mice and humans and finding that immunity to intestinal fungi is important in models of intestinal inflammation.[6] More recently, we examined intestinal mucosa-associated fungal populations in patients with Crohn's disease and healthy controls.[7] We observed a strong association of *Malassezia* spp. (*M. restricta* & *M. globosa*) with Crohn's disease. Subsequently, Miller and coworkers observed a remarkable appearance of gut-derived *Malassezia* in pancreatic tumors[8], further supporting the notion that *Malassezia* is associated with intestinal dysbiosis-driven diseases. In Crohn's disease, we found that increases in intestinal *Malassezia* were especially prevalent in patients carrying a previously-characterized single nucleotide polymorphism in *CARD9* that confers increased risk of developing Crohn's disease.[9–11] CARD9, a signaling adaptor protein connecting C-type lectin receptor signaling with activation of NF-κB, is essential for host defense against fungi, and the disease-associated change is a nonsynonymous polymorphism in exon 2 of *CARD9* leading to production of a CARD9^{S12N} variant of the protein[12]. Together, these data led us to hypothesize that *Malassezia* might exacerbate intestinal inflammation in a CARD9-dependent manner, and we observed this to be true in the mouse acute DSS model of colitis.

Malassezia spp. are members of the Basidiomycota phyla of fungi and, while all other members of the Ustilaginomycotina subdivision are plant pathogens, *Malassezia* are commensal skin microbes found on nearly all warm-blooded animals. In humans, *Malassezia* is known to cause folliculitis and dandruff.[13,14] An important feature of *Malassezia* genomes is the loss of key enzymes required for lipid metabolism, including fatty acid synthase, Δ^9desaturase, and $\Delta^{2,3}$enoyl CoA isomerase.[15] Therefore, they cannot produce fatty acids themselves and need lipids from the environment for growth. In the skin, they harvest lipids from sebum in hair follicles through secretion of a host of lipases and phospholipases. These enzymes can release unsaturated free fatty acids from sebum lipids including oleic acid and arachidonic acid that can be inflammatory.[16] That *Malassezia* spp. might be important in intestinal inflammation is unexpected.

Due to the prevalence of *Malassezia* spp. on skin and its association with skin disorders, most of our limited understanding of how immune cells respond to *Malassezia* is focused on skin responses and, for technical and historical reasons, mostly on species other than *M. globosa* and *M. restricta*.[17–20] To extend this work and focus on gut-associated species and systemic immune cells, we have here systematically characterized inflammatory responses induced by *M. restricta* and *M. globosa* in bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). While *Malassezia* yeasts induce proinflammatory cytokine

production from both macrophages and dendritic cells, the levels of production from BMDC were more pronounced. Both *M. restricta* and *M. globosa* activated inflammatory cytokine production from BMDC primarily through Dectin2 and CARD9 signaling, however, the data indicate there may be some redundancy in the recognition with other innate immune receptors. Both *M. restricta* and *M. globosa* were able to stimulate production of pro-IL-1β as well as activate the NLRP3 inflammasome in BMDM and BMDC. NLRP3 inflammasome activation by *Malassezia* fungi requires SYK signaling, potassium efflux and actin rearrangement. From these data we are able to better understand the inflammatory properties of these gut-associated *Malassezia* spp.

MATERIALS AND METHODS

Fungal and Bacterial Growth

All experiments using *S. aureus* were preformed using mutant *S. aureus* (ΔoatA-SA) kindly provided by Fiedrich Götz.[21,22] *S. aureus* was grown in THB media overnight at 37°C with agitation. *S. aureus* was then subcultured in THB at a dilution of 1 to 100 for 2.5 hours at 37°C with agitation until the culture reaches log phase growth. Bacteria were washed and diluted to OD_{600}=0.4, determined to be 6.7×10^7cfu/ml. *M. restricta* (ATCC MYA-4611) and *M. globosa* (ATCC MYA-4612) were grown under static conditions for 2–3 days at 30ºC in modified Dixon media containing glycerol monostearate. *C. albicans* (ATCC 90028) was grown overnight under static conditions at 30ºC in Sabouraud dextran broth. Live yeasts were harvested and washed 3 times in media before counting. Fixed yeasts were harvested, washed 3 times with sterile PBS and fixed in 2% paraformaldehyde for 1 hour at room temperature. Cells were then washed 6 times with media and counted on a hemocytometer.

Mice and Cell culture

Dectin1$^{-/-}$, Dectin2$^{-/-}$, and CARD9$^{-/-}$ mice were bred and housed under SPF conditions in the Cedar-Sinai Medical Center animal facility and handled in accordance with approved IACUC procedures. C57BL/6 and MyD88$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Bone marrow derived macrophages (BMDM) and dendritic cells (BMDC) were generated from femurs and tibias by culturing for 7–9 days in complete RPMI 1640 (5 mM glucose, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine; Mediatech) supplemented with 50 ng/ml human recombinant M-CSF or mouse recombinant GM-CSF (Peprotech). BMDM and BMDC were plated the day before experiments 1×10^5/well on 96-well plate, 4×10^5/well on 24-well plate, or 2.5×10^5/well 48-well plate. For inflammasome experiments cells were primed with 100 ng/ml LPS (*S. minnesota*, Invivogen) for 4 hr. Then stimulated with ATP 5 mM (Sigma), 1 μg/ml pdA:dT (Invivogen) was complexed with Lipofectamine 2000 (ThermoFisher) for 30 min at room temperature and 10ul was added per well (96-well plate). Fungi were washed with PBS and then hand counted to determine the fungi/ml. Infections were done by adding indicated MOIs of bacteria or fungi onto cells and spinning at 450xg for 1 min. The *S. aureus* infection was allowed to progress for 30 min, then 100 μg/ml gentamycin was added to the media to limit growth of extracellular bacteria, supernatants were harvested at designated times, cytokine levels were measured by IL-1β, TNFα, and IL-6 ELISA (Biolegend) or Mouse Macrophage/Microglia 13-plex LegendPlex (Biolegend) (limit of

detection according to the assay instructions. The limit of detection for all ELISAs is 15.625 pg/ml. The limit of detection for the LegendPlex assay is 2.44 pg/ml.

Immunoblotting

At designated timepoints, cells were lysed in 1x Nupage SDS loading buffer (ThermoFisher) or cells were stimulated in Optimem serum-free media and supernatants were harvested. Supernatants were concentrated using 5 μl of Strataclean resin (Agilent Technologies) per 500 μl of supernatant the agitated at low speed for 30 min at room temperature, resin was pelleted at 2500xg for 2 min, then boiled in 20 μl 1x Nupage SDS loading buffer. Samples were run on Nupage precast gels in MOPS running buffer and transferred to PVDF. Membrane was blocked in Odyssey blocking buffer (Li-Cor) 1–2 hr at room temperature. Blots were stained with primary antibodies overnight at 4°C. Antibodies: 1:1000 anti-IL-1β (AF-401-NA, R&D), 1:1000 anti-NLRP3 (Cryo-2, Adipogene), 1:500 anti-caspase-1 p10 (M-20,Santa Cruz), 1:2000 anti-tubulin (TUB2.1, Sigma).

Phagocytosis assay

BMDCs were plated the day before at 4×10^5/well on 24-well non-tissue culture treated plates (Falcon). Live yeasts (400×10^6/ml) were labelled with cell proliferation dye eFluor™ 670 (eBioscience) at 5 μM final concentration in PBS for 10 minutes at 37ºC followed by washing 4 times in media. Labelled yeasts were added to BMDC at a MOI of 5 and spun down onto cells at 450xg for 1 min and then incubated at 37°C for 5, 15, or 30 minutes. After the incubation time, wells were washed 3 times with PBS and BMDCs were lifted from the wells using PBS with Ca^{2+} containing proteinase K (1:100) (Sigma P5568). Cells were stained with FITC-labeled anti-CD45 (1:200) (Biolegend) for 30min on ice. Cells were then fixed in 2% paraformaldehyde at RT for 20 minutes washed, resuspended in FACS buffer and analyzed on a BD LSR2 flow cytometer (BD). Flow cytometry analysis was performed using FlowJo software (TreeStar).

Reactive oxygen assay

BMDC were plated in opaque white NUNC 96-well plates at 5×10^4/well. Cells were stimulated with live fungi at an MOI of 5 in the presence of 100μM luminol (Sigma). Luminescence was measured for 5 seconds every 9 minutes for 20 cycles on a Clariostar plate reader.

Statistics

All statistics are done by Student t-test with triplicated stimulated wells of cells* $p\leq.05$, ** $p\leq0.01$,*** $p\leq0.001$. All experiments were done a minimum of three independent times unless stated in the figure legend.

RESULTS

Gut-associated Malassezia species induce proinflammatory cytokine products from macrophages and dendritic cells

In our previous work we found increased burdens of *Malassezia restricta* and *Malassezia globosa* in the intestinal mucosa of patients with Crohn's disease, and we noted in vitro that fixed *M. restricta* could induce TNFα and IL-6 from mouse BMDM and BMDC.[7] Here we have extended these observations to evaluate responses to live *M. restricta* and *M. globosa* yeasts. When we stimulated BMDM or BMDC with *M. restricta* or *M. globosa* compared to *Candida albicans* we find that all the fungal species induced production TNFα in a time dependent manner (Fig. 1A). To more thoroughly characterize the inflammatory responses, we performed LegendPlex multiplex cytokine and chemokine analysis (Biolegend) of supernatants from cells stimulated with live fungi. *Malassezia* species consistently induced TNFα and CXCL1 (KC) from both macrophages and dendritic cells (Fig. 1B). We only detected measurable increase in production of IL-12p40, IL-6 and IL-1β in BMDC (Fig. 1B). We did not detect production of TGF-β1, IL-18, or IL-12p70. Together the data show that macrophages and dendritic cells encountering live *M. restricta* and *M. globosa* mount individual proinflammatory responses.

Inflammatory responses to live Malassezia restricta and globosa is predominantly dependent on Dectin2 and CARD9 signaling

Our initial examination of the inflammatory responses to *M. restricta* and *M. globosa* showed that inflammatory cytokine production was more prominent in dendritic cells than in macrophages. Because of this, and together with the fact that dendritic cell subsets have been shown to be vital for sensing and inflammatory signaling in response to fungi in the gut[23,24], we focused our subsequent experiments on dendritic cell responses.

To determine which fungal receptors might contribute to the inflammatory response to *Malassezia*, we infected BMDC deficient for the C-type lectin receptors Dectin1 or Dectin2 or the signaling molecule CARD9 with live *M. restricta*, *M. globosa*, or *C. albicans*. Following exposure to live *M. restricta* we found that TNFα, IL-6, IL-12p40 and CXCL1 were only partially dependent on Dectin1 signaling but were strongly regulated by both Dectin2 and CARD9 (Fig. 2A). In addition, we also found that Dectin2 and Card9 were predominantly responsible for G-CSF and IL-10 production (Supplemental material Fig. 1A).

Dectin-1 is known to trigger phagocytosis and activation of reactive oxygen production, so we tested whether these receptors might be involved in these responses to *Malassezia*. We observed only the mildest effect on efficiency of phagocytosis of *M. restricta* or *M. globosa* in Dectin2$^{-/-}$ cells, but no observable impact in the absence of Dectin1 or CARD9 in dendritic cells (Fig. 2B and Supplemental Material Fig. 1B). We also observed that both *M. restricta* or *M. globosa* trigger production of reactive oxygen species by BMDC and found CARD9 to be dispensable for this response in dendritic cells (Fig. 2C and Supplemental Material Fig. 1C). However, we did see a consistent decrease in ROS production in Dectin2$^{-/-}$ BMDC. The importance of Dectin1 was less clear as it varied between

experiments but looks to play the mildest role in *M. restricta* and *M. globosa* ROS production. In contrast, it is clearly important for detection of depleted-zymosan, a predominantly β-glucan particle (Fig. 2C and Supplemental Material Fig. 1C). Together these data indicate that multiple receptors contribute to the varied responses of dendritic cells to *M. restricta* and *M. globosa* and that additional dominant phagocytic receptor(s) have yet to be identified.

Live *Malassezia* fungi induce cytokine production that is not dependent on TLR signaling and induce significantly less cell death than *C. albicans*

While C-type lectins are the dominant phagocytic receptors for the detection of fungi, inflammatory responses often include signaling through Toll-like receptors (TLR). To further characterize the receptors involved in responses to live *Malassezia* we infected BMDC from mice deficient for myeloid differentiation primary response 88 (MyD88), an adaptor molecule that is essential for all but intracellular TLR4 and TLR3 signaling[25]. To our surprise TLR signaling through MyD88 plays little role in the detection of *Malassezia,* at least for the production of TNFα and IL-6 (Fig. 3A–B). It is possible that MyD88-TLR signaling is important for specific cytokines, and this will need to be addressed in future work. We also assessed if *Malassezia* is toxic to BMDC. It is well known that *C. albicans* in cell culture media shifts to a hyphal form of growth that can disrupt cell integrity after phagocytosis. While most *Malassezia* spp. can form hyphae, this is kind of growth is rare, and we did not see any visual evidence of hyphal formation in our cell culture experiments. However, after infection for 24 hours, cell death, as measured by LDH release, was elevated in *Malassezia*-treated BMDC compared to untreated (UT) or LPS-treated BMDC (Fig. 3C). The cell death induced by *Malassezia* was significantly less than that caused by infection with *C. albicans*, which consistently induced 100% cell death by 24 hours (Fig. 3C). These results help to clarify dynamics of the myeloid cell responses to *Malassezia* infection.

Live Malassezia fungi induce a more potent IL-6 and IL-1β inflammatory response than killed Malassezia fungi

In our previous work observed that formaldehyde fixed *Malassezia* induced inflammatory cytokines from macrophages and dendritic cells suggesting that yeast do not need to be alive to stimulate responses.[7] However, to directly assess the contribution of yeast viability to innate immune sensing, we compared inflammatory cytokine production by BMDC and BMDM stimulated with live or fixed *M. restricta* or *M. globosa*. We found that live and dead yeasts stimulated similar production of TNFα from dendritic cells (Fig. 4A). However, IL-6 production is reduced when *M. restricta* and *M. globosa* are fixed (Fig. 4B). In contrast, TNFα production in response to C. albicans was reduced by more than 60% when the fungi were fixed but IL-6 was slightly reduced by fixation (Fig. 4A–B). These data suggest that the availability of ligands may be different in live compared to dead fungi as well as between different fungal species, which may contribute to the unique inflammatory responses elicited by different fungi.

Production of IL-1β, however, was strongly influenced by yeast viability. During our multiplex cytokine experiments we observed small amounts of IL-1β production in the presence of *Malassezia* (Fig. 1B), so we performed a time course experiment in unprimed

BMDC to extend the results. Compared to live *C. albicans*, which was a potent inducer of IL-1β, live *M. globosa* stimulated low levels of IL-1β secretion by unprimed BMDC (Fig. 4C), and *M. restricta* induced little or no IL-1β. In contrast, dead (fixed) *M. globosa* failed entirely to induce IL-1β production (Fig. 4D). *S. aureus*, used as a control in this experiment, does not need to be live to induce IL-1β secretion.

*IL-1*β production is important for induction and maintenance of Th17 T cells, which are important in colitis, and we had previously observed elevation of Th17 T cell responses in the intestines of mice gavaged with *Malassezia* prior to DSS-induced colitis. [7] We were therefore interested to more fully understand the mechanisms by which *Malassezia* induce or fail to induce IL-1β.

Malassezia fungi inflammasome priming and activation occur at discordant times

Production of mature functional IL-1β is a two-step process, first requiring activation of transcriptional and translational production of cytosolic immature pro-IL-1β protein that is subsequently processed into mature cytokine and secreted upon activation of any one of a number of NLR proteins leading to the assembly of the caspase-1-activating, multi-protein signaling aggregate, called the inflammasome.[26–32] Given the poor induction of IL-1β from BMDC by *Malassezia* fungi that we observed, we examined whether *Malassezia* adequately "prime" the cells for expression of pro-IL-1β and the necessary inflammasome components. When we treated BMDC with live *Malassezia*, *C. albicans* or LPS for 4 hours we observed expression of pro-IL-1β, NLRP3, and pro-caspase-1 in the cell lysates (Fig. 5A). The induction by the fungal species was not as potent as LPS, however all the necessary inflammasome components were produced.

To specifically examine the capacity of each of the fungi to induce inflammasome assembly and IL-1β cleavage, we "primed" a cohort of BMDM with LPS for 4 hours to induce uniform pro-IL-1β production and expression of inflammasome components. We then treated the cells with live, heat-killed (HK), or PFA-fixed (PFA) fungi, *S. aureus*, or ATP (positive control). In this context, we found that *M. restricta* and *M. globosa* were as good or better than *C. albicans* at inducing secretion of mature IL-1β (Fig. 5B). As has previously been reported for *C. albicans*,[30] we noted that heat-killed or PFA-fixed *Malassezia* induced inflammasome activation and secretion of mature IL-1β much less efficiently than live organisms.

When we examined the kinetics of inflammasome activation and IL-1β secretion from LPS-primed BMDC following treatment with live fungi, we found that *Malassezia* rapidly (within 3 hours) induced maximum IL-1β levels (Fig. 5C). In contrast, *C. albicans* and *S. aureus* more slowly triggered inflammasome activation. In both cases, we confirmed that the IL-1β being released into the supernatants was mature cleaved IL-1β p17 by immunoblotting (Fig. 5D).

Together, these results suggest that *Malassezia* fungi induce a rapid and transient inflammasome activation signal. This could explain the relatively poor induction of IL-1β secretion in unprimed cells given that the early and transient inflammasome activation signal would precede expression of pro-IL-1β protein.

Malassezia fungi activate the NLPR3 inflammasome by a partially Dectin2 and CARD9-dependent mechanism

Previous work with *C. albicans* fungi found that activation of the NLRP3 inflammasome was largely responsible for IL-1β production.[28–32] We similarly observed that secretion of mature IL-1β in LPS-primed BMDC response to live *M. restricta* and *M. globosa* was lost in NLRP3$^{-/-}$ cells (Fig. 6A). By contrast, activation of the AIM2 inflammasome by poly(dA:dT) was not affected by loss of NLRP3. NLRP3 deficiency did not affect priming by LPS as determined by LPS-induced secretion of TNFα in culture supernatants (Fig. 6A) and expression of pro-IL-1β in cell lysates (Fig. 6B). We also confirmed that the NLRP3-dependent IL-1β secreted in response to the yeasts was mature IL-1β by measuring IL-1β p17 by immunoblotting culture supernatants (Fig. 6C).

To try to understand which receptors might trigger activation of the inflammasome in response to *M. restricta* and *M. globosa*, we measured IL-1β secretion from LPS-primed BMDC from Dectin1, Dectin2, or CARD9 deficient mice (Fig. 6D). IL-1β production was not affected by Dectin-1 deficiency, but it was partially blocked in Dectin-2- and CARD9-deficient cells. Dectin-1, Dectin-2, and CARD9 deficiency did not affect priming of the cells by LPS as determined by LPS-induced secretion of TNFα in culture supernatants (Fig. 6D) and expression of pro-IL-1β and inflammasome components in cell lysates (Fig. 6E).

Together, these data suggest that in addition to signaling for cytokine production via a C-type lectin/CARD9 pathway, *M. restricta* and *M. globosa* also trigger some of the observed NLRP3 inflammasome activation via this pathway.

Inflammasome activation by Malassezia is dependent on SYK-signaling, potassium efflux and actin rearrangement

There are conflicting reports describing how fungi can activate the NLRP3 inflammasome and production of IL-1β, so we examined the importance of several mechanisms implicated inflammasome activating pathways. Previous work has suggested that the tyrosine-protein kinase SYK (spleen tyrosine kinase) activated downstream of C-type lectin receptors may play a role in NLRP3 activation.[19,24,28] After LPS-priming, we pretreated BMDC with the SYK inhibitor, piceatannol, before exposing them to live fungi and found that SYK was important specifically for responses to both *M. restricta* and *M. globosa*-induced IL-1β production but not *S. aureus*- or ATP-induced IL-1β production (Fig. 7A).

One model of activation of NLRP3 inflammasome assembly involves the efflux of potassium from cells.[33] We tested this model by incubated LPS-primed BMDC in isotonic Ringer's buffer containing either normal concentrations of extracellular potassium (5 mM K^+) or concentrations of potassium equivalent to the cytosol (150 mM K^+) disrupting the gradient of potassium and thus preventing any potassium efflux. When LPS-primed BMDC treated with live *M. restricta* and *M. globosa* were both inhibited by high concentrations of extracellular potassium (Fig. 7B). This is the same response seen with the NLRP3 activator ATP, previously shown to be dependent on potassium efflux, but contrasts with *S. aureus*, which we have previously shown is potassium efflux-independent (Fig. 7B).[34]

We also examined whether phagocytosis of yeasts was important for NLRP3 activation in response to *Malassezia*. We treated LPS-primed BMDC with the actin polymerization inhibitor cytochalasin B before infecting with live *M. restricta* or *M. globosa*. We observed a significant inhibition of IL-1β production (Fig. 7C) suggesting that phagocytosis, or at the very least actin rearrangement, is necessary for the activation of NLRP3 by *Malassezia* yeasts.

DISCUSSION

Several recent studies have found that under certain conditions the skin fungus *Malassezia* becomes abundant in the intestinal tract where it is associated with exacerbated inflammation[7] and can transit to the pancreas and exacerbate pancreatic cancer.[8] While more is known about immune responses to *Malassezia* in the skin, little is currently known about how systemic phagocytes recognize and respond to the major gut-associated *Malassezia* spp., *M. restricta* and *M. globosa*. We have examined the inflammatory responses of conventional bone marrow-derived macrophages and dendritic cells to the live *M. restricta* and *M. globosa* and found that they induced a broad range of proinflammatory cytokines and chemokines including TNFα, IL-6, IL-1β IL-12p40, IL-10 and CXCL1. While BMDM were able to produce some cytokines, the induction was substantially lower than from BMDC. This may be a consequence of reduced levels of CARD9 expression in macrophages or other regulatory factors known to increase the inflammatory response in GM-CSF-treated cells.[35] Cytokine and chemokine production was largely, although not entirely, dependent on signaling by the C-type lectin receptor Dectin-2 via the signaling protein CARD9. We expected TLR signaling might synergize with the C-type lectin receptors as has been shown for responses to some microbes[36,37], however, when we examined responses in MyD88$^{-/-}$ BMDC that will be deficient in most TLR signaling, we saw little to no impact on *Malassezia*-induced TNFα and IL-6 production. Given that we do not see complete abrogation of responses to *Malassezia* in Dectin2$^{-/-}$ BMDC, we imagine additional pattern recognition receptors are involved, but further work will need to be done to determine the identity of those receptors.

Upon engaging *M. restricta* and *M. globosa* BMDC phagocytose the yeasts and activate production of reactive oxygen species. While phagocytosis of *Malassezia* was not impacted in Dectin1, Dectin2 or Card9 knockout BMDC, we observed reduced reactive oxygen species production in Dectin-2$^{-/-}$ cells. Together these observations illustrate the requirement for coordinated engagement of multiple innate immune receptors to orchestrate the full responses of macrophages and dendritic cells to *Malassezia*.

Malassezia can induced production of pro-IL-β from dendritic cells, however, in unprimed cells production of active IL-1β is low and requires a minimum of 10 hours of stimulation. Our observations suggest that priming (stimulation of transcription and translation of pro-IL-1β) is a relatively slow process, requiring 10 or more hours. In contrast, triggering inflammasome activation by *M. restricta* and M. *globosa* is relatively fast and transient, being complete in 3 hours or less. Together, the data suggest that IL-1β production is poor in unprimed cells exposed to the yeasts because priming and inflammasome activation signals are misaligned in time. However, in dendritic cells previously exposed to a priming stimulus,

as for example during repeated exposure to fungi or other microbial stimuli, *Malassezia* can trigger NLRP3 inflammasome activation and release of mature IL-1β. Also, freshly isolated peritoneal macrophages, intestine tissue and keratinocytes exhibit an already primed state expressing pro-IL1β, raising questions as to how often the timing of a "priming" step is relevant during the course of infection and host defense *in vivo*.[38–40]

Our observations raise several questions that will require further study. First, the data suggest that *Malassezia* might be an excellent model for dissecting how multiple pattern recognition receptors coordinate the different responses of dendritic cells to contact with the microbe. Identifying the family of dominant receptors influencing these processes will be illuminating and may provide deeper insights to mechanisms influencing diseases of the gut. Second, it is unclear if tissue specific myeloid cells will respond differentially to different fungi or if they utilize different subsets of receptors to detect fungi. While there have been comparisons different species within *Candida* and *Malassezia* genus[13,41], there have not been comparisons in different tissue-specific myeloid cells, which will be important for understanding the different responses seen at different sights of infection. Third, the mechanisms by which SYK signaling, actin polymerization, and, to a lesser degree, CARD9 signaling influence NLRP3 inflammasome activation by *M. restricta* and *M. globosa* warrant further study. Previous work on C-type lectin receptor signaling suggests that blocking phagocytosis with cytochalasin results in prolonged SYK activation[42], so we might have expected if SYK signaling is important for inflammasome activation that cytochalasin B would have enhanced rather than blocked inflammasome activation and IL-1β production. However, it is also possible that actin rearrangement is necessary for steps downstream of SYK signaling for inflammasome activation. SYK signaling in response to *C. albicans* was shown to been important for NLRP3 activation[28], however, *C. albicans* induced NLRP3 activation was also dependent on hyphae formation.[30] We did not observe hyphae formation during *M. restricta* and *M. globosa* infection *in vitro* during our studies suggesting that these fungi may activate the NLRP3 inflammasome by a different combination of signals. It is also unclear how *Malassezia* triggers potassium efflux from cells. These topics will need to be addressed in future experiments.

The data presented here further our understanding of the innate pro-inflammatory responses of myeloid phagocytes to *M. restricta* and *M. globosa* yeasts and some of the underlying mechanisms. In the context of colitis, the increased presence of *Malassezia* in the gut could result in increased inflammation when the yeast come in contact with phagocytic cells, contributing to the persistent inflammation that is the hallmark of inflammatory bowel diseases. Our recent work noted a specific enrichment of *Malassezia* in the colonic mucosa of patients with Crohn's disease,[7] although in other studies and models additional fungi have been implicated in colonic inflammation including *C. tropicalis* and *C. albicans*[6,43,44]. Given the complex nature of the microbiome, it is unclear if specific fungal species drive colitis, or if the disruption of the gut epithelial layer and dysbiosis allow for fungal species to expand where they would not normally. It is also possible that the balance of fungal to bacterial recognition leads to improper levels of inflammation in colitis. It will be important in the future to examine whether tissue specific subsets of macrophages and dendritic cells respond differently to different fungi. Understanding these interactions and the roles of gut fungi in

colitis may lead to development of novel strategies for targeted therapeutic interventions in select patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

IBD	inflammatory bowel disease
BMDM	bone marrow-derived macrophages
BMDC	bone marrow-derived dendritic cells
HK	heat-killed
PFA	paraformaldehyde fixed

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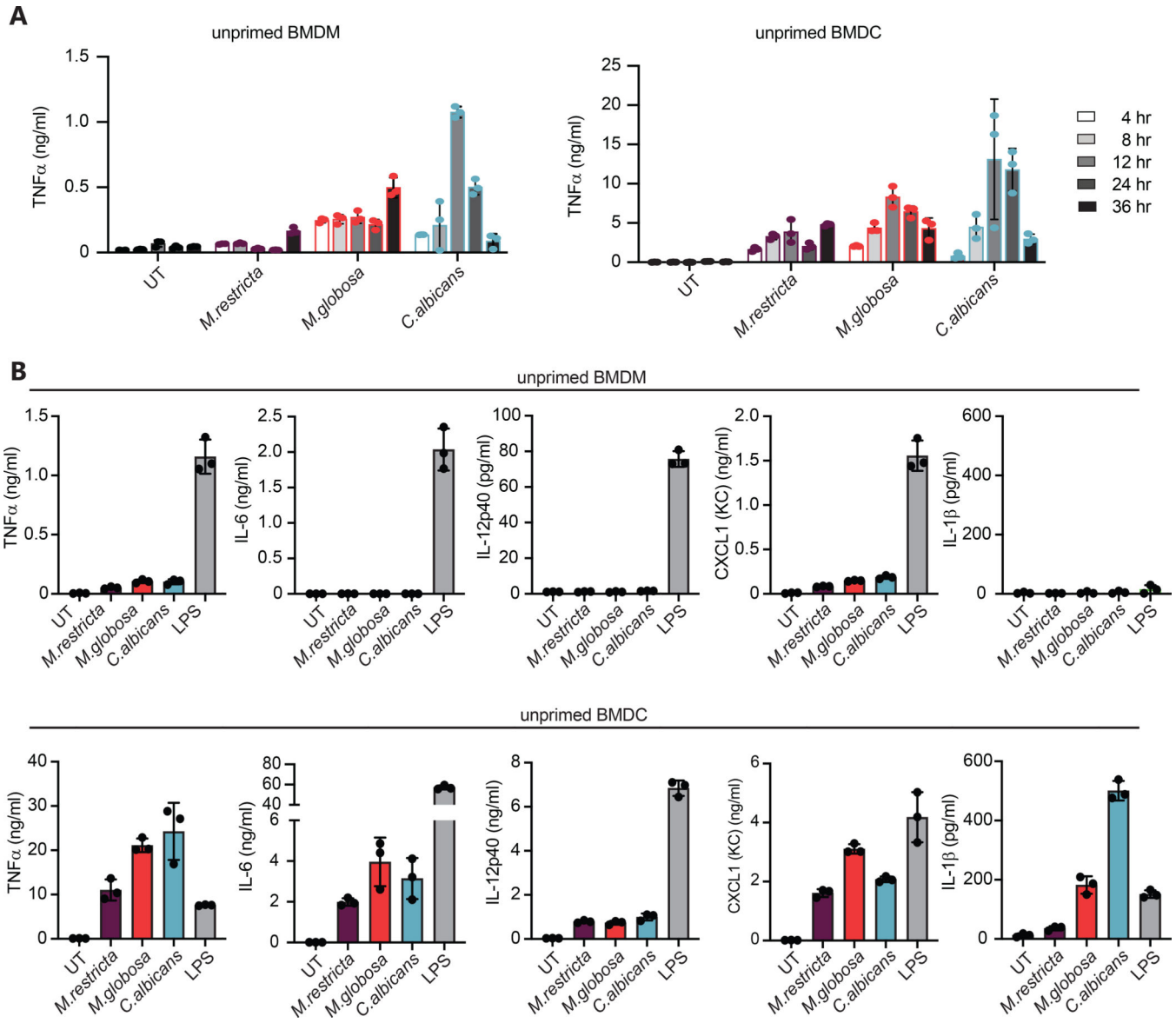

Figure 1. Malassezia induces a limited panel of cytokines from macrophages and dendritic cells.

(**A**) BMDM and BMDC treated with live *M. restricta*, *M. globosa*, or *C. albicans* at MOI=5. Supernatants were collected at designated time points to determine cytokine by ELISA. (**B**) Production of the indicated cytokines was assessed by LegendPlex multiplex analysis in supernatants from cells stimulated as in A, or with 100 ng/ml LPS, for 24 hr. LegendPlex data are representative of two independent experiments.

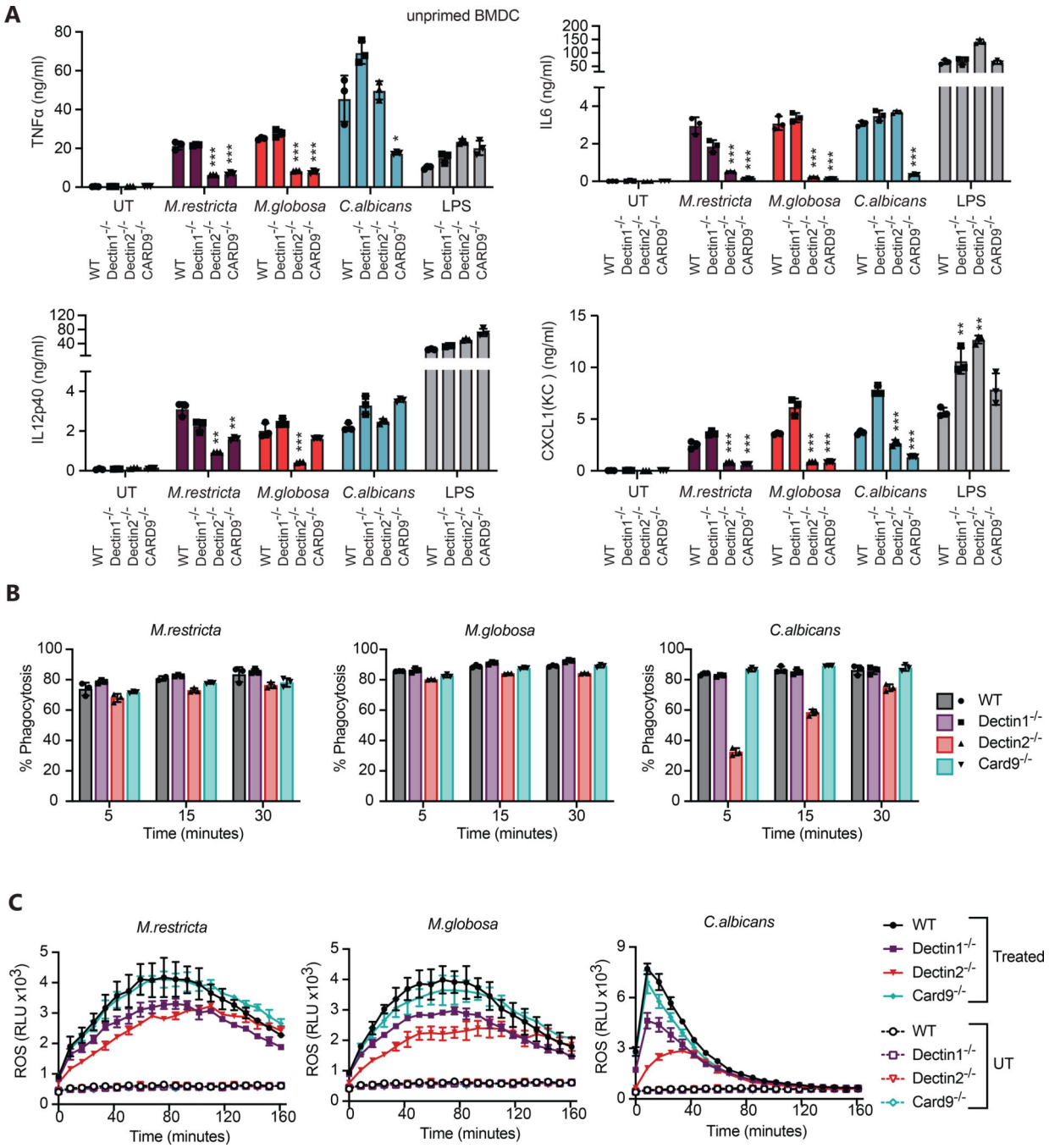

Figure 2. Role of CARD9 signaling in dendritic cell responses to *Malassezia*.

(**A**) BMDC from wild type and the indicated knockout mice were treated with live *M. restricta*, *M. globosa*, or *C. albicans* at MOI=5 or with 100 ng/ml LPS. Supernatants were collected after 24 hr, and production of the indicated cytokines was measured by LegendPlex. (**B**) BMDC from wild type and the indicated knockout mice were incubated for the indicated periods of time with eFluor 670 fluorescently labeled yeasts, and phagocytosis was quantified by flow cytometry. (**C**) BMDC from wild type and the indicated knockout mice were treated with live *M. restricta* or *M. globosa* (MOI=5) or not (UT = untreated), and

production of reactive oxygen species (ROS) was measured by luminol-enhanced chemiluminescence (RUL=relative light units). The experiments in this figure are representative of two independent experiments. All data represent measurements done in triplicate ± SD, and statistics were done by student t-test * $p \leq 0.05$, ** $p \leq 0.01$,*** $p \leq 0.001$.

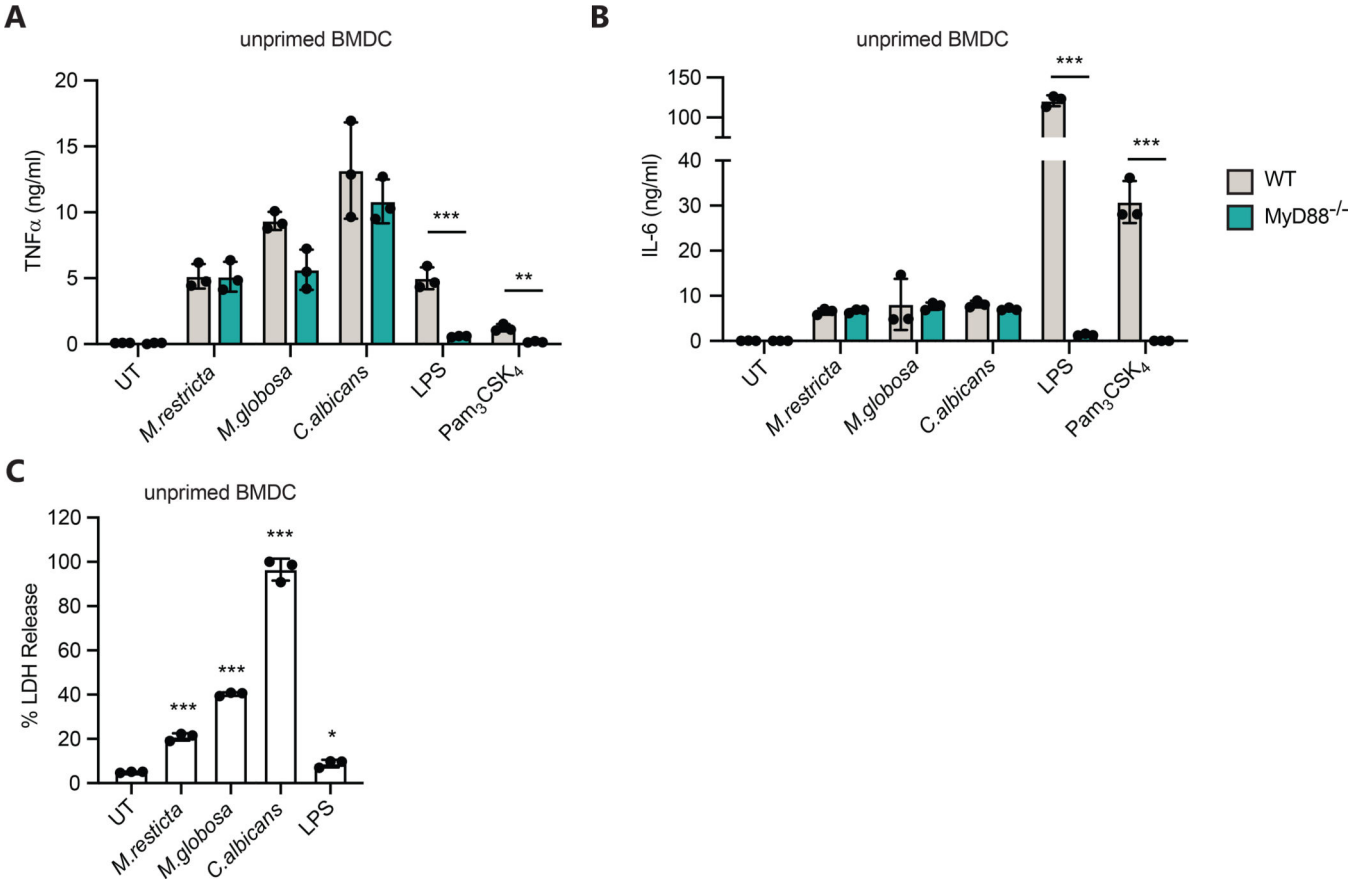

Figure 3. Cytokine responses to live *Malassezia* fungi do not require TLR signaling.

(**A**-**C**) BMDC from the wild type and MyD88$^{-/-}$ mice were treated with live *M. restricta*, *M. globosa*, or *C. albicans* at MOI=5 or with 100 ng/ml LPS or 1 μg/ml Pam_3CSK_4. Supernatants were collected after 24 hr and production of TNFα (**A**) and IL-β (**B**) was measured by ELISA, or cell death was measured by lactate dehydrogenase release (LDH) (**C**). All data represent measurements done in triplicate ± SD, and statistics were done by student t-test * p≤0.05, ** p≤0.01, *** p≤0.001.

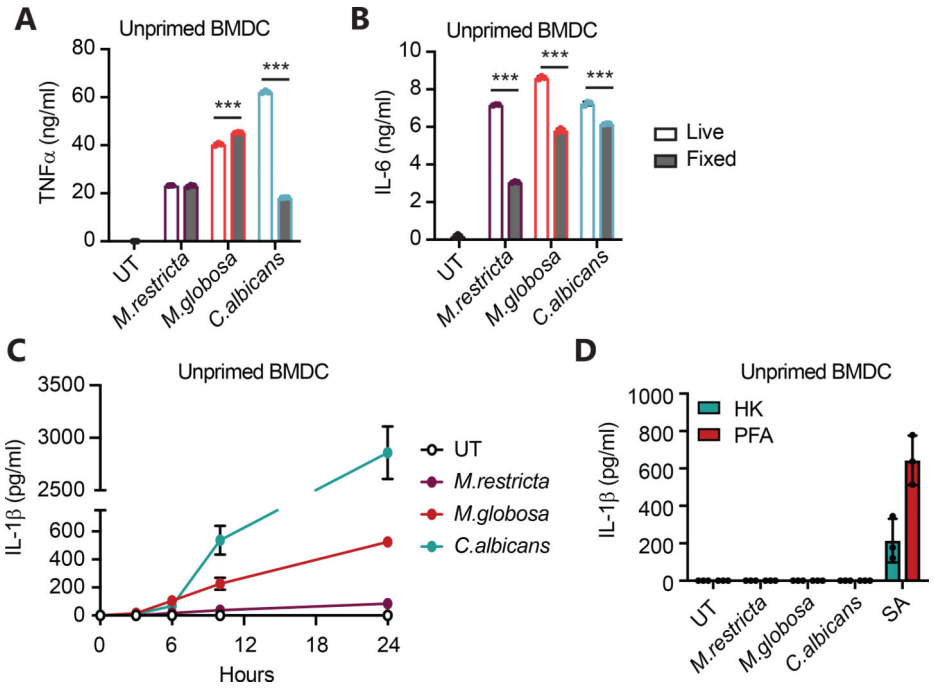

Figure 4. Live *Malassezia* fungi are more potent cytokine producers than dead fungi.

(**A-B**) BMDC were treated with live or PFA-fixed fungi at MOI=5 for 24 hr (6 hr. live *C. albicans*) and cytokines were determined by LegendPlex. (**C**) Unprimed BMDC were treated with the indicated fungi or not (UT = untreated) at MOI=5, supernatants were collected at designated time points, and IL-1β was measured by ELISA. (**D**) Unprimed BMDC were treated with either heat-killed (HK) or paraformaldehyde fixed (PFA) fungi or with *S. aureus* (SA) at MOI=5 for 6 hr, and IL-1β was measured in supernatants by ELISA. All data represent measurements done in triplicate ± SD, and statistics were done by student t-test * p≤0.05, ** p≤0.01, *** p≤0.001.

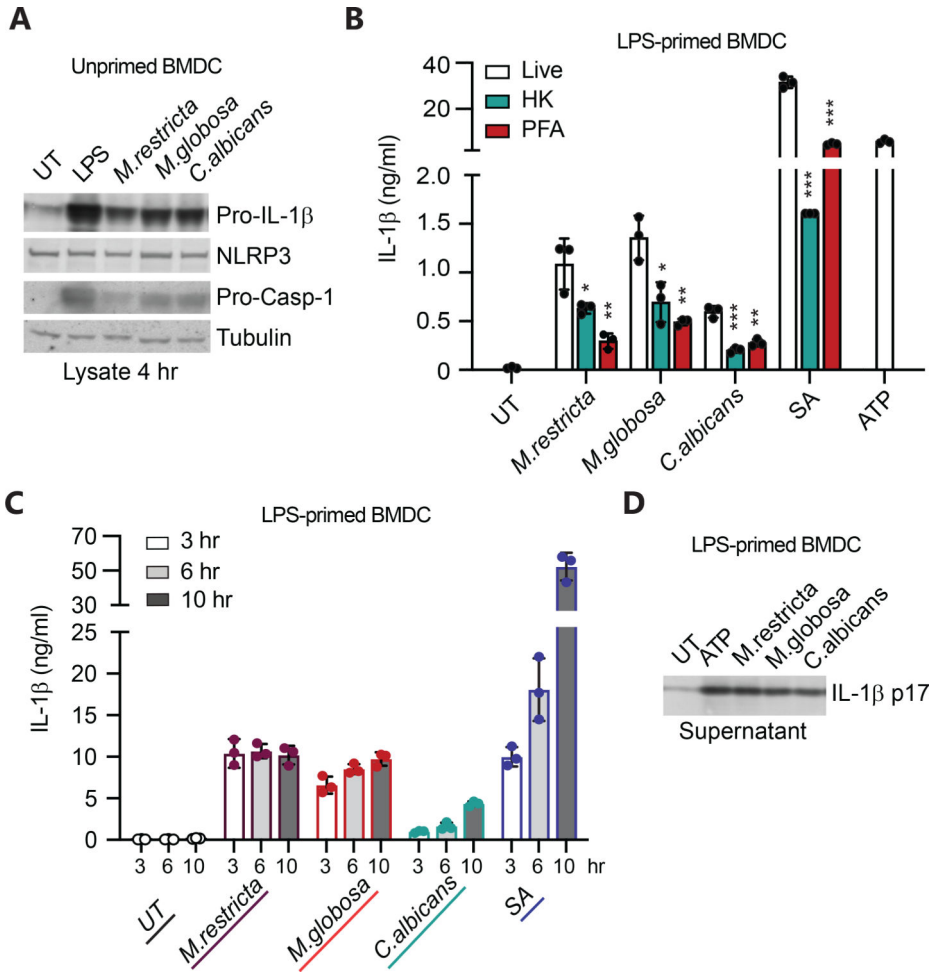

Figure 5. *Malassezia* fungi can prime an inflammasome response and induce caspase-1 activation, but in the timing is discordant.

(**A**) Unprimed BMDM were left untreated (UT) or treated with 100 ng/ml LPS or fungi at MOI=5. Cell lysates were harvested at 4 hr, and protein expression determined by immunoblotting. (**B**) LPS-primed BMDC (4 hr) were treated with live or killed *M. restricta*, *M. globosa*, or *C. albicans* at MOI=5 for 3 hr, *S. aureus* (SA) MOI=5 for 6 hr, or 5 mM ATP for 2 hr. IL-1β was measured in the supernatants by ELISA. (**C**) LPS-primed BMDC (4 hr) were treated with live fungi or *S. aureus* as in B and supernatants were collected at designated time points. (**D**) LPS-primed BMDC (4 hr) were treated with 5 mM ATP for 2h or live fungi at MOI=5 for 3 hr. Cleaved IL-β p17 was assessed in the supernatants by immunoblotting. HK=heat-killed, PFA=1% paraformaldehyde fixed, SA= *S. aureus.* All data represent measurements done in triplicate ± SD and statistics were done by student t-test * p≤0.05, ** p≤0.01, *** p≤0.001.

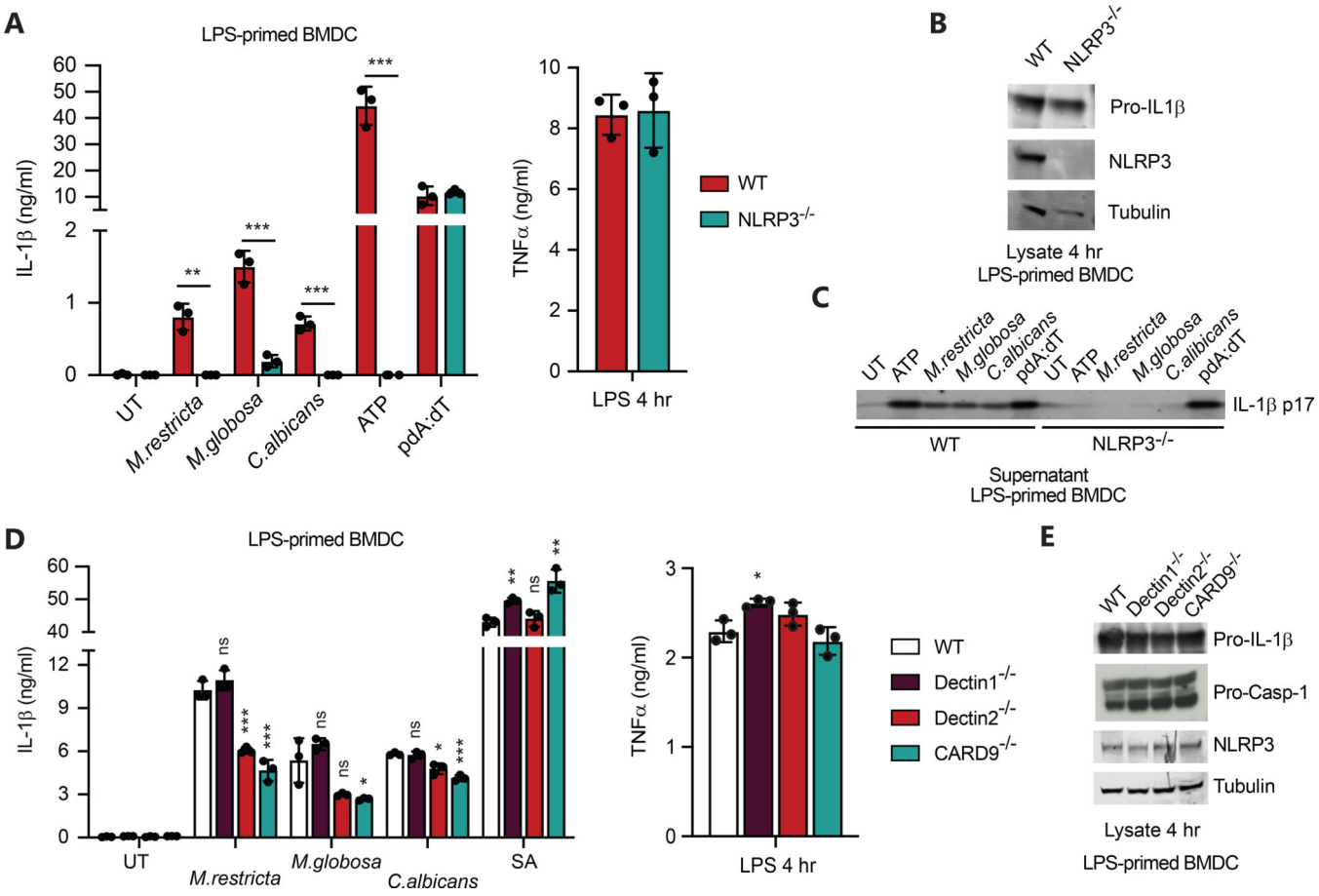

Figure 6. *Malassezia* induces production of IL-1β through the NLRP3 inflammasome and is partially dependent on Dectin-2 and CARD9.

(**A**) BMDC from wild type and NLRP3 knockout mice were primed with LPS (100 ng/ml, 4 hr) and stimulated with the indicated yeasts (MOI=5) for 3 hr or *S. aureus* (MOI=5) for 6 hr, and IL-1β and TNFα secretion in the supernatants was measured by ELISA. (**B**) Cell lystates from LPS-primed cells prepared as in (A) were immunoblotted for the indicated proteins. (**C**) Cell culture supernatants from cells stimulated as in (A) were immunoblotted for mature IL-1β p17. (**D**) BMDC from wild type and the indicated knockout mice were primed with LPS (100 ng/ml, 4 hr) and stimulated as in (A), and IL-1β and TNFα secretion in the supernatants was measured by ELISA. (**E**) Cell lysates from LPS-primed cells prepared as in A were immunoblotted for the indicated proteins. All data represent measurements done in triplicate ± SD, and statistics were done by student t-test * p≤0.05, ** p≤0.01, *** p≤0.001.

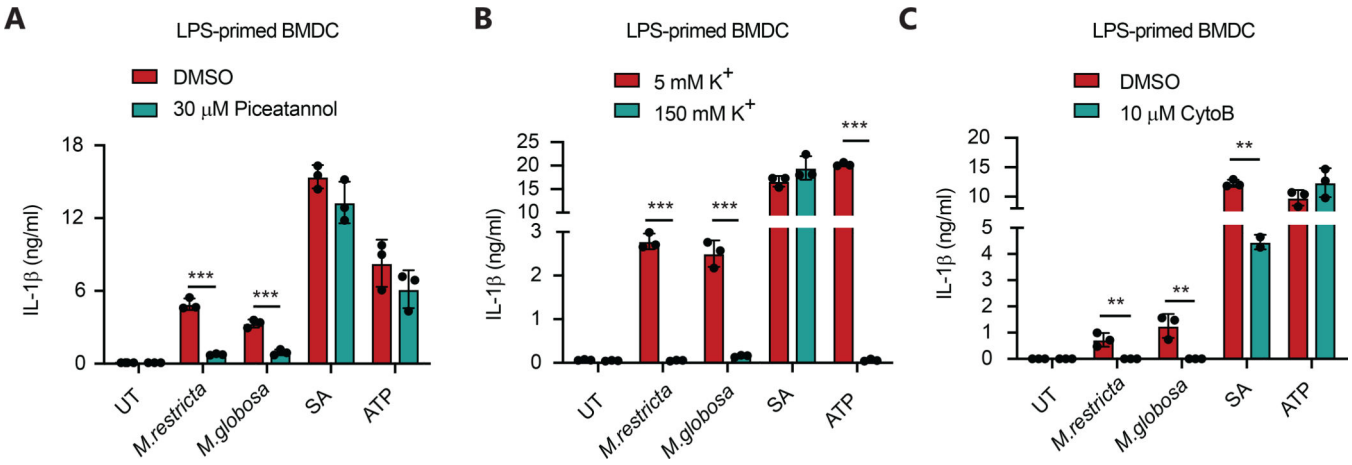

Figure 7. *Malassezia*-induced inflammasome activation is partially dependent on SYK signaling, potassium efflux and actin rearrangement.

(**A**) BMDC from wild type mice were primed with LPS (100 ng/ml, 4 hr) and stimulated with the indicated yeasts (MOI=5) for 3 hr or *S. aureus* (MOI=5) for 6 hr, or ATP (5 mM) for 30 min in the presence or absence of the SYK inhibitor Piceatannol. IL-1β secretion in the supernatants was measured by ELISA. (**B**) BMDC from wild type mice were primed with LPS (100 ng/ml, 4 hr) and stimulated as in (A) in the presence of the indicated levels of extracellular potassium. IL-1β secretion in the supernatants was measured by ELISA. (**C**) BMDC from wild type mice were primed with LPS (100 ng/ml, 4 hr) and stimulated as in (A) in the presence or absence of the actin polymerization inhibitor cytochalasin B. IL-1β secretion in the supernatants was measured by ELISA. All data represent measurements done in triplicate ± SD and statistics were done by student t-test * $p \leq 0.05$, ** $p \leq 0.01$,*** $p \leq 0.001$.