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Regulation of Cytosolic Phospholipase A_2 Activation and Cyclooxygenase 2 Expression in Macrophages by the β-Glucan Receptor*

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Phagocytosis of non-opsonized microorganisms by macrophages initiates innate immune responses for host defense against infection. Cytosolic phospholipase A_2 is activated during phagocytosis, releasing arachidonic acid for production of eicosanoids, which initiate acute inflammation. Our objective was to identify pattern recognition receptors that stimulate arachidonic acid release and cyclooxygenase 2 (COX2) expression in macrophages by pathogenic yeast and yeast cell walls. Zymosan- and *Candida albicans*-stimulated arachidonic acid release from resident mouse peritoneal macrophages was blocked by soluble glucan phosphate. In RAW264.7 cells arachidonic acid release, COX2 expression, and prostaglandin production were enhanced by overexpressing the β-glucan receptor, dectin-1, but not dectin-1 lacking the cytoplasmic tail. Pure particulate (1, 3)-β-D-glucan stimulated arachidonic acid release and COX2 expression, which were augmented in a Toll-like receptor 2 (TLR2)-dependent manner by macrophage-activating lipopeptide-2. However, arachidonic acid release and leukotriene C_4 production stimulated by zymosan and *C. albicans* were TLR2-independent, whereas COX2 expression and prostaglandin production were partially blunted in TLR2$^{-/-}$ macrophages. Inhibition of Syk tyrosine kinase blocked arachidonic acid release and COX2 expression in response to zymosan, *C. albicans*, and particulate (1, 3)-β-D-glucan. The results suggest that cytosolic phospholipase A_2 activation triggered by the β-glucan component of yeast is dependent on the immunoreceptor tyrosine-based activation motif-like domain of dectin-1 and activation of Syk kinase, whereas both TLR2 and Syk kinase regulate COX2 expression.

Macrophages play an important role in innate immunity, serving as a first line of defense against microorganisms (1, 2). Resident macrophages are widely distributed in tissues and are one of the first cell types to sense microbial invaders. Macrophages recognize and engulf opsonized microorganisms through immunoglobulin and complement receptors, but they also possess a number of phagocytic receptors that mediate non-opsonic uptake by binding to specific molecular components of microbial surfaces (3–5). Examples of phagocytic pattern recognition receptors include scavenger receptors, the mannose receptor, a variety of C-type lectin receptors, and the C-type lectin-like β-glucan receptor dectin-1, which is a receptor for zymosan and fungi (6). Phagocytosis of microorganisms is accompanied by activation of the non-phagocytic Toll-like receptors (TLR),[3] which participate in promoting inflammatory responses (7, 8). Distinct signaling pathways are triggered during phagocytosis depending on the receptors engaged by microorganisms influencing functional responses and the survival of intracellular pathogens (8, 9).

Zymosan particles are cell walls of *Saccharomyces cerevisiae* that have been used extensively as a model to study innate immune responses to fungal infections (10). Intraperitoneal injection of zymosan into mice induces acute peritonitis and generalized inflammation that can lead to multiple organ failure (11–13). Non-opsonized zymosan is a potent activator of mononuclear phagocytes inducing production of pro-inflammatory mediators including eicosanoids and cytokines (14–18). The production of prostaglandin (PG) E_2 and leukotriene C_4 (LTC_4) by resident mouse peritoneal macrophages occurs within minutes of intraperitoneal zymosan administration and plays a role in mediating early vascular permeability (11, 19–22).

The release of arachidonic acid for production of eicosanoids by resident mouse peritoneal macrophages during phagocytosis of zymosan is initiated by the activation of Group IVA cytosolic phospholipase A_2 ($cPLA_2$) based on results demonstrating that these responses are blunted in $cPLA_2^{-/-}$ macrophages and by treatment with pyrrolidine-1, an inhibitor of Group IV $cPLA_2$ (23–25). Arachidonic acid released by $cPLA_2$ is metabolized by 5-lipoxygenase to leukotrienes and by constitutive cyclooxygenase (COX) 1 or inducible COX2 for prostanoid production. $cPLA_2$ is regulated post-translationally by increases in intracellular calcium levels and by phosphorylation (26, 27). Calcium binds to an N-terminal C2 domain that promotes translocation of $cPLA_2$ from the cytosol to the membrane for access to phospholipid substrate (28–31). In resident mouse peritoneal macrophages, zymosan activates mitogen-activated protein kinases, leading to phosphorylation of $cPLA_2$ on Ser-505 (24, 25). We have shown that $cPLA_2$ translocates to the

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[3] The abbreviations used are: TLR2, Toll-like receptor-2; $cPLA_2$, cytosolic phospholipase A_2; COX, cyclooxygenase; P-βG, particulate (1,3)-β-D-glucan; MALP-2, macrophage-activating lipopeptide-2; PG, prostaglandin; LTC_4, leukotriene; ITAM, immunoreceptor tyrosine-based activation motif; ROS, reactive oxygen species; SIGNR1, specific ICAM (intercellular adhesion molecule)-3-grabbing nonintegrin-related 1; DMEM, Dulbecco's modified Eagle's medium; m.o.i., multiplicity of infection; IL, interleukin; TNF, tumor necrosis factor.

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forming phagosome during internalization of zymosan (32). The receptors engaged by zymosan that promote cPLA$_2$-mediated release of arachidonic acid have not been identified in macrophages and are the focus of this study.

Zymosan is composed primarily of the carbohydrate polymers glucan and mannan, which are recognized by a variety of receptors on macrophages (33, 34). The macrophage mannose receptor, complement receptor 3, scavenger receptors, the C-type lectin-like receptor, dectin-1, and C-type lectin receptor, specific ICAM (intercellular adhesion molecule)-3-grabbing nonintegrin-related 1 (SIGNR1), have been shown to play a role in binding and internalization of zymosan (35–40). It has previously been reported that monocytes produce eicosanoids during phagocytosis of zymosan through an unidentified β-glucan receptor (41). Although the complement receptor 3 can mediate uptake of zymosan through recognition of both glucose and mannose moieties, engagement of complement receptor 3 does not induce arachidonic acid release in resident mouse peritoneal macrophages (42). The β-glucan receptor dectin-1 has been shown to mediate zymosan-induced production of cytokines and reactive oxygen species (ROS) in macrophages (43, 44). It has recently been demonstrated that dectin-1 acts cooperatively with TLR2 for mediating production of TNFα and IL-12 but can also act independent of TLR2 and mediate production of IL-2, IL-10, and ROS by a pathway involving Syk kinase (45, 46). To further understand the mechanisms leading to activation of cPLA$_2$ and eicosanoid production, we investigated the role of the β-glucan receptor and TLR2 in mediating zymosan- and *Candida albicans*-induced arachidonic acid release and COX2 expression.

EXPERIMENTAL PROCEDURES

Materials—Pathogen-free ICR mice were obtained from Harlan Sprague-Dawley and used for all experiments unless otherwise specified. The TLR4 mutant mouse strain C3H/HeJ and control strain C3H/HeOuJ were obtained from The Jackson Laboratory. TLR2$^{-/-}$ mice (C57BL/6) were generated as previously described (47), and age-matched control C57BL/6 mice were obtained from The Jackson Laboratory. All mice were used for macrophage isolation at 4–6 weeks of age. Zymosan was purchased from Sigma and boiled in phosphate-buffered saline three times before use. Fluorescein- and Texas Red-labeled zymosan particles were obtained from Molecular Probes. Particulate β-glucan was purified from *S. cerevisiae* and structurally characterized by NMR (48). Endotoxin-free water-soluble glucan phosphate was prepared from particulate β-glucan as previously described (49). Macrophage-activating lipopeptide-2 (MALP-2) was from Alexis Biochemicals. Mannan was purchased from Sigma. ERTR9 blocking monoclonal antibody to a murine analogue of human DC-SIGN (SIGNR1) was obtained from Bachem. Lipopolysaccharide (*Escherichia coli* 0111:B4) was from List Biologicals. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific activity 100 Ci/mmol) was from PerkinElmer Life Sciences. Fetal bovine serum (Gemini Bio-Products) was heat-inactivated at 56 °C for 30 min before use. Dulbecco's modified Eagle's medium (DMEM) was from Cambrex BioScience Walkersville, Inc. Human serum albumin was obtained from Intergen. The Group IVA cPLA$_2$ inhibitor, pyrrolidine-2, was generously provided by Dr. Michael Gelb. The Src and Syk kinase inhibitors PP2 and piceatannol, respectively, were obtained from Calbiochem. Rabbit polyclonal antibody to Syk (N-19) was obtained from Santa Cruz Biotechnology, Inc. Phospho-Syk antibody was from Cell Signaling Technology, Inc. Polyclonal antibody to murine COX2 was obtained from Caymen Chemical Co.

Macrophage Cultures and Arachidonic Acid Release Assay—Resident mouse peritoneal macrophages were obtained by peritoneal lavage using 5 ml of DMEM containing 10% heat-inactivated fetal bovine serum, 10 units/ml heparin, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.29 mg/ml glutamine (supplemented DMEM). Cells were plated at a density of 0.5×10^6/cm^2 (48-well plate) and incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Wells were washed twice with calcium- and magnesium-free Hanks' balanced salts solution to remove non-adherent cells. The adherent macrophages were incubated in supplemented DMEM containing [^{3}H]arachidonic acid (0.1 μCi/250 μl/well) and incubated for 16–18 h at 37 °C. The cells were washed twice with phosphate-buffered saline followed by 1 wash with serum-free DMEM containing 0.1% human serum albumin (stimulation medium) to remove unincorporated [^{3}H]arachidonic acid and then incubated in stimulation medium. After stimulation of the macrophages, the culture medium was removed and centrifuged, and the amount of radioactivity released into the medium was measured by scintillation counting. The cell-associated radioactivity was measured after solubilization of the monolayer with 0.1% Triton X-100. The amount of radioactivity released is expressed as the percent of the total radioactivity incorporated (cell-associated plus medium).

RAW264.7 macrophages (ATCC number TIB 71) expressing SIGNR1, murine dectin-1, or dectin-1 with a truncated cytoplasmic tail were generated as previously described (43). They were plated at 1.5×10^5/2 cm^2 well (24-well plate) in RPMI containing 10% heat-inactivated fetal bovine serum, 0.6 mg/ml G418, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.29 mg/ml glutamine (supplemented RPMI). After ~6 h they were washed once, incubated in supplemented RPMI containing [^{3}H]arachidonic acid, and further processed for measuring arachidonic acid release as described above for mouse peritoneal macrophages.

C. albicans Culture—*C. albicans* (ATCC 10231) was grown on Sabouraud dextrose agar plates and maintained at 4 °C. The day before the experiment *C. albicans* was streaked onto a fresh plate and incubated overnight at 37 °C. Yeast cells were scraped from the plate and washed twice in endotoxin-free phosphate-buffered saline.

Phagocytosis Assay—Resident mouse peritoneal macrophages were plated at 1×10^6 cells/2 cm^2, adhered for 1 h, and washed to remove non-adherent cells. After overnight incubation in supplemented DMEM, the cells were washed and then incubated in stimulation medium containing fluorescein-labeled zymosan (25 particles/cell) for 1 h at 37 °C. After thorough washing to remove unbound zymosan, the cells were fixed with 1% paraformaldehyde for 15 min. The percentage of macrophages with cell-associated (bound and internalized) fluorescein-labeled zymosan was determined by fluorescence microscopy and counting 75–100 cells.

Eicosanoid Analysis and Western Blots—Resident mouse peritoneal macrophages and RAW264.7 cells were plated as described for arachidonic acid release assays. After incubation overnight in supplemented DMEM, the cells were stimulated for 6 h, and amounts of PGE_2, PGD_2 and LTC_4 in the culture medium were quantified by enzyme-linked immunosorbent assay (Elisa Tech, Aurora, CO). PGD_2 was measured as the methoxime derivative as outlined by Cayman Chemical Co. LTC_4 was quantified using cysteinyl leukotriene assay reagents, which detect LTC_4, LTD_4, and LTE_4, using LTC_4 as a standard.

For Western blots the cell monolayers were washed twice in ice cold phosphate-buffered saline and then scraped in lysis buffer (50 mM Hepes, pH 7.4, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 200 μM sodium vanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 300 nM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 30 min, lysates were

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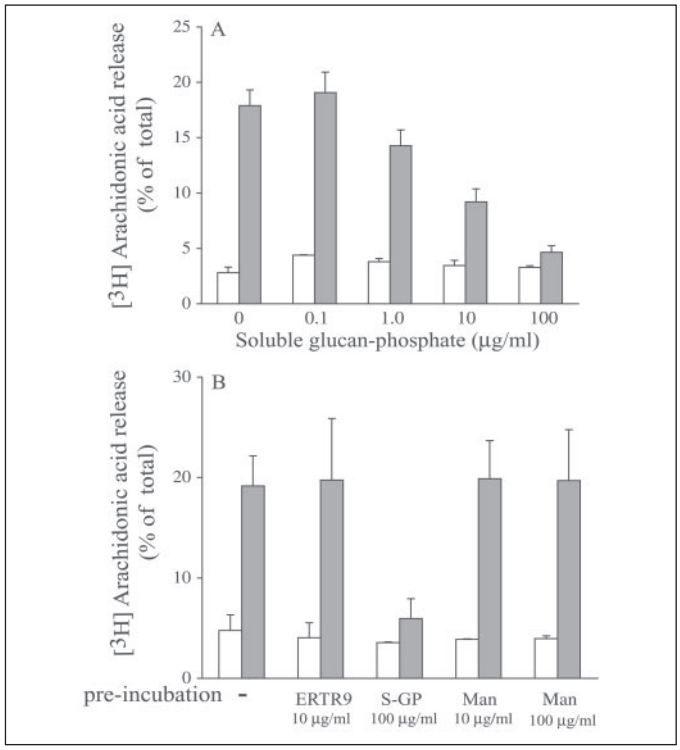

FIGURE 1. **β-Glucan receptor mediates zymosan-stimulated arachidonic acid release.** Mouse peritoneal macrophages labeled with [^{3}H]arachidonic acid were preincubated for 15 min with soluble glucan phosphate (*A*) or blocking monoclonal antibody (*ERTR9*) to SIGNR1, soluble glucan phosphate (*S-GP*), or mannan (*Man*) (*B*). After an additional incubation for 45 min in the absence (*open bars*) or presence (*gray bars*) of zymosan (20 particles/cell), the amount of [^{3}H]arachidonic acid released was determined and expressed as the % of the total label incorporated (cells plus medium). The results are the mean of three experiments ±S.E. (*A*) or the average of two experiments ±S.D. (*B*).

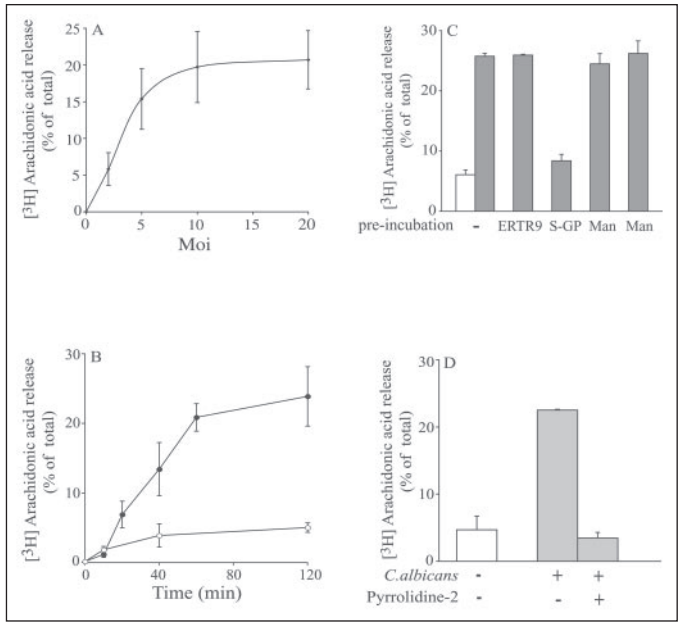

FIGURE 2. ***C. albicans* stimulates cPLA$_2$-mediated arachidonic acid release through a β-glucan receptor.** The release of [^{3}H]arachidonic acid was determined after a 60-min incubation of mouse peritoneal macrophages with the indicated m.o.i. of *C. albicans* (*A*), at the indicated times in control (*open circles*) or *C. albicans* (m.o.i. 5)-infected macrophages (*closed circles*) (*B*), after preincubation (using concentrations shown in Fig. 1*B*) for 15 min with blocking monoclonal antibody (*ERTR9*) to SIGNR1, soluble glucan phosphate (*S-GP*), or mannan (*Man*) followed by a 60-min incubation without (*open bar*) or with *C. albicans* (m.o.i. 5) (*gray bars*) (*C*), or after a 15-min preincubation with 5 μM pyrrolidine-2 followed by 60-min incubation without (*open bars*) or with *C. albicans* (m.o.i. 5) (*gray bars*) (*D*). The amount of [^{3}H]arachidonic acid released in *A* is expressed as the % of the total label incorporated (cells plus medium) after subtracting the % release from unstimulated cells. Results in *A* and *B* are the mean of three experiments ±S.E., those in *C* are the average of triplicate samples ±S.D. of a representative experiment, and those in *D* are the average of two experiments ±S.D.

centrifuged at 15,000 rpm for 15 min, and protein concentration in the supernatant was determined by the bicinchoninic acid method. Lysates were boiled for 5 min after addition of Laemmli electrophoresis sample buffer, and then proteins were separated on 10% SDS-polyacrylamide gels. For COX2 analysis, 8 μg of total lysate protein was used, and for Syk and phospho-Syk analysis, 30 μg of protein was used. After transfer to nitrocellulose membrane, samples were incubated in blocking buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween (TTBS)) containing 5% nonfat milk for 1 h and then incubated overnight at 4 °C with polyclonal antibodies (1:1000) to COX2, Syk, or phospho-Syk in TTBS. The membranes were incubated with anti-rabbit IgG horseradish peroxidase antibody (1:5000) in TTBS for 30 min at room temperature. The immunoreactive proteins were detected using the Amersham Biosciences ECL system.

Statistics—Statistics were calculated in GraphPad using unpaired *t* test to obtain two-tailed *p* values.

RESULTS

β-Glucan Receptor Mediates Arachidonic Acid Release Induced by Zymosan and C. albicans—To identify the receptors that mediate zymosan-induced arachidonic acid release, resident mouse peritoneal macrophages were incubated with a variety of agents that block recognition of zymosan by specific receptors. As shown in Fig. 1*A*, arachidonic acid release induced by zymosan was dose-dependently blocked by a highly purified preparation of soluble β-(1,3)-glucan phosphate (49). The number of macrophages undergoing phagocytosis of fluorescein isothiocyanate-zymosan decreased from 44 ± 2.6 to 17 ± 1.3% after preincubation with soluble glucan phosphate (100 μg/ml). It has recently been shown that resident peritoneal macrophages also contain a mannose binding (mannan-inhibitable) receptor, SIGNR1, which plays a role in non-opsonic uptake of zymosan and *C. albicans* and can act cooperatively with β-glucan receptor (40). To determine whether SIGNR1 plays a role in zymosan-induced arachidonic acid release, macrophages were preincubated with mannan or a blocking monoclonal antibody (ERTR9) to SIGNR1 (Fig. 1*B*). Arachidonic acid release was unaffected by mannan or the blocking monoclonal antibody ERTR9. In additional experiments, zymosan-induced arachidonic acid release was not blocked by polyinosinic acid (data not shown), an inhibitor of macrophage scavenger receptor, which can directly bind β-(1,3)-glucan polymers (39).

The ability of the live fungal pathogen *C. albicans* to stimulate arachidonic acid release in resident mouse peritoneal macrophages was also investigated. Arachidonic acid release was induced in macrophages infected with *C. albicans* using multiplicities of infection (m.o.i.) from 2 to 10 (Fig. 2*A*). The time course of arachidonic acid release induced by *C. albicans* (m.o.i. 5) was determined (Fig. 2*B*). After the addition of *C. albicans*, there was a 10-min lag phase followed by steady accumulation of arachidonic acid in the medium up to 60 min. Arachidonic acid release induced by *C. albicans* was inhibited by preincubation with soluble glucan phosphate but not by mannan or ERTR9 (Fig. 2*C*). As shown in Fig. 2*D*, the cPLA$_2$ inhibitor pyrrolidine-2 blocked *C. albicans*-induced arachidonic acid release to basal levels, implicating a role for Group IVA cPLA$_2$ (50). The results demonstrate that a receptor that recognizes β-glucan on zymosan and *C. albicans* mediates cPLA$_2$ activation and arachidonic acid release.

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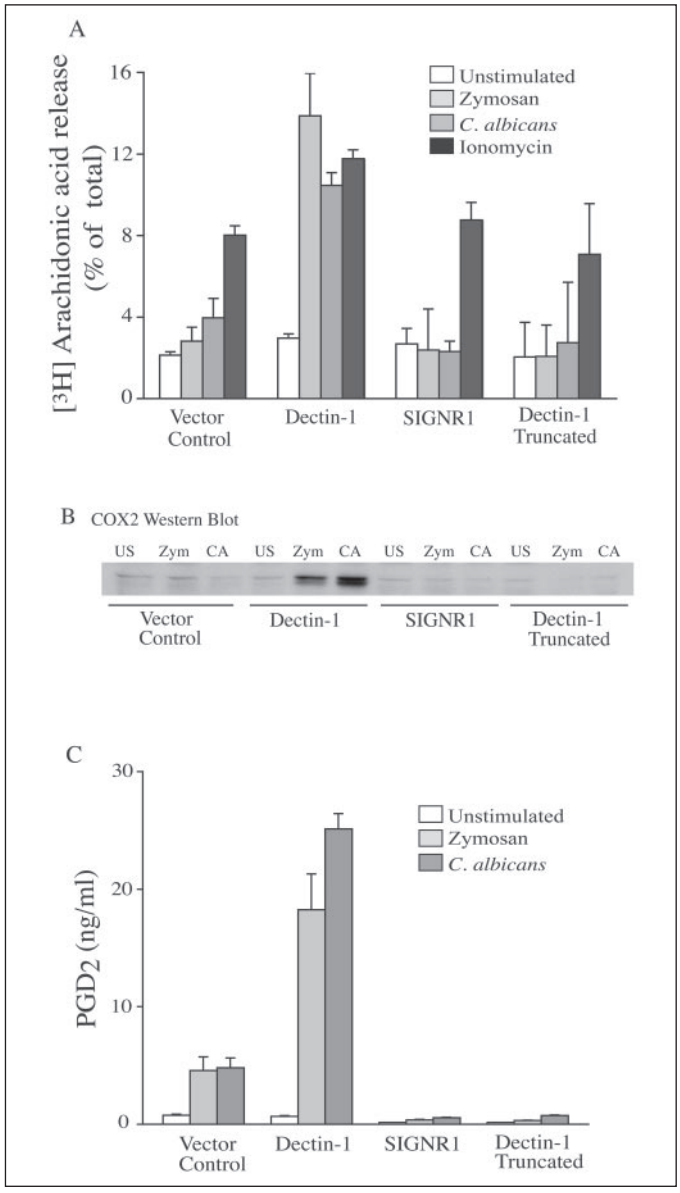

FIGURE 3. **Dectin-1 mediates zymosan- and *C. albicans*-induced arachidonic acid release, COX2 expression, and prostanoid production.** *A*, RAW264.7 cells expressing dectin-1, SIGNR1, truncated dectin-1, and vector controls were labeled overnight with [^{3}H]arachidonic acid. After washing, the amount of [^{3}H]arachidonic acid release at 60 min from unstimulated cells (*open bars*), cells stimulated with zymosan (20 particles/cell) (*light gray bars*), *C. albicans* (m.o.i. 5) (*dark gray bars*), or 1 μM ionomycin (*black bars*) was determined. Results are the mean of three experiments ±S.E. RAW264.7 cells expressing dectin-1, SIGNR1, truncated dectin-1 vector controls were incubated either without (unstimulated (*US*)) or with zymosan (*Zym*) (20 particles/cell) or *C. albicans* (*CA*; m.o.i. 5), and the level of COX2 expression in cell lysates was determined by Western blot (*B*), or the amount of PGD$_2$ in the culture supernatant was determined after stimulation of cells for 6 h (*C*).

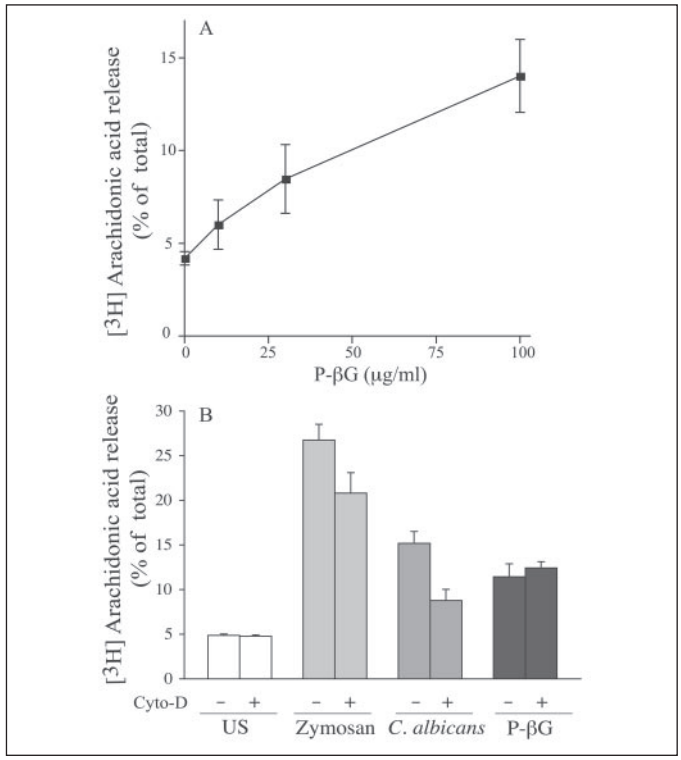

FIGURE 4. **Stimulation of cPLA$_2$-mediated arachidonic acid release by purified P-βG.** The release of [^{3}H]arachidonic acid was determined after a 60-min incubation of mouse peritoneal macrophages with the indicated concentrations of P-βG (*A*) or after a 30-min preincubation with 4 μM cytochalasin D followed by a 60-min incubation without (unstimulated (*US*)) or with zymosan (20 particles/cell), *C. albicans* (m.o.i. 5), or P-βG (100 μg/ml) (*B*). The results are the mean of three experiments ±S.E.

Role of Dectin-1 in Mediating Arachidonic Acid Release, COX2 Expression, and Prostaglandin Production—Dectin-1 has been identified as the β-glucan receptor on macrophages that mediates the production of pro-inflammatory cytokines induced by zymosan and live fungi (38, 43). RAW264.7 cells lines stably expressing dectin-1 or SIGNR1 were used to determine whether these receptors mediate arachidonic acid release in response to zymosan. RAW264.7 cells normally express very low levels of these receptors (43). As shown in Fig. 3, arachidonic acid release was stimulated by ionomycin to a similar extent in vector control cells and in RAW264.7 cells overexpressing dectin-1 and SIGNR1. In contrast, arachidonic acid release in response to zymosan and *C. albicans* was greater in RAW264.7 cells overexpressing dectin-1 compared with vector control cells or cells expressing SIGNR1 or truncated dectin-1. Only in RAW264.7 cells overexpressing dectin-1 was expression of COX2 induced by zymosan and *C. albicans* (Fig. 3*B*). RAW264.7 cells overexpressing dectin-1 also produced more PGD$_2$, the major prostanoid made by this macrophage cell line, than cells overexpressing SIGNR1 or truncated dectin-1. Zymosan and *C. albicans* stimulated a low level of arachidonic acid release and PGD$_2$ production in vector control cells but not in cells expressing SIGNR1 or truncated dectin-1. The reason for this is not known, but since SIGNR1 and truncated dectin bind the fungal agents but do not signal for arachidonic acid release, they may have a dominant negative effect. The lack of stimulation in cells expressing truncated dectin-1 suggests that the ITAM-like motif in the cytoplasmic tail of dectin-1 is required for cPLA$_2$ activation and COX2 expression in response to zymosan and *C. albicans*.

Zymosan and *C. albicans* are complex agonists that may engage multiple receptors to provide signals for activating cPLA$_2$. To investigate the ability of the β-glucan receptor to mediate arachidonic acid release, we used a highly purified endotoxin-free preparation of particulate β-glucan (P-βG) that had been thoroughly characterized by NMR (48). P-βG stimulated a dose-dependent release of arachidonic acid from mouse peritoneal macrophages, resulting in a 14% release at a concentration of 100 μg/ml, demonstrating that cross-linking the β-glucan receptor with purified particulate β-glucan induces signals for cPLA$_2$ activation (Fig. 4*A*). To determine whether particle internalization is required for cPLA$_2$-mediated arachidonic acid release by P-βG, zymosan, and *C. albicans*, resident mouse peritoneal macrophages were treated with cytochalasin D (Fig. 4*B*). The release of arachidonic acid induced by

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C. albicans and zymosan was inhibited by 60 and 27%, respectively, in macrophages treated with cytochalasin D, but release induced by P-βG was unaffected. Cytochalasin D did not inhibit arachidonic acid release from macrophages stimulated with ionomycin (not shown). The 1-h pretreatment of peritoneal macrophages with 4 μM cytochalasin D and maintenance at this concentration during stimulation prevented internalization of Texas Red zymosan (data not shown) as previously reported (51). The results demonstrate that particle internalization is not essential for cPLA$_2$ activation through the β-glucan receptor, although internalization of *C. albicans* and to a lesser extent zymosan enhances arachidonic acid release.

Role of TLRs in Mediating Arachidonic Acid Release, COX2 Expression, and Eicosanoid Production—Dectin-1 promotes β-glucan-dependent internalization of zymosan and mediates TLR2-dependent and -independent responses in macrophages and dendritic cells. TNFα and IL-12 production triggered by dectin-1 are TLR2-dependent, whereas IL-2, IL-10, and ROS production are independent of TLR2 (43–46). Our results using purified P-βG suggest that engagement of the β-glucan receptor itself provides signals that activate cPLA$_2$; however, it is possible that TLR2 contributes to cPLA$_2$ activation in response to zymosan and *C. albicans*. To investigate whether a soluble TLR2 agonist can induce arachidonic acid release or enhance the response to engagement of the β-glucan receptor, peritoneal macrophages were treated with synthetic MALP-2, a lipoprotein from *Mycoplasma fermentans* that has been shown to be a specific activator of TLR2 (52). As shown in Fig. 5*A*, MALP-2 was a very weak agonist and induced only a small increase in arachidonic acid release at 0.1–1.0 ng/ml. When MALP-2 was added together with P-βG, a greater than additive effect on arachidonic acid release was observed. The stimulation of arachidonic acid release by P-βG and P-βG together with MALP-2 was inhibited by pyrrolidine-2, confirming a role for cPLA$_2$ (Fig. 5*B*). The results suggest that engagement of the β-glucan receptor by purified P-βG more effectively triggers cPLA$_2$ activation than engagement of TLR2, but signals from both receptors can act together to enhance activation of cPLA$_2$.

The contribution of TLRs in mediating cPLA$_2$ activation in response to zymosan, *C. albicans*, and P-βG was investigated by using resident mouse peritoneal macrophages isolated from TLR2 and TLR4 knockout mice. Both TLR2 and TLR4 have been implicated in murine defenses against *C. albicans* (53, 54). As shown in Fig. 6*A*, TLR4 was not required for arachidonic acid release, which was slightly enhanced in TLR4$^{-/-}$ macrophages in response to P-βG, zymosan, and *C. albicans*. We did not observe significant stimulation of arachidonic acid release from wild type or TLR4$^{-/-}$ macrophages in response to lipopolysaccharide, consistent with previous reports that endotoxin is a poor inducer of arachidonic acid release in resident mouse peritoneal macrophages (55). The reason for the enhanced response is not known, but a similar enhancement of IL-6 production in TLR4-deficient macrophages infected with *Helicobacter* has also been observed (56).

Arachidonic acid release was also measured in peritoneal macrophages isolated from WT and TLR2$^{-/-}$ mice (Fig. 6*B*). TLR2 was not required for arachidonic acid release in response to zymosan, *C. albicans*, or P-βG. However, the ability of the soluble TLR2 agonist MALP-2 to enhance arachidonic acid release when used together with P-βG was dependent on TLR2, since the extent of arachidonic acid release in response to MALP-2 and P-βG in TLR2$^{-/-}$ macrophages was significantly attenuated to the level observed with P-βG alone.

The ability of the particulate agonists (zymosan, *C. albicans*, P-βG) and MALP-2 to induce COX2 expression and eicosanoid production in wild type and TLR2$^{-/-}$ macrophages was compared. All the agonists

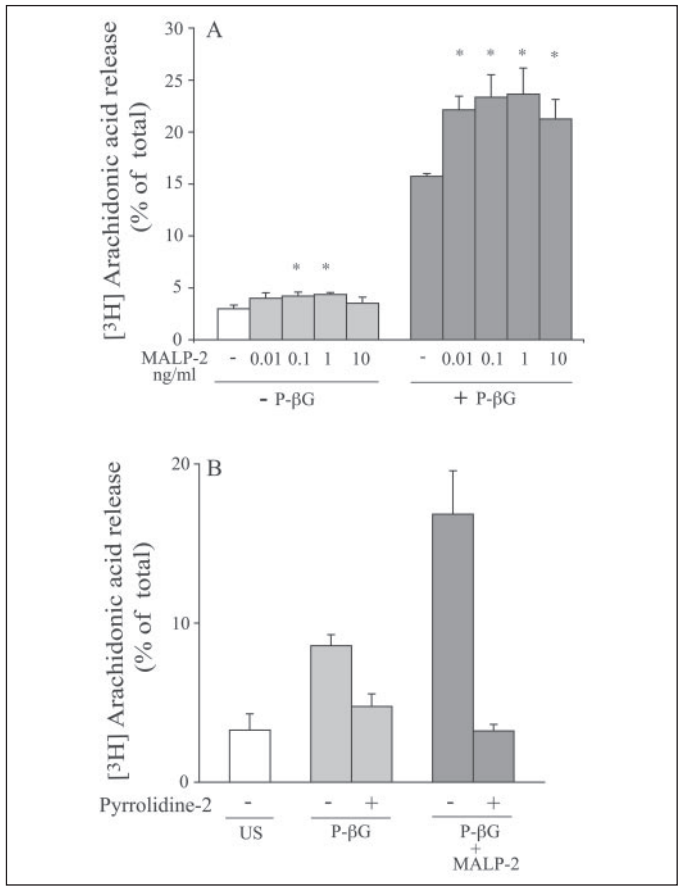

FIGURE 5. **Augmentation of P-βG-induced arachidonic acid release by the TLR2 agonist MALP-2.** The release of [^{3}H]arachidonic acid was determined after a 60-min incubation of mouse peritoneal macrophages with the indicated concentrations of MALP-2 either alone or together with 100 μg/ml P-βG (*A*) or after a 15-min preincubation with 5 μM pyrrolidine-2 followed by a 60-min incubation without (unstimulated (*US*)) or with 100 μg/ml P-βG alone and together with 1 ng/ml MALP-2 (*B*). The results are the mean of three experiments ±S.E. In *A*, the *asterisk* indicates a significant increase ($p < 0.05$) for cells treated with MALP-2 alone compared with untreated control cells (*open bar*) and for cells treated with MALP-2 together with P-βG compared with cells treated with P-βG alone.

increased COX2 expression in TLR2$^{+/+}$ macrophage after treatment for 6 h (Fig. 7*A*). COX2 expression was partially suppressed in TLR2$^{-/-}$ macrophages treated for 6 h with zymosan, *C. albicans*, and P-βG (Fig. 7*A*), which correlated with decreased PGE$_2$ production (Fig. 7*B*). MALP-2 induced COX2 expression in a TLR2-dependent manner (Fig. 7*A*); however, MALP-2 did not significantly increase PGE$_2$ production (Fig. 7*B*) presumably due to its poor ability to activate cPLA$_2$ and provide arachidonic acid. However, as observed for arachidonic acid release, COX2 expression and PGE$_2$ production were greater in macrophages treated with both P-βG and MALP-2 than with either agonist alone, and these enhanced responses by MALP-2 were TLR2-dependent. The effect of the agonists on LTC$_4$ production was also determined. The relative levels of LTC$_4$ produced by TLR2$^{+/+}$ macrophages was similar to the relative amounts of arachidonic acid released (compare with Fig. 6*B*). Also similar to arachidonic acid release, LTC$_4$ production was independent of TLR2 in response to zymosan, *C. albicans*, and P-βG but was lower in TLR2$^{-/-}$ macrophages in response to P-βG together with MALP-2. The results indicate that transcriptional regulation of COX2 is partially dependent on TLR2, but regulation of cPLA$_2$ and 5-lipoxygenase, which involves posttranslational processes, is TLR2-independent.

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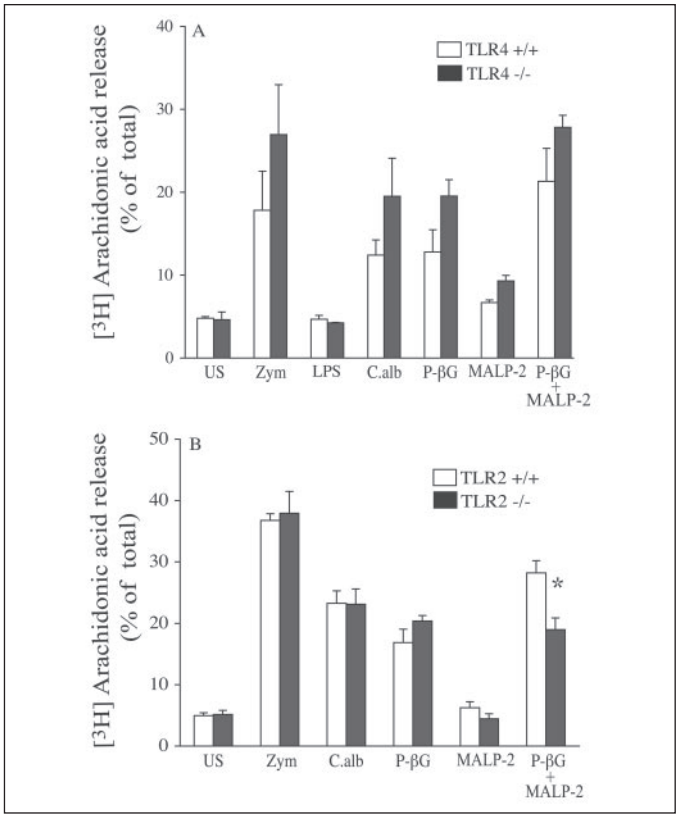

FIGURE 6. **Role of TLRs in regulating arachidonic acid release.** Resident mouse peritoneal macrophages isolated from TLR4 (*A*) or TLR2 (*B*) knock-out (*black bars*) and wild type (*open bars*) mice were labeled with [^{3}H]arachidonic acid and then stimulated with zymosan (*Zym*; 20 particles/cell), lipopolysaccharide (*LPS*; 0.5 μg/ml), *C. albicans* (*C.alb*) (m.o.i. 5), P-βG (100 μg/ml), MALP-2 (1 ng/ml), or P-βG (100 μg/ml) together with MALP-2 (1 ng/ml). Release of [^{3}H]arachidonic acid was measured at 60 min from unstimulated (*US*) and stimulated cells. The *asterisk* in *B* indicates a significant decrease ($p < 0.005$) for TLR2$^{-/-}$ macrophages treated with P-βG plus MALP-2 compared with TLR2$^{+/+}$ macrophages.

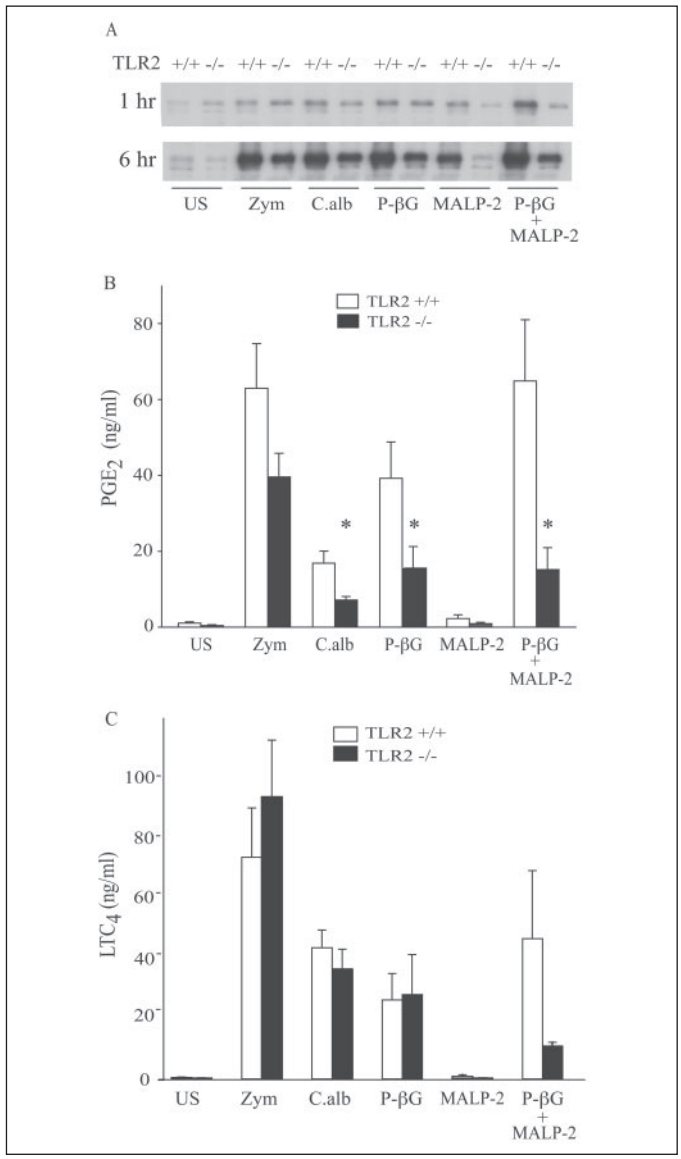

FIGURE 7. **Role of TLR2 in regulating COX2 expression and eicosanoid production.** Resident peritoneal macrophages isolated from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice were incubated overnight as described under "Experimental Procedures" and then stimulated for 1 or 6 h with zymosan (*Zym*; 20 particles/cell), *C. albicans* (*C.alb*) (m.o.i. 5), P-βG (100 μg/ml), MALP-2 (1 ng/ml), or P-βG (100 μg/ml) together with MALP-2 (1 ng/ml). *A*, cells stimulated for 6 h were analyzed for COX2 by Western blotting and for levels of PGE_2 (*B*) and LTC_4 (*C*) in the culture medium. The results in *B* and *C* are the average of 5 and 3 experiments ±S.E., respectively. The *asterisk* indicates a significant decrease ($p < 0.05$) in PGE_2 production in cells from TLR2$^{-/-}$ compared with TLR2$^{+/+}$ mice. *US*, unstimulated.

Inhibition of Src and Syk Kinases Blocks Arachidonic Acid Release and COX2 Expression—It has recently been shown that TLR2-independent responses induced by zymosan engagement of dectin-1 in dendritic cells and in certain populations of macrophages require Syk kinase (45, 46). In macrophages, activation of ROS production by zymosan requires Src and Syk kinases; however, phagocytosis of zymosan is Syk-independent (46, 57). Because we found that TLR2 was not required for β-glucan receptor-dependent activation of arachidonic acid release and only partially required for COX2 expression, the role of Src and Syk kinases was investigated. As shown in Fig. 8*A*, the Src kinase inhibitor PP2 suppressed arachidonic acid release in response to zymosan. In contrast, arachidonic acid release stimulated by A23187 was not affected by PP2 (data not shown). The Syk kinase inhibitor piceatannol inhibited arachidonic acid release and COX2 expression in peritoneal macrophages treated with zymosan, *C. albicans*, and P-βG (Fig. 8, *B* and *C*). We confirmed that zymosan stimulated phosphorylation of Syk, a measure of Syk activation, and this was blocked by piceatannol (Fig. 8*B*, *inset*). The results suggest that cPLA$_2$ activation and COX2 expression stimulated by the β-glucan receptor involves tyrosine phosphorylation of the ITAM-like motif of dectin-1 and activation of Syk kinase.

DISCUSSION

The polysaccharide-rich cell walls of yeast are potent inducers of acute inflammation, as reflected in the ability of zymosan to promote complement activation and production of eicosanoids, ROS, and cytokines (15, 33, 58–64). The zymosan-induced mouse peritonitis model has all the key features of acute inflammation including increased vascular permeability, pain, leukocyte influx, and eicosanoid production (11, 12, 20). In the peritonitis model the production of eicosanoids by resident peritoneal macrophages peaks within minutes after zymosan administration and contributes to pain, edema formation, and leukocyte recruitment (20–22). *In vitro* studies have confirmed the ability of resident peritoneal macrophages to release large amounts of arachidonic acid and eicosanoids during phagocytosis of zymosan (62, 63). In fact resident mouse peritoneal macrophages produce much greater amounts of eicosanoids than elicited/activated peritoneal macrophages, suggesting that lipid mediator production by resident tissue macrophages that first encounter microorganisms plays an important role in initiating acute inflammatory responses and innate immunity (65–67).

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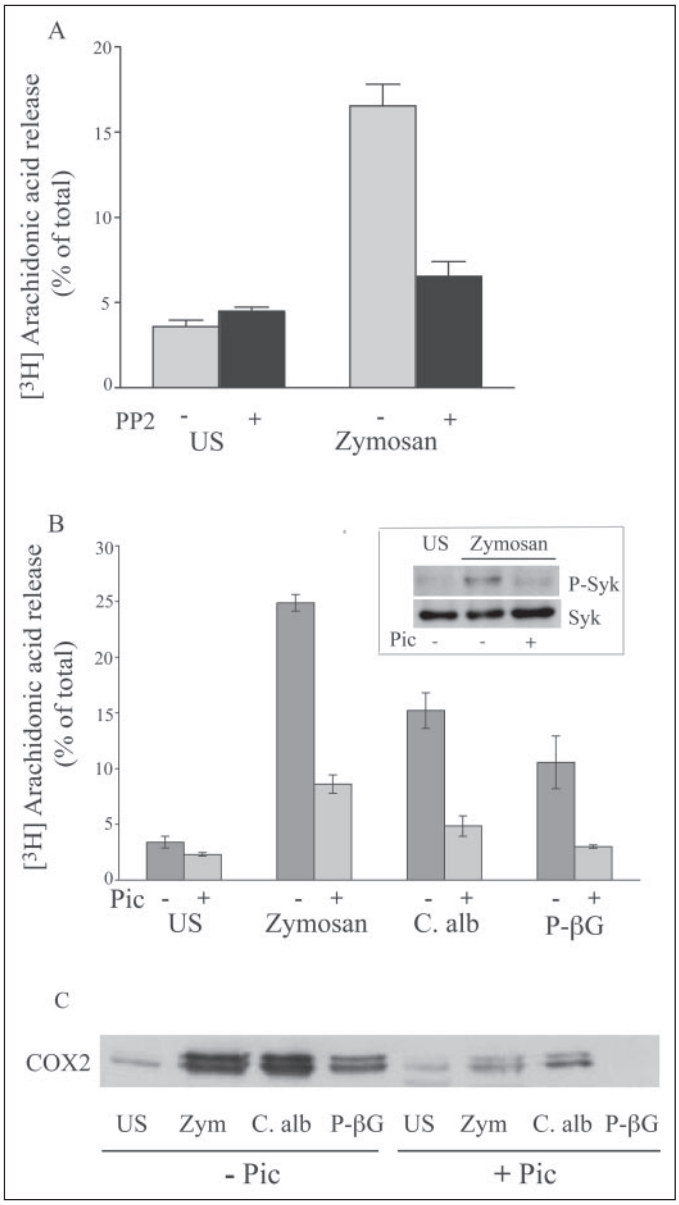

FIGURE 8. **Inhibition of Syk blocks arachidonic acid release and COX2 expression.** Peritoneal macrophages (from ICR mice) labeled with [^{3}H]arachidonic acid were preincubated for 30 min with 10 μM PP2 (*A*) or 25 μM piceatannol (*Pic*) (*B*) and then stimulated with zymosan (10 particles/cell), *C. albicans* (*C.alb*) (m.o.i. 5), or 100 μg/ml P-βG. Release of [^{3}H]arachidonic acid was measured at 60 min from unstimulated (*US*) and stimulated cells. *B*, *inset*, phospho (*P*)-Syk or total Syk was analyzed by Western blot in lysates of unstimulated or zymosan-stimulated (10 particles/cell, 60 min) macrophages that had been preincubated for 15 min with or without piceatannol (25 μM). *C*, peritoneal macrophages were stimulated for 6 h with zymosan (*Zym*; 10 particles/cell), *C. albicans* (*C.alb*) (m.o.i. 5), or 100 μg/ml P-βG in the presence or absence of piceatannol, and cell lysates were analyzed for COX2 expression by Western blotting.

Zymosan-induced release of arachidonic acid from peritoneal macrophages is mediated by cPLA$_2$, which is the only *sn*-2 arachidonic acid-selective mammalian PLA$_2$ that has been identified (27, 68).

A number of receptors on macrophages have been implicated in mediating phagocytosis of non-opsonized zymosan through recognition of carbohydrate moieties in the yeast cell wall. A β-glucan receptor was previously shown to be responsible for zymosan-induced arachidonic acid release and eicosanoid production in rodent and human mononuclear phagocytes, as we observed in resident mouse peritoneal macrophages (64, 69). Complement receptor 3, scavenger receptor AI/II, and lactosylceramide on macrophages can bind β-glucan; however, recent studies demonstrate that the C-type lectin-like receptor dectin-1 is the principal β-glucan receptor on macrophages that mediates biological responses such TNFα and ROS production (36, 39, 43, 44, 51, 70). Our findings that soluble glucan-phosphate blocks zymosan- and *C. albicans*-induced arachidonic acid release and that overexpression of dectin-1 in RAW264.7 macrophages enhances arachidonic acid release suggests a role for dectin-1 in cPLA$_2$ activation by zymosan and *C. albicans*. In addition, our results demonstrate that engagement of dectin-1 by zymosan and *C. albicans* promotes expression of COX2 and increased prostaglandin production. Dectin-1 is widely expressed in human and mouse monocyte/macrophage populations (71, 72). However, resident mouse peritoneal macrophages express relatively low levels compared with mouse alveolar macrophages or thioglycolate-elicited macrophages (72, 73). Dectin-1 is increased on resident mouse peritoneal macrophages after 1 day in culture (72); however, we observed that zymosan-stimulated arachidonic acid release is blocked to a similar extent by soluble glucan phosphate in resident mouse peritoneal macrophages cultured for 4 (not shown) or 24 h after isolation.

C. albicans and other opportunistic fungal pathogens have been shown to induce eicosanoid production by macrophages. Arachidonic acid release from mouse alveolar macrophages and human monocytes incubated with live *C. albicans* was shown to be partially blocked by α-mannan purified from *C. albicans* and blocked to a lesser extent with β-glucan, suggesting a role for mannose and β-glucan receptors (74, 75). We found that soluble glucan phosphate blocked arachidonic acid release from resident mouse peritoneal macrophages incubated with *C. albicans* to a large extent (90%), but α-mannan had no effect, suggesting a primary role for the β-glucan receptor. It was originally thought that the mannose receptor on alveolar macrophages was the primary receptor for the opportunistic fungus *Pneumocystis carinii*, which also stimulates arachidonic acid release (76). However, the absence of the mannose receptor in mice does not increase susceptibility to either *P. carinii* or *C. albicans* (77, 78), and a role for the β-glucan receptor, dectin-1, in the recognition of *P. carinii*, *C. albicans*, and *Coccidioides posadasii* has emerged (79, 80). Unlike zymosan particles, the β-glucan component of live *C. albicans* is buried beneath the outer cell wall components; however, it has recently been shown that soluble dectin-1 binds to discrete patches on the yeast form of *C. albicans* corresponding to bud scars, suggesting that these are regions of exposed β-glucan (81).

Tyrosine phosphorylation of an ITAM-like motif in the cytoplasmic tail of dectin-1 is required for internalization of zymosan (43, 44). We found that the internalization of zymosan was not essential for arachidonic acid release, similar to results for zymosan-induced TNFα production (43). When particle internalization was blocked with cytochalasin D, arachidonic acid release was attenuated to a greater extent with *C. albicans* (60%) than with zymosan (27%), and the response to P-βG was completely unaffected. These results suggest a correlation with accessibility of β-glucan and the requirement for internalization. Although there is evidence that discrete regions of *C. albicans* bind dectin-1 (81), internalization of *C. albicans* may expose more β-glucan for interaction with dectin-1, enhancing activation of cPLA$_2$.

Dectin-1 cooperates with TLR2, which is recruited to macrophage phagosomes containing zymosan and is required for production of TNFα (43, 44). In addition, TLR2 is required for host defense to pathogenic fungus and mediates TNFα production by the *C. albicans* cell wall component phospholipomannan (82). Zymosan has also been shown to directly bind to TLR2 (83). However, the component on zymosan that activates TLR2 has not been identified. Hot alkali treatment depletes the TLR2 activating component of zymosan, which eliminates NFκB acti-

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vation but not dectin-1 binding or zymosan internalization (44). The ability of a soluble TLR2 agonist to synergize with depleted zymosan for induction of cytokine production demonstrates that signals from dectin-1 and TLR2 can act together for enhanced responses (44). Similarly we found that the soluble TLR2 agonist MALP-2, which on its own is a poor activator of cPLA$_2$, augments P-βG-induced arachidonic acid release in a TLR2-dependent manner. However, stimulation of cPLA$_2$-mediated arachidonic acid release by zymosan, *C. albicans*, and P-βG is independent of TLR2. The ability of MALP-2 but not the TLR2-activating component of zymosan to contribute to cPLA$_2$ activation may be explained by the recent finding that CD36 is a TLR2 adaptor protein required for sensing the diacylated bacterial lipopeptide MALP-2 but not triacylated lipopeptides or the TLR2 activating component of zymosan (84). Consequently, our results suggest that co-engagement of CD36 and TLR2 promotes signals that can augment cPLA$_2$ activation. This is consistent with our previous observation that oxidized low density lipoprotein, a CD36 ligand, induces a small increase in arachidonic acid release in mouse peritoneal macrophages (85).

The production of LTC$_4$ by resident peritoneal macrophages in response to zymosan, *C. albicans*, and P-βG is also independent of TLR2. There are similarities in the regulation of cPLA$_2$ and 5-lipoxygenase, which contains a C2-like domain and exhibits calcium-dependent membrane binding and may be regulated by phosphorylation. In contrast, the expression of COX2 and PGE$_2$ production induced by zymosan, *C. albicans*, and P-βG is partially dependent on TLR2. That the TLR2-dependent effect on COX2 expression was observed with purified P-βG suggests "cross-talk" between the β-glucan receptor and may not be dependent on the TLR2 ligand in zymosan or *C. albicans*.

The cytoplasmic tail of dectin-1 is required for zymosan-induced arachidonic acid release, but internalization of zymosan is not essential, suggesting a role for the ITAM-like motif of dectin-1 in providing signals for cPLA$_2$ activation. We also demonstrated that zymosan induces phosphorylation of endogenous Syk in resident peritoneal macrophages. Although dectin-1 does not have a classic ITAM, engagement of dectin-1 by zymosan activates Src and Syk kinases, which are required for zymosan-induced ROS production in macrophages (46). Thus, NADPH oxidase and cPLA$_2$ are activated by similar mechanisms in macrophages through the β-glucan receptor involving the Src and Syk kinase pathway but not TLR2 (44, 46). Syk kinase has also been reported to be required for cPLA$_2$ activation in mast cells stimulated by antigen via the immunoglobulin E receptor (86). Syk activation by antigen promotes increases in intracellular calcium and activation of mitogen-activated protein kinases, both of which are important regulators of cPLA$_2$ (86, 87). Zymosan also induces an increase in intracellular calcium and activation of mitogen-activated protein kinases, which are involved in regulating cPLA$_2$ in mouse peritoneal macrophages (25, 88, 89). Therefore, Syk kinase is a key regulator for cPLA$_2$ activation by classical ITAM in immunoreceptors and the atypical ITAM in the lectin-like receptor, dectin-1.

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