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Dectin-1 Stimulation by *Candida albicans* Yeast or Zymosan Triggers NFAT Activation in Macrophages and Dendritic Cells[1]

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Dectin-1 Stimulation by *Candida albicans* Yeast or Zymosan Triggers NFAT Activation in Macrophages and Dendritic Cells[1]

Helen S. Goodridge, Randi M. Simmons, and David M. Underhill[2]

Innate immune pattern recognition receptors play critical roles in pathogen detection and initiation of antimicrobial responses. We and others have previously demonstrated the importance of the β-glucan receptor Dectin-1 in the recognition of pathogenic fungi by macrophages and dendritic cells and have elucidated some of the mechanisms by which Dectin-1 signals to coordinate the antifungal response. While Dectin-1 signals alone are sufficient to trigger phagocytosis and Src-Syk-mediated induction of antimicrobial reactive oxygen species, collaboration with TLR2 signaling enhances NF-κB activation and regulates cytokine production. In this study we demonstrate that Dectin-1 signaling can also directly modulate gene expression via activation of NFAT. Dectin-1 ligation by zymosan particles or live *Candida albicans* yeast triggers NFAT activation in macrophages and dendritic cells. Dectin-1-triggered NFAT activation plays a role in the induction of early growth response 2 and early growth response 3 transcription factors, and cyclooxygenase-2. Furthermore, we show that NFAT activation regulates IL-2, IL-10 and IL-12 p70 production by zymosan-stimulated dendritic cells. These data establish NFAT activation in myeloid cells as a novel mechanism of regulation of the innate antimicrobial response. ***The Journal of Immunology,*** **2007, 178: 3107–3115.**

Innate immune receptors, including TLRs and lectins, play critical roles in the recognition of pathogens and their products, and the induction of antimicrobial responses. The β-glucan receptor Dectin-1 is a C-type lectin that we and others have previously demonstrated plays a crucial role in the detection of zymosan and live pathogenic fungi (*Candida albicans*, *Aspergillus fumigatus*, *Pneumocystis carinii*) by macrophages and dendritic cells (1–8). A recent report also implicated Dectin-1 in macrophage activation by mycobacteria (9). Together these reports demonstrate that Dectin-1 collaborates with TLRs in the induction of proinflammatory cytokines. We have previously reported collaboration between Dectin-1 and TLR2 in the activation of NF-κB (2). Additionally, a recent report has suggested that Dectin-1 can directly activate NF-κB in dendritic cells via the signaling adaptor molecule CARD9 (10). Recognition of zymosan and live *C. albicans* yeast by Dectin-1 also triggers phagocytosis and the production of reactive oxygen species (ROS)[3] by mechanisms independent of TLR signaling (1–3, 11). Thus, Dectin-1 is a key coordinator of macrophage/dendritic cell antimicrobial responses.

Dectin-1 has an ITAM-like motif in its intracellular tail and mutation of this motif results in loss of its signaling activity (1–3, 11, 12). ITAM-based signaling is classically associated with lymphocyte Ag receptors (TCR and BCR) ligation, resulting in activation of Src and Syk kinases. We recently demonstrated that Dectin-1 ligation triggers Src-Syk activation in macrophages and that Syk signaling is required for ROS induction (11). Rogers et al. also observed zymosan-stimulated Syk activation in their recent investigation of Dectin-1 signaling in dendritic cells (3). Dectin-1 ligation triggered Syk-dependent induction of IL-2 and IL-10.

We now report that recognition of zymosan and live *C. albicans* yeast by Dectin-1 triggers activation of NFAT in macrophages and dendritic cells. The NFAT family of transcription factors comprises 4 closely related members with overlapping functions (NFATc1 through NFATc4) and the distantly related NFAT5, which is important for cellular responses to hypertonic stress (reviewed in Ref. 13). The role of NFAT activation has been defined most comprehensively in T cells: thymocyte development, T cell differentiation, T cell activation, and anergy are all regulated by NFAT (reviewed in Ref. 13). In T cells, NFAT proteins are normally inactive and highly phosphorylated, and TCR stimulation triggers dephosphorylation by the calcium-dependent phosphatase calcineurin. Dephosphorylated NFAT proteins enter the nucleus where they form strong collaborative complexes on DNA with a variety of transcription factors to integrate signaling pathways. In addition, NFAT transcription factors also regulate cell differentiation outside the immune system, with established roles in processes such as cardiac valve development, skeletal muscle differentiation, and osteoclastogenesis (reviewed in Ref. 14).

A role for NFAT activation in the innate antimicrobial response has not previously been demonstrated. In this study we show that Dectin-1-mediated NFAT activation in macrophages and dendritic cells regulates the induction of early growth response (Egr) family transcription factors Egr2 and Egr3, as well as key inflammatory mediators cyclooxygenase-2 (COX-2), IL-2, IL-10, and IL-12 p70.

Materials and Methods

Mice and cells

Wild-type (WT) C57BL/6, MyD88$^{-/-}$, and TLR2$^{-/-}$ mice were maintained at the Institute for Systems Biology (Seattle, WA) and Cedars-Sinai Medical Center (Los Angeles, CA). NFAT-luciferase reporter transgenic mice (15) were a gift from Dr. J. Molkentin (University of Cincinnati, Cincinnati, OH). Bone marrow-derived macrophages and dendritic cells

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[3] Abbreviations used in this paper: ROS, reactive oxygen species; COX-2, cyclooxygenase-2; Egr, early growth response; miRNA, microRNA; PGE_2, prostaglandin E_2; WT, wild type; CsA, cyclosporin A.

were prepared from the femurs of WT, MyD88$^{-/-}$, TLR2$^{-/-}$, and NFAT-luciferase reporter transgenic mice by culture of bone marrow cells in complete medium (RPMI 1640, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; Mediatech) supplemented with 50 ng/ml recombinant human M-CSF (macrophages) or 10 ng/ml recombinant mouse GM-CSF (dendritic cells) (PeproTech). RAW264.7 murine macrophage cell lines were cultured in complete RPMI 1640. RAW264.7 cells stably expressing WT Dectin-1, signaling-deficient Dectin-1Δ38, and streptavidin binding peptide (SBPc)-tagged Dectin-1 were described previously (2, 11). Dectin-1 expression was suppressed in RAW264.7 cells by retroviral expression (pMSCV-LMP; Open Biosystems) of a microRNA (miRNA) targeting Dectin-1. The target sequence was CTATTTAGCTTCTCAGGAA. Control cells were also generated by transduction with the parental retrovirus. Stable populations of cells were generated without cloning to prevent artifacts arising from differential behavior of RAW264.7 subclones. Identical results were obtained with two independently derived Dectin-1 miRNA-expressing populations (data not shown).

Reagents and Candida albicans *cultures*

Pam_3CSK_4 was from InvivoGen, and the soluble β-glucan laminarin from *Laminaria digitata* was from Sigma-Aldrich. Cyclosporin A and 11R-VIVIT were from EMD Biosciences. Zymosan (Sigma-Aldrich) was prepared as described previously (16). Depleted zymosan was prepared by boiling 250 μg of zymosan in 1 ml of 10 M sodium hydroxide for 30 min and washing three times with sterile PBS. The absence of TLR-stimulating capability of depleted zymosan was verified by stimulation of RAW264.7 cells stably expressing an ELAM-luciferase reporter (2) (data not shown). Fresh *Candida albicans* yeast and hyphae were prepared as described previously (4). Briefly, yeast were seeded into Sabouraud dextrose broth and incubated with shaking overnight at 37°C; hyphae were prepared by overnight culture of *C. albicans* in RPMI 1640 at 37°C with shaking.

Streptavidin-coated bead stimulation

Streptavidin-coated beads from Dynal (Invitrogen Life Technologies) were washed with complete DMEM (Mediatech) before use. Cells were plated in complete DMEM for bead stimulation because RPMI contains biotin which interferes with the assays.

Microarray

Bone marrow-derived macrophages from MyD88$^{-/-}$ mice were cultured in 6-well dishes and stimulated with 100 μg/ml zymosan for 2 h. Total RNA was then isolated using TRIzol (Invitrogen Life Technologies). Two independent samples each for unstimulated and zymosan-stimulated macrophages were prepared. Gene expression was analyzed on Affymetrix GeneChip Mouse 430 2.0 microarray chips according to the manufacturer's instructions. The data have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE6376. Data were analyzed using TIGR MeV: MultiExperiment Viewer software.

Real-time RT-PCR

RNA purification, reverse transcription and TaqMan real-time RT-PCR were performed as described previously (2). Primer/probe sets for murine Egr1, Egr2, Egr3, Dectin-1, and COX-2 were from Applied Biosystems. EF1α primer and probe sequences were as follows: forward 5′-GCAAAAACGACCCACCAATG-3′, reverse 5′-GGCCTGGATGGTTCAGGATA-3′, probe 5′-FAM-CACCTGAGCAGTGAAGCCAGCTGCT-TAMRA-3′.

Measurement of reactive oxygen production

Cells were pretreated with 25 U/ml murine IFN-γ (PeproTech) for 18 h before stimulation, and the production of reactive oxygen species was assayed by luminol-ECL as described previously (2).

NFAT-luciferase assays

RAW264.7 cells in 24-well plates (1×10^5 cells/well) were rested overnight before LipofectAMINE 2000 (Invitrogen Life Technologies) transfection with 0.6 μg of pNFAT-luciferase (Stratagene) and 0.2 μg pTK-*Renilla* luciferase (Promega) for 18 h according to the manufacturer's directions. Following 2-h stimulations, cells were lysed, and luciferase activity was assessed using a Dual Luciferase Reporter Assay (Promega) and a Veritas microplate luminometer (Turner BioSystems), according to the manufacturers' instructions. NFAT-luciferase reporter activity in bone marrow-derived macrophages from NFAT-luciferase reporter transgenic mice was assayed using a Steady-Glo Luciferase Assay (Promega).

Table I. *Top 10 definitively identified genes most strongly up-regulated by zymosan in MyD88$^{-/-}$ macrophages*[a]

Gene	Fold Induction
*Egr3**	63.8
CSF1	21.1
Heat shock protein 1A	14.7
*Egr2**	11.5
*PG-endoperoxide synthase (COX-2)**	11.2
Heat shock protein 1B	10.9
Chemokine (CXC motif) ligand 1 (GroA)	10.5
Egr1	9.5
Myeloperoxidase	9.4
*CD69 Ag**	9.0

[a] Asterisks indicate genes known to be regulated by NFAT in T cells (20, 23, 24).

Western blotting

Whole cell lysates were assessed by Western Blotting using an anti-COX-2 Ab from Cayman Chemical and an anti-Erk1/2 Ab from Cell Signaling Technology.

Prostaglandin E_2 (PGE_2) and cytokine assays

PGE2 levels in culture supernatants were measured using an EIA kit from Cayman Chemical. Cytokine levels in culture supernatants were assayed using ELISA kits from R&D Systems according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using a student's *t* test. Unless otherwise indicated in the figures, analyses were performed comparing experimental samples to unstimulated or control samples.

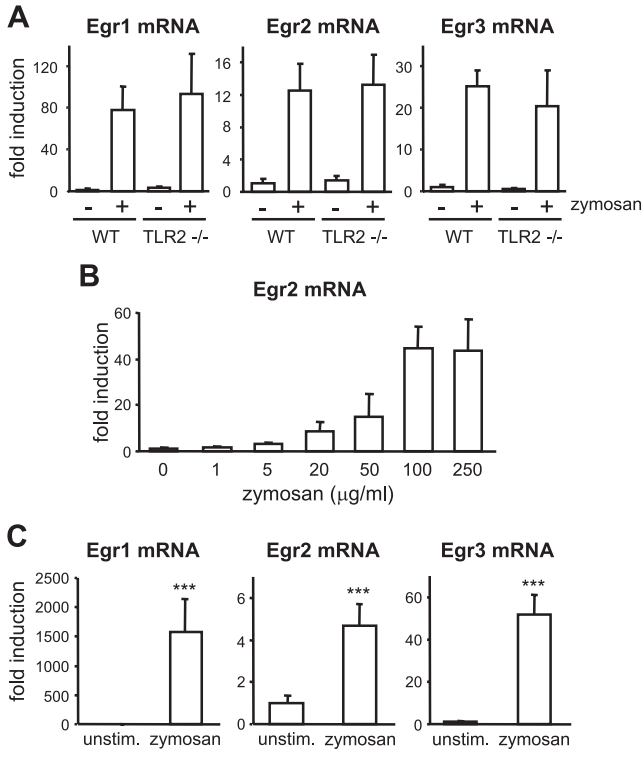

FIGURE 1. Zymosan stimulation triggers Egr transcription factor gene expression in macrophages and dendritic cells. *A*, WT and TLR2$^{-/-}$ bone marrow-derived macrophages were stimulated for 1 h with 100 μg/ml zymosan. *B*, WT bone marrow-derived macrophages were stimulated with the indicated concentrations of zymosan for 1 h. *C*, WT bone marrow-derived dendritic cells were stimulated for 1 h with 100 μg/ml zymosan. Egr mRNA levels were assessed by real-time RT-PCR, normalized to EF1α, and are expressed as fold induction relative to unstimulated controls. ***, $p < 0.001$. Data are representative of three independent experiments.

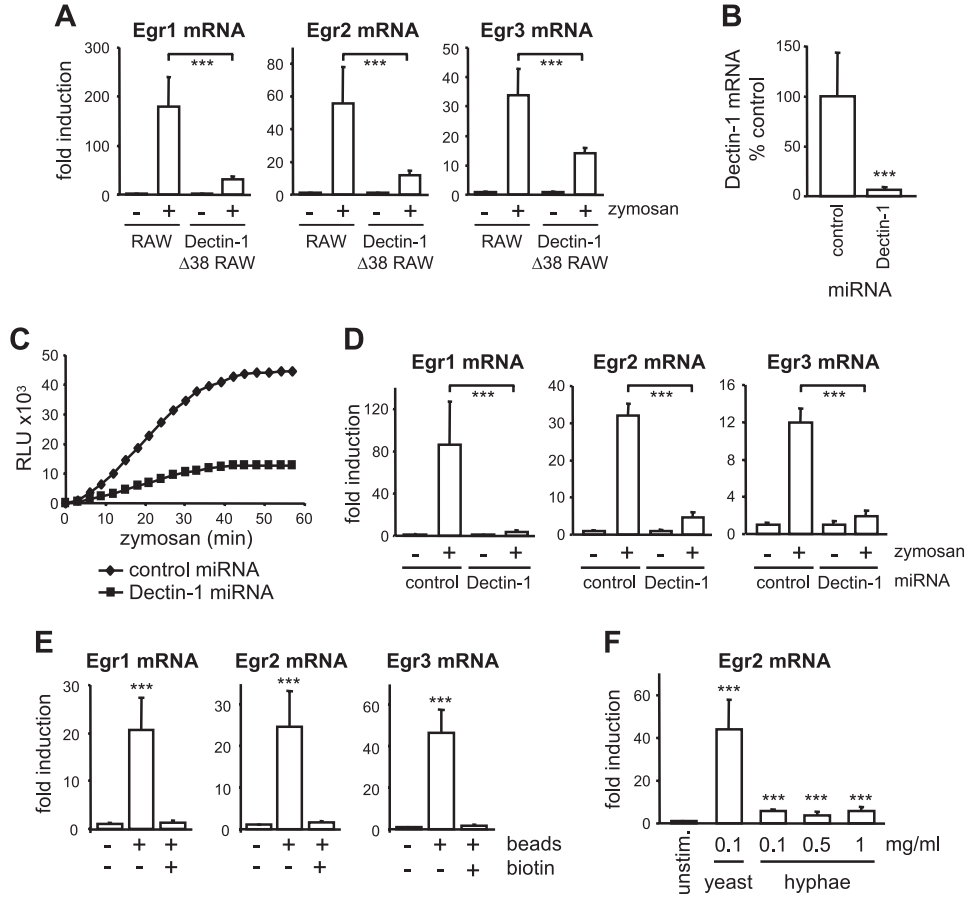

FIGURE 2. Dectin-1 signaling is both necessary and sufficient for zymosan-triggered Egr gene induction. *A*, Parental RAW264.7 cells and RAW264.7 cells stably expressing signaling-deficient Dectin-1Δ38 were stimulated with 100 μg/ml zymosan for 1 h. Egr mRNA levels were assessed by real-time RT-PCR, normalized to EF1α mRNA, and are expressed as fold induction relative to unstimulated controls. *B*, Dectin-1 levels in unstimulated control (empty vector) and Dectin-1 miRNA-expressing RAW264.7 cells were assessed by real-time RT-PCR, normalized to EF-1α mRNA, and are expressed as a percentage of control (empty vector) cells. *C* and *D*, Control (empty vector) and Dectin-1 miRNA-expressing RAW264.7 cells were stimulated with 100 μg/ml zymosan as indicated (*C*) or for 1 h (*D*). ROS production was assayed by luminol-ECL (*C*) and Egr mRNA levels were assessed as in *A* (*D*). *E* and *F*, RAW264.7 cells stably expressing SBPc-tagged Dectin-1 were stimulated with 170 μg/ml streptavidin beads for 1 h in the presence or absence of 100 μM biotin (*E*) and bone marrow-derived macrophages were stimulated for 1 h with *C. albicans* yeast or hyphae at the concentrations indicated (*F*). Egr mRNA levels were assessed as in *A*. ***, $p < 0.001$. Data are representative of at least three independent experiments.

Results

Microarray analysis of TLR-independent gene regulation in zymosan-treated macrophages

The transcriptional response of macrophages to zymosan and live pathogenic fungi has largely been attributed to the TLR-mediated activation of NF-κB, although we and others have demonstrated that Dectin-1 signals can collaborate with TLR signals to amplify the transcriptional response (1–3, 17, 18). However, a recent paper suggested that Dectin-1 signals can directly activate NF-κB via CARD9 in dendritic cells (10), indicating that TLR signals may not be absolutely required for gene induction in response to zymosan/fungi. We therefore investigated macrophage transcriptional responses to zymosan in the absence of TLR signaling by performing Affymetrix microarray analysis of gene induction in macrophages from MyD88$^{-/-}$ mice. Over 200 genes were induced or repressed >2-fold following zymosan stimulation of MyD88-deficient bone marrow-derived macrophages for 2 h (Supplementary Table I).[4] The top 10 most strongly up-regulated MyD88-independent zymosan-induced genes are listed in Table I. These data demonstrate that non-TLR innate immune signaling in response to zymosan leads to a robust transcriptional response.

Induction of Egr transcription factors by zymosan and Candida albicans *yeast is mediated by Dectin-1 and not by TLR2*

Strikingly, three of the top 10 most strongly up-regulated MyD88-independent genes (Table I) are members of the Egr family of transcription factors—Egr1, Egr2, and Egr3. TaqMan real-time RT-PCR analysis of bone marrow-derived macrophages confirmed that zymosan induces mRNA for Egr1, Egr2, and Egr3 (Fig. 1*A*). Egr induction was maximal at a dose of 100 μg/ml zymosan (Fig. 1*B*), which we have also previously determined to be optimal for the induction of reactive oxygen species and TNF-α production. All three Egrs were also induced by zymosan stimulation of bone marrow-derived dendritic cells (Fig. 1*C*). Time course analysis indicated that mRNA levels for these transcription factors are maximal ~1 h after zymosan stimulation (data not shown). Transcript levels for all three Egrs were comparable in zymosan-treated macrophages from WT and TLR2$^{-/-}$ mice (Fig. 1*A*). These data indicate that while some transcriptional responses to zymosan such as TNF-α and IL-12 p40 induction are highly dependent on TLR2

[4] The online version of this article contains supplemental material.

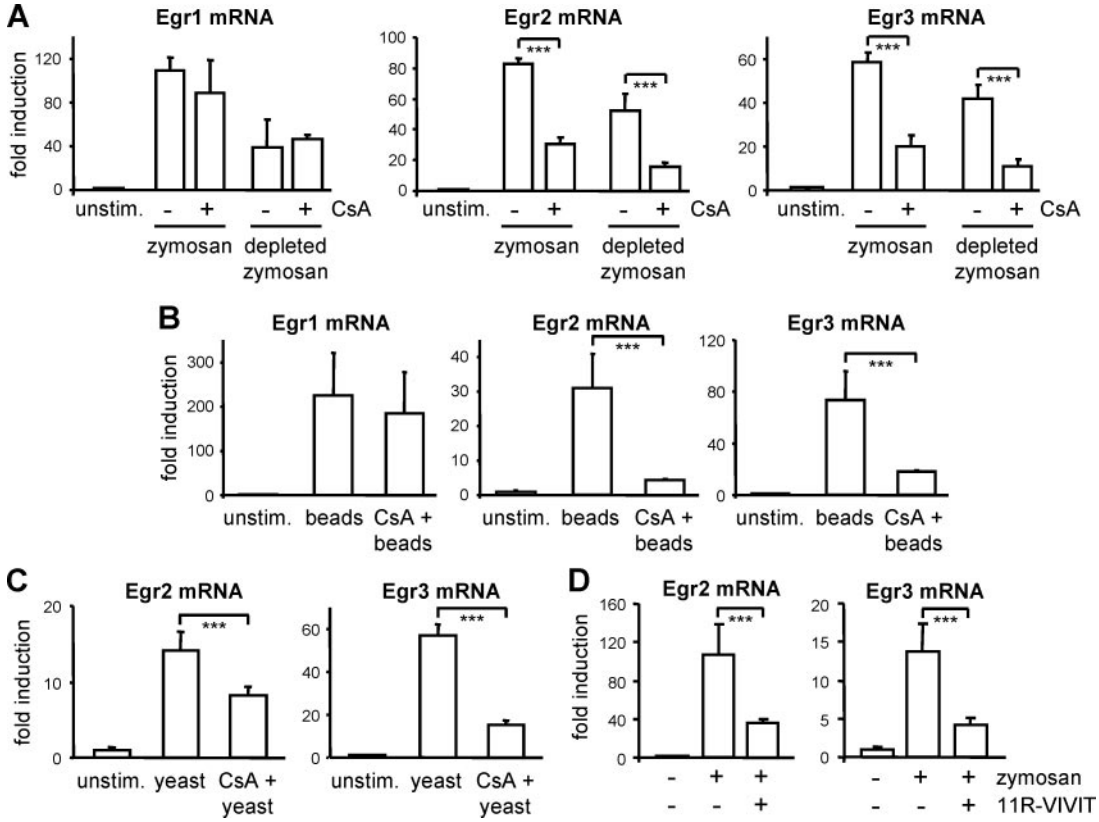

FIGURE 3. Induction of Egr2 and Egr3, but not Egr1, by zymosan and live *C. albicans* yeast is NFAT dependent. *A*, RAW264.7 cells stably expressing WT Dectin-1 were stimulated with 100 μg/ml zymosan or depleted zymosan for 1 h in the presence or absence of 1.5 μM CsA. *B*, RAW264.7 cells stably expressing SBPc-tagged Dectin-1 were stimulated with 170 μg/ml streptavidin beads for 1 h in the presence or absence of 1.5 μM CsA. *C*, RAW264.7 cells stably expressing WT Dectin-1 were stimulated with 100 μg/ml live *C. albicans* yeast for 1 h in the presence or absence of 1.5 μM CsA. *D*, RAW264.7 cells stably expressing WT Dectin-1 were stimulated with 100 μg/ml zymosan for 1 h in the presence or absence of 5 μM 11R-VIVIT. Egr mRNA levels were assessed by real-time RT-PCR, normalized to EF1α mRNA, and are expressed as fold induction relative to unstimulated controls. Identical data were obtained using bone marrow-derived macrophages and dendritic cells (data not shown). ***, $p < 0.001$. Data are representative of at least three independent experiments.

(2, 16), non-TLR pattern recognition receptors can independently stimulate specific genes. We therefore investigated whether Dectin-1 transduces signals responsible for Egr induction.

First we examined Egr induction in a RAW264.7 murine macrophage cell line stably expressing a signaling-deficient form of Dectin-1 that lacks the cytoplasmic tail (Dectin-1Δ38) which we have previously demonstrated interferes with signaling through endogenous Dectin-1(2). Induction of all three Egrs by zymosan was dramatically reduced in Dectin-1Δ38 RAW264.7 cells (Fig. 2*A*). As an alternative approach, we generated RAW264.7 cells in which Dectin-1 expression was specifically suppressed by a retrovirally transferred microRNA (miRNA) targeting Dectin-1. Real-time RT-PCR analysis confirmed the knockdown of Dectin-1 mRNA in these cells (Fig. 2*B*), and measurement of zymosan-induced ROS production verified the reduction of Dectin-1 signaling (Fig. 2*C*). Zymosan-induced expression of all three Egrs was abolished by the Dectin-1 miRNA (Fig. 2*D*), confirming that Dectin-1 is necessary for Egr induction.

To determine whether Dectin-1 signals are sufficient for Egr induction, we used RAW264.7 cells stably expressing Dectin-1 tagged extracellularly with a streptavidin-binding peptide (SBPc-Dectin-1 RAW264.7 cells). We have previously demonstrated that stimulation of these cells with streptavidin-coupled beads specifically triggers Dectin-1-mediated phagocytosis of the beads and ROS production (11). Exposure of SBPc-Dectin-1 RAW264.7 cells to streptavidin beads resulted in induction of all three Egrs (Fig. 2*E*). The addition of biotin, which binds to the beads and blocks their association with the SBPc-tagged receptor, completely inhibited Egr induction (Fig. 2*E*).

We also investigated whether live pathogenic fungi trigger Egr induction. We have previously shown that Dectin-1 recognizes β-glucan exposed on the surface of the yeast form of the dimorphic fungus *Candida albicans*, triggering the production of ROS (4). In contrast, the filamentous growth form of *C. albicans* (hyphae), which lacks exposed β-glucan on its surface, does not interact with Dectin-1 and therefore fails to trigger ROS production. We therefore investigated whether *C. albicans* yeast and hyphae induce Egr synthesis. Stimulation of bone marrow-derived macrophages with live *C. albicans* yeast induced Egr1, Egr2, and Egr3, but an equivalent mass of hyphae stimulated only very low levels of Egr induction, and increasing the mass of hyphae up to 10-fold did not increase Egr induction (Fig. 2*F* and data not shown).

Taken together, these data indicate that Dectin-1 is necessary and sufficient for the induction of Egr transcription factors by zymosan and live pathogenic yeast.

Induction of Egr2 and Egr3, but not Egr1, is suppressed by the NFAT inhibitors cyclosporin A and 11R-VIVIT

Egr1 has been reported to be induced by a variety of stimuli, including LPS (19). In contrast, few studies have investigated the induction of Egr2 and Egr3. In T cells, induction of Egr2 and Egr3, but not Egr1, following ligation of the TCR, has been shown to be

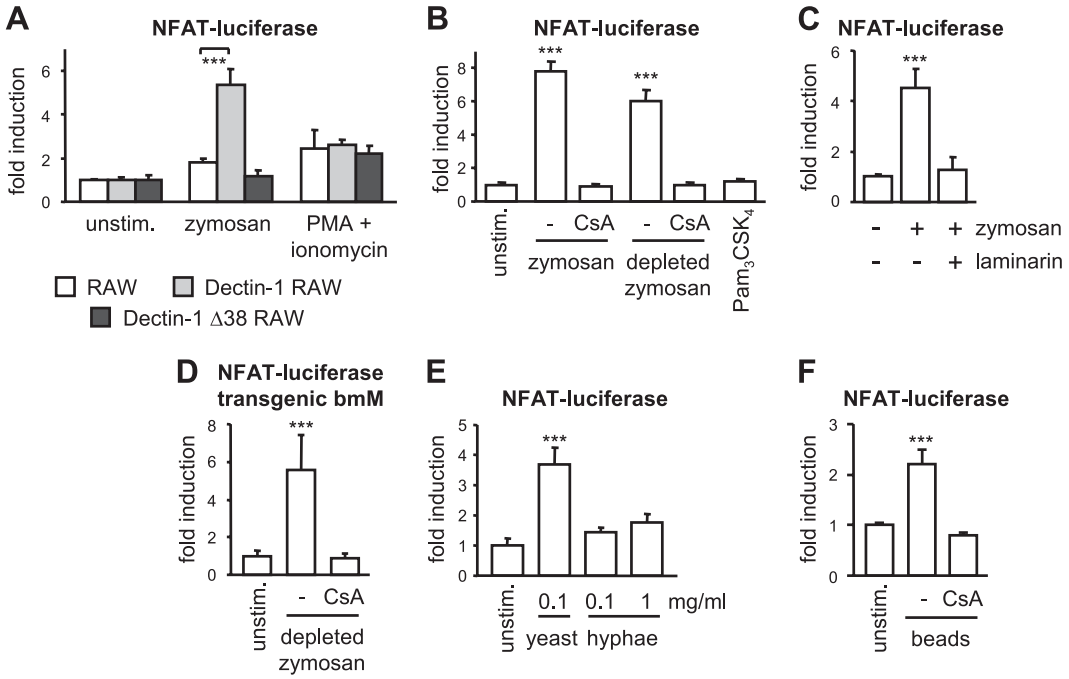

FIGURE 4. Dectin-1 signaling activates NFAT transcription factors. *A*, Parental RAW264.7 cells and RAW264.7 cells stably expressing WT Dectin-1 or Dectin-1Δ38 were transfected overnight with an NFAT-luciferase reporter and a thymidine kinase-*Renilla* luciferase (TK-RL) control reporter before stimulation with 100 μg/ml zymosan, or 200 nM PMA plus 5 μM ionomycin, for 2 h. NFAT-luciferase activity was assessed using a dual luciferase assay, normalizing NFAT reporter-driven firefly luciferase activity to TK-RL activity, and is expressed as fold induction relative to unstimulated cells. *B* and *C*, NFAT-luciferase/TK-RL-transfected Dectin-1 RAW264.7 cells were stimulated for 2 h with 100 μg/ml zymosan or depleted zymosan, or 100 ng/ml Pam_3CSK_4, in the presence or absence of 1.5 μM CsA or 500 μg/ml laminarin as indicated. NFAT-luciferase reporter activity was assessed as in *A*. *D*, Bone marrow-derived macrophages from NFAT-luciferase reporter transgenic mice were stimulated for 2 h with 100 μg/ml depleted zymosan in the presence or absence of 1.5 μM CsA. NFAT-luciferase reporter activity in transgenic macrophages is expressed as fold induction relative to unstimulated cells. *E* and *F*, NFAT-luciferase/TK-RL-transfected Dectin-1 RAW264.7 cells were exposed to live *C. albicans* yeast or hyphae at the concentrations indicated for 2 h (*E*) and NFAT-luciferase/TK-RL-transfected RAW264.7 cells stably expressing SBPc-tagged Dectin-1 were stimulated for 2 h with 170 μg/ml streptavidin beads in the presence or absence of 1.5 μM CsA (*F*). NFAT-luciferase reporter activity was assessed as in *A*. ***, $p < 0.001$. Data are representative of at least three independent experiments.

mediated by NFAT transcription factors (20). To explore the possibility that zymosan-induced Egr induction in macrophages is mediated by NFAT we stimulated macrophages with zymosan in the presence of cyclosporin A (CsA), which blocks the ability of calcineurin to dephosphorylate NFAT, thereby blocking NFAT nuclear translocation and gene transactivation. CsA strongly inhibited the induction of Egr2 and Egr3, but not Egr1, in WT Dectin-1-expressing RAW264.7 cells stimulated with zymosan (Fig. 3*A*). Furthermore, stimulation with "depleted zymosan," an alkali-treated β-glucan particle that we have previously used to specifically activate β-glucan receptors and not TLRs (2), also triggered CsA-sensitive Egr2 and Egr3, and CsA-insensitive Egr1 induction (Fig. 3*A*). Identical results were obtained using zymosan-stimulated bone marrow-derived macrophages and dendritic cells (data not shown). To determine directly that Dectin-1-triggered induction of Egr2 and Egr3, but not Egr1, is dependent on NFAT, we cross-linked SBPc-tagged Dectin-1 with streptavidin beads to specifically activate Dectin-1, and observed CsA-inhibitable Egr2 and Egr3 induction (Fig. 3*B*). Furthermore, Egr2 and Egr3 induction by live *C. albicans* yeast was also suppressed by CsA treatment (Fig. 3*C*).

Although CsA is a well-established and widely used inhibitor of NFAT activation, concerns have been voiced about its specificity. Therefore, to verify the CsA data we also used a peptide inhibitor of NFAT activation, called VIVIT. VIVIT is a 16 amino acid, affinity-selected peptide, which binds with high affinity and specificity to the NFAT docking site on calcineurin, thereby preventing the association of NFAT isoforms with calcineurin and blocking their activation (21). We treated macrophages with an 11-arginine-conjugated VIVIT peptide (11R-VIVIT), which is readily internalized into cells (22). 11R-VIVIT treatment dramatically reduced Egr2 and Egr3 induction, while having little effect on Egr1 (Fig. 3*D* and data not shown). Collectively, the above data demonstrate that full induction of Egr2 and Egr3 by Dectin-1 requires NFAT.

Dectin-1 signaling triggers NFAT activation in macrophages and dendritic cells stimulated with zymosan and C. albicans *yeast*

Although NFAT transcription factors are well-known regulators of gene induction in adaptive immunity, as well as having roles in cardiac development, skeletal muscle differentiation and osteoclastogenesis (reviewed in Ref. 14), a role in innate immunity has not previously been demonstrated. We therefore next verified directly that Dectin-1 activates NFAT in macrophages.

We assessed NFAT activity by transfecting an NFAT-luciferase reporter into parental RAW264.7 cells, or RAW264.7 cells stably expressing either WT Dectin-1 or Dectin-1Δ38. Reporter activity was relatively low in zymosan-stimulated parental RAW264.7 cells, which express low levels of Dectin-1(1), but NFAT activity was dramatically enhanced by WT Dectin-1 overexpression (Fig. 4*A*). In contrast, expression of the signaling-deficient Dectin-1Δ38 failed to enhance reporter activity (Fig. 4*A*). Activation of NFAT by PMA/ionomycin was not affected by the expression of either WT Dectin-1 or Dectin-1Δ38 (Fig. 4*A*). NFAT activity was maximal at a dose of 100 μg/ml zymosan (data not shown). NFAT activity was also induced by depleted zymosan, but not by the pure

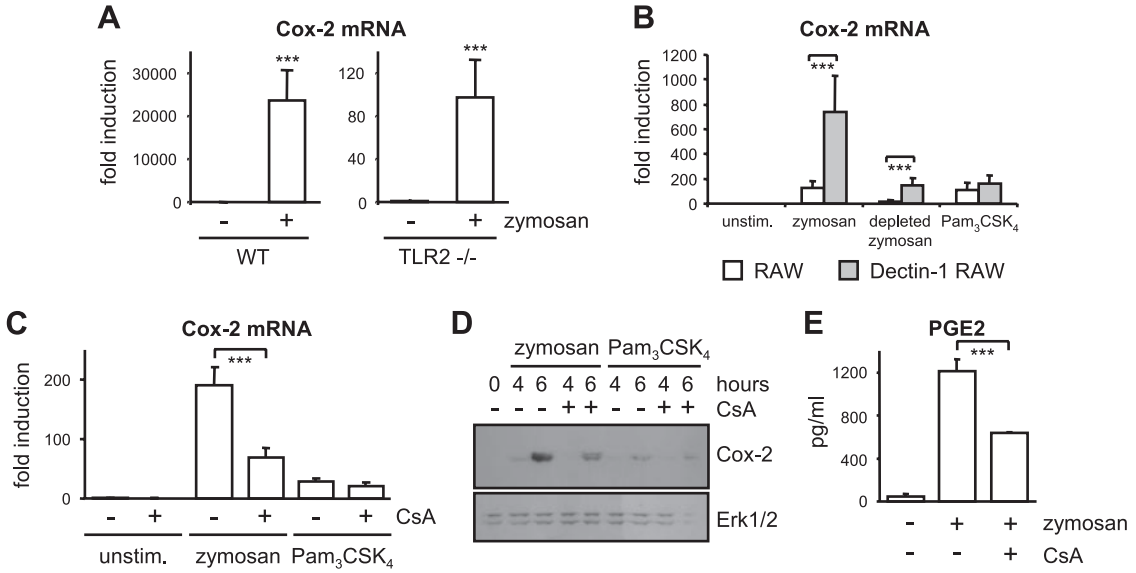

FIGURE 5. Zymosan-induced COX-2 and PGE_2 production are regulated by NFAT. *A* and *B*, Bone marrow-derived macrophages from WT and $TLR2^{-/-}$ mice were stimulated with 100 μg/ml zymosan for 4 h (*A*) and parental RAW264.7 cells and RAW264.7 cells stably expressing WT Dectin-1 were stimulated with 100 μg/ml zymosan or depleted zymosan or 100 ng/ml Pam_3CSK_4 for 4 h (*B*). COX-2 mRNA levels were assessed by real-time RT-PCR, normalized to EF1α mRNA, and are expressed as fold induction relative to unstimulated controls. *C–E*, RAW264.7 cells stably expressing WT Dectin-1 were stimulated with 100 μg/ml zymosan or 100 ng/ml Pam_3CSK_4 in the presence or absence of 1.5 μM CsA. COX-2 mRNA levels after 4 h stimulation were assessed as in *A* (*C*), COX-2 protein levels in whole cell lysates at the times indicated were assessed by Western blotting with Erk1/2 as a loading control (*D*), and PGE_2 secretion was assessed by analyzing 6-h culture supernatants using a PGE_2 EIA assay (*E*). ***, $p < 0.001$. Data are representative of at least three independent experiments.

TLR2 agonist Pam_3CSK_4 (Fig. 4*B*), and was inhibited by the soluble β-glucan laminarin (Fig. 4*C*). To directly assess whether β-glucan particles activate NFAT in primary cells, we treated macrophages and dendritic cells derived from the bone marrow of NFAT-luciferase reporter transgenic mice (15). Luciferase activity was detected in both macrophages and dendritic cells following stimulation with zymosan or depleted zymosan (Fig. 4*D* and data not shown).

Consistent with a role for Dectin-1, and not TLR signaling, in zymosan-triggered NFAT activation, live *C. albicans* yeast, but not hyphae, activated the NFAT-luciferase reporter (Fig. 4*E*). Furthermore, stimulation of RAW264.7 cells stably expressing SBPc-tagged Dectin-1 with streptavidin beads also induced NFAT activity (Fig. 4*F*), directly demonstrating that Dectin-1 ligation is sufficient for NFAT activation. Taken together, these data demonstrate that Dectin-1 signaling uniquely activates NFAT and that TLR signaling does not. These data raise the possibility that NFAT activation may participate in collaborative regulation of genes by Dectin-1 and TLRs.

NFAT activation contributes to the induction of COX-2 by zymosan

Of the 10 genes most strongly up-regulated by zymosan in the absence of TLR signaling, 4 are known to be regulated by NFAT in T cells (Table I), including Egr2 and Egr3, as well as PG-endoperoxide synthase 2, which is also known as cyclooxygenase 2 (COX-2) (20, 23, 24). COX-2 is an inducible enzyme that catalyzes the metabolism of arachidonic acid to generate eicosanoids, including PGs, prostacyclins, and thromboxane, which are key inflammatory mediators with a variety of effects, including regulation of vascular permeability and leukocyte recruitment (25). Arachidonic acid release and eicosanoid production by macrophages is observed within 30 min of intraperitoneal injection of mice with zymosan (26).

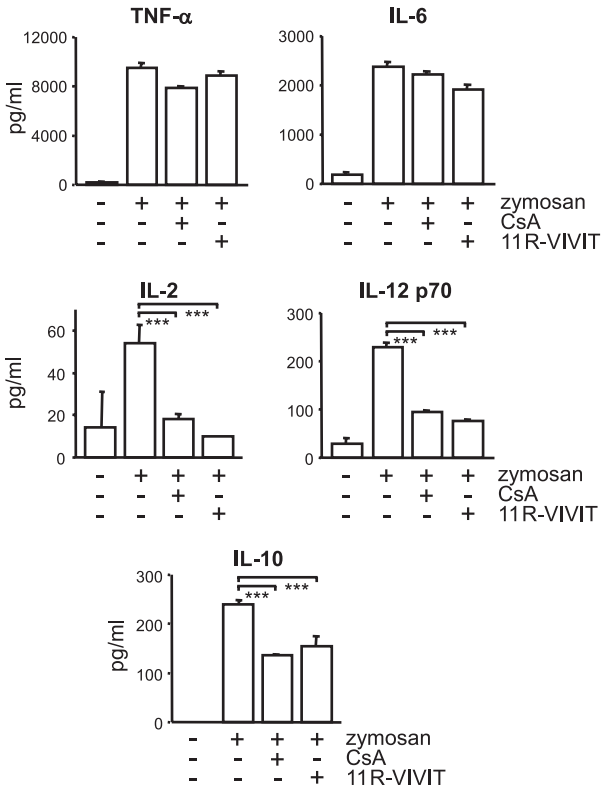

FIGURE 6. Zymosan-induced IL-2, IL-10, and IL-12 p70 production is regulated by NFAT. Bone marrow-derived dendritic cells were stimulated in quadruplicate for 24 h with 100 μg/ml zymosan in the presence or absence of 1.5 μM CsA or 5 μM 11R-VIVIT. Cytokine levels in culture supernatants were assessed by ELISA. ***, $p < 0.001$. Data are representative of at least three independent experiments.

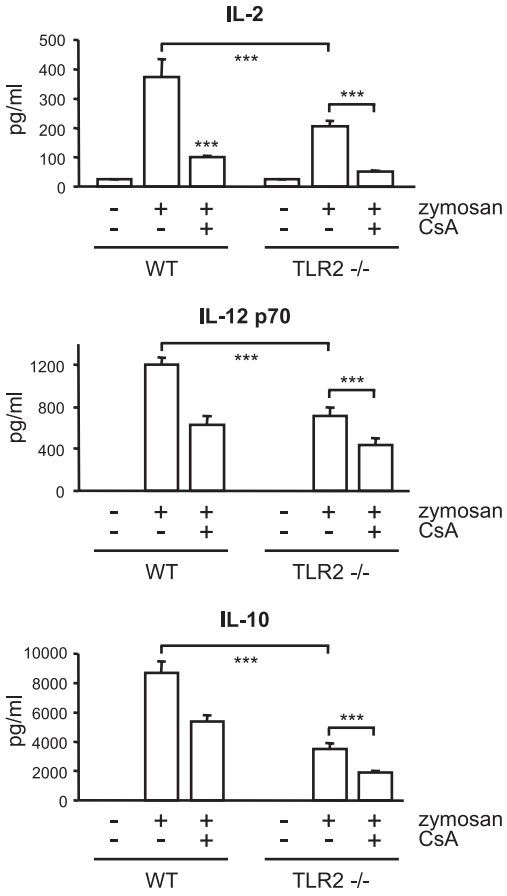

FIGURE 7. Zymosan-induced IL-2, IL-10, and IL-12 p70 production is dependent on both TLR2 and NFAT signals. Bone marrow-derived dendritic cells from WT or TLR2$^{-/-}$ mice were stimulated in quadruplicate for 24 h with 100 μg/ml zymosan in the presence or absence of 1.5 μM CsA. Cytokine levels in culture supernatants were assessed by ELISA. ***, $p < 0.001$. Data are representative of three independent experiments.

Real-time PCR analysis confirmed that zymosan can induce COX-2 expression in bone marrow-derived macrophages from TLR2$^{-/-}$ mice, although at much lower levels than WT macrophages, indicating that while TLR2 plays a major role in COX-2 production, Dectin-1 may contribute to its induction (Fig. 5*A*). Consistent with this possibility, stimulation of RAW264.7 cells with zymosan or depleted zymosan induced low levels of COX-2 expression, and overexpression of Dectin-1 enhanced COX-2 levels without affecting COX-2 induction by the pure TLR2 agonist Pam_3CSK_4 (Fig. 5*B*). Furthermore, depleted zymosan failed to induce COX-2 expression in RAW264.7 cells in which Dectin-1 expression was specifically suppressed by a retrovirally transferred miRNA targeting Dectin-1 (data not shown). Consistent with these observations, a recent report by Suram et al. (18) demonstrated Dectin-1/TLR2 collaboration for COX-2 gene induction. Since COX-2 expression has previously been demonstrated to be regulated by NFAT in T cells (23), we assessed whether zymosan-induced COX-2 production by macrophages is CsA sensitive. CsA treatment reduced the induction of COX-2 by zymosan at both the mRNA and protein levels (Fig. 5, *C* and *D*). In contrast, CsA treatment had no effect on COX-2 induction by Pam_3CSK_4 (Fig. 5, *C* and *D*). Release of PGE_2, a product of arachidonic acid metabolism by COX-2, into culture supernatants was also triggered by zymosan treatment in a CsA-sensitive manner (Fig. 5*E*).

Zymosan-stimulated production of IL-2, IL-10, and IL-12 p70, but not IL-6 or TNF-α, by dendritic cells is regulated by NFAT

Zymosan stimulation of dendritic cells triggers the release of a variety of cytokines, including IL-2, IL-6, IL-10, IL-12 p70, and TNF-α (1–3, 17). Since NFAT is a key regulator of IL-2 production by T cells and scattered reports in the literature indicate the involvement of this transcription factor in the induction of other cytokines, we measured cytokine production by bone marrow-derived dendritic cells stimulated with zymosan in the presence of CsA or 11R-VIVIT. As shown in Fig. 6, inhibition of NFAT had no effect on the induction of TNF-α and IL-6. However, CsA and 11R-VIVIT strongly inhibited the induction of IL-2, IL-12 p70, and IL-10, indicating a key role for NFAT in production of these cytokines (Fig. 6). Consistent with a role for both Dectin-1-mediated NFAT activation and TLR2 signaling in triggering IL-2, IL-12 p70, and IL-10 production in response to zymosan stimulation, induction of these cytokines was reduced in dendritic cells from TLR2-deficient mice and further suppressed by CsA treatment (Fig. 7). Similar data were obtained using dendritic cells from MyD88$^{-/-}$ mice (data not shown).

Discussion

Several previous studies have highlighted the importance of Dectin-1 as a key innate microbial receptor (1–7, 9, 11, 12, 17, 18, 27). Dectin-1 collaborates with TLR2, as well as acting independently, to initiate a range of antimicrobial responses (1–3, 11, 12, 18). We began this study by investigating the TLR-independent transcriptional response of macrophages to zymosan, observing the regulation of over 200 genes in MyD88-deficient macrophages, including three members of the Egr family of zinc finger-containing transcription factors. This led us to the discovery that zymosan and live *C. albicans* yeast induce TLR-independent activation of NFAT transcription factors in macrophages and dendritic cells. NFAT and Egr induction was completely dependent on Dectin-1 signaling. Furthermore, *C. albicans* hyphae, which are not detected by Dectin-1 due to the absence of exposed β-glucan on their surface (4), failed to induce NFAT activation and Egr gene expression, and the pure TLR2 agonist Pam_3CSK_4 also failed to trigger NFAT activation. Collectively, these data demonstrate an independent role for Dectin-1 in NFAT activation.

NFAT activation in lymphocytes is a well-characterized consequence of ITAM signaling following ligation of the T and B cell AgRs (TCR and BCR), with key roles in the adaptive immune response. The ITAM-containing signaling components of the TCR and BCR complexes are phosphorylated by Src family kinases, enabling the recruitment and activation of Syk kinases. Src-Syk signaling results in the activation of phospholipase C-γ, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. While 1,2-diacylglycerol activates protein kinase C, inositol 1,4,5-trisphosphate triggers the release of calcium (Ca^{2+}) ions from intracellular stores. The rise in cytoplasmic Ca^{2+} levels, which is further enhanced and sustained by Ca^{2+} influx, enables activation of the Ca^{2+}-dependent phosphatase calcineurin. Calcineurin dephosphorylates NFAT transcription factors at multiple sites, permitting their nuclear entry and regulation of gene expression. Given that Dectin-1 has an ITAM-like motif in its intracellular tail, which it requires for signaling (1–3, 11, 12), and that Dectin-1 signals trigger Src-Syk signaling in macrophages and dendritic cells (3, 11), and that zymosan has been previously noted to trigger calcium fluxes in macrophages (28, 29), it seems likely that NFAT activation in these cells would occur by similar mechanisms to those used in lymphocytes.

However, Zhu et al. (30) have previously demonstrated that NFAT in macrophages is constitutively found in the nucleus. We have similarly observed that macrophage NFAT is constitutively nuclear and capable of binding to an NFAT consensus DNA binding site (data not shown). Furthermore, inhibition of calcineurin activity results in enhanced phosphorylation of NFAT, indicating that in resting cells there is calcineurin-dependent cycling of NFAT phosphorylation. Zhu et al. reported that constitutive nuclear NFAT is associated with the IL-12 p40 promoter and that it is required to enable IFN consensus sequence-binding protein to bind to the IL-12 p40 promoter in LPS-stimulated macrophages (30). However, it was unclear whether LPS stimulation actually induced NFAT transcriptional activity. We have observed that NFAT itself becomes transcriptionally active upon stimulation of Dectin-1. These data suggest complex mechanisms of regulation of NFAT activation in macrophages. In T cells, NFAT is known to be regulated by kinases in the nucleus, including JNK, Pim kinase 1, and Cot kinase, which phosphorylate single residues (distinct from the multiple residues dephosphorylated by calcineurin) to enhance its transactivation activity (31–33). Similar mechanisms may trigger NFAT transcriptional activity in phagocytes.

NFAT transcription factors are key regulators of T cell activation, promoting the induction of numerous genes, most notably IL-2. The pivotal role of NFAT in lymphocyte responses is clearly demonstrated by the success of immunosuppressive therapies that target NFAT activation, most notably CsA and FK506. However, in recent years it has become clear that NFAT activation also promotes the induction of genes that induce T cell anergy (reviewed in Ref. 13). In vitro and in vivo studies have reported Dectin-1-dependent production of both proinflammatory (TNF-α, MIP2, IL-12, IL-2, COX-2, and PGE_2) and anti-inflammatory (IL-10 and TGF-β) effectors by macrophages and dendritic cells in response to stimulation with zymosan and live fungi (1–7, 17, 18). Zymosan injection triggered severe chronic arthritis in genetically susceptible mice in a Dectin-1-dependent manner (34), and intratracheal administration of *Aspergillus fumigatus* conidia induced proinflammatory cytokine production, which was blocked by coadministration of either a soluble Dectin-1-Fc fusion protein or a Dectin-1 blocking Ab (6, 7). In contrast, a recent study examining the phenotype of dendritic cells exposed to zymosan in vitro and in vivo demonstrated induction of a regulatory dendritic cell phenotype, characterized by secretion of abundant IL-10, but little IL-6 or IL-12 p70, and resulting in the poor response of Ag-specific $CD4^+$ T cells (17). In parallel with its role in promoting both activation and anergy in T cells, our data suggest that NFAT activation in macrophages and dendritic cells promotes both proinflammatory (IL-2, IL-12, and COX-2) and anti-inflammatory (IL-10) responses.

Consistent with the previous reports of collaboration between Dectin-1 and TLR2 in the transcriptional response to zymosan and pathogenic fungi (1–3, 17), Dectin-1-triggered NFAT signaling appears to collaborate with TLR signals, as well as having TLR-independent effects. Thus, TLR2 and Dectin-1 signals are integrated at the promoters of individual genes. Induction of IL-2, IL-12 p70, and IL-10, for example, is dependent on TLR2 signaling (Refs. 2 and 3, and Fig. 7) but is also strongly influenced by Dectin-1/NFAT signaling. In contrast, Dectin-1 collaboration with TLR2 to induce IL-6 and TNF-α production is likely mostly mediated by NF-κB (1, 2) and not influenced by NFAT activation.

TLR-independent, Dectin-1-dependent induction of Egr2 and Egr3, but not Egr1, was CsA/11R-VIVIT sensitive, indicating that Dectin-1-triggered Egr1 induction occurs through a pathway distinct from the NFAT pathway required to induce Egr2 and Egr3 production. A recent report demonstrated that Egr induction is similarly regulated by NFAT in T cells (20). TCR engagement triggered synthesis of Egr1, Egr2 and Egr3, but only Egr2 and Egr3 induction was CsA sensitive.

The significance of Egr induction in zymosan/yeast-stimulated macrophages/dendritic cells remains unclear. While Egr1 is known to be induced by a variety of stimuli (19), Egr2/Egr3 induction has been less widely reported and comparatively few target genes have been identified. Although all Egr family members bind to the same consensus sequence, variation in target gene binding has been noted. For example, Egr1 associates with the human IL-2 promoter, whereas Egr2 and Egr3 do not (35). Hence, regulation of Dectin-1-triggered IL-2 production by NFAT is likely independent of Egr2/Egr3 induction. However, NFAT-independent Egr1 induction may contribute to Dectin-1-stimulated IL-2 production. Indeed, Egr1 promotes T cell activation by associating with NFAT to induce IL-2 and TNF-α production (36, 37). In contrast, Egr2 and Egr3 have recently been implicated in the induction of the E3 ligase Cbl-b and the establishment of T cell anergy (20). Dectin-1-induced Egr2/Egr3 may similarly be involved in restricting the macrophage/dendritic cell inflammatory response.

In addition to triggering cellular antimicrobial responses, including phagocytosis and ROS production, we have now demonstrated that Dectin-1 signaling regulates inflammatory transcriptional responses in part through NFAT. Dectin-1-activated NFAT participates in gene regulation directly, and by integrating signals through TLR2, which does not itself activate NFAT. Whether other pattern recognition receptors can activate NFAT, and whether NFAT is important for modulating TLR-stimulated responses to nonfungal microbes, remains to be determined. However, this study demonstrates that NFAT activation, like Src-Syk activation, is not only a central component of adaptive immune signaling, but also a key regulator of innate immunity.

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Disclosures

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