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Calcium-Activated Pathways and Oxidative Burst Mediate Zymosan-Induced Signaling and IL-10 Production in Human Macrophages

Erin K. Kelly,* Lu Wang,† and Lionel B. Ivashkiv†,‡

Outside of the TLR paradigm, there is little understanding of how pathogen recognition at the cell surface is linked to functional responses in cells of the innate immune system. Recent work in this area demonstrates that the yeast particle zymosan, by binding to the β-glucan receptor Dectin-1, activates an ITAM-Syk–dependent pathway in dendritic cells, which is required for optimal cytokine production and generation of an oxidative burst. It remains unclear how activation of Syk is coupled to effector mechanisms. In human macrophages, zymosan rapidly activated a calcium-dependent pathway downstream of Dectin-1 and Syk that led to activation of calmodulin-dependent kinase II and Pyk2. Calmodulin-dependent kinase and Pyk2 transduced calcium signals into activation of the ERK–MAPK pathway, CREB, and generation of an oxidative burst, leading to downstream production of IL-10. These observations identify a new calcium-mediated signaling pathway activated by zymosan and link this pathway to both inflammatory and anti-inflammatory responses in macrophages. ***The Journal of Immunology*****, 2010, 184: 5545–5552.**

Macrophages differentiate endogenous self-proteins from invading microorganisms via a diverse set of proteins, collectively referred to as pattern recognition receptors (PRRs), which bind molecules containing conserved pathogen-associated molecular patterns (PAMPs) (1). Receptor binding triggers changes in cell function and gene expression, which are in turn translated into innate and adaptive immune responses highly specific for the detected pathogen. The prototypical PRRs, the TLRs, recognize well-characterized PAMPs and signal via conserved pathways leading to the production of inflammatory cytokines and mediators. However, intact pathogens are much more complex than a single TLR ligand and often bind to a combination of both TLR and non-TLR PRRs. Only recently has it been appreciated that PRRs, such as the C-type lectin Dectin-1, have the capacity to signal independently of TLRs, and thus the molecular details of these pathways are just beginning to be explored.

Zymosan is a particulate derivative of yeast cell walls that has been used for over half a century to study pathogen–host interactions and acute inflammation in phagocytic cells (2). Zymosan is composed of β-glucan, mannan, protein, and lipid, making it an ideal model of a complex microorganism displaying several PAMPs. Dectin-1, a receptor for β-glucan, collaborates with TLR2 in mediating zymosan-induced production of inflammatory mediators, such as TNF-α (3–5). However, genetic deletion of Dectin-1 in macrophages or dendritic cells (DCs) only partially blocks inflammatory cytokine production in response to zymosan while severely impairing IL-10 production and reactive oxygen species (ROS) generation (6, 7), suggesting that Dectin-1 can function both independently and in conjunction with TLR2 to mediate distinct effects of zymosan.

The ability of Dectin-1 to signal in an autonomous manner was suggested after the discovery of an atypical ITAM in its cytoplasmic tail. ITAMs are traditionally found in the signaling chains of Ag receptors in lymphocytes and FcRs in myeloid cells. Upon engagement of the associated receptor, tyrosines within the ITAM are phosphorylated by Src family tyrosine kinases, followed by recruitment of Syk or ZAP70 kinases and downstream signaling (8). Syk can also be recruited to the intracellular region of Dectin-1, in a manner dependent on a single membrane-proximal tyrosine within the ITAM-like motif (9). Furthermore, zymosan-induced IL-10 and IL-2 production is completely abrogated in Syk-deficient DCs (9). However, the role of Syk in zymosan signaling differs depending on cell type and context, because Syk is required for phagocytosis of zymosan in mouse DCs but not in mouse macrophages (10–12). In mouse macrophages, zymosan-induced phosphorylation of Syk is observed in a small subset of cells and is required for generation of ROS (12). Thus, both Syk-dependent and Syk-independent pathways are involved in zymosan signaling in mouse macrophages.

It is currently unclear how Syk activation is coupled to IL-10 production, although knowledge of traditional ITAM signaling provides some clues. ITAM-dependent signaling activates a calcium flux via activation of phospholipase Cγ (PLC-γ) and uses caspase recruitment domain adaptor proteins Carma1 (in lymphocytes) or CARD9 (in DCs, downstream of Dectin-1) to activate NF-κB and MAPK pathways (10, 13, 14). The role of CARD9 in activating MAPKs or NF-κB differs depending on the type of cell and the exact stimulus, as does its role in MyD88-mediated signaling (14–16). Of downstream signaling molecules activated by ITAMs, a key role for ERK in zymosan-induced IL-10 production has been well established (17, 18). The role of ERK in zymosan-induced responses in macrophages has not been fully addressed, and it is entirely unclear how ERK is coupled to upstream signaling.

*Graduate Program in Cell Biology and Genetics and ‡Graduate Program in Immunology and Microbial Pathogenesis, Weill Cornell Graduate School of Medical Sciences; and †Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY 10021

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Address correspondence and reprint requests to Dr. Lionel B. Ivashkiv, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail address: ivashkivl@hss.edu

Abbreviations used in this paper: BHA, butylated hydroxyanisole; bis I, bisindolylmaleimide I; CaM, calmodulin; CaMK, calmodulin-dependent kinase; DC, dendritic cell; PAMP, pathogen-associated molecular pattern; PKC, protein kinase C; PLC-γ, phospholipase Cγ; PRR, pattern recognition receptor; ROS, reactive oxygen species; siRNA, short interfering RNA.

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In classical ITAM-based signaling, Syk activates PLC-γ, resulting in the generation of inositol 1,4,5-trisphosphate and release of calcium ions from intracellular stores into the cytoplasm. Thus, the activation of Syk by zymosan confers the potential for induction of calcium-mediated responses, which would distinguish zymosan signaling from signaling by TLRs, which activate calcium signaling only weakly or indirectly. Supporting a role for calcium pathways in mediating zymosan responses, a recent study identified a role for the calcium-dependent, calcineurin-activated transcription factor NFAT in zymosan-induced gene expression (19). We reasoned that calcium signaling pathways distinct from the calcineurin–NFAT pathway may contribute to zymosan-induced cytokine production. In this study, we have characterized zymosan-induced calcium-mediated signaling in human blood-derived macrophages. Zymosan activated a calcium-dependent calmodulin (CaM)-dependent kinase (CaMK)–Pyk2–ERK signaling pathway that was coupled to generation of an oxidative burst, activation of CREB, and IL-10 production. These results define a new signaling pathway downstream of zymosan and demonstrate the function of this pathway in regulating cytokine production.

Materials and Methods

Cell culture and reagents

PBMCs were isolated by density-gradient centrifugation using Ficoll (Invitrogen, Carlsbad, CA), and monocytes were further purified by positive magnetic separation of $CD14^+$ cells (Miltenyi Biotec, Auburn, CA). Human monocytes were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FBS (HyClone, Logan, UT) and M-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ) for 2 d to obtain monocyte-derived macrophages, unless otherwise noted. BAPTA, W7, KN93, KN92, AG17, PP1, piceatannol, UO126, and bisindolylmaleimide I (bis I) were purchased from Calbiochem (San Diego, CA). Butylated hydroxyanisole (BHA) and laminarin were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal Ab to human Dectin-1 was purchased from R&D Systems (Minneapolis, MN). Macrophages were preincubated with inhibitors or Ab 1 h prior to treatment with 400 μg/ml zymosan (Invitrogen), unless otherwise indicated.

mRNA isolation and real-time PCR

RNA was extracted from whole-cell lysates with an RNeasy Mini kit (Qiagen, Valencia, CA), and 0.5 μg total RNA was reverse transcribed with a First-Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). Real-time PCR was performed in triplicate wells with an iCycler IQ thermal cycler and detection system (Bio-Rad, Hercules, CA) using *IL10*-specific primers. Threshold cycle numbers were normalized to duplicate samples amplified with primers specific for GAPDH.

Immunoblot analysis

Whole-cell lysates were prepared by direct lysis in SDS loading buffer. For immunoblot analysis, lysates were separated by 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane for probing with Ab. Ab to Pyk2 was purchased from Upstate Biotechnology (Lake Placid, NY), and Ab to p38 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs specific for Pyk2 phosphorylated at Tyr^{402}, CamKII phosphorylated at Thr^{286}, ERK1/2 phosphorylated at Thr^{202}/Tyr^{204}, MEK1/2 phosphorylated at $Ser^{217/221}$, p38 phosphorylated at Thr^{180}/Tyr^{182}, JNK phosphorylated at Thr^{183}/Tyr^{185} and CREB, and CREB phosphorylated at Ser^{133} were from Cell Signaling Technology (Beverly, MA).

RNA interference

Three different prevalidated short interfering RNA (siRNA) oligos targeting Pyk2 and two different control nontargeting oligos were purchased from Dharmacon (Lafayette, CO) and Invitrogen. Purified human monocytes were transfected using Lipofectamine RNAiMAX (Invitrogen) immediately following isolation from donor blood, according to the manufacturers' instructions. Transfections were repeated 24 h later, and cells were harvested after 4 d for experiments. Knockdown efficiency was determined by Western blotting of whole-cell lysates for relative Pyk2 protein levels and ranged between 50 and 90%.

Measurement of reactive oxygen production

Macrophages were incubated with the ROS indicator aminophenyl fluorescein (Invitrogen) 30 min prior to preincubation with inhibitors and stimulation with zymosan. Cells were harvested, washed once, and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Mean fluorescence of individual cells was determined using Cell Quest software (BD Biosciences).

Results

Zymosan-induced IL-10 production is mediated by calcium signaling pathways

Zymosan generates 10-fold more IL-10 protein production than soluble TLR ligands alone (17, 18, 20). We wished to identify zymosan-induced signaling pathways that promote high IL-10 production. We treated primary human macrophages with a variety of specific inhibitors directed against potentially important signaling proteins and screened for decreased IL-10 production by ELISA. Several inhibitors that target calcium-mediated signaling pathways consistently and selectively blocked production of IL-10 by human macrophages (Fig. 1). Depletion of intracellular calcium with the calcium chelator BAPTA effectively inhibited zymosan-stimulated production of IL-10; mean inhibition was 91% in five independent experiments with different blood donors (Fig. 1*A* and data not shown). In some donors, inhibition of IL-10 production by BAPTA was partial, suggesting the involvement of calcium-independent pathways in IL-10 production (data not shown). Inhibition of CaM, a Ca^{2+} sensor that binds calcium and mediates many calcium-dependent cellular processes, using the antagonist W-7 also strongly blocked IL-10 production in response to zymosan (Fig. 1*B*). Carrier controls treated with DMSO alone were performed at the initiation of the project and displayed no effect on cytokine production (data not shown). In all donors tested, these calcium inhibitors had minimal effects on IL-8

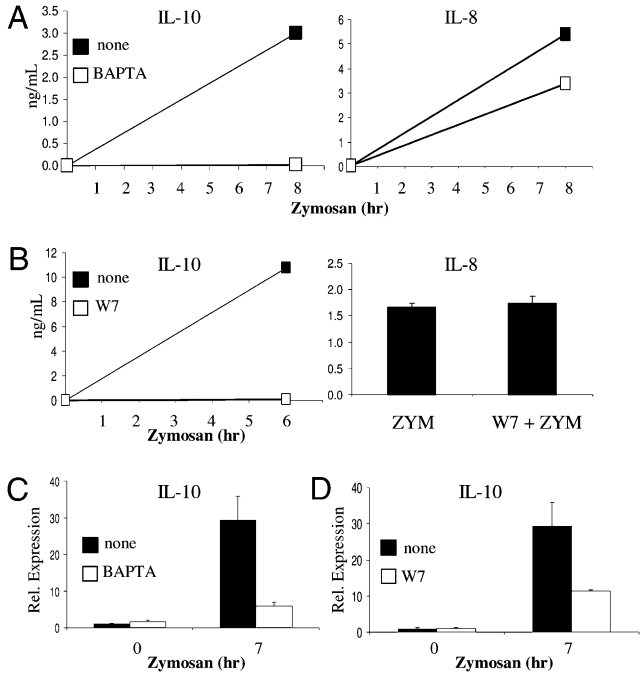

FIGURE 1. Ca^{2+}-dependent signaling pathways regulate zymosan-induced IL-10 production. *A* and *B*, ELISA of culture supernatants from primary human macrophages pretreated for 1 h with or without 10 μM BAPTA-AM (*A*) or 25 μM W7 (*B*) and then stimulated with 400 μg/ml zymosan. *C* and *D*, Real-time PCR of IL-10 mRNA from human macrophages pretreated for 1 h with 10 μM BAPTA-AM (*C*) or 25 μM W7 (*D*) and then stimulated with 400 μg/ml zymosan for 7 h. Values are normalized relative to GAPDH and expression in stimulated macrophages relative to control macrophages (set at 1) is shown. Error bars represent SD of triplicate wells. Data are representative of at least three independent experiments.

production, showing selectivity in inhibition of cytokine production and that these inhibitors were not toxic (Fig. 1*A*, 1*B*). In some experiments, W-7 and BAPTA partially inhibited TNF-α production, especially at early time points (data not shown), suggesting that regulation of cytokine production by calcium pathways is selective but not entirely specific for IL-10. Induction of IL-10 mRNA by zymosan was suppressed by calcium and CaM inhibitors (Fig. 1*C*, 1*D*), indicating that calcium-dependent signaling pathways regulate *IL10* gene expression.

Zymosan activates a CaMK–Pyk2 pathway that contributes to IL-10 production

Calcium pathways can regulate cytokine production via two major downstream effector pathways: activation of the phosphatase calcineurin and its target transcription factor NFAT and activation of CaMK that contributes to MAPK activation (21). A role for NFAT in mediating zymosan-induced gene expression in mouse DCs, including expression of IL-10, was described recently (19). We confirmed these results in human macrophages, where we observed a modest (<50%) decrease in IL-10 production when calcineurin or NFAT was inhibited (data not shown). The modest effect of the NFAT pathway inhibitors, compared with the >75% reduction of IL-10 by BAPTA and W7 (Fig. 1*A*, 1*B*), suggested that a calcium-mediated, NFAT-independent pathway may also contribute to zymosan-induced cytokine production. To address this possibility, we used KN-93, a potent and specific inhibitor of CaMKs. Preincubation with KN-93, but not its inactive structural homolog KN-92, suppressed IL-10 induction by zymosan (Fig. 2*A* and data not shown). This result implies that calcium-dependent signaling mechanisms distinct from the calcineurin/NFAT pathway are also important in zymosan-stimulated IL-10 production. We wished to delineate the calcium-dependent signaling pathway downstream of CaMK that mediates IL-10 production. First, we confirmed that zymosan activated CaMK by inducing phosphorylation on the activating Thr^{286} (Fig. 2*B*). As expected, activation of CaMK was dependent on calcium as zymosan did not induce Thr phosphorylation in the presence of BAPTA (Fig. 2*B*). CaMK contributes to the activation of the calcium-dependent protein tyrosine kinase Pyk2 (21) that can transmit signals to MAPKs and potentially *IL10*, and thus, we tested whether Pyk2 was activated in response to zymosan. Stimulation of human macrophages with zymosan resulted in rapid, calcium-dependent Tyr phosphorylation of Pyk2 (Fig. 2*B*). Preincubation with KN-93, but not KN-92 (Fig. 2*C*), and with W7 (data not shown), suppressed Pyk2 phosphorylation by zymosan, showing that zymosan induced a calcium-dependent CaMK–Pyk2 pathway in primary human monocytes.

Pyk2 can also be indirectly regulated by calcium via protein kinase C (PKC), which is activated by calcium and diacyl glycerol. To determine the role of PKC in Pyk2 activation, we preinucbated cells with the pan-PKC inhibitor bis I and measured zymosan-induced Pyk2 activation by Western blot. Bis I modestly inhibited Pyk2-phosphorylation but more weakly than the CaMK inhibitor KN-93 (Fig. 2*D*). Consistent with this signaling result, bis I only modestly suppressed IL-10 production (<30%), whereas KN-93 essentially completely blocked IL-10 production (Fig. 2*A*). Thus, PKC contributes to Pyk2 activation, but CaMKs play a more prominent role.

The importance of Pyk2 in cell morphology, motility, and adhesion in a variety of cells including macrophages has been widely investigated (22), yet its role in inflammation is only beginning to be explored. To determine whether Pyk2 is required for zymosan-induced IL-10 production in macrophages, we used both pharmacologic and genetic approaches to inhibit Pyk2 function in our system. Preincubation of macrophages with the Pyk2 inhibitor AG17 blocked induction of IL-10 protein (Fig. 3*A*) and IL-10 mRNA (Fig. 3*B*) by zymosan. AG17 had essentially no effect on zymosan-induced IL-6 or TNF-α (Fig. 3*A*), indicating that Pyk2 selectively regulates IL-10 production. To substantiate these results, we used three different siRNA oligonucleotides to knock down expression of endogenous Pyk2 in primary human macrophages, reducing Pyk2 protein levels by >75% relative to cells treated with a control nontargeting siRNA (Fig. 3*C*, *inset*, and data not shown). Human macrophages transfected with siRNA duplexes specific for Pyk2 demonstrated diminished IL-10 production in response to zymosan; this suppressive effect was partial and variable depending on the donor but statistically significant ($p < 0.005$; Student paired *t* test) (Fig. 3*C*). The partial effects of Pyk2 knockdown may be secondary to incomplete silencing of expression, compensation by a redundant kinase (such as closely related family member FAK), or a parallel calcium-mediated pathway induced by zymosan that is not dependent on Pyk2.

Pyk2 activation is dependent on Dectin-1, Src kinases, and Syk

Dectin-1 plays a major role in mediating zymosan-induced IL-10 production. To determine whether Dectin-1 activates the Pyk2–IL-10 pathway described above, we preincubated human macrophages

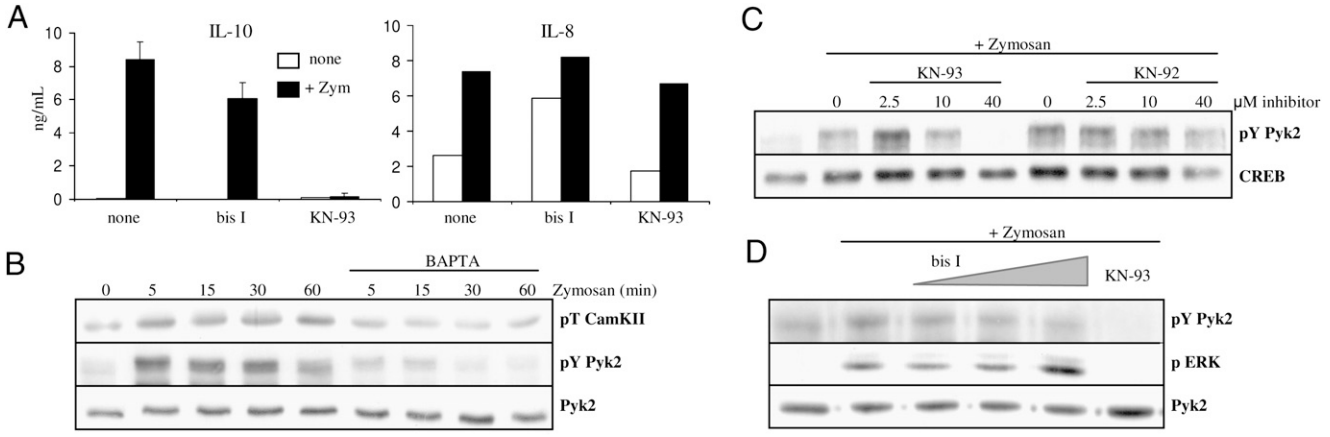

FIGURE 2. CaMKII and Pyk2 are activated by zymosan. *A*, Macrophages pretreated for 1 h with 5 μM bis I or 20 μM KN93 were stimulated for 7 h with 400 μg/ml zymosan and IL-10 and IL-8 in culture supernatants were measured by ELISA. *B–D*, Immunoblot of lysates of primary human macrophages pretreated for 1 h with 10 μM BAPTA (*B*), 2.5–40 μM KN-93 or 2.5–40 μM KN-92 (*C*), 1–10 μM bis I or 20 μM KN-93 (*D*), and stimulated for the indicated times (*B*) or 10 min (*C*, *D*) with 400 μg/ml zymosan. Blots were probed with Abs specific for CaMKII phosphorylated on Thr^{286} or Pyk2 phosphorylated on Tyr^{402} or total Pyk2 or CREB.

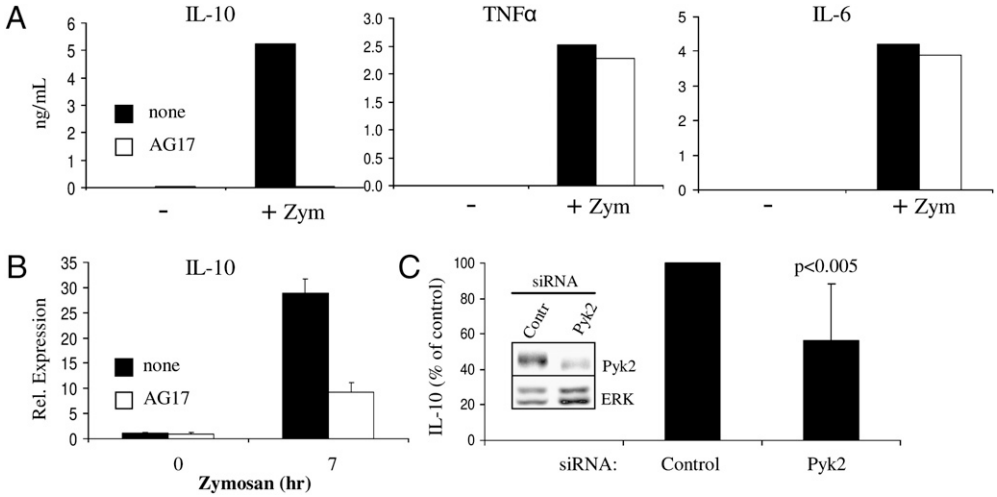

FIGURE 3. Pyk2 is required for optimal IL-10 production. *A* and *B*, Macrophages were pretreated for 1 h with 10 μM AG17 and stimulated for 7 h with 400 μg/ml zymosan. IL-10, TNF-α, and IL-6 levels were measured by ELISA (*A*) or real-time PCR (*B*). *C*, Human monocytes were transfected with control or Pyk2-specific siRNA and 4 d later were stimulated for 24 h with zymosan. Pyk2 levels in cell lysates were measure by immunoblotting, and IL-10 in culture supernatants was measured using ELISA. Cumulative data from five independent blood donors are shown. $p < 0.005$, as determined by Student paired *t* test. Three different Pyk2-specific RNA duplexes were used individually with comparable results.

with a polyclonal Ab against Dectin-1, which blocks recognition of zymosan by Dectin-1 (23, 24). The Dectin-1 blocking Ab strongly suppressed zymosan-induced phosphorylation of Pyk2 (Fig. 4*A*) and suppressed zymosan-induced Thr phosphorylation of CaMKII (Fig. 4*B*), suggesting that Pyk2 activation, as well as upstream calcium signaling, is mediated by Dectin-1. We further observed that the low m.w. β-glucan, laminarin, can also impede activation of Pyk2 by zymosan (data not shown). This result supports a role for Dectin-1 but is subject to the caveat that small β-glucans may bind to and block receptors other than Dectin-1. To further corroborate a role for Dectin-1 and ITAM-dependent signaling in activation of Pyk2, we used inhibitors of the Src and Syk kinases that mediate proximal signaling by Dectin-1 via its ITAM-like motif (9, 12, 25); Src kinases can also associate with Pyk2 and contribute to its activation (22). In human macrophages, activation of Syk and Pyk2 was impaired in the presence of the Src inhibitors PP1 and PP2 but was unaffected by the inactive analog, PP3 (Fig. 4*C*, 4*D* and data not shown). The Syk inhibitor piceatannol also effectively inhibited Pyk2 phosphorylation (Fig. 4*E*). Furthermore, piceatannol suppressed zymosan-dependent IL-10 production while having no effect on TNF-α generation (Fig. 4*F*). These results support a role for Dectin-1 and proximal Src and Syk signaling molecules in the activation of Pyk2 by zymosan and in downstream IL-10 production.

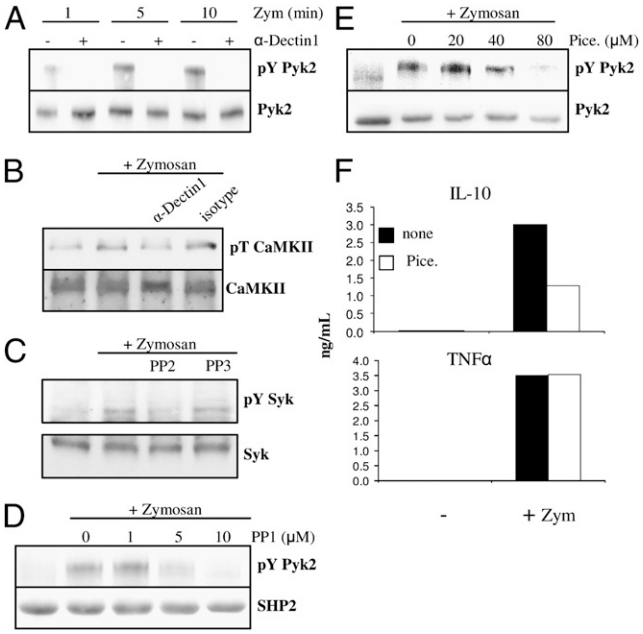

FIGURE 4. Dectin-1, Src, and Syk are required for Pyk2 phosphorylation by zymosan. *A–E*, Immunoblot of lysates of primary human macrophages pretreated for 1 h with 20 μg/ml hDectin-1 Ab (*A*, *B*), 1–10 μM PP1, PP2, or PP3 (*C*, *D*), or 20–80 μM piceatannol (*E*) prior to stimulation with zymosan for the indicated times (*A*) or 10 min (*B–E*). *F*, ELISAs of IL-10 and TNF-α in culture supernatants of human macrophages pretreated for 1 h with 40 μM piceatannol and stimulated with 400 μg/ml zymosan for 7 h. Data are representative of at least three different individual experiments.

Syk typically activates calcium signaling via activation of PLC-γ, and PLC-γ2 has very recently been shown to play a critical role in Dectin-1–dependent cytokine production in mouse DCs (26). However, we believe that induction of PLC-γ2 likely does not play a role in the macrophage-specific pathway described in this paper, because we could not detect its activation in response to zymosan in human macrophages or in mouse bone marrow-derived macrophages (data not shown). Consistent with the recent literature, we have observed zymosan-dependent activation of PLC-γ2 in mouse DCs, suggesting cell-type and species-specific differences in calcium-dependent signaling. Differing signaling pathways are consistent with the Dectin-1 literature, which delineates divergent pathways among macrophages and DCs.

Zymosan-mediated calcium signaling induces IL-10 production via ERK and CREB

We wished to connect zymosan-mediated calcium pathways with IL-10 production at the molecular level. A role for ERK in IL-10 production is well established (27–29), including in zymosan-induced IL-10 production in DCs (17, 18). Consistent with these reports, we found that inhibition of ERK activation resulted in a substantial decrease in zymosan-induced IL-10 production in human macrophages while having no effect on IL-8 levels (Fig. 5*A*). This result confirms an important role for ERK in IL-10 production in our system, and we next tested whether ERK is regulated by the calcium–Syk–CaMK–Pyk2 pathway that is

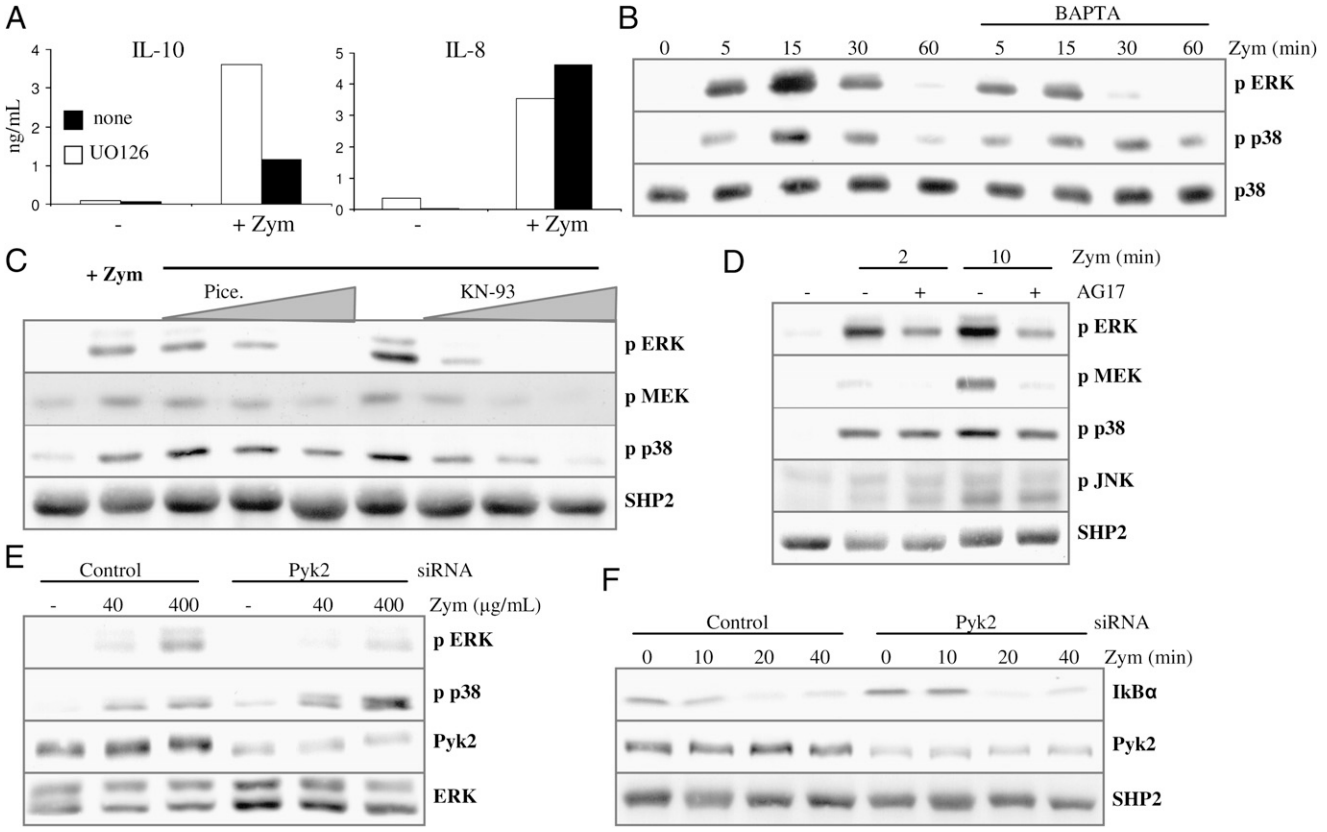

FIGURE 5. Zymosan-induced ERK phosphorylation requires Ca^{2+}-dependent signaling and Pyk2. *A*, ELISAs of IL-10 and IL-8 production from primary human macrophages pretreated with 20 μM UO126 and then stimulated with zymosan for 7 h. *B–D*, Immunoblots of lysates of macrophages pretreated for 1 h with 10 μM BAPTA (*B*), 20–80 μM piceatannol or 10–40 μM KN-93 (*C*), or 10 μM AG17 (*D*) and then stimulated with 400 μg/ml zymosan for 10 min or indicated times. *E* and *F*, Immunoblots of lysates of human monocytes transfected with control or Pyk2-specific siRNA and 4 d later stimulated for 10 min or indicated times with zymosan. Data are representative of at least three different individual experiments.

activated by zymosan. As expected, phosphorylation of ERK occurred rapidly upon stimulation with zymosan in human macrophages (Fig. 5*B*). Zymosan-induced ERK phosphorylation was largely suppressed in cells pretreated with BAPTA (Fig. 5*B*), KN-93 (Fig. 5*C*), or AG17 (Fig. 5*D*). Consistent with recent results in Syk-deficient mouse DCs (18), ERK phosphorylation was also suppressed by the Syk inhibitor piceatannol (Fig. 5*C*). Phosphorylation of MEK, which lies upstream of ERK in MAPK signaling pathways, was also inhibited (Fig. 5*C*, 5*D*). In contrast, zymosan-induced phosphorylation of p38 and JNK was mostly resistant to inhibition by BAPTA, piceatannol, and AG17 (Fig. 5*B–D*), showing a selective regulation of the MEK–ERK pathway relative to other MAPK pathways.

Supporting the pharmacological data, macrophages in which Pyk2 levels were decreased by siRNA exhibited diminished ERK activation in response to zymosan but normal activation of p38 (Fig. 5*E*). Pyk2-depleted macrophages demonstrated similar degradation of IκBα upon stimulation with zymosan as macrophages treated with control siRNA duplexes, suggesting that Pyk2 does not regulate NF-κB activation (Fig. 5*F*). These results provide evidence for selective regulation of the ERK–MAPK pathway by the zymosan-induced Syk–CaMK–Pyk2 signaling pathway in human macrophages and suggest that activation of ERK may represent one mechanism by which zymosan induces production of high levels of IL-10.

The transcription factor CREB is regulated by calcium (30) and a role for CREB in IL-10 gene expression has been described previously (31, 32). Thus, we investigated the regulation of CREB by zymosan-induced, calcium-mediated signaling pathways. CREB was phosphorylated on the activating Ser^{133} residue upon stimulation of macrophages with zymosan, and this phosphorylation was suppressed in cells pretreated with BAPTA, W7 or KN-93 but not KN-92 (Fig. 6*A*, 6*B*). Zymosan-induced CREB phosphorylation

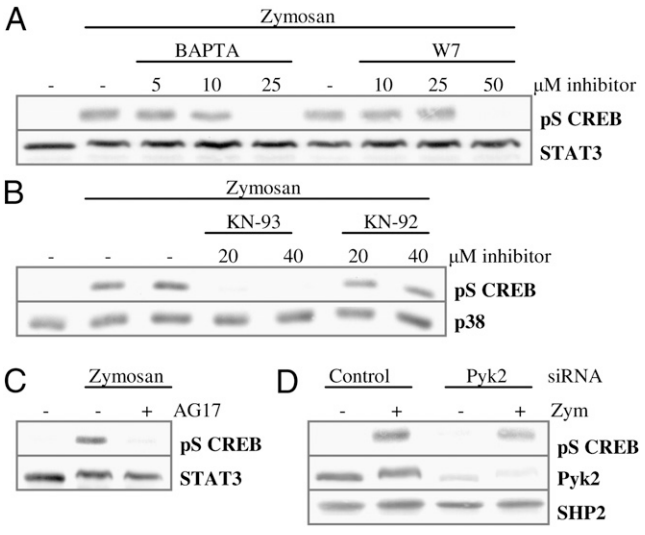

FIGURE 6. Zymosan activates CREB in a manner dependent on calcium, CaMK, and Pyk2. *A–C*, Immunoblot of lysates of primary human macrophages pretreated for 1 h with 5–25 μM BAPTA or 10–50 μM W7 (*A*), 20–40 μM KN-93 or KN-92 (*B*), or 10 μM AG17 (*C*) and then stimulated with 400 μg/ml zymosan for 10 min. *D*, Immunoblot of lysates of human monocytes transfected with control or Pyk2-specific siRNA and 4 d later stimulated for 10 min with 400 μg/ml zymosan. Data are representative of two (*D*) or three (*A–C*) different individual experiments.

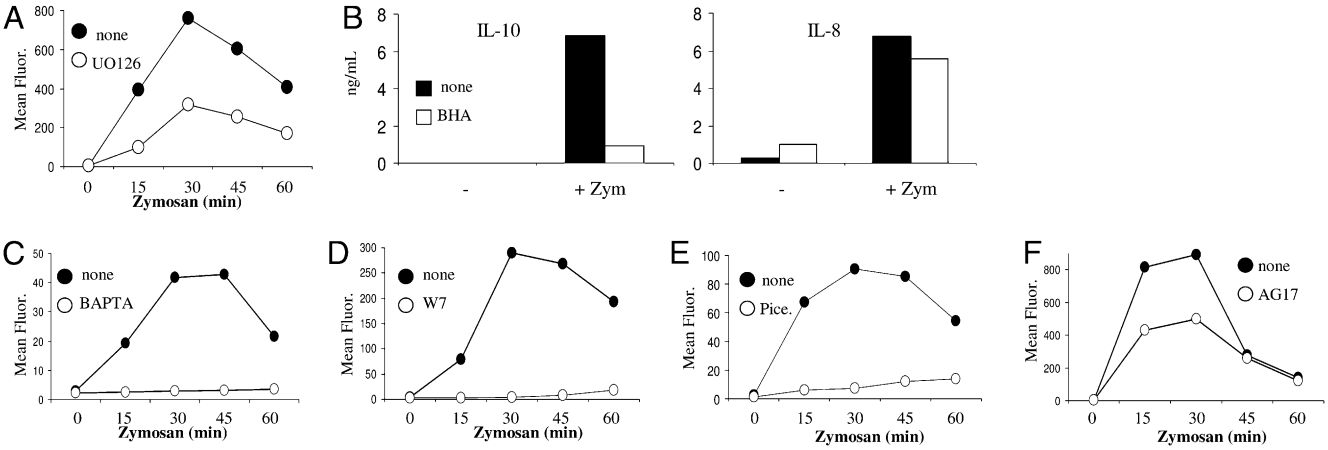

FIGURE 7. ROS are important for IL-10 production and require Ca^{2+}-dependent signaling pathways. *A* and *C–F*, Primary human macrophages were pretreated for 1 h with 20 μM UO126 (*A*), 10 μM BAPTA (*C*), 25 μM W7 (*D*), 40 μM piceatannol (*E*), or 10 μM AG17 (*F*), labeled with aminophenyl fluorescein for 30 min, and then stimulated with zymosan for indicated times. Data represent mean fluorescence intensity of labeled cells as detected by flow cytometry. *B*, ELISAs of IL-10 and IL-8 production from macrophages pretreated with 50 μM BHA and then stimulated with zymosan for 7 h. Data are representative of at least three different individual experiments.

was also suppressed by the Pyk2 inhibitor AG17 (Fig. 6*C*) and was modestly diminished in macrophages in which Pyk2 expression was decreased using siRNA (Fig. 6*D*). These results show that the zymosan-induced calcium–CaMK–Pyk2 signaling pathway regulates CREB activation in primary human macrophages and, together with previous findings (31, 32), suggest a role for CREB in mediating zymosan-induced IL-10 production. Although CREB is a downstream target of ERK in other systems, zymosan-induced CREB phosphorylation was not consistently suppressed by the MEK-ERK inhibitor U0126 (data not shown), suggesting that calcium signaling may promote IL-10 production by two or more distinct pathways downstream of Pyk2.

Calcium- and ERK-dependent respiratory burst mediates IL-10 production

Unlike soluble TLR ligands, zymosan can generate ROS (4, 6, 7). The function of ROS in cellular responses to zymosan is not known. We tested whether ROS play a role in zymosan-induced IL-10 production. We detected zymosan-induced ROS production in human macrophages that peaked 30–45 min following stimulation (Fig. 7*A*). In the presence of U0126, ROS induction was suppressed (Fig. 7*A*). This suggests a role for ERK upstream of ROS generation by zymosan, which is consistent with MAPK-dependent ROS generation in neutrophils (33, 34). To determine whether ROS play a role in ERK-mediated IL-10 production induced by zymosan, we used the ROS scavenger BHA. Inhibition of ROS generation resulted in a substantial decrease in zymosan-induced IL-10 production (Fig. 7*B*), suggesting that ROS contribute to IL-10. BHA had no effect on IL-8 production, demonstrating that the antioxidant selectively blocks IL-10 levels and is not toxic (Fig. 7*B*). Similar to ERK activation, zymosan-induced ROS generation was dependent on intracellular calcium, CaM, Syk, and Pyk2 (Fig. 7*C–F*). Thus, ROS contribute to the induction of IL-10 production by calcium-mediated pathways that are activated by zymosan (Fig. 8).

Discussion

Recent work has highlighted the role of Dectin-1 in mediating phagocytosis and macrophage activation by zymosan, but the signaling pathways that are activated by Dectin-1 and how they are linked to cellular functions remain unclear. In this study, we have identified a zymosan-activated, calcium-dependent CaMKII-Pyk2-ERK–ROS signaling pathway emanating from Dectin-1. This pathway branched at the level of Pyk2, as zymosan activated CREB in a calcium- and Pyk2-dependent manner but independently of ROS (Fig. 8). We linked calcium signaling with the production of IL-10, a salient characteristic of macrophage and DC responses to zymosan, the regulation of which is complex and not well understood. Our results showing that blocking Dectin-1 or inhibiting the Src and Syk kinases that mediate proximal ITAM signaling suppressed calcium-dependent IL-10 production implicate a role for Dectin-1 and its ITAM-like motif in generating calcium-mediated responses in human macrophages. Thus, our results identify a new signaling pathway that is activated by zymosan and Dectin-1 and provide a function for this pathway in specifically regulating the production of an anti-inflammatory cytokine.

The role of calcium signaling pathways in lymphocyte activation is well established, but less is known about the role of these pathways in innate immune cells, such as macrophages and DCs. Basal calcium signaling has been recently shown to modulate IFN responses (35) and to downregulate TLR signaling (36), but calcium-activated pathways are not directly triggered by prototypic PRRs, such as TLRs. Calcium flux in response to zymosan has been demonstrated in both mouse macrophages and mouse DCs,

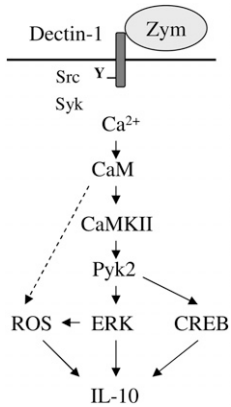

FIGURE 8. In human primary macrophages, zymosan activates a calcium-dependent CaMKII–Pyk2–ERK signaling pathway downstream of Dectin-1 and ITAM-associated kinases Src and Syk. Calcium pathways also induce CREB phosphorylation and generation of ROS. ERK, CREB, and ROS contribute to calcium-mediated IL-10 production.

but we were unable to detect an inducible flux in human macrophages (E. K. Kelly, data not shown). Although this difference may be secondary to technical challenges of detecting calcium signals in our system where zymosan interacts asynchronously with human macrophages, it may also reflect species-specific differences in calcium signaling in which basal calcium-mediated signaling is important in human macrophages, whereas inducible signaling occurs in murine cells. Such differences are also supported by the differential zymosan-induced phosphorylation of PLC-γ in human and murine cells. Therefore, the mechanism linking Syk to CaMK/Pyk2 activation will need to be investigated in future work.

Taken together with previous reports that established a role for ERK and CREB in IL-10 production (17, 18, 27, 29, 31, 32), our results link zymosan-induced calcium signaling to IL-10 generation via activation of ERK and CREB and for the first time identify a function for ROS in inducing production of this cytokine. These results provide insight into mechanisms by which Dectin-1 may augment IL-10 production in response to TLRs, which do not directly engage calcium signaling pathways. The data also suggest that calcium pathways may contribute to induction of IL-10 by other ITAM-coupled receptors, for example, FcγRs that activate ERK to augment TLR-induced IL-10 production (27, 29).

Interestingly, the zymosan-induced calcium signal leading to IL-10 production was transduced by CaMKII and Pyk2, whose roles in macrophage function and cytokine production are just emerging. Pyk2, known to be important in cell motility and adhesion (22), has been mostly unexplored in immune function and inflammation; our results implicate Pyk2 in zymosan-induced activation of ERK and downstream IL-10 production in human macrophages. In contrast, we found that Pyk2 was dispensable for IL-10 production in mouse bone marrow-derived macrophages (E.K. Kelly, unpublished data). Consistent with this result, Pyk2 was not strongly activated by zymosan in bone marrow-derived macrophages, further supporting a species-specific role for this pathway in human macrophages.

In combination with previous reports showing that Syk can activate Pyk2 in other systems (37, 38), our data support a Dectin-1–Src–Syk–CaMKII–Pyk2 upstream signaling pathway activated by zymosan in human macrophages (Fig. 8). Pyk2 links this pathway to activation of ERK, ROS, and CREB, which in turn induce IL-10 production. The exact mechanism of regulation of ERK by Pyk2 is still unclear, but previous work has shown that in other cell types Syk and Pyk2 can activate ERK via either Ras-Raf or the MAPKKK Tpl2 (39, 40). Raf-1 has recently been shown to be necessary for Dectin-1–dependent activation of NF-κB in human DCs but in a Syk-independent manner (41). However, we did not detect activation of Raf proteins in macrophages in response to zymosan, and a Tpl2 inhibitor had no effect (E.K. Kelly, unpublished data); thus, future work will be required to understand the mechanism by which Pyk2 activates ERK.

Zymosan interaction with murine macrophages and DCs generates ROS in a manner dependent on Dectin-1 and Syk (4, 6, 12, 42). The role of reactive oxygen in microbial killing and in signal transduction is well established (43, 44), but the role of ROS in mediating macrophage responses to zymosan has not been identified. In this paper, we show that zymosan-stimulated generation of ROS contributes to the production of IL-10. ROS have been shown to modulate transcriptional responses to inflammatory cytokines by activating signaling mediators, such as NF-κB and MAPK (43). The mechanism by which ROS activate production of the anti-inflammatory IL-10 remains to be clarified but appears unlikely to be mediated by MAPKs or CREB, because the antioxidant BHA had no effect on zymosan-induced phosphorylation of these proteins (E.K. Kelly, unpublished data).

Although traditionally thought to be an inflammatory stimulus, zymosan induces robust amounts of the anti-inflammatory cytokine IL-10 that can attenuate cytokine production and inflammation and modulate the differential induction of Th subsets during the emergence of acquired immunity. Our results link zymosan-induced IL-10 production with calcium-mediated signaling that occurs at least in part downstream of the ITAM-containing Dectin-1 receptor. Various innate immune receptors contain ITAM and ITAM-like motifs (45, 46). Our results suggest that calcium signaling downstream of these receptors may play a role in regulating IL-10 and cytokine production.

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Disclosures

The authors have no financial conflicts of interest.

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