Notch- and Transducin-like Enhancer of Split (TLE)-dependent Histone Deacetylation Explain Interleukin 12 (IL-12) p70 Inhibition by Zymosan

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The fungal analog zymosan induces IL-23 and low amounts of IL-12 p70. This study addresses the molecular mechanisms underlying this cytokine pattern in human monocyte-derived dendritic cells. The transcriptional regulation of il23a, one of the chains of IL-23, depended on the activation of c-Rel and histone H3 phosphorylation, as judged from the association of c-Rel with the il23a promoter and the correlation between IL-23 production and Ser-10-histone H3 phosphorylation. Consistent with its reduced ability to produce IL-12 p70, zymosan induced a transient occupancy of the il12a promoter by c-Rel, blocked the production of IL-12 p70 and the transcription of il12a induced by other stimuli, and triggered the expression and nuclear translocation of the transcriptional repressors of the Notch family and enhancer of split (Hes)-1, Hes5, hairy/ enhancer-of-split related with YRPW motif protein (Hey)-1, and transducin-like enhancer of split (TLE). Zymosan also induced the interaction of Hes1 and TLE with histone H3 phosphorylated on Ser-10 and deacetylated on Lys-14. Inhibition of class III histone deacetylases increased the production of IL-12 p70 and partially blunted the inhibitory effect of zymosan on the production of IL-12 p70 elicited by LPS and IFN-γ. These results indicate that the selective induction of IL-23 by β-glucans is explained by the activation of c-Rel associated with Ser-10-histone H3 phosphorylation in the il23a promoter mediated by mitogen- and stress-activated kinase and/or protein kinase A and inhibition of il12a transcription by a mechanism involving activation of several corepressors with the ability to bind TLE and to promote histone deacetylation.

IL-12 p70 (1) and IL-23 (2) are cytokines released by antigen-presenting cells that are involved in the induction or amplification of the T-helper type 1 and type 17 responses, respectively. IL-12 p70 and IL-23 share a common chain IL-12 p40 (il12/23b) and differ by another chain, IL-12 p35 (il12a) in the case of IL-12 p70 and IL-12 p19 (il23a) in IL-23. The production of IL-12 p70 and IL-23 is regulated at the transcriptional level and determined by their heterodimeric structure. il12a/23b regulation depends on NF-κB activation (3), whereas the transcriptional regulation of il12a also requires a type I interferon autocrine-paracrine loop involving interferon regulatory factors (IRF)-1, IRF-3, and IRF-8/ICSBP (3–6). Stimulation of Toll-like receptor (TLR)3-4 induces an IL-12/IL-23 balance different from that elicited through the TLR2 and C-type lectin routes (7, 8). At first glance, this may be explained because, unlike TLR2, TLR4 ligands activate the MyD88 and the TIR domain-containing adapter-inducing interferon-β routes and trigger both the NF-κB pathway and the type I IFN autocrine-paracrine loop. However, this does not explain why TLR2 ligands may behave as negative modulators of IL-12 p70 production (7, 9) nor the molecular mechanisms underlying the distinct patterns of IL-23/IL-12 p70 response. Whereas LPS induces both cytokines, Mycobacterium tuberculosis (7) and zymosan, an extract of the cell wall of Saccharomyces cerevisiae mainly composed of β-glucans that is recognized by at least the C-type lectin receptor dectin-1 (10) and TLR2 (11, 12), induce IL-23 and a low amount of IL-12 p70 (12–15). Moreover, coligation of the β-glucan receptor dectin-1 and TLR2 enhances IL-23 and down-regulates IL-12 p70, but there is no mechanistic explanation for this finding (8, 16). Addressing whether the effect of zymosan occurs via inhibition of il12a transcription or through a sole stimulation of il23a transactivation has pathophysiological relevance, because IL-23 takes part in the defense against pathogens and in the development of autoimmunity. Several hypotheses can be put forward to explain the distinct IL-12/IL-23 balances elicited by different stimuli as follows. (i) il23a induction could be explained through the activation of transcription factors of the family ATF-2/CREB. In fact, competition between CRE-binding protein (CREB) and NF-κB for the coactivator CREB-binding protein (CBP) explains the IL-12 p70/−/IL-10+/+ pattern induced by zymosan in dendritic cells (DC) (17). A corollary to this finding is that stimuli with a
Notch and Histones in the IL-12/IL-23 Balance

strong ability to activate CREB should produce a parallel reduction of κB-dependent transcription. In keeping with this mechanism, enhancement of IL-23 production by chemicals acting on the protein kinase A (PKA)/CREB route has been reported (18, 19).

(ii) Differential activation of NF-κB family elements could explain differences in IL-12 p70 and IL-23 production. For instance, c-Rel-p50 complexes regulate genes involved in T cell function, including IL-12 p70, whereas the RelA/p65 subunit regulates genes encoding inflammatory cytokines (20). This can be explained by different promoter κB-site sequences, but it may also depend on additional factors such as the capacity of RelA to be phosphorylated by PKA and to interact with CBP (21, 22), which allows acetylation of Lys-310, a site critical for transcriptional activity (23), or the unique ability of c-Rel/c-Rel homodimers to bind with high affinity to a broader range of NF-κB recognition sequences than do RelA-p65-containing complexes. This fact seems to be specially relevant regarding the transcriptional regulation of il12a in DC stimulated with LPS (24). In addition, a differential combination of NF-κB subunits could explain the occurrence of distinct transcriptional programs because activation of the β-glucan receptor dectin-1 allows for the formation of inactive RelA–RelB complexes (25), and an exchange of dimers allows fine-tuning of the response over time (26). Whether the reported enhancement of IL-23 production by cyclic AMP is associated with NF-κB signaling should be experimentally tested, taking into account that Ser-276 phosphorylation of p65 can also be elicited by mitogen- and stress-activated protein kinase-1 (MSK1) (27), thus suggesting alternative mechanisms of activation.

(iii) TLR and Notch routes cooperate in the activation of canonical Notch target genes. However, TLR- and Notch-induced hairy and enhancer of split (Hes)-1 and hairy/ enhancer-of-split related with YRPW motif protein (Hey)-1 also attenuate IL-6 and IL-12 p70 production via a feedback inhibitory loop that is abrogated by IFN-γ (28).

After testing all of these hypotheses, we have observed the unique involvement of c-Rel in the regulation of both il12a and il23a. Whereas LPS produced binding of c-Rel to both promoters, zymosan induced binding and early histone H3 phosphorylation of the il23a promoter as well as a transient binding of c-Rel to il12a promoter. The production of IL-23 correlated with the extent of histone H3 phosphorylation and was highly sensitive to H89, an inhibitor of the nucleosomal response (29). Addition of zymosan prior to the stimulation with LPS and IFN-γ induced a near complete inhibition of IL-12 p70 production. Zymosan inhibited IFN-γ signaling at different steps and also induced the expression of the transcriptional repressors Hes1, Hes5, Hey1, and the coexpressor transducin-like enhancer of split (TLE), which may bind the set of N- and E-boxes in the il12a promoter. In addition, zymosan induced the association of TLE and Hes1 with histone H3 showing increased Ser-10 phosphorylation and reduced Lys-14 acetylation. These data indicate that zymosan stimulates il23a transcription via c-Rel and histone H3 phosphorylation and activates transcriptional repressors of il12a that ultimately produce Lys-14-histone H3 deacetylation.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Mice—Zymosan, mannann from Saccharomyces cerevisiae, curdlan from Alcaligenes faecalis, H89, and the catalytic subunit of PKA were from Sigma. Depleted zymosan from S. cerevisiae was from InvivoGen (San Diego). Endotoxin levels in the reagents were below 1 ng/ml as determined by the limulus amebocyte lysate assay (Cambrex Bio Sciences, Walkersville, MD). Moreover, addition of 200 μg/ml polymyxin B did not modify the effect of the different stimuli. This negates the possible involvement of LPS in the responses studied. IL-12 p70 and IL-6 were assayed with reagents from Thermo Scientific Pierce and IL-23 with reagents from R&D Systems (Minneapolis, MN). IFN-β was assayed with reagents from PBL. InterferonSource (Piscataway, NJ). Recombinant histone H3.3 was from New England Biolabs (Ipswich, MA). Recombinant active MSK1, anti-Ser(P)-10-histone H3 (catalog no. 04-817), anti-Ac-Lys-14-histone H3 (catalog no. 07-353), anti-histone H3 (catalog no. 05-928), and PKA inhibitor peptide were from Upstate Biotechnology (Lake Placid, NY). DC were obtained from human monocytes as reported previously (15). Bone marrow-derived DC from mice were obtained by culture in the presence of murine recombinant GM-CSF.

Because deletion of hes1 in mice results in embryonic or neonatal lethality (30), hes1 conditional knock-out mice were generated by crossing hes1floxflox animals, kindly provided by Dr. Ryoiichiro Kageyama, with Mx1-Cre transgenic mice (the Jackson Laboratory). Littermates with hes1floxflox;Mx1-Cre (KO) or hes1+/+;Mx1-Cre (WT control) genotypes were intraperitoneally injected with 200 μg/mouse of poly(I:C) three times in 5 days to induce deletion, and mice were used for experiments 2 weeks later. hey1-null mice were kindly provided by Dr. Manfred Gessler (31), and hey1+/+ and hey1−/− littermates were used for experiments. Unless otherwise stated, LPS was used at a concentration of 10 μg/ml, zymosan at 1 mg/ml, and IFNs at 1000 units/ml.

Immunobots—Proteins were separated by electrophoresis in SDS-PAGE and transferred to nitrocellulose membranes. The membranes were used for immunodetection of c-Rel (Santa Cruz Biotechnology sc-70), RelB (Santa Cruz Biotechnology sc-226), p50 (Santa Cruz Biotechnology sc-7178), RelA/p65 (Millipore catalog no. 06-418), Ser(P)-276-p65 (Cell Signaling catalog no. 3031), Ser(P)-536-p65 (Cell Signaling catalog no. 3037), Stat1 (Cell Signaling catalog no. 9172), Tyr(P)-701-Stat1 (Cell Signaling catalog no. 9171S), Ser(P)-727-Stat1 (Cell Signaling catalog no. 9177), Hes1 (Santa Cruz Biotechnology sc-25392), Hes5 (Millipore, AB5708), and TLE (pan-TLE goat polyclonal, Santa Cruz Biotechnology sc-13373). Quantitative of the blots was carried out using Quantity One gel imaging software (Bio-Rad). For immunoblot directed to assay nuclear proteins, the nuclear extracts were obtained by using a nuclear extract kit (Active Motif, Carlsbad, CA).

Real Time RT-PCR—Purified RNA was depleted of genomic DNA by treatment with DNase (Turbo-DNA free™, Ambion) and used for RT reactions. The sets of primers for PCR are shown in Table 1. Cycling conditions were adapted to each set of primers. gapdh was used as a housekeeping gene to assess the relative abundance of the different mRNA, using the compara-
tive $C_T$ (cycle threshold) method. In the case of reactions directed to assay the il12a promoter and TLE 1, the PCR medium was supplemented with 1 mM betaine in view of the high GC content of the amplified sequences.

**IRF-3 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 of IRF-3 in the nuclear extracts were quantitated with TransAM (Active Motif) and expressed as the absorbance at 450 nm.

**ChIP Assay**—ChIP assays were conducted with reagents and Ab from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), as reported previously (17). PCRs were carried out with primers designed from both the il23a and il12a promoters (Table 1).

**Expression of the Rel Homology Domain (RHD) of Human c-Rel**—The RHD of human c-Rel(1–309) was constructed by PCR from a full-length c-Rel cDNA construct provided by Dr. Tse-Hua Tan (Baylor College of Medicine, Houston, TX) using PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA) and fused in-phase to the N-terminal histidine tag using SalI-NotI sites of a pET-28a vector. The histidine tag was purified with nickel beads and used for in vitro kinase assays.

**In Vitro Kinase Assay**—A 1-μg aliquot of substrate proteins was incubated at 30 °C for 20 min in reaction buffer (12 mM MOPS/NaOH, pH 7, 0.3 mM EDTA, 0.01% β-mercaptoethanol, 0.5% glycerol, 0.01 Brij-35, and 0.1 mg BSA) containing 15 ng of PKAc or active MSK1 and supplemented with 10 mM Mg(Ac)$_2$, 20 μM ATP, and 1 μCi of $[γ-^32P]$ATP. The reaction was stopped by addition of Laemmli buffer, and the phosphorlated substrate proteins were separated by SDS-PAGE in 15% acrylamide. Phosphate incorporation was analyzed using PhosphorImager technology. Phosphorylation of c-Rel was also assayed by Western blot using anti-Ser(P)276-p65 phosphospecific antibody.

**Immunoprecipitation of TLE and Hes1**—Immunoprecipitation of TLE, Hes1, Ser(P)10-histone H3, and Ac-Lys-histone H3 was carried out using the nuclear complex Co-IP kit (Active Motif). The clarified lysates were preabsorbed on protein G-Sepharose and then incubated overnight with anti-pan-TLE precipitating goat Ab or anti-Hes1 rabbit Ab, followed by 2 h of incubation with protein A/G Plus-agarose beads. Immune complexes were extensively washed, suspended in Laemmli sample buffer, and subjected to SDS-PAGE. Blots were stained to assess the input protein and the coimmunoprecipitation of Ser(P)-10- and Ac-Lys-14-histone H3.
RESULTS

Production of IL-23 by Different Stimuli—Zymosan and LPS were strong stimuli of IL-23 production in human monocyte-derived dendritic cells, whereas ovalbumin/anti-ovalbumin equivalence immune complexes and IFN-γ were inactive (Fig. 1A). Zymosan induced a higher production of IL-23 than LPS, but no significant differences were found. Maximal response to zymosan was observed with concentrations higher than 0.5 mg/ml (Fig. 1B), thus agreeing with previous results showing that optimal production is obtained with concentrations higher than 0.2 mg/ml and that different dose-response patterns can be observed for each cytokine (7). In line with recent reports stressing that ultrapure LPS does not consistently induce detectable quantities of IL-12 and IL-23 from human DC (32, 33), LPS response was not saturated with concentrations as high as 20 µg/ml (Fig. 1C), thus suggesting that the process of differentiation of monocytes into DC, which is associated with a decrease of the surface display of CD14, might explain these findings. Alternatively, optimal response to LPS might depend on the concomitant presence of signals elicited by other agonists, for instance IFNs (4, 34). Because zymosan is a β-glucan polymer that also contains mannose moieties, experiments were conducted with mannan, the pure β-glucan curdlan, and a combination thereof. Similar to zymosan, curdlan induced a predominant production of IL-23 (Fig. 1D), which was also observed with combinations of stimuli. Maximal concentrations of cytokines were observed at 24 h, with a trend of IL-23 production to decrease after this time when zymosan or combination of curdlan and mannan were employed (Fig. 1, E and F). Attempts to ascertain whether the induction of cytokines is
transcriptionally regulated were carried out by real time RT-PCR. As shown in Fig. 2, A and B, all of the stimuli induced the mRNA of *il23a*, without significant differences in the amounts elicited by LPS and zymosan (Fig. 2A). In contrast, the increase of *il12a* mRNA induced by LPS was 66-fold as high as that elicited by zymosan (Fig. 2C). The induction of *il12a/23b* mRNA elicited by LPS was 10-fold as high as that produced by zymosan and 5-fold as high as that observed in response to a combination of curdlan and mannan when the time points of maximal induction were considered (Fig. 2D).

### Transcriptional Regulation of *il23a* by the NF-κB Route—Nonselective inhibition of the NF-κB route with pyrrolidine dithiocarbamate and N-acetyl-leucinyl-leucinyl-norleucinal diminished IL-23 protein production, whereas SN50, an inhibitor of NF-κB1/p50 nuclear export, was inactive (Fig. 2E), thus confirming prior reports on the involvement of NF-κB proteins and in particular c-Rel in the regulation of *il23a* (35–37).

Involvement of the cyclic AMP/PKA route in the regulation of IL-23 stems from two different types of observations. First, the regulation of NF-κB transactivating activity by PKA-mediated phosphorylation of Ser-276-RelA (21) and, likely, a similar consensus sequence in c-Rel (see below). Second, the enhancement of IL-23 production by agents that increase intracellular cyclic AMP levels (18, 19). The PKA and nucleosomal response inhibitor H89 produced an almost complete inhibition of IL-23 production in response to zymosan and LPS (Fig. 2F). In contrast, the cell-permeable analog 8-bromo-cAMP and activation of adenyl cyclase with prostaglandin E2 did not increase IL-23 production (data not shown). This finding is consistent with an effect of H89 on c-Rel and/or RelA phosphorylation or on another target, rather than on the PKA/CREB system, because the catalytic subunit of PKA is tightly bound to ακB and RelA and does not require prior dissociation from its regulatory subunit by cyclic AMP to become active (21), but it differs from results reported in Schwann cells, where both Ser-276-RelA/p65 phosphorylation and κB-dependent transcriptional activity were induced by treatment with forskolin and dibutyryl-cAMP (22). Zymosan induced a rapid and lasting translocation of c-Rel, RelA/p65, and RelB into nuclear fractions, which increased up to at least 4 h, and a similar result was observed with the combination of mannan and curdlan (Fig. 3A). In contrast, p50 levels in nuclear fractions did not show significant changes upon cell stimulation (Fig. 3B), which agrees with the reported negative regulation of NF-κB target genes by p50 homodimers in resting cells (38). The phosphorylations of NF-κB elements were addressed with phosphospecific antibodies reactive to Ser(P)-276-RelA (this Ab could also recognize Ser(P)-267-c-Rel epitope) and Ser(P)-536-RelA. As shown in Fig. 3B, zymosan induced a faint phosphorylation of Ser-276-RelA and a more intense phosphorylation of Ser-536-RelA.

**Role of c-Rel and Histone H3 Phosphorylation**—c-Rel contains a sequence around Ser-267 similar to that around Ser-276 of RelA/p65 that plays a central role in the transcriptional activity (Fig. 4A). Because the use of phosphospecific antibodies to assess these phosphorylations has shown some limitations (39), we proceeded to carry out in vitro kinase assays with MSK1 and PKA catalytic subunit (PKAc), i.e. protein kinases inhibited by H89 (40). As shown in Fig. 4B, in vitro phosphorylation of the RHD of c-Rel and the S267A mutant did not induce any significant change of the reactivity with the phosphospecific antibody. This differs from the massive phosphorylation that has been reported when RelA/p65 was used as a substrate in *in vitro* kinase assays using the same Ab for immunoblotting (39). To confirm this issue, radioactive phosphate incorporation into the recombinant RHD of c-Rel was assessed. As shown in Fig. 4, C and D, both PKAc and MSK1 induced similar incorporation of phosphate into the RHD in both the wild type and the S267A mutant, thus suggesting that any of the 10 Ser and 14 Thr of the RHD phosphorylated in the wild type (Fig. 4A) is a target of MSK1, whereas in the S267A mutant, none of the 10 Ser and 14 Thr of the RHD was phosphorylated (Fig. 4B). Notably, although 1 μM H89 and PKAi completely inhibited histone H3.3 phosphorylation by PKAc, this was not observed with MSK1 (Fig. 4D, right panels). Further assessment of the effect of H89 on histone H3.3 phosphorylation by PKAc and MSK1 was conducted with different concentrations of H89. As shown in Fig. 4E, H89 still inhibited PKAc activity at 0.1 μM (IC₅₀ ~ 0.08 μM), thus showing a good correlation with the concentrations required to inhibit IL-23 production, whereas concentrations above 1 μM (IC₅₀ ~ 6 μM) were needed to inhibit MSK1 activity.

We next looked at the binding of NF-κB to *il12a* and *il23a* promoters using ChIP assays. Zymosan induced a significant

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**FIGURE 3. Nuclear translocation of NF-κB proteins in response to different stimuli.** DC were incubated with different ligands and at the times indicated were collected for the separation of nuclear fractions and the immunodetection of c-Rel, RelA/p65, RelB, p50, and the phosphorylated forms of RelA/p65. Results are representative of at least three independent experiments with similar results. TATA box-binding protein (TBP) was used as a load control.
binding of c-Rel to the il23a promoter (Fig. 5A). This binding was more prominent than that elicited by LPS and was accompanied by MSK1. Zymosan also elicited binding of the coactivator CBP to the il23a promoter, and this was inhibited by H89. Notably, histone H3 phosphorylation in the il23a promoter could be observed as soon as 30 min after zymosan addition and was inhibited by H89 (Fig. 5A, bottom panel). A weak and transient binding of c-Rel to the il12a promoter could be detected only 20 min after zymosan addition, whereas LPS-induced binding was detectable for at least 4 h (Fig. 5B). Although RelB binding was detected on both promoters, this was neither reproducible nor was it very different from that observed in resting cells. As regards RelA/p65 binding, we did not observe differences between resting and stimulated cells (data not shown). Contrary to the il23a promoter, the il12a promoter showed some degree of basal Ser-10-histone H3 phosphorylation, but this was not enhanced by zymosan nor showed consistent inhibition by H89, at least in the time frame of the experiments (Fig. 5B, bottom panel). Together, these results show distinct degrees and time patterns of c-Rel binding and Ser-10-histone H3 phosphorylation of il12a and il23a promoters that might explain the distinct responses elicited by the different stimuli.

Role of IFN and IFN Regulatory Factors—As shown in supplemental Fig. S1A, most stimuli induced a low amount of IFN-β protein, with the combination of LPS and poly(I:C) being the most active. A similar pattern of response was observed when the miRNA levels were assayed (supplemental Fig. S1A, lower panel). These results are consistent with the notion that
type I IFNs can robustly induce gene expression at low or even undetectable levels of signaling (41). Notably, zymosan was consistently found to inhibit IFN-β production. Further assessment of the IFN signaling loop was carried out by looking at the activation of IRF-1, IRF-3, and IRF-8. LPS was not found to enhance significantly IRF-3 binding activity, whereas this activity was clearly detected with a control protein (supplemental Fig. S1B). Induction of IRF-1 and IRF-8 was observed with IFNs and combinations of LPS and IFN/poly(I:C). Again, zymosan significantly inhibited IRF-1 and IRF-8 production (supplemental Fig. S1C).

**Stat1 phosphorylation** was addressed to assess downstream effects of IFN receptor activation. IFN-β was the most potent activator of Tyr-701-Stat1 phosphorylation (Fig. 6A). Zymosan produced a significant inhibition of Tyr-701-Stat1 phosphorylation at 1 h, but this did not reach statistical significance at 2 h. In contrast, all of the stimuli induced a similar degree of Ser-727-Stat1 phosphorylation at 1 h, which was not inhibited by zymosan (Fig. 6B). Taken together, these data suggest an inhibitory effect of zymosan at different levels of the IFN autocrine-paracrine loop that might explain a portion of its effect on the IL-23/IL-12 balance.

**Zymosan Inhibits the Effect of LPS and IFN-γ**—Preincubation of DC with zymosan induced a dose-dependent inhibition of IL-12 p70 production without significantly affecting IL-23 levels. This was observed with a preincubation of 1 h, but was more intense after overnight incubation (Fig. 7A). This inhibition was detectable with concentrations higher than 0.5 mg/ml and was more effective when depleted zymosan was used, thus suggesting that the inhibition is independent of TLR-activating components (Fig. 7B). The inhibitory effect of zymosan was at the transcriptional level because il12a mRNA was decreased (Fig. 7C). Attempts to associate the effect of zymosan with the β-glucan receptor dectin-1 were carried out by adding 10 μg/ml anti-dectin-1 mAb 30 min before zymosan. As shown in supplemental Fig. S2A, the inhibitory effect of zymosan on the production of IL-12 p70 elicited by combination of LPS and IFN-γ was also observed in the DC pretreated with the blocking mAb. Further evidence that dectin-1 is not the main receptor involved in the inhibitory effect of zymosan was obtained in bone marrow-derived DC from *dectin-1−/−* mice. As shown in supplemental Fig. S2B, *dectin-1−/−* mice showed a lower production of IL-12 p70 in response to combination of LPS and IFN-γ as compared with *dectin-1+/+* mice; however, zymosan exerted a similar inhibition in both cases. These findings indicate that TLR2 is not involved in the mediation of the inhibitory effect of zymosan on IL-12 p70 production and that the sole activation of

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**FIGURE 5. Promoter binding and histone H3 phosphorylation.** DC were stimulated in the presence and absence of H89 and at the times indicated were fixed with 1% formaldehyde. After separation of the nuclear pellet, solubilization of chromatin, and pre-clearing, antibodies were added for ChIP assays. Semiquantitative PCRs were carried out for 40 cycles with primers from both il23a (A) and il12a promoters (B). Reactions with il12a primers were conducted in media supplemented with 1 mM betaine. Sequences of *il12a* and *il23a* κB sites are shown for comparison with functional sites that preferentially bind RelB and c-Rel (C).
Notch and Histones in the IL-12/IL-23 Balance

Mechanism of Zymosan-induced Inhibition of il12a Transcription—We next addressed the activation by zymosan of the transcriptional repressors Hes1 and Hey1, which have been reported to block IL-6 and IL-12 p70 production by TLR ligands (28). In keeping with this notion, zymosan and depleted zymosan inhibited the production of IL-6 (Fig. 8A). Stimulation of DC with zymosan and LPS produced a marked induction of hes1 mRNA, whereas hey1 mRNA was only induced by zymosan (Fig. 8B). This was accompanied by nuclear translocation of Hes1 protein in response to zymosan (Fig. 8C). Hes1 nuclear translocation was also observed with depleted zymosan and when IFN-γ was added 1 h before zymosan (Fig. 8D). IFN-γ reduced LPS-induced hes1 and hey1 mRNA expression and to a lesser extent the effect of zymosan (supplemental Fig. S3). However, this inhibition was not observed when combination of LPS and IFN-γ was used, thus suggesting a synergistic effect of LPS and zymosan that maximal inhibition of IL-12 p70 production is associated with maximal induction of hes1 and hey1 and with nuclear translocation of Hes1 (Fig. 9A). Transcriptional repression by Hes1 and Hey is most frequently due to interaction with TLE. Consistent with this mechanism, TLE protein expression was observed 1 h after addition of zymosan followed by nuclear translocation at 3 and 6 h (Fig. 9B). The mRNA encoding tle1, the most abundant isoform in myeloid cells, was increased 1 h after the addition of zymosan (Fig. 9C). In contrast, LPS and IFN-γ did not induce TLE protein nor mRNA expression (Fig. 9, B and C). The effect of TLE on transcription depends on its interaction with histone H3 and the ensuing recruitment of histone deacetylases. In line with this, TLE1 binds both class I (42) and class III histone deacetylases (43), whereas Hes1 and Hey2 bind class III deacetylases (44). Zymosan produced the interaction of both TLE and Hes1 with Ser(P)-10-histone H3 as shown in coimmunoprecipitation experiments (Fig. 9D), which agrees with the aforementioned involvement of Ser-10-histone H3 phosphorylation in IL-23 production and with the occurrence of transcriptional repression linked to the association of TLE with the N-terminal residues of histone H3 (45). Unlike combination of LPS and IFN-γ (Fig. 9D, rightmost lower panel), zymosan decreased the amount of TLE- and Hes1-associated Ac-K14-histone H3 (Fig. 9D), which is consistent with the notion that zymosan enhances the recruitment by TLE and Hes1 of histone deacetylases to selected promoters, even though it might exert mild effects on global histone H3 acetylation (see input and nuclear fractions in Fig. 9D). Unlike the il23a promoter, zymosan also reduced the amount of Ac-Lys-14-histone H3 in the il12a promoter (Fig. 9E), although to a lower extent than in the coimmunoprecipitation experiments, thus suggesting that ChIP assays may detect acetylated histone H3 along the promoter, whereas coimmunoprecipitation experiments pull down histone H3 in close contact with TLE and/or Hes1. Experiments with inhibitors of lysine deacetylases showed that the inhibitor of class I and II enzymes trichostatin (TSA) induced a complete blockade of IL-12 production, whereas the class III deacetylase inhibitor indole EX527 (46) enhanced the production of IL-12 p70 and reduced the inhibitory effect of zymosan (Fig. 9F). Because the function of TLE is not restricted to interaction with elements of the Notch route, as it can also interact with Runt domain transcription factors and proteins involved in Wnt signaling as Lef/Tcf, the effect of zymosan in hes1−/− and hey1−/− animals was addressed. As shown in Fig. 10, A–C, individual ablation of these genes did not suppress the inhibitory effect of zymosan, thus suggesting that there is redundancy between elements of the Notch route and/or that proteins of other families may interact with TLE. In keeping with the first hypothesis is the finding of strong induction and nuclear translocation of Hes5 in response to zymosan (Fig. 10D). Taken together, these data indicate that zymosan induces a TLE-dependent blockade of il12a transcriptional expression that might be exerted through Notch-dependent routes, and it ultimately depends on histone deacetylation reactions most likely dependent on class III histone deacetylases (sirtuins).

DISCUSSION

The present results confirm the strong induction of IL-23 expression and the low production of IL-12 p70 elicited by zymosan, which differs from the response elicited by the LPS/TLR4 route. The largest differences between the effect of LPS and zymosan were observed on il12a mRNA expression, thus
suggesting that the low induction of il12a elicited by zymosan may be the main factor explaining IL-23 polarization. Most reports on the transcriptional regulation of il23a have focused on the role of NF-κB and ATF/CREB factors, although there is at least one report showing the involvement of AP-1 (47) and a recent report stressing the involvement of C/EBP homologous protein (CHOP) (33). Our data are consistent with a role for NF-κB in the regulation of il23a but are less conclusive as regards ATF/CREB factors. On the one hand, pharmacological modulation of intracellular cyclic AMP levels did not influence IL-23 production, thus differing from the results observed on IL-10 production, which is strongly dependent on CREB activity (17, 48). On the other hand, the purported PKA inhibitor H89 produced a complete inhibition of IL-23 production. Because H89 inhibits both PKA and MSK activities and these kinases phosphorylate histone H3 and nonhistone chromosomal protein HMG-14 (26, 49) in addition to both CREB (50) and RelA/p65, H89 effects should be scrutinized before being assigned to any specific target. The involvement of MSK1 in IL-23 production is supported by its location to the il23a promoter in response to zymosan and by the complete inhibition of IL-23 production by the p38 MAPK inhibitor SB203580, as MSK1 and MSK2 are activated downstream of p38 MAPK (51).

c-Rel has been involved in the control of murine il12a, because this promoter contains the sequence 5’-GGG-AATCC-3’ found in functional κB sites that preferentially bind c-Rel-containing dimers (3, 6, 52). Notably, this sequence is also found in the il23a promoter. Zymosan enhanced the binding of

FIGURE 7. Zymosan inhibits IL-12p70 production. DC were incubated with 1 mg/ml zymosan or left untreated for 1 h or overnight prior to the addition of the indicated stimuli. After an additional period of 24 h, IL-12 p70 and IL-23 were assayed in the supernatants. This is a representative experiment out of three with a similar trend (A). The effect of depleted zymosan and the TLR2 ligand Pam3CSK is shown in B. The effect of zymosan on the mRNA encoding if12a is shown in C.
c-Rel to the il23a promoter together with MSK1 and CBP, and it increased early Ser-10-histone H3 phosphorylation, thus suggesting optimal conditions for transcriptional activation. Unlike zymosan, LPS induced c-Rel binding to the il12a promoter at least from 30 min to 4 h after addition of the stimulus. As to the role played by Ser(P)-267-c-Rel in the transcriptional regulation of il23a, our data do not support the involvement of the canonical RRPS sequence around Ser-267. This agrees with the report by Yu et al. (53), who showed that stimulation by PKAc/H9252 of the transcriptional activity of c-Rel was not affected by S267A mutation, thus indicating that there are other sites in c-Rel that could be phosphorylated by PKAc. Alternatively, these findings suggest that the effect of PKAc on c-Rel-dependent transcription might depend on Ser-10-histone H3 phosphorylation in view of the strong interaction of c-Rel and PKA-Cβ disclosed in that study, which was maintained in the S267A mutant and is reminiscent of the PKA translocation associated with protein kinase A-anchoring proteins that regulate local protein phosphorylation (54). Altogether, the present results are consistent with a central role for c-Rel and Ser/Thr kinases in the regulation of il23a transcription; however, the lack of formal proof of Ser-267-Rel RHD phosphorylation and the correlation between the concentrations of H89 required to inhibit IL-23 production and histone H3.3 phosphorylation, point to histone H3 phosphorylation as the target of PKA and/or MSK. It seems possible that both kinases might exert a redundant function, but the finding that inhibition of histone H3.3 phosphorylation by PKA shows better correlation with inhibition of IL-23 production than inhibition of MSK on the same substrate suggests a preferential involvement of PKA.

Our data indicate that the differences in the production of IL-12 p70 elicited by LPS and zymosan can be the result of differential binding of c-Rel to the κB sites of il12a, but an explanation for this difference is not obvious in view of the similar sequences of the κB sites in il12a and il23a promoters (Fig. 5C). Although we did not disclose a potent effect of zymosan on IFN signaling, a reduction of the IFN signature was observed, thus agreeing with previous reports on ligands of receptors containing ITAM motifs (55, 56). Notably, our findings agree most with the recently reported attenuation of IL-12 p70 production by the transcriptional repressors Hes1 and Hey1. TLR2 and TLR4 signaling induces the canonical Notch target genes hes1 and hey1 (28), which in turn behave as feedback inhibitors of IL-6 and IL-12 family cytokine genes. Another mechanism of hes1 induction has been reported in hepatocytes, where CREB-mediated induction of hes1 inhibits peroxisome proliferator-activated receptor-γ expression in the fasted state (57). Whether this route is also implicated in the immune system is worth noting in view of the prominent ability of zymosan to activate the CREB route. In contrast, IFN-γ sig-
naling blocks this route by interfering with Notch intracellular domain expression, which is upstream of Hes1 and Hey1 induction. The mechanism whereby TLR signaling induces Hes1 depends on p38 MAPK, IKK, and phosphorylation of Ser-10-histone H3 in the hes1 promoter (supplemental Fig. S4). Hes1 and Hey1 inhibit transcription by binding to N- and E-boxes in proximal promoters. In silico analysis of human il12a promoter disclosed three E-boxes upstream of the /H9260 B and IFN-/H9253 -activated sites. Notably, the nearest one to these sites matches the perfect palindrome sequence CACGTG optimal for Hes1 and Hey1 binding. One of the best established mechanisms whereby mammal Hes proteins repress transcription is by their ability to recruit the Groucho homologs TLE1–4 to generate a transcriptional repressor complex (58). TLE interacts with the N-terminal residues 1–46 of histone H3 and attracts further corepressors like class I and III histone deacetylases and members of the Sin3 complex, thus leading to strong transcriptional repression (45). The present results extend the aforementioned model of negative feedback modulation by showing strong induction of Hes1, Hes5, Hey1, and TLE, as well as physical interaction of TLE and Hes1 with Ser(P)-10-histone H3 and deacetylated Lys-14-histone H3 in response to zymosan. Experiments with knock-out mice indicate that isolated genetic abrogation of hes1 and hey1 does not eliminate the inhibitory effect of zymosan. Because zymosan also induces the expression of Hes5 and TLE, and Hes5 has been found to be the main player in the development of pathophysiological conditions such as pulmonary arterial hypertension (59), it seems likely that il12a repression might occur through a redundant mechanism involving various partners at initial steps, but ultimately depending on TLE and histone deacetylase-mediated repression. As judged from the outcome of pharmacological experiments, class III deacetylases targeted by the indole compound EX527, rather than TSA-sensitive class I and II enzymes, are involved. This is consistent with the capacity of both TLE and Hes1 to bind the class III lysine deacetylase Sirt1 and with the finding that both TLE and Sirt1 are required for inhibition of NF-/H9260 B activity (43). However, inhibition of IL-12 p70 production by TSA can be best explained by targeting transcription factors, rather than histones, because both TSA-dependent and -independent deacetylases are involved in the modulation of NF-κB and IFN regulatory factor activity (60–63), and TSA blocks IL-12 p70 production at concentrations that only slightly affect global histone H3 acetylation (Fig. 9D). In addition to its interaction with

FIGURE 9. Effect of zymosan on the expression of Hes1 and TLE, and histone H3 phosphorylation and acetylation elicited by different ligands. Hes1 protein was assayed 6 h after incubation with the indicated additions (A). The induction and the nuclear translocation of TLE by zymosan is shown in B. TATA box-binding protein (TBP) was used as a load control of nuclear proteins. The expression of the mRNA encoding tle1 after incubating DC for 1 h with different additions is shown in C. Coimmunoprecipitation of TLE and Hes1 with Ser(P)-10-histone H3 and Ac-Lys-14-histone H3, DC were incubated in the presence and absence of 1 mg/ml zymosan for 5 h, and then 500 μg of protein from the nuclear fractions were used for immunoprecipitation with 5 μg of either pan-anti-TLE Ab or anti-Hes1 Ab. Anti-Ser(P)-10-histone H3, anti-Ac-Lys-14-histone H3, and anti-histone H3 Ab were used for blotting. Input nuclear fractions are shown in the right panels. The effect of combination of LPS and IFN-γ and 100 nM trichostatin on global Ac-Lys-14-histone H3 and on the acetylation of histone H3 coimmunoprecipitated with Hes1 in DC treated with LPS + IFN-γ is shown in the lower panel (D). Changes in the acetylation state of Lys-14-histone H3 in the il12a and il23a promoters after 5 h of stimulation with the indicated additions or treatment with 100 nM TSA are shown in E. The effect of TSA and indole EX527 on the production of IL-12 p70 is shown in F. Results show mean ± S.D. of four experiments. *, p < 0.05 as compared with controls in the absence of drug.
the Notch route, TLE may exert transcriptional repression through binding to Pax/BASP, Six homeobox proteins, Runt transcription factors, and Lef/Tcf. Notably, the E-box nearest to the transcription start of the *il12a* promoter overlaps with a consensus site for Runx binding (supplemental Fig. S4). Because Runx proteins are expressed in DC and may exert TLE-dependent repression (64), a plausible explanation might be that wherever TLE induction takes place, its interaction with context-specific transcription factors gives rise to the formation of high order complexes with gene-specific sequence repression. This agrees with current views regarding Gro/TLE proteins as versatile corepressors that can sense and integrate different cellular inputs to regulate the expression of large sets of genes (65). These data suggest a combinatorial formation of complexes from distinct protein families and the possibility of allowing the transition from activation to repression for transcription factors capable of interacting with TLE. To the best of our knowledge, this is the first description of the induction of TLE during the innate immune response.

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