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The Induction of IL-10 by Zymosan in Dendritic Cells Depends on CREB Activation by the Coactivators CREB-Binding Protein and TORC2 and Autocrine PGE₂

Yolanda Alvarez,* Cristina Municio,* Sara Alonso,* Mariano Sánchez Crespo,²* and Nieves Fernández†

Stimulation of human monocyte-derived dendritic cells with the yeast extract zymosan is characterized by a predominant production of IL-10 and a strong induction of cyclooxygenase-2, but the molecular mechanisms underlying this response are only partially understood. To address this issue, the activation of transcription factors that may bind to the il10 proximal promoter was studied. Binding activity to Sp1, Sp3, NF-Y, and CAMP response element (CRE) sites was detected in the nuclear extracts of dendritic cells; however these binding activities were not influenced by zymosan. No binding activity to Stat1, Stat3, and c/EBP sites was detected. Notably, zymosan activated β3-binding activity, but inhibition of NF-κB was associated with enhanced IL-10 production. In sharp contrast, treatments acting on CREB (CRE binding protein), including 8-Br-cAMP, PGE₂, and inhibitors of PKA, COX, and glycogen-synthase kinase-3β showed a direct correlation between CREB activation and IL-10 production. Zymosan induced binding of both P-CREB and CREB-binding protein (CBP) to the il10 promoter as judged from chromatin immunoprecipitation assays, whereas negative results were obtained with Ab reactive to Sp1, Sp3, c-Maf, and NF-Y. Zymosan also induced nuclear translocation of the CREB coactivator transducers of regulated CREB activity 2 (TORC2) and interaction of TORC2 with P-CREB coincidental with the association of CREB to the il10 promoter. Altogether, our data show that zymosan induces il10 transcription by a CRE-dependent mechanism that involves autocrine secretion of PGE₂ and a network of interactions of PKA, MAP/ERK, glycogen-synthase kinase-3β, and calcineurin, which regulate CREB transcriptional activity by binding the coactivators CBP and TORC2 and inhibiting CBP interaction with other transcription factors. The Journal of Immunology, 2009, 183: 1471–1479.

Fungal infections are very common due to the high number of people with a compromised immune system and the widespread use of antibiotics, the ubiquitous Candida being the most prevalent infectious fungus. The first line of defense against fungi is the innate immune system, which is mainly composed of phagocytic cells endowed with a set of germline encoded receptors that recognize conserved microbial structures. These receptors, so called pattern recognition receptors (PRR), display broad substrate specificity, enabling the host to recognize a wide range of pathogens without the need for somatic mutation. The structures expressed in pathogens, which in turn are recognized by PRR, are called pathogen-associated molecular patterns (PAMP) and, in the case of Candida, α-mannans and β-glucans are the most important PAMP. The PRR that recognize α-mannans are the mannose receptor and DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin), whereas the recognition of β-glucans is conducted by dectin-1 (1) and TLR2 (2–4). The recognition of fungal PAMP by their cognate PRR results in a phagocytic and inflammatory response directed to the elimination of the pathogen. However, the secretion of inflammatory mediators that ensues engagement of PRR may differ according to the nature of the receptors involved and has important consequences for the outcome of the infection.

Among the distinct types of phagocytes, dendritic cells (DC) regulate the immune response to infection because of the array of cytokines they release. A response dominated by IL-12 p70 is generally protective by promoting Th1 immunity, whereas IL-10 prevents excessive activation of the immune response and limits immune pathology, but alternatively may prevent the complete eradication of pathogens.

Previous studies in macrophages and DC have disclosed that fungal PAMP acting through dectin-1 and DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) induce a cytokine response characterized by a high production of IL-10 and IL-23, and a low secretion of IL-12 p70 (5–8), as compared with the effect of archetypal TLR4 agonists. This may have pathophysiological consequences for the persistence of fungal infection and raises the question of the signaling pathways involved in the predominant IL-10 response. The regulation of IL-10 production has been the subject of intense research. However, most studies have been conducted in archetypical TLR4 models using LPS as a ligand and...
both transcriptional and posttranscriptional mechanisms have been reported. As regards transcriptional regulation, many transcription factors have been considered as master regulators, namely Stat3 (9–12), Sp1 and Sp3 (12–14), c-Maf (15), NF-Y (16), NF-kB (17–19), Pbx1b (pre-B cell leukemia transcription factor-1b) (20), eEBP (21), NFAT (22, 23), and cyclic AMP response element (CRE) binding protein (CREB) (24, 25). In addition, posttranscriptional regulation of IL-10 message has also been proposed because of the high number of AU-rich elements in the 3′-UTR of IL-10 mRNA (26) and their binding by the RNA-binding protein tristetraprolin, which destabilizes the message (27). In this study, we have analyzed the signaling mechanisms whereby IL-10 is induced in DC by zymosan, an extract of Saccharomyces cerevisiae cell wall, the composition of which mimics the structure of Candida.

After addressing the possible involvement of several transcription factors and mRNA stability, we found a major role of a CRE (cyclic AMP response element) site and have analyzed the protein kinase network associated with the activation of CREB (CRE-binding protein) and the role of CREB coactivators CBP (CREB-binding protein) and TORC2 (transducer of regulated CREB binding protein) activity 2, also known as CREB-regulated transcriptional coactivator 2, CRTC2). In addition, we have found a cycloxygenase (COX)-dependent autocrine feed-forward mechanism and a negative regulation of IL-10 by NF-kB activation, most likely due to a competition of both CREB and NF-kB for a limited amount of CBP.

Materials and Methods
Reagents and cells
Zymosan, mannan from Saccharomyces cerevisiae, H-89, LiCl, and OptiPrep were from Sigma-Aldrich. SV-50 was purchased from Calbiochem. IL-10 and TNF-α were assayed with Biotrack ELISA systems from Amersham Biosciences. Mononuclear cells were collected from buffy coats of healthy donors by centrifugation on Ficoll-Hypaque cushions. The separation of monocytes and their differentiation into DC has been reported previously (7). Anti-dectin-1 mAb was a gift from Dr. Gordon D. Brown (Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa). Anti-mannose receptor mAb (no. 559592) was from BD Pharmingen and anti-DC-SIGN mAb (no. MAB161) was from R&D Systems.

Immunoblots
Proteins were separated by electrophoresis in SDS/PAGE and transferred to nitrocellulose membranes. The membranes were used for immunodetection of MAPK/ERK and p38 MAPK with anti-ACTIVE MAPK and p38 polyclonal Abs from Promega, and with reagents from Cell Signaling Technology for the assay of Stat1, Stat3, and glycogen synthase kinase (GSK)-3β-Ser9 phosphorylation. Quantitation of the blots was conducted using BioRad Quantity One gel imaging software (Bio-Rad). For immuno-oblots directed to assay nuclear translocation of TORC2, nuclear extracts were obtained by using a nuclear extract kit (Active Motif). Chromatin precipitation of P-CREB and TORC2 was conducted using the Nuclear Complex Co-IP kit of Active Motif. Anti-TORC2 Ab was from Santa Cruz Biotechnology.

Real time RT-PCR of IL-10, IL-12 p40, IL-12 p35, and COX-2
Purified RNA was depleted of genomic DNA by treatment with DNase (Turbu-DNA free, Ambion) and used for reverse transcriptase reactions. The resulting cDNA was amplified in a PTC-200 apparatus equipped with a Chromo4 detector (Bio-Rad) using SYBR Green I mix containing Hot Start polymerase (ABgene). The sets of primers for PCR are shown in supplemental Table S1.1 Cycling conditions were adapted to each set of primers, but in most cases the following conditions were: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and extension at 72°C for 30 s. GAPDH was used as a housekeeping gene to assess the relative abundance of the different mRNA, using the comparative Ct (threshold cycle) method.

Nuclear transcription factor DNA-binding activity
Nuclear extracts of DC were obtained from cell cultures grown in 10-cm tissue culture dishes by using a nuclear extract kit. Levels of transcription factor binding activity in the nuclear extracts were quantitated by TransAM Sp1/3, Stat Family, NF-YA, c/EBPα/β, and pCREB assay kits (Active Motif) and expressed as the OD at 450 nm. The assay of NF-kB binding activity was conducted by conventional methods as previously described (28). For this purpose, DC were washed with ice-cold hypotonic lysis buffer. Unbroken cells were eliminated by centrifugation at 1,000 × g for 10 min, and the nuclei were collected by centrifugation at 15,000 × g for 1 min. The nuclear pellet was resuspended in high salt extraction buffer and the nuclear extract was obtained by pelleting for 30 min at 105,000 × g. Double-stranded oligonucleotide probes were end labeled with [γ-32P]ATP using T4 polynucleotide kinase. A total of 10 μg nuclear protein was incubated for 20 min on ice with radiolabeled oligonucleotide probes (2–6 × 104 cpm) in a 25 μl reaction buffer containing 2 μg poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% Ficoll, and 4% glycerol. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4% nondenaturing PAGE. The oligonucleotide sequences used for the detection of binding activity to the NF-kB sites from the COX-2 promoter are 5′-GGGGGATGGGGGACTACCCCCTCT-3′ (163–154 Site I) and 5′-GGGGGAAGGGGATTCCTGGCCG-3′ (388–379 Site II).

Chromatin immuno precipitation (ChIP) assay
ChIP assays were conducted with reagents and Ab from Santa Cruz Bio technology, except anti-P-CREB Ab, which was from Upstate Biotechnol ogy and anti-TBP (TATA box binding protein), which was from Diage node. In brief, DC were stimulated and then washed twice with PBS containing 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated by 0.125 M glycine and after washing, cells were resuspended in lysis buffer. Crude nuclear extracts were collected by microcentrifugation at 2,000 rpm for 5 min and resuspended in a lysis buffer containing a high salt concentration. Chromatin sonication was conducted using a Bioruptor device (Diagenode) set at maximal power for three intervals of 10 min, including 30 s sonication and 30 s pause. This procedure yielded chromatin fragments of 200–600 bp. The chromatin solution was preclarified by adding 50 μl Protein A/G PLUS-Agarose for 30 min at 4°C under continuous rotation. After elimination of the beads, Ab was added for overnight incubation at 4°C, and then Protein A/G PLUS-Agarose was added and incubated for an additional period of 2 h at 4°C. Beads were harvested by centrifugation at 12,000 rpm and sequentially washed with lysis buffer (high salt), wash buffer, and elution buffer. Cross-links were reversed by heating at 67°C in a water bath, and the DNA bound to the beads isolated by extraction with phenol/chloroform/isoamylalcohol. PCR were conducted with primers designed from both the il10 and the cox2 promoters.

Results
Zymosan is a strong inducer of IL-10 production by DC
Zymosan is a potent stimulus for IL-10 production through a Syk-dependent signaling pathway (5, 6). This route conveys signals different from those triggered by TLR2, which may lead to IL-12 p70 induction, and explains why zymosan is a less potent stimulus for IL-12 p70 production than genuine TLR4 ligands such as LPS. In a previous study conducted in DC, we have found that zymosan induces about a fifth of the amount of IL-12 p70 produced by LPS, whereas it is a strong stimulus for COX-2 induction (7). Because the production of IL-10 and IL-12 p70 by DC defines the polarization of T cells, we scrutinized the molecular mechanisms underlying the response to a fungal stimulus. Zymosan was a more potent stimulus than LPS to induce IL-10 production in 11 independent experiments (Fig. 1A), although the difference did not reach statistical significance due to the dispersion of the data. As compared with stimuli signaling through other pathways, zymosan was a stronger stimulus than immune complexes, which archetypically transduce signals through Syk-dependent mechanisms. IFN-γ, which acts through the Jak/Stat signaling pathway, failed to induce IL-10. The production of IL-10 elicited by zymosan was induced by 20 μg/ml, whereas in the

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*The online version of this article contains supplemental material.*
case of zymosan plateaued at 1 mg/ml and was accompanied by a strong stimulation of TNF-α production (Fig. 1D). To address whether the effect of zymosan could be explained by either β-glucans or α-mannans, additional experiments were conducted using curdian, a pure β-glucan, and combination of curdian and mannan, as well as mAb reactive to dectin-1, DC-SIGN, and the mannose receptor. As shown in Fig. 1E, mannan showed a synergistic effect with curdian, whereas anti-dectin-1 mAb produced a complete inhibition of the IL-10 response and anti-DC-SIGN mAb inhibited IL-10 production by ~50%. In contrast, anti-mannose receptor mAb showed no effect. These results suggest that similar to what has been described regarding arachidonic acid release (7), receptors for both β-glucan and α-mannan, in particular DC-SIGN, cooperate to induce IL-10, although dectin-1 seems to be the main receptor involved.

To address whether the distinct effect of zymosan on DC can be explained by an effect on transcription, the levels of the mRNA encoding IL-12 p40, IL-12 p35, IL-10, and COX-2 were assayed in cells treated with LPS, zymosan, and IC. As shown in Fig. 2, LPS was the strongest stimuli for the induction of IL-12 p40 and IL-12 p35, whereas zymosan was the strongest stimulus for both IL-10 and COX-2 mRNA induction, what agrees with the notion that differences in the expression of cytokines and COX-2 elicited by the different stimuli can be explained by transcriptional mechanisms. Experiments directed to address the influence of message stability were conducted in cells treated with the transcription inhibitor actinomycin D (Fig. S1). Under these conditions, IL-10 mRNA decreased to 38% 2 h after the addition of the drug, whereas in control cells only decreased to 86%, thus suggesting that stabilization of the mRNA does not play a major role in the control of IL-10 production.

**Transcriptional regulation of IL-10**

Because the regulation of IL-10 expression is best explained by transcriptional mechanisms, the study of the transcription factors implicated in this regulation was addressed by several approaches. Computer analysis of human and mouse il10 promoters was conducted using the MatInspector program and the TRANSFAC database (Genomatics Software) to detect binding sites for transcription factors. In addition, both sequences were aligned with DNA Block Aligner software to define conserved areas, because these regions are more likely to represent functionally relevant elements (Fig. S2A). Several of the sites detected have been previously associated with the transcriptional regulation of il10, but there are some discrepancies regarding their functional relevance and studies using fungal stimuli have not been reported so far. Our first approach was to search for the presence of binding activities to the consensus strings of the transcription factors found on the human il10 promoter. As shown in Fig. 3, A, B, and G, no binding activity to Stat and C/EBP consensus sequences was observed in the nuclear extracts of cells treated with zymosan. However, binding activity to Stat1 and Stat3 was elicited by IFN-α and c/EBP binding activity could be detected in a control nuclear extract from rat liver. Notably, constitutive binding activity to Sp sites compatible with both Sp1 and Sp3 was detected (Fig. 3, C and D), as well as binding activity to NF-Y (Fig. 3E) and CRE (Fig. 3F) consensus sequences. As regards NF-Y, binding activity was not detected in monocytes, whereas high amounts were found in lymphocytes, Jurkat cells, and DC (Fig. 3E). These findings agree with previous reports in monocytic cells where it has been reported that the A
subunit of NF-Y, which is indispensable for DNA binding, is expressed after monocyte differentiation. NF-κB is activated by zymosan and has been associated to the regulation of \textit{il10} in mouse macrophages (17–19), as well as to the regulation of \textit{cox2}. Taking into account that the expression of COX-2 parallels IL-10 induction, experiments were conducted using probes containing the κB sites from the human \textit{cox2} promoter that have been found of functional relevance. As shown in Fig. 4, zymosan and LPS were strong activators of NF-κB binding activity both at early and late times after the addition of the stimuli, because peaks of activity were observed both 1 and 24 h after addition of the stimuli (Fig. 4A), thus suggesting that both direct and indirect mechanisms might be involved in this response. In contrast, IC displayed a nonsignificant activation. The κB-binding activity was also observed in response to curdlan and mannan (Fig. 4B, \textit{top}), thus agreeing with the involvement of both β-glucan and α-mannan components in zymosan response. The response to zymosan was dose-dependent and binding was competed by the

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FIGURE 3. Presence of transcription factor binding activity in nuclear extracts. DC were stimulated with different additions for 30 min as indicated. After this time, nuclear extracts were obtained and used for the binding assay to consensus sequences using TransAM assay. The specificity of binding was confirmed by carrying out the reaction in the presence of an excess of wild type (WT) and mutated probe. Positive and negative controls have been included as indicated on each panel. These are representative experiments of at least two for each transcription factor.

FIGURE 4. Nuclear extracts from cells incubated in the presence of different stimuli contain binding activities to the NF-κB sites of COX-2 promoter. A, DC were incubated with 1 mg/ml zymosan, 10 μg/ml LPS, and 100 μg/ml IC. At the times indicated, cell lysates were collected and the nuclear extracts assayed for binding to probes containing the κB-binding sites of the COX-2 promoter. Lanes marked “Competitor” indicate that the binding reaction was conducted in the presence of a 100-fold excess of unlabeled oligonucleotide probes. B, To address the dose-dependency of the induction of κB binding activity, nuclear extracts from cells stimulated for 45 min in the presence of different concentrations of stimuli were used. These are representative experiments of three with similar results.
unlabeled sequence (Fig. 4B); however, in some cases maximal response was observed with 0.1 mg/ml, what could be explained by differences linked to different donors, basal activation of the DC, or variations in the time-course of NF-κB activation. However, as the sequence involved in NF-κB-dependent regulation of il10 expression in the mouse is not conserved in the human il10 promoter, the simple presence of κB-binding activity in the nuclear extracts upon zymosan challenge is not a proof of the involvement of NF-κB in the regulation of IL-10 expression in human DC. Altogether, the above-mentioned results do not support the involvement of Stat1, Stat3, and c/EBP in the regulation of IL-10 induction, and additional experiments were conducted focusing on the possible involvement of NF-κB, Sp1, Pbx1b, and CREB.

**Effect of the pharmacological modulation of CREB and NF-κB activity on IL-10 production**

Because the activity of CREB and NF-κB can be modulated by pharmacological tools, experiments were conducted with 8-Br-cAMP, a cell permeable analog of cyclic AMP, PGE2, and the protein kinase A (PKA) inhibitor H-89. As shown in Fig. 5, increase of the intracellular levels of cyclic AMP by both PGE2 and 8-Br-cAMP had on its own a limited effect on IL-10 production. In contrast, stimulation of DC with zymosan in the presence of these compounds produced a synergistic increase of IL-10 production. Notably, the opposite effect was observed in the presence of the protein kinase A inhibitor H-89 (Fig. 5A). An enhancing effect on IL-10 production was also observed in the presence of LiCl, a compound which has been found to increase IL-10 production by inhibiting GSK3β, an enzyme that positively regulates NF-κB activity and negatively regulates CREB activity (25, 29). The effect of LiCl and 8-Br-cAMP was not additive, thus indicating that both compounds produced a significant increase of the intracellular levels of cyclic AMP by both PGE2 and protein kinase A (PKA) inhibitor H-89. As shown in Fig. 5, in contrast, stimulation of DC with zymosan in the presence of these compounds produced a synergistic increase of IL-10 production.

**FIGURE 5.** Effect of different additions on the production of IL-10. DC were preincubated for 30 min with the different additions as indicated and then stimulated with zymosan and IC. Supernatants were collected after 48 h in culture for the assay of IL-10. Results represent mean ± SEM of five to six independent experiments. *, p < 0.05.

The regulation of CREB activity has been related to calcium/calmodulin kinases and CRE coactivators, the activity of which depends on a sensor of both Ca^{2+} and cyclic AMP levels (30), the Ca^{2+}-dependence of IL-10 production was addressed. IL-10 production was blunted by Ca^{2+}-chelation (Fig. 5C). Ionomycin induced a limited production of IL-10, thus suggesting that intracellular Ca^{2+} levels are not the only factor determining IL-10 production. However, low micromolar concentrations of cyclosporin induced a significant decrease of IL-10 production, thus pointing to the involvement of calcineurin in the regulation of IL-10 production (Fig. 5C). Because E prostanoid receptors type 2 and 4 are involved in the regulation of the intracellular levels of cyclic AMP and zymosan is a strong inducer of COX-2 and PGE2 production, the effect of inhibiting COX-1 with the specific inhibitor SC560 0.3 μM and COX-2 with the specific inhibitor NS398 1 μM was addressed. As shown in Fig. 5D, the isolated addition of any of those compounds did not show any significant effect on IL-10 production, although NS398 significantly inhibited IL-10 production when tested at 5 μM. Notably, combination of both compounds produced a significant inhibition, thus suggesting that both COX isoforms might be involved in the autocrine production of PGE2 that regulate intracellular cyclic AMP levels and zymosan-induced IL-10 production. Attempts to modulate the production of IL-10 induced by IC showed again enhancement by 8-Br-cAMP and inhibition by H-89, thus suggesting a similar mechanism of regulation as that involved in response to zymosan (Fig. 5E). To obtain further insight into the signaling mechanisms involved in IL-10 induction by zymosan, the effect of LiCl and H-89 on GSK3β-Ser9 phosphorylation, IκBα degradation, MAP/ERK activation, and COX-2 induction was addressed. Unlike H-89, LiCl and zymosan were potent inducers of GSK3β-Ser9 phosphorylation (Fig. S3), a posttranslational modification associated with inhibition of GSK3β activity (31). Notably, LiCl also induced a slight induction of COX-2 and enhanced the effect of zymosan, thus pointing to a parallel regulation of IL-10 and COX-2 induction, and explaining the enhancing effect of GSK3β inhibition on both IL-10 and COX-2 expression. The degradation of IκBα induced by zymosan was not influenced by the pharmacological treatments, including SN50, which blocks NF-κB p50 nuclear translocation, but does not inhibit IκBα degradation. In addition, the activation of MAP/ERK was not influenced by those treatments (Fig. S3). Taken together, these results suggest that the polarization of DC cytokine response vs IL-10 production might depend on the balance between NF-κB and CREB activity, and that mechanisms other than the simple activation of the DNA-binding activity of these factors by separation of IκB inhibitors and phosphorylation of CREB, respectively, regulate the final response.

**The MAPK cascade and IL-10 production**

IL-10 production has been shown to be highly dependent on signaling cascades regulated by both the MAPK/ERK (32, 33) and the p38 MAPK (34). Triggering the MAPK/ERK pathway could activate kinases involved in the phosphorylation of CREB (30, 32, 33) or in the phosphorylation of histones (35), thus allowing binding of transcription factors to the promoters. The effect of p38...
MAPK has been associated with the activation of mitogen and stress kinases (MSK), which in turn phosphorylate CREB in murine cells treated with LPS (36). IL-10 production was inhibited by the MAPK/ERK inhibitor U0126 (Fig. 5A), but not by the p38 inhibitor SB203580 (data not shown). As shown in Fig. S4, all of the stimuli activated the p38 MAPK pathway, although zymosan was the most active activator of the MAPK/ERK route. These data are consistent with the involvement of MAPK pathways in the regulation of IL-10 production and support the preferential involvement of the MAPK/ERK route in response to zymosan.

The role of distinct transcription factors on IL-10 induction
ChIP assays were conducted using Abs reactive to P-CREB, CBP, c-Maf, NF-YA, Sp1, Pbx1, and TBP. As shown in Fig. 6A, significant binding of P-CREB to the il10 promoter was observed in DC stimulated with zymosan, but not in control cells nor in samples treated with an irrelevant Ab. Pretreatment with the PKA inhibitor H-89 inhibited P-CREB binding to the il10 promoter (Fig. 6A, right). Notably, P-CREB binding to the il10 promoter was associated with a 64-fold increase of the amount of CBP bound to the il10 promoter (Fig. 6B). ChIP was negative when the PCR was conducted using primers from the il12p35 promoter, which is known not to include CRE sites (data not shown). P-CREB binding was also detected in the cox2 promoter upon zymosan stimulation (Fig. 6A, bottom), which agrees with the presence of two CRE sites in this promoter (Fig. S2B) and with the functional relevance of these sites in cox2 transcriptional regulation (37, 38).

Notably, stimulation of DC with both zymosan and curdlan was associated with binding of TBP to the il10 promoter (Fig. 6C, left), which suggests association of P-CREB and CBP binding to the promoter with recruitment of the RNA polymerase II and activation of transcription. Curdlan also enhanced the binding of CBP to the il10 promoter, which agrees with the notion that the β-glucan receptor is involved in at least a part of the response (Fig. 6C, right). Binding of P-CREB and CBP to the promoters was coincidental with the detection of TORC2 in the nuclear extracts, which was observed between 30 and 60 min after zymosan addition, and to a reduced extent at 4 h. In contrast, the subcellular distribution of the nuclear protein TBP and the cytoplasmic protein β-actin was not modified under these conditions (Fig. 6D), thus pointing to the selectivity of the effect of zymosan on TORC2 translocation. Moreover, immunoprecipitation of the nuclear extracts with anti-P-CREB Ab showed that TORC2 coimmunoprecipitated with P-CREB in DC treated with zymosan (Fig. 6E). Taken together, these findings suggest that zymosan induces binding of P-CREB to CRE sites, recruitment of the coactivators CBP and TORC2, binding of components of the RNA II polymerase complex to the il10 promoter, and that these responses require PKA activity.

As regards the possible involvement of c-Maf, we found the expression of the mRNA encoding both the long and the short form of c-Maf in DC (Fig. S5A), the induction of which by LPS and IL-4 has been reported in monocytes (15), but we could not show binding to the il10 promoter in ChIP assays (data not shown). Regarding Sp1 and Sp3, the detection of binding activity in resting cells was not accompanied by binding to the il10 promoter, which agrees with the notion that this family of transcription factors behaves as a constitutive activator of housekeeping genes and other TATA-less genes. ChIP assays with anti-Pbx1 Ab showed immunoprecipitation of <0.001% of the input, without difference between resting and zymosan-treated DC. Stat3 has been associated with il10 transcriptional activation, specially in response to ligands of TLR4, which differ from zymosan because of their capacity to activate the Jak/Stat pathway by TRIF (TIR-domain-containing adapter-inducing IFN-β-dependent mechanisms). In addition to our inability to show Stat-binding activity in nuclear extracts from stimulated DC, which was further scrutinized in EMSA assays with the consensus Stat sequence (data not shown), we did not find tyrosine-phosphorylated Stat1 in DC stimulated with zymosan, whereas it was induced upon LPS and IFN-γ treatment or after

![A](http://www.jimmunol.org/Downloaded-from/http://www.jimmunol.org/)
washing the culture medium and addition of IL-4 (Fig. S5B), thus indicating that although zymosan does not activate Stat1, it may be temporarily activated by cytokines present in the medium (39). Stat3 showed tyrosine phosphorylation in resting cells, which was increased by IFN-γ but not by other treatments. Taken collectively, our results show a major role for CREB in the positive transcriptional regulation of \( \text{il10} \) in response to the fungal stimulus zymosan. A diagram of the signaling routes involved in IL-10 regulation in DC stimulated with fungal stimuli is shown in Fig. 7.

**Discussion**

The present results confirm the major effect of zymosan on IL-10 production and show that this effect can be explained via transcrip-
induce different responses on inflammatory cells and/or use distinct routes to elicit the same responses. Because most studies have been conducted using LPS as a stimulus, the control of IL-10 expression by Stat3 has been considered a general paradigm so far (9–12). Further evidence that distinct stimuli might use distinct transcription factors has recently been reported by showing that during the ingestion of apoptotic cells by murine macrophages, the synthesis of IL-10 is under the control of Pbx1b (20) and by the involvement of CREB in the regulation of IL-10 production by murine macrophages stimulated with adiponectin (47).

Recent studies have disclosed that TLR4 stimulation activates CREB by a mechanism linked to the p38 MAPK-dependent kinases MSK1 and MSK2 (36), thus making it likely that CREB might cooperate with Stat3 to induce IL-10 expression upon LPS challenge. Taking this finding into account and in view of the failure of our attempts to show the involvement of the Stat3 in the induction of IL-10 in response to zymosan, we put forward the hypothesis of the involvement of CREB in the transcriptional regulation of IL-10. Our results support this notion on the basis of several findings. First, nuclear extracts of DC showed high levels of CRE binding activity in an assay for P-CREB, even in the absence of stimulus, which agrees with previous reports on murine (38) and human monocyte cell lines (48) and point to mechanisms other than CREB phosphorylation for transactivating activity to occur. In keeping with this concept, we found that zymosan promotes P-CREB binding to the CRE site and recruitment of the coactivator CBP, as well as translocation of TORC2 to the nucleus and formation of P-CREB/TORC2 nuclear complexes. Current concepts on CRE-driven transcriptional activation include the involvement of cyclic AMP-mediated signals, Ca²⁺/calcinurin-mediated signals, and MAPK/ERK mediated signals. These signals may be modulated by GSK3β and by autocrine production of PGE₂. A model of integration of these signals could be the extension of the paradigm delineated in liver and pancreas where both Ca²⁺ and cyclic AMP signals act on a common sensor, TORC2, which is also expressed in hemopoietic cells (49, 50). TORC2 is a CREB coactivator retained in the cytoplasm of resting cells by interaction with 14-3-3 proteins. This depends on tonic phosphorylation under the control of kinases of the AMP-activated protein kinase family, in particular salt-inducible kinases, the activity of which is enhanced by GSK3β and inhibited by PKA (51). In contrast, TORC2 is dephosphorylated by calcineurin. This sensitivity of TORC2 to both cyclic AMP- and Ca²⁺-dependent signals makes it a dual sensor for cyclic AMP and intracellular Ca²⁺ levels (30). Our findings suggest a mechanism of regulation of CREB similar to that depicted in TORC-dependent systems on the basis of the enhancement of IL-10 production by cyclic AMP analogues and GSK3β inhibitors, inhibition by PKA and calcineurin blockers, and the occurrence of nuclear translocation of TORC2 and interaction with P-CREB following zymosan stimulation. In addition, the PKA inhibitor H-89 blocked P-CREB binding to the il10 promoter. LiCl on its own induced COX-2 and IL-10 production and enhanced zymosan effect. This agrees with its ability to enhance GSK3β-Ser9 phosphorylation and also with the recently reported ability of LiCl to enhance CREB-mediated transcription by favoring the interaction of TORC with the bZIP domain of CREB (52). The parallel induction of COX-2 and IL-10 points to close links for both proteins as regards mechanism of regulation and functional role. Human cox2 has two κB sites and two CRE sites (37, 38). In view of its ability to activate both NF-κB and CREB, zymosan is an optimal stimulus for COX-2 induction, which can occur even when both transcription factors compete for a limited amount of CBP, because blunting of κB-dependent transcription might be compensated by an increase of CREB-dependent transcription. Our findings also show a functional cooperation of COX-2 and IL-10 in the response to fungal stimuli, because endogenous PGE₂ enhances the effect of zymosan on IL-10 induction and the opposite effect is observed upon COX inhibition. The production of IL-10 by PGE₂ has been reported previously (53), and more recently the cooperation of IL-23 and PGE₂ to induce IL-17 has been described (54). Whether this cooperation can be explained on the basis of similar mechanisms to those described in this study is an open question in view of the strong induction of IL-23 elicited by zymosan (8, 55). It remains to be ascertained whether similar mechanisms of regulation of IL10 expression are operative in monocytes and macrophages. In preliminary experiments in macrophages derived from human monocytes differentiated for 14 days in the presence of serum, we observed lower levels of IL-10 protein induction upon zymosan challenge than in DC, whereas mannan was a potent stimulus. An explanation for this finding could be the different array of receptors expressed in DC and macrophages. For instance, macrophages do not show a level of DC-SIGN expression as high as DC, but it should also be taken into account that the expression of inhibitors of dectin-1 signaling may explain the lack of some responses to β-glucan signaling in some types of myeloid cells (56). Taken collectively, our data show that zymosan is a strong activator of CRE-dependent transcription in DC. This explains a production of cytokines dominated by IL-10 rather than by IL-12 p70. As to the mechanism of regulation of CREB activation, our results disclose an autocrine role for PGE₂ and a network of interactions of PKA, MAPK/ERK, GSK3β, and calcineurin that point to TORC2 as a central regulator of IL-10 expression.

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References


