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Abbreviations used

AcK ₃₁₀ p65:	Acetylated lysine 310 residue of NF-κBp65
ARF:	Acute respiratory failure
CBP:	CREB-binding protein
CDK9:	Cyclin-dependent kinase 9
ChIP:	Chromatin immunoprecipitation
COPD:	Chronic obstructive pulmonary disease
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HAT:	Histone acetyltransferase
HDAC:	Histone deacetylase
MIP:	Macrophage inflammatory protein
NF-κB:	Nuclear factor κB
Pol II:	Polymerase II
PRL:	Prolactin
pS ₂₇₆ p65:	LPS-induced phosphorylation of the nuclear factor κB p65 serine 276 residue
pS ₂ Pol II:	Pol II phosphorylated at the Ser2 residue of the C-terminal domain
qPCR:	Quantitative real-time PCR
STAT:	Signal transducer and activator of transcription
TLR:	Toll-like receptor

mechanisms regulating cytokine gene transcription.¹¹ Recruitment of histone acetyltransferases (HATs), such as p300, CREB-binding protein (CBP), and p300/CBP-associated factor, to cytokine/chemokine promoters is crucial for transcriptional activation. Conversely, histone deacetylases (HDACs) play a central role in the negative regulation of inflammatory responses by inducing deacetylation of histones, chromatin condensation, and transcriptional repression.¹²

On the basis of these premises, we investigated the molecular mechanisms through which IL-10 directly inhibits the transcription of CXCL8 and TNF-α induced by LPS in human monocytes. Specifically, we focused on IL-10-induced covalent modifications of histones and transcription factors embedded in the CXCL8 and TNF-α promoters. Our results suggest a model whereby IL-10 regulates CXCL8 and TNF-α transcriptional output by acting on 2 different rate-limiting steps: phosphorylation of nuclear factor κB (NF-κB) p65Ser₂₇₆ residue induced by LPS and histone H4 deacetylation mediated by HDAC2. Finally, in support of our *in vitro* findings, in peripheral blood monocytes from patients with COPD, we observed a deficiency in HDAC2 intracellular contents, which dampened CXCL8 suppression by IL-10.

METHODS**Cell purification and culture**

Monocytes were purified from buffy coats of healthy donors by means of density centrifugation and cultured as described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

PBMCs were isolated from venous blood from 16 subjects recruited for this study. All the patients' and healthy donors' features are described in the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) experiments were performed, as described elsewhere,⁹ and carried out with the antibodies listed in the [Methods](#) section in this article's Online Repository. Purified DNA was analyzed by

using promoter-specific primers (see [Fig E1](#) in this article's Online Repository at www.jacionline.org).

Quantitative real-time PCR

Gene-specific primer pairs used in quantitative real-time PCR (qPCR) for cytokine primary transcript and mRNA expression are listed in [Table E2](#) in this article's Online Repository at www.jacionline.org. Data are expressed as mean normalized expression units after glyceraldehyde-3-phosphate dehydrogenase (GAPDH) normalization.

Immunoblots

Preparation of cell lysates and Western blot analysis were conducted as described in detail in the [Methods](#) section in this article's Online Repository. The blots were incubated with the antibodies anti-HDAC1 (sc-7872) and anti-HDAC2 (sc-7899; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-signal transducer and activator of transcription (STAT) 3 (#9139; Cell Signaling, Danvers, Mass), anti-Ach4 (06-866; Upstate Biotechnology, Lake Placid, NY), or anti-GAPDH (#4300; Life Technologies, Grand Island, NY), and detection was performed and quantified by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Neb).

Laser confocal microscopy

Cytospin preparations of 0.1×10^6 cells were fixed and permeabilized, as previously described¹³ and detailed in the [Methods](#) section in this article's Online Repository. Preparations were visualized by using confocal laser scanning microscopy (Leica-Microsystems, Wetzlar, Germany). Image analysis and signal quantification were performed with LAS Advanced Fluorescence Lite software (Leica-Microsystems).

Detection of CXCL8 release

CXCL8 concentrations in cell-free supernatants were measured by using a specific ELISA kit (Mabtech, Nacka Strand, Sweden), according to the manufacturer's instructions.

Chemotaxis assay

Human granulocyte chemotaxis was assessed by using 96-transwell Boyden chambers of 3/5-mm pore size (Corning Costar, Corning, NY), as described in detail in the [Methods](#) section in this article's Online Repository.

Statistical analysis

Statistical evaluation was performed with the Student *t* test, 1-way ANOVA followed by the Tukey posttest, or 2-way ANOVA followed by the Bonferroni posttest. *P* values of less than .05 were considered statistically significant. Data are expressed as means ± SEMs.

Study approval

Human samples were obtained after informed written consent by healthy donors and patients. The study has been cleared by the Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy).

RESULTS**IL-10 inhibits LPS-induced activation of the CXCL8 and TNF-α promoter-bound RNA polymerase II**

Preliminary experiments confirmed that in LPS-stimulated monocytes IL-10 inhibits CXCL8 and TNF-α production and secretion (see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org), as well as CXCL8 and TNF-α mRNA expression (see [Fig E2, B](#)), by acting at the transcriptional level

(see Fig E2, C).⁴ Polymerase II (Pol II) recruitment and activation in monocytes cultured for 1 hour with LPS in the presence or the absence of IL-10 were analyzed by using the ChIP assay to start elucidating the molecular basis of the CXCL8 and TNF- α transcriptional inhibition directly mediated by IL-10. qPCR of the immunoprecipitated DNA showed a robust Pol II enrichment on LPS stimulation that was not affected by IL-10 (Fig 1, A). Conversely, LPS-induced increased occupancy of CXCL8 and TNF- α promoters by “activated” Pol II phosphorylated at the Ser2 residue of the C-terminal domain (pS₂Pol II) was found to be significantly inhibited by IL-10 (Fig 1, B). Consistent with these data, IL-10 inhibited also LPS-induced increase of Pol II occupancy at genomic sequences located at the intron-exon 4 boundary of the *CXCL8* and *TNFA* genes at levels comparable with pS₂Pol II (Fig 1, C). In parallel, the recruitment of cyclin-dependent kinase 9 (CDK9; Fig 1, D) and the bromodomain-containing protein Brd4 (Fig 1, E), which are required during the transition from initiation of transcription to elongation,¹⁴⁻¹⁷ was significantly reduced in the presence of IL-10. Conversely, no inhibition of LPS-induced recruitment of these same proteins to the $\text{I}\kappa\text{B}\alpha$ promoter, a canonical LPS-induced NF- κB -responsive gene, was observed (see Fig E3, A-E, in this article’s Online Repository at www.jacionline.org). The promoter of prolactin (PRL; Fig 1, A-E) and ChIP performed with isotype matching control (see Fig E3, F) were used as negative controls. Notably, such an IL-10-mediated mechanism selectively targets LPS. In fact, in monocytes activated for 1 hour with TNF- α or other Toll-like receptor (TLR) agonists, including R848 (a TLR7/8 agonist), Pam3CSK4 (a TLR2 agonist), and polyinosinic:polycytidylic acid (a TLR3 agonist), IL-10 was found to inhibit CXCL8 and TNF- α transcription through a rapid and direct inhibition of Pol II recruitment to the respective promoters (see Fig E4 in this article’s Online Repository at www.jacionline.org). Altogether, these data demonstrate that IL-10 inhibits Pol II activation and CXCL8 and TNF- α transcript elongation through inhibition of LPS-induced Brd4 and CDK9 recruitment but without interfering with Pol II recruitment.

IL-10 inhibits the LPS-induced Brd4-docking sites at the CXCL8 and TNF- α promoter level

Several studies have established that acetylated lysine 310 residue of NF- κB p65 (AcK₃₁₀p65) represents a specific docking site for Brd4 recruitment.^{18,19} Acetylation of K₃₁₀p65 occurs after recruitment of a HAT, which in turn depends on prior NF- κB p65 phosphorylation on the serine 276 residue (pS₂₇₆p65).^{20,21} In our system LPS-induced NF- κB p65 recruitment to the CXCL8 and TNF- α promoters remained unmodified in the presence of IL-10 (Fig 2, A), which is consistent with previous knowledge.^{9,22} Nonetheless, IL-10 significantly inhibited the levels of LPS-induced pS₂₇₆p65 recruitment to the CXCL8 (by 74% $\pm$ 12%, $P = .015$, $n = 3$) and TNF- α (by 60% $\pm$ 12%, $P = .026$, $n = 3$) promoters (Fig 2, B). Also, the levels of p300 (Fig 2, C), but not CBP (Fig 2, D), recruited to the CXCL8 and TNF- α promoters in response to LPS were significantly inhibited by IL-10. Consistent with these results, IL-10 inhibited also the levels of the promoter-bound AcK₃₁₀p65 induced by LPS by 51.5% $\pm$ 7.6% ($P = .005$, $n = 3$) for the CXCL8 promoter and by 39.2% $\pm$ 4.6% ($P = .04$, $n = 3$) for the TNF- α promoter (Fig 2, E). Interestingly, confocal microscopy experiments showed that IL-10 does not modify LPS-induced nuclear

translocation of p65 (Fig 3, A and C) but rather inhibits the upstream LPS-induced phosphorylation of S₂₇₆p65 in the cytosol (Fig 3, B and D). Altogether, these data suggest that IL-10-mediated inhibition of pS₂₇₆p65 recruited to CXCL8 and TNF- α promoters in response to LPS determines a reduction in the levels of promoter-bound p300, which in turn is likely responsible for the decrease in levels of the Brd4-docking molecule AcK₃₁₀p65.

IL-10-mediated inhibition of LPS-stimulated H4 acetylation and CXCL8 transcription is dependent on HDAC2 recruitment

Previous studies have shown that, in addition to AcK₃₁₀p65, acetylated lysine residues of histones might function as Brd4-docking sites.²³ Fig 4, A, shows that LPS-triggered H4 acetylation was reduced by IL-10 at the CXCL8, but not TNF- α , promoter, suggesting that H4 deacetylation might play a role in suppression of CXCL8, but not TNF- α , transcription. Notably, such an IL-10-mediated mechanism is once again selective for LPS, as in monocytes activated for 1 hour with TNF- α or other TLR agonists, no reduction of AcH4 at the CXCL8 promoter was observed in the presence of IL-10 (see Fig E4, D). Because the observed reduction in levels of CXCL8 promoter-bound AcH4 determined by using IL-10 (Fig 4, A) might derive from either reduced p300 HAT (Fig 2, C) or increased HDAC function, we subsequently analyzed the effect of various HDAC inhibitors. Accordingly, monocytes were pretreated for 45 minutes with 10 $\mu\text{mol/L}$ MC-1568, an inhibitor of type II HDACs (which include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), or 10 $\mu\text{mol/L}$ CI-994, which selectively inhibits the type I HDACs (HDAC1 and HDAC2), and subsequently incubated for 1 hour with LPS or LPS plus IL-10 for AcH4 (Fig 4, B), Brd4 (Fig 4, C), CDK9 (Fig 4, D), and pS₂Pol II (Fig 4, E) ChIP assays, as well as CXCL8 and TNF- α transcriptional rate analysis (Fig 4, F). Notably, CI-994, but not MC-1568, abrogated the ability of IL-10 to inhibit LPS-induced H4 acetylation at the level of the CXCL8 promoter (Fig 4, B), thus suggesting that the activity of type I HDACs is required to accomplish an IL-10-mediated effect. As a result of the failure by IL-10 to reduce AcH4 levels in the presence of CI-994, the downstream IL-10-mediated inhibition of Brd4 (Fig 4, C) and CDK9 (Fig 4, D) recruitment, Pol II activation (Fig 4, E), and CXCL8 transcription (Fig 4, F) was lost. Consistently, the release of biologically active CXCL8 by LPS-stimulated monocytes in the presence of CI-994 was not inhibited by IL-10 (see Fig E5 in this article’s Online Repository at www.jacionline.org). In parallel and consistent with the lack of effect of IL-10 on TNF- α promoter-bound AcH4 (Fig 4, A), HDAC inhibitors did not affect the ability of IL-10 to inhibit any of the subsequent LPS-induced events (Fig 4, right panels).

Collectively, these data strongly suggest that type I HDAC activity is required for IL-10-mediated inhibition of the recruitment and assembly of the preinitiation complex triggered by LPS at the CXCL8 promoter. They also suggest that suppression by IL-10 proceeds in an HDAC-independent manner for the TNF- α promoter.

Levels of CXCL8 promoter-bound HDAC1 and HDAC2 were investigated to directly ascertain the role of type I HDAC in the IL-10-mediated suppression of CXCL8 transcription (Fig 5). Although a constitutive HDAC1/HDAC2 occupancy of the CXCL8 and TNF- α promoters was observed in unstimulated monocytes, confirming previous observations,²⁴⁻²⁶ a decrease in

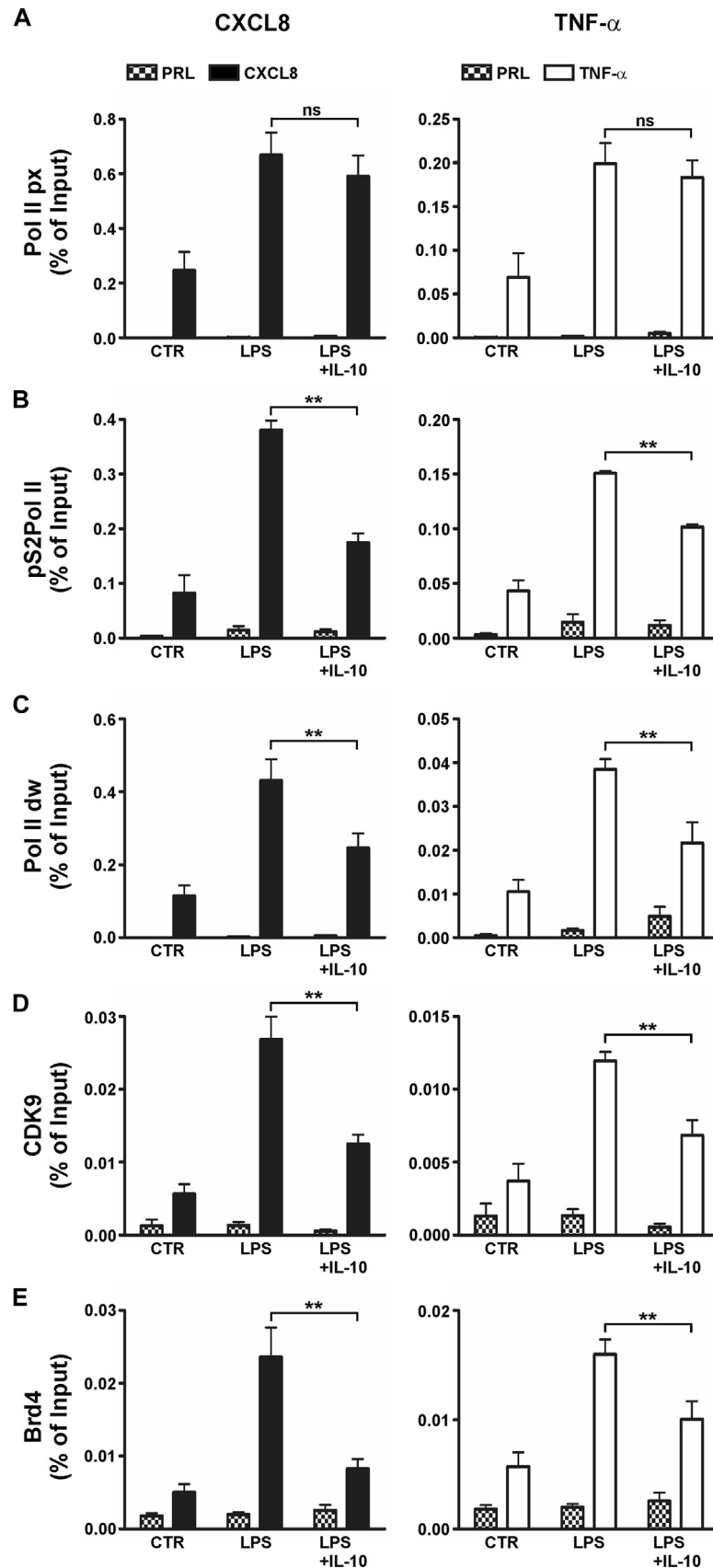


FIG 1. IL-10 inhibits LPS-induced Pol II activation and CDK9 and Brd4 recruitment to the CXCL8 and TNF- α promoters. Monocytes cultured for 1 hour as indicated were processed for ChIP assay with anti-Pol II (A and C), anti-pS2Pol II (B), anti-CDK9 (D), and anti-Brd4 (E) antibodies. Data are expressed as percentage over input DNA and are displayed as means $\pm$ SEMs ($n = 4$). ** $P < .01$. CTR, Control; ns, not significant.

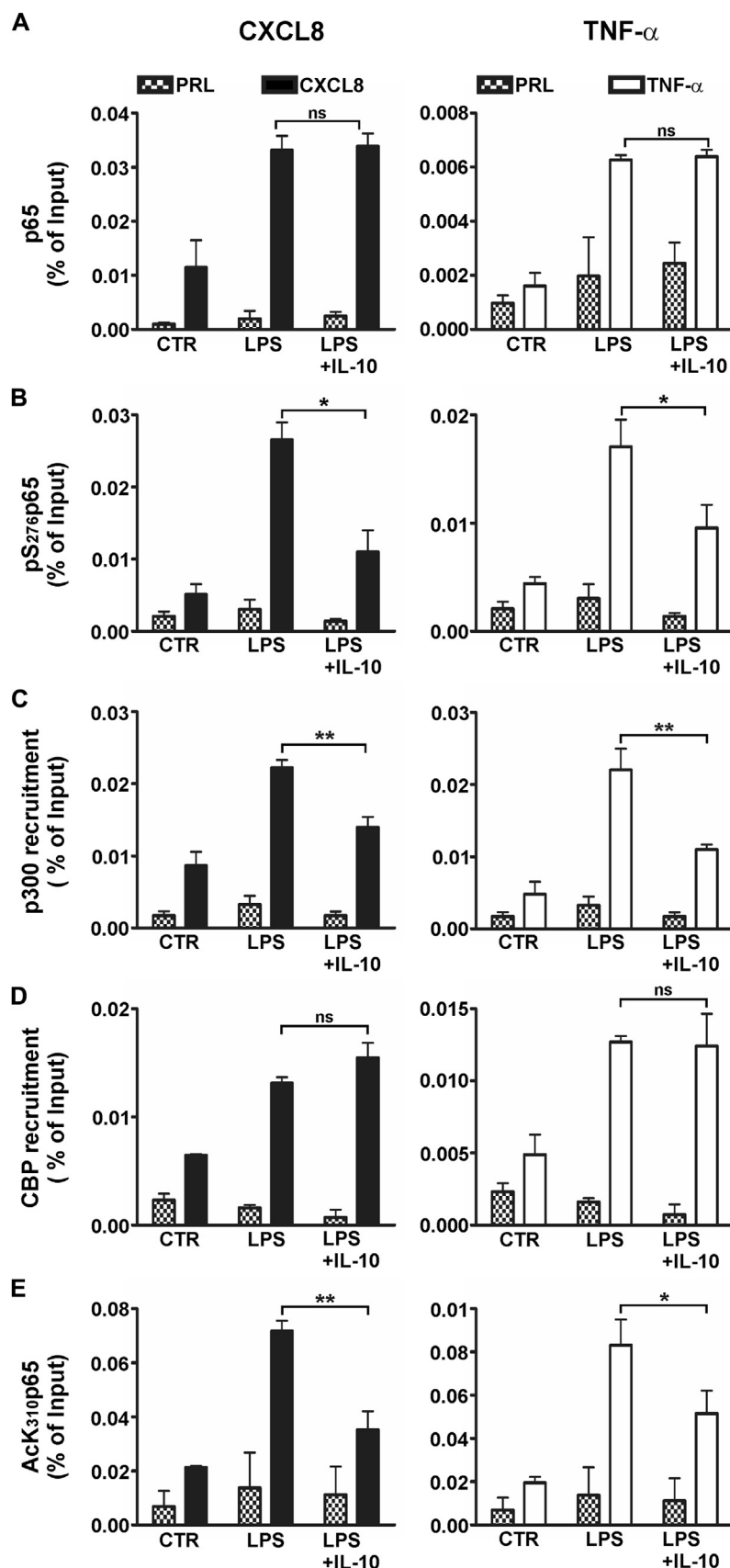


FIG 2. Effect of IL-10 on LPS-induced p65 activation and HAT recruitment. Monocytes cultured for 1 hour as indicated were subjected to ChIP analysis with anti-p65 (A), anti-pS₂₇₆p65 (B), anti-p300 (C), anti-CBP (D), and anti-AcK₃₁₀p65 (E) antibodies. Data from qPCR are expressed as percentage over input DNA and displayed as means $\pm$ SEMs (n = 4). **P* < .05 and ***P* < .01. CTR, Control; ns, not significant.

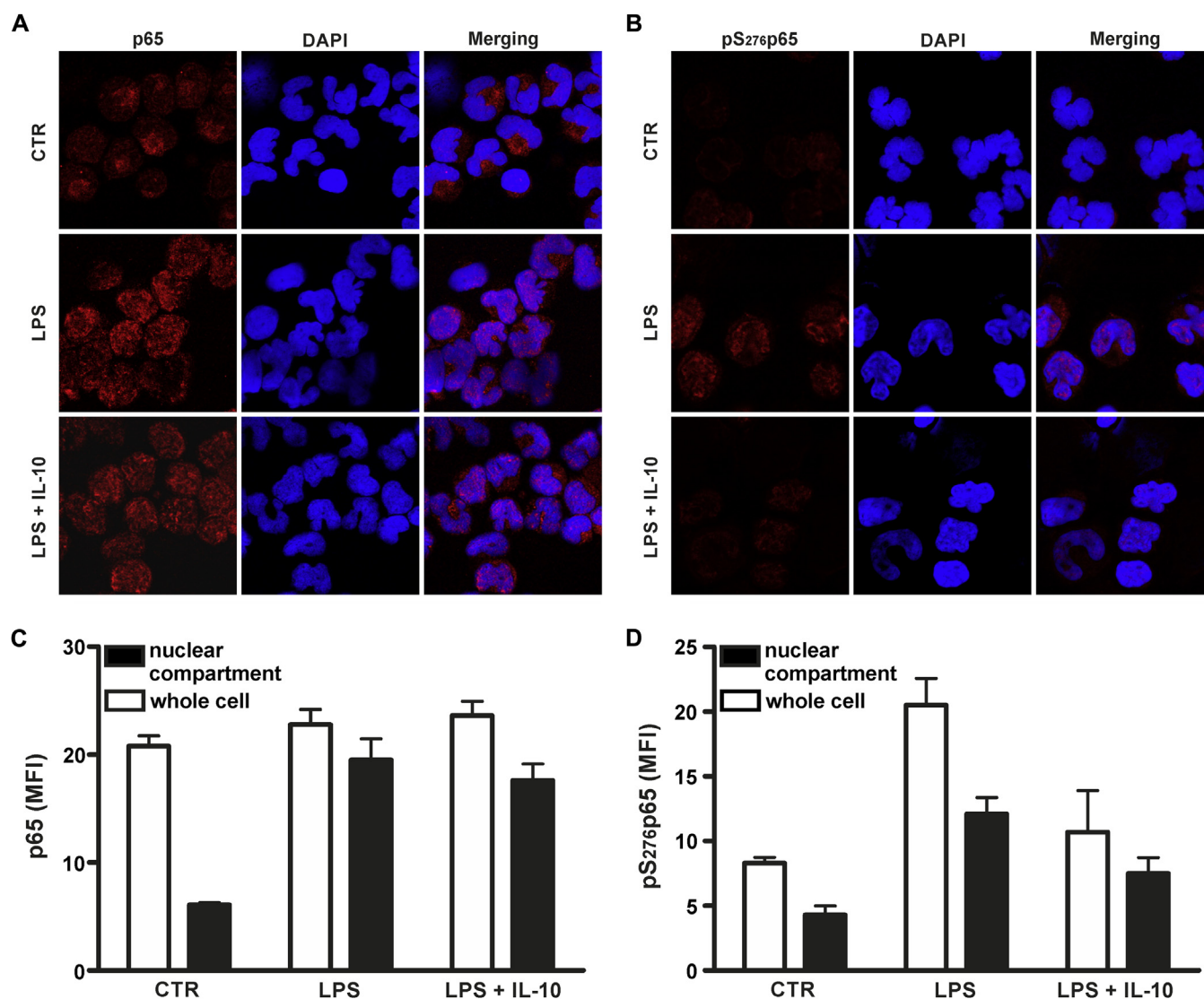


FIG 3. IL-10 reduces LPS-induced phosphorylation of Ser₂₇₆p65. **A** and **B**, Monocytes cultured for 1 hour were processed for confocal laser scanning microscopy with 4'-6-diamidino-2-phenylindole dihydrochloride (in blue) and anti-p65 (Fig 3, A) or anti-pS₂₇₆p65 (Fig 3, B) antibodies, followed by goat anti-rabbit Cy3 (in red) staining. One experiment representative of 3 is shown. The fluorescent signal was quantified with LAS Advanced Fluorescence Lite software (Leica-Microsystems) in 10 different fields for each condition. **C** and **D**, The mean fluorescence intensity (MFI) $\pm$ SEM of each field is reported. CTR, Control.

levels of CXCL8, but not TNF- α , promoter-associated HDAC1 (Fig 5, A) and HDAC2 (Fig 5, B), was induced by LPS stimulation. However, the latter effect was substantially counteracted by IL-10. Notably, the differences in the levels of promoter-bound HDAC1 and HDAC2 were not due to changes in their total protein (see Fig E6, A, in this article's Online Repository at www.jacionline.org) and mRNA (see Fig E6, B) expression. Taken together, these data demonstrate that the suppression of LPS-induced CXCL8 transcription relies on IL-10's ability to restore HDAC1/HDAC2 association to the CXCL8 promoter.

IL-10 does not inhibit LPS-induced CXCL8 transcription in PBMCs from patients with COPD

Because it has been recently shown that the levels and activity of HDAC2 in the lung parenchyma, bronchial biopsy

specimens, alveolar macrophages, and peripheral blood monocytes of patients with COPD are greatly reduced compared with those in healthy subjects,²⁷⁻³⁰ we examined whether such HDAC2 reduction impairs the ability of IL-10 to selectively suppress LPS-induced CXCL8 transcription. Thereby PBMCs from 6 patients with COPD (GOLD stage 4), 4 patients with acute respiratory failure (ARF) not consequent to COPD, and 6 healthy control subjects were isolated and incubated for 1 hour with or without LPS in the presence or absence of IL-10. First, Western blot analysis (Fig 6, A) and related quantification (Fig 6, B) confirmed that PBMCs from patients with COPD express lower HDAC2 levels than PBMCs from patients with ARF or healthy subjects. HDAC2 content is reduced not only at the protein level (Fig 6, A) but also at the mRNA (Fig 6, C) level, thus suggesting that an impaired transcription of the HDAC2 gene occurs systemically in these subjects. Under

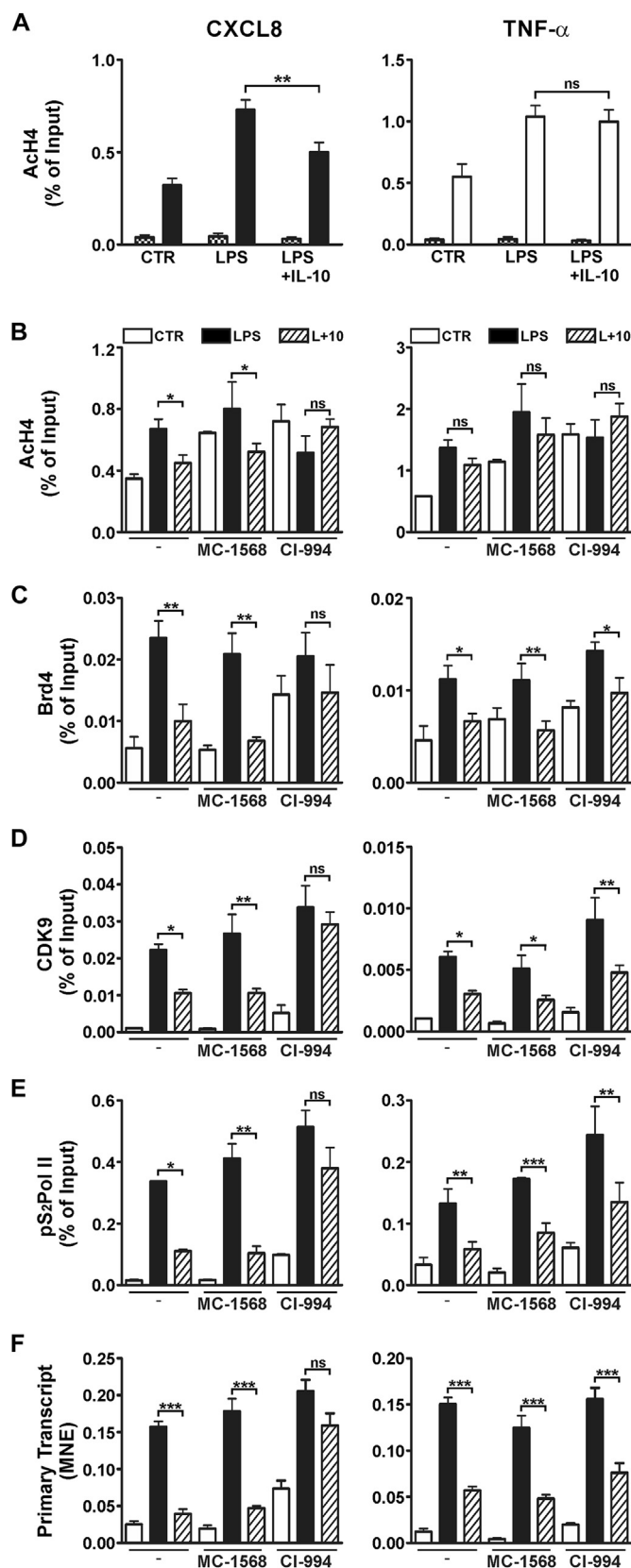


FIG 4. Inhibition of type I HDAC activation reverts the ability of IL-10 to inhibit LPS-stimulated CXCL8 transcription. Freshly purified monocytes (A) or monocytes pretreated for 45 minutes with 10 μ M CI-994 or MC-1568 (B-F) were cultured with LPS or LPS plus IL-10 for 1 hour. Cells were

these experimental conditions, responsiveness of PBMCs from patients with COPD and healthy control subjects to IL-10 was comparable, as demonstrated by activation of Tyr₇₀₅-STAT3 phosphorylation (Fig 6, D and E).

Moreover, in line with previous studies,^{28,30,31} RT-qPCR analysis showed that reduction of HDAC2 content in PBMC samples from patients with COPD induces a dramatically enhanced CXCL8 mRNA transcription in response to LPS compared with that observed in PBMCs from patients with ARF and healthy control subjects (Fig 7, A). Most importantly and further supporting a role for HDAC2 in mediating the suppressive activity of IL-10, a defect in the ability of IL-10 to inhibit LPS-induced CXCL8 transcription was observed (Fig 7, A). In fact, IL-10 inhibited LPS-induced CXCL8 transcription by $40\% \pm 5\%$ in samples from patients with COPD compared with $71\% \pm 6\%$ in patients with ARF and $73\% \pm 2.7\%$ in PBMCs from healthy control subjects (Fig 7, F). Consistently, levels of HDAC2 directly correlate with IL-10 suppressive activity on CXCL8 mRNA transcription (Fig 7, M). Finally, a defect in the ability of IL-10 to inhibit LPS-induced H4 acetylation at the level of the CXCL8 promoter was observed in PBMCs from patients with COPD (Fig 7, R), thus suggesting that the activity of HDAC2 is required to accomplish the IL-10-mediated effect.

Remarkably, PBMCs from patients with COPD showed no defect of IL-10 inhibitory activity on TNF- α , as well as on CCL3/macrophage inflammatory protein (MIP) 1 α , CCL4/MIP-1 β , and CCL20/MIP-3 α (Fig 7, B-E and G-L) transcription, thus demonstrating that PBMCs from patients with COPD are perfectly responsive to IL-10 and that HDAC2 is distinctively required by IL-10 to suppress only CXCL8 transcription. No significant correlation between HDAC2 expression levels and the degree of IL-10 transcriptional inhibition of TNF- α or any of the other chemokines tested (Fig 7, N-Q) were in fact detected. In addition and in line with the dispensable role of HDAC2 in IL-10-mediated inhibition of TNF- α transcription, IL-10 modified the levels of AcH4 in PBMCs isolated from patients with COPD and healthy control subjects neither at the TNF- α promoter (Fig 7, S) nor at any of the chemokine promoters investigated (Fig 7, T-V).

Collectively, these data uncover that the mechanism whereby IL-10 suppresses CXCL8 transcription induced by LPS strictly depends on HDAC2 levels and function.

DISCUSSION

In this work we have investigated the molecular mechanisms through which IL-10 directly inhibits LPS-induced CXCL8 and TNF- α transcription in human monocytes. In agreement with the well-established model of proinflammatory cytokine gene expression,²³ we show that LPS-induced CXCL8 and TNF- α transcript elongation and processing depend on CBP/p300-Brd4-P-TEFb (positive transcription elongation factor b)

processed for ChIP assay with anti-AcH4 (Fig 4, A and B), anti-Brd4 (Fig 4, C), anti-CDK9 (Fig 4, D), and anti-pS2Pol II (Fig 4, E) antibodies. In parallel, CXCL8 and TNF- α primary transcript expression was quantified by means of RT-qPCR (Fig 4, F). Data are displayed as means $\pm$ SEMs ($n = 3$) * $P < .05$, ** $P < .01$, and *** $P < .001$. MNE, Mean normalized expression; ns, not significant.

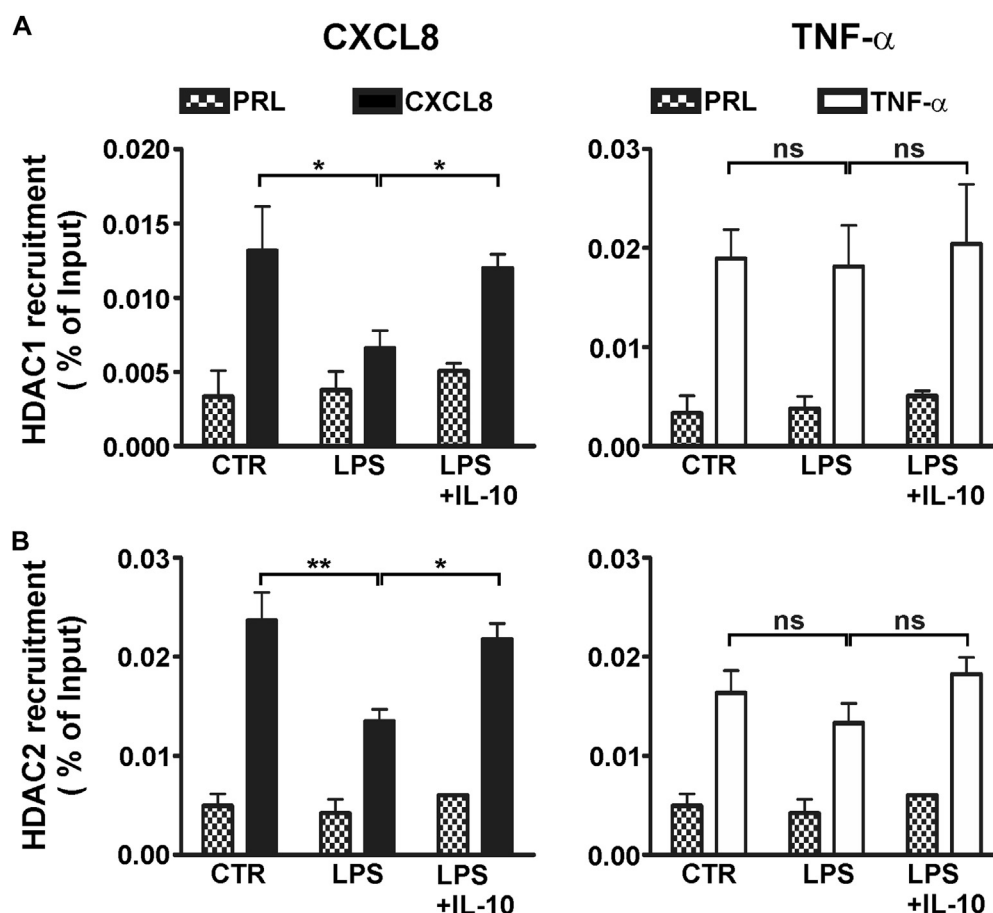


FIG 5. Effect of IL-10 on CXCL8 and TNF- α promoter-bound HDAC1 and HDAC2. ChIP analysis with anti-HDAC1 (A) or anti-HDAC2 (B) antibodies of monocytes treated for 1 hour as indicated is shown. Data are expressed as percentage over input DNA (mean $\pm$ SEM, $n = 3$). * $P < .05$ and ** $P < .01$. CTR, Control; ns, not significant.

complex assembly. In this scenario we demonstrate that IL-10 specifically inhibits upstream events, leading to Pol II activation, namely LPS-induced Brd4-CDK9 recruitment to the CXCL8 and TNF- α promoters. Remarkably, this mechanism is selective for LPS because IL-10 rapidly and directly inhibits CXCL8 and TNF- α promoter enrichment of Pol II induced by TNF- α and other TLR agonists. Because Brd4 functions as a “reader” of both acetylated histones and transcription factors,^{18,19,23,32} we reasoned that its reduced recruitment must be consequent to an IL-10-mediated diminishment in the levels of either of these 2 Brd4-docking molecules. Accordingly, our data show that LPS-induced enrichment of promoter-bound acetylated NF- κ Bp65 (AcK₃₁₀p65), which has been shown to recruit Brd4,^{18,19} is significantly reduced by IL-10 at both the CXCL8 and TNF- α promoters. Our experiments also show that the degree of LPS-induced recruitment to the CXCL8 and TNF- α promoter of pS₂₇₆p65 (which is essential for the recruitment of CBP and p300 coactivatory molecules that mediate acetylation of promoter-bound K₃₁₀p65),^{20,21,33} as well as of p300 but not unphosphorylated p65, is significantly reduced in the presence of IL-10.

Finally, using confocal microscopy, we demonstrate that IL-10-mediated inhibition of LPS-induced S₂₇₆p65 phosphorylation

occurs in the cytoplasm. Evidence indicates that signal-induced phosphorylation of NF- κ B is crucial for its function, whereas the loss of phosphorylation of p65 influences both its DNA-binding and transactivation activities.³⁴ The IL-10-mediated event or events regulating p65 phosphorylation might proceed through the inhibition of the kinase or kinases involved in p65 phosphorylation or, alternatively, the activation of phosphatases. In this regard IL-10 has been shown to inhibit LPS-induced TNF- α translation through activation of the Src homology domain 2-containing inositol phosphatase 1 phosphatase.³⁵ Whether this mechanism is active also in IL-10-mediated transcriptional inhibition is currently under investigation.

Altogether, our data suggest that IL-10, by inhibiting the initial cytoplasmic S₂₇₆p65 phosphorylation, limits the downstream events of p300 recruitment, K₃₁₀p65 acetylation, Brd4-CDK9 anchoring, and, ultimately, Pol II activation. It is noteworthy that IL-10 has been shown to suppress LPS-induced TNF- α transcript elongation, Pol II activation, and CDK9 recruitment to the TNF- α promoter also in human monocyte-derived macrophages through a mechanism that involves a widespread block in p65 recruitment to κ B sites at the TNF- α promoter.¹⁰ The discrepancy of the mechanisms proposed might reflect the use of circulating monocytes rather than macrophages derived from monocytes cultured for

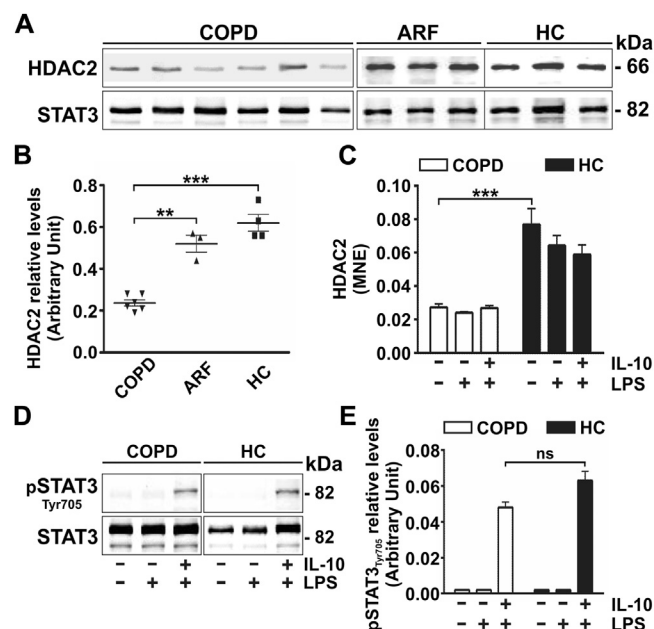


FIG 6. Peripheral blood monocytes from patients with COPD show reduced levels of intracellular HDAC2 protein. PBMCs from 6 patients with COPD, 4 subjects given a diagnosis of ARF, and 6 healthy control subjects (HC) were collected. **A–C**, HDAC2 protein levels (Fig 6, A) and relative quantification (Fig 6, B), together with HDAC2 mRNA expression (Fig 6, C), are shown. **D** and **E**, pTyr705-STAT3 protein levels were detected (Fig 6, D) and quantified (Fig 6, E) in total cell lysates from PBMCs cultured for 20 minutes in the presence or absence of LPS or LPS plus IL-10. ** $P < .01$ and *** $P < .001$. MNE, Mean normalized expression; ns, not significant.

3 days with 100 ng/mL granulocyte colony-stimulating factor,¹⁰ thus highlighting not only the gene-specific but also the cell-specific nature of the IL-10 anti-inflammatory response.

Because acetylated histones, in addition to AcK₃₁₀p65, can provide a Brd4-docking site as well,²³ we subsequently explored whether IL-10 targets promoter-bound histone acetylation. Remarkably, we found that IL-10 inhibits LPS-induced histone H4 acetylation only at the CXCL8, but not TNF- α , gene promoter and that such inhibition strictly correlates with the IL-10-dependent increase in levels of HDAC2 recruited to the CXCL8 promoter. Additionally, selective inhibition of HDAC2 by CI-994 reverts IL-10-induced suppression of AcH4, Brd4, and CDK9 recruitment, Pol II activation, and CXCL8 transcript elongation, thus indicating that these 2 events are causally linked. Most importantly, the latter notion has a relevant translational significance, particularly in the context of COPD. In fact, and in agreement with Chen et al,²⁸ PBMCs from patients with COPD display consistently reduced HDAC2 gene expression and intracellular protein content, which significantly affects the IL-10 suppressive activity on CXCL8 gene expression. Conversely, PBMCs from healthy subjects or patients affected by respiratory insufficiency consequent to cardiovascular disease did not show any defect in the levels of intracellular HDAC2 and were found to be perfectly responsive to IL-10-mediated transcriptional inhibition of CXCL8 expression. Notably, this observation further supports the recent concept that COPD can no longer be judged as a disease only of the lungs but must be considered a chronic systemic inflammatory syndrome.³⁶ In addition, it demonstrates that the defective

CXCL8 suppression by IL-10 is not secondary to respiratory insufficiency of any origin but is strictly specific for COPD.

Based on our findings, HDAC2 activity seems to be a requirement for IL-10 to keep CXCL8 promoter-bound histone H4 in a hypoacetylated condition, in this manner precluding the establishment of the Brd4-docking site. On the contrary, HDACs are dispensable for IL-10 suppression of LPS-induced transcription of TNF- α and other chemokines (namely CCL3/MIP-1 α , CCL4/MIP-1 β , CCL20/MIP-3 α). Taken together, these data emphasize that CXCL8 transcriptional suppression by IL-10 proceeds through an HDAC2-dependent mechanism, whereas suppression of TNF- α and several other LPS-induced chemokines by IL-10 proceeds in an HDAC2-independent manner. In addition, they demonstrate that AcH4 is likely not working as a Brd4-docking site at the TNF- α locus, they demonstrate that PBMCs from patients with COPD are wholly responsive to IL-10, and finally, they highlight the potential mechanism leading to constitutively high local and systemic expression of CXCL8 in patients with COPD.

Notably, the recruitment of HDAC2 to activated inflammatory genes represents a major anti-inflammatory mechanism used by glucocorticoids to suppress inflammatory gene transcription.³⁷ In fact, the markedly reduced HDAC2 activity and expression in both PBMCs and alveolar macrophages from patients with COPD renders these patients poorly responsive to glucocorticoid therapy.^{28,30} Moreover, HDAC2 reduction in patients with COPD strictly correlates with disease severity and increased histone acetylation and inflammatory cytokine gene expression.^{38,39} Thus based on our data, it appears that the mechanisms through which IL-10 suppresses CXCL8 transcription closely resemble those used by glucocorticoids because both IL-10 and glucocorticoids converge on the HDAC2 requirement to fulfill their anti-inflammatory actions. The molecular events leading to the selective IL-10-induced HDAC2 recruitment to the CXCL8 promoter are presently under investigation. Remarkably, the ability of IL-10 to induce covalent histone modification at the promoter of other cytokines has been already described. For instance, IL-10 has been found to mediate histone deacetylation of the IL-12 promoter in peritoneal mouse macrophages through HDAC3 and to consequently attenuate IL-12 transcription.⁴⁰ Conversely, the ability of IL-10 to potentiate IL-1 receptor antagonist transcription in LPS-stimulated human monocytes has been found to rely on its ability to increase acetylated histone H4 levels associated with the IL-1 receptor antagonist promoter, thus activating local chromatin remodeling events that render the NF- κ B site accessible to LPS-activated p65/p50 NF- κ B heterodimers.⁹ Based on the widely accepted notion that IL-10 requires STAT3 activation to accomplish its anti-inflammatory actions, a very intriguing hypothesis is that IL-10-activated STAT3 can function as a rheostat that is able to modulate gene transcription by promoting or inhibiting histone acetylation.

In summary, we provide novel insights into the mechanisms that IL-10 uses to suppress LPS-induced recruitment and assembly of the preinitiation complex at the CXCL8 and TNF- α promoters through inhibition of pS₂₇₆p65. We also uncover the existence of an additional HDAC2-dependent pathway, which is highly specific for the suppression of CXCL8 transcript elongation (Fig 7). Finally, the finding that PBMCs from patients with acute-phase COPD are intrinsically resistant to the ability of IL-10 to suppress CXCL8 expression because of their reduced intracellular HDAC2 levels indicates that modulating these mechanisms might be a novel approach for developing broader-spectrum anti-inflammatory therapies.

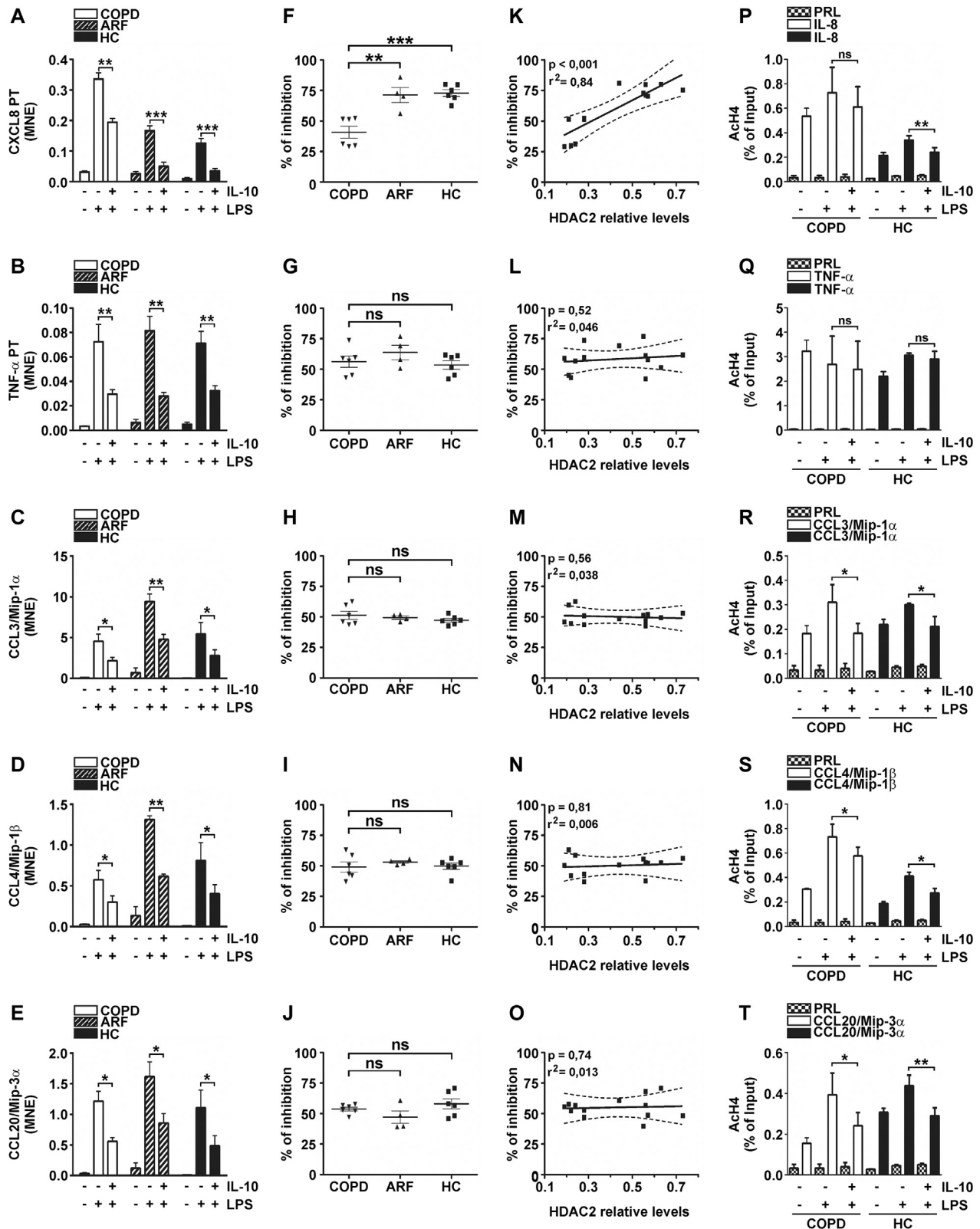


FIG 7. IL-10 does not inhibit LPS-induced CXCL8 transcription in PBMCs from patients with COPD. **A-E**, Normalized CXCL8, TNF- α , CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL20/MIP-3 α primary transcript (PT) expression from PBMCs cultured for 1 hour with 100 ng/mL LPS in the presence or absence of 200 U/mL IL-10 is shown (mean $\pm$ SEM, $n = 4$). **F-M**, Percentage of IL-10-induced inhibition of CXCL8, TNF- α , CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL20/MIP-3 α (Fig 7, **F-J**) and correlation analysis between HDAC2 levels and the percentage of inhibition (Fig 7, **K-O**) are shown. **P-T**, PBMCs cultured for 1 hour were subjected to ChIP analysis with anti-ACh4 antibodies. Data from qPCR are expressed as percentage over input DNA and displayed as means $\pm$ SEMs ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$. MNE, Mean normalized expression; ns, not significant.

Key messages

- IL-10 inhibits LPS-induced CXCL8 and TNF- α transcript elongation targeting phosphorylated NF- κ Bp65, a Brd4-docking molecule.
- IL-10 selectively controls CXCL8 transcript elongation through HDAC2-dependent chromatin modifications, disrupting assembly of the transcriptional machinery.
- HDAC2-dependent suppression of CXCL8 transcription is defective in patients with COPD.

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METHODS

Cell purification and culture

Monocytes were isolated from PBMCs after centrifugation over Percoll gradients, as already described.^{E1} After purification, monocytes were suspended at 3×10^6 /mL in RPMI 1640 medium (BioWhittaker, Walkersville, Md) supplemented with 10% low endotoxin FCS (BioWhittaker; <0.06 EU/mL by using the Limulus amoebocyte lysate assay), plated in tissue-culture plates (BioWhittaker), and cultured in the presence or absence of 100 ng/mL ultrapure *Escherichia coli* LPS (0111:B4 strain; Alexis Biochemicals, Lausen, Switzerland), 10 ng/mL TNF- α (PeproTech, Rocky Hill, NJ), 5 μ M R848 (InvivoGen, San Diego, Calif), 1 μ g/mL Pam3CSK4 (InvivoGen), and 50 μ g/mL polyinosinic:polycytidylic acid (InvivoGen) in the presence or absence of 200 U/mL recombinant human IL-10 (kindly provided by Dr Rene de Waal Malefyt; Schering-Plough Biopharma, Palo Alto, Calif). In selected experiments 10 μ M/L MC-1568 (type II HDAC inhibitor) or 10 μ M/L CI-994 (type I HDAC inhibitor, Santa Cruz Biotechnology) was added to freshly purified monocytes 45 minutes before incubation with LPS or LPS plus IL-10. After the indicated times, ice-cold PBS was added, and cells were collected and centrifuged twice at 500g for 5 minutes at 4°C and processed for further investigation. All reagents were of the highest available grade, and buffers were prepared with pyrogen-free water for clinical use.

Patients

Sixteen subjects were recruited for this study: 6 patients affected by COPD (GOLD stage 4, FEV₁/FEV₁ percentage, Tiffenau index <70%), 4 patients with ARF nonconsequent to COPD (3 with heart failure in postischemic dilative cardiomyopathy and 1 with sepsis), and 6 healthy control subjects. All the patients' and donors' features, together with leukocyte frequencies, are summarized in Table E1. Venous blood (10 mL) was collected from each subject and diluted 1:1 with sterile PBS. PBMCs were then isolated by means of density centrifugation, as described above. Monocyte frequency in the PBMC fraction from patients with COPD and healthy donors were comparable.

ChIP assay

ChIP experiments were performed as described elsewhere.^{E2} Immunoprecipitations were carried out overnight at 4°C with 5 μ g of antibodies toward Pol II (sc-899), CDK9 (sc-8338), NF- κ Bp65 (sc-372), p300 (sc-585), CBP (sc-369, Santa Cruz Biotechnology); phospho-Ser₂ Pol II (pS₂Pol II, ab5095) and acetyl-K₃₁₀NF- κ Bp65 (AcK₃₁₀NF- κ Bp65, ab19870; Abcam, Cambridge, United Kingdom); Brd4 (HPA015055; Sigma-Aldrich, St Louis, Mo); and acetyl-histone H4 (06-866), HDAC1 (06-720), HDAC2 (17-10237), and phospho-Ser₂₇₆NF- κ Bp65 (pS₂₇₆NF- κ Bp65, AB3375) from Upstate (Lake Placid, NY). Immune complexes were processed, as previously described.^{E2} DNA was then extracted with the QIAquick PCR Purification Kit (Qiagen, Crawley, United Kingdom), and one twentieth of the immunoprecipitated DNA was used in each qPCR reaction. Sequences and localization of promoter-specific primers (purchased from Invitrogen, Carlsbad, Calif) used in qPCR are listed in Fig E1. In all experiments 10% of the input chromatin was removed before addition of the antibodies and used to normalize the amount of immunoprecipitated DNA (specified in the text as "input" DNA). Data were expressed as a percentage of input according to the following formula:

$$10 \times 2^{-(\text{input Ct} - \text{sample Ct})}$$

In all experiments protein recruitment to the PRL promoter was quantified and used as a control for the specificity of the assay.

Immunoblots

Monocytes (5×10^6) were harvested; incubated at 4°C for 5 minutes with 1 mmol/L diisopropyl fluorophosphate; lysed in 40 mmol/L Tris, 1% SDS, and

7.5% glycerol; and boiled for 10 minutes at 95°C. Fifty micrograms of total cell lysate was resolved on 10% SDS-PAGE and transferred to nitrocellulose (Hybond; GE Healthcare, Franklin Lakes, NJ). The blots were incubated with the antibodies anti-HDAC1 (sc-7872) and anti-HDAC2 (sc-7899, Santa Cruz Biotechnology) and anti-STAT3 (#9139; Cell Signaling, Danvers, Mas), anti-Ach4 (06-866, Upstate), and anti-GAPDH (#4300, both from Life Technologies). Detection was carried out with Alexa Fluor 680-conjugated goat anti-rabbit antibodies (Invitrogen) or IRDye 800-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, Pa) secondary antibodies. Blotted proteins were detected and quantified with the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification was performed with the analysis software provided by the manufacturer.

Laser confocal microscopy

Cytospin preparations of 0.1×10^6 cells cultured as previously described were fixed and then permeabilized as previously described.^{E3} Thereafter, samples were washed with PBS and 0.2% BSA, blocked with 5% heat-inactivated human serum, and incubated with 5 μ g/mL anti-NF- κ Bp65 (sc-109, Santa Cruz Biotechnology) or anti-pSer₂₇₆NF- κ Bp65 (AB3375, Upstate) diluted 1:1000 in PBS overnight at 4°C. Subsequently, cytospin preparations were stained with anti-rabbit IgG (goat)-Cy3 (Amersham Biosciences, Piscataway, NJ) at a dilution of 1:1000 for 30 minutes at room temperature. After washing, nuclear staining was done with 4'-6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). Preparations were mounted in anti-bleaching medium and visualized by using confocal laser scanning microscopy (Leica-Microsystems). Image analysis and signal quantification were performed with LAS Advanced Fluorescence Lite software (Leica-Microsystems).

Chemotaxis assay

Highly purified granulocytes (>99%) were isolated under endotoxin-free conditions from buffy coats of healthy donors by means of centrifugation on Ficoll-Paque plus (GE Healthcare), as previously described,^{E4} and resuspended in RPMI medium containing 10% FBS. Briefly, granulocytes were cultured for 30 minutes in the presence or absence of 500 nmol/L SCH 563705 (a potent dual CXCR2/CXCR1 antagonist; MedChemExpress, Monmouth Junction, NJ) and subsequently seeded (300×10^3 cells/well) into the upper transwell inserts (5-mm pore filter) of a 96-well chemotaxis chamber. Two hundred thirty-five microliters of 1:40 diluted supernatant from monocytes cultured as specified was added to the bottom chamber. After 1 hour, transmigrated CD16⁺ cells were quantified by means of flow cytometry. The migration index was calculated as the percentages of cells in the lower chamber over input (PMNs initially present in the cell suspension).^{E5}

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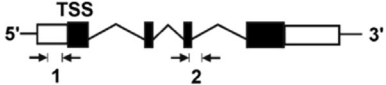
ChIP	Location	Sequence	
Primers	(relative to the gene TSS)	Forward Primer	Reverse Primer
CXCL8 			
px #1	-196 to -86	TCAGTTGCAAATCGTGGAAT	TTGTGCCTTATGGAGTGCTC
dw #2	+1320 to +1467	GTCTGGACCCCAAGGAAAAC	AAAGGCAGATACCTAATGACG
TNF-α 			
px #1	-160 to -73	GATGGAGAAGAAACCGAGAC	AAAGAATCATTCAACCAGCGG
dw #2	+1011 to +1235	GGCAGTCAGTAAGTGTCTCCAA	TTACCTACAACATGGGCTACAGG
IKBα 			
	-384 to -300	CGTCCTTGGGATCTCAGC	CCTGCACCCTGTAATCCTGT
PRL 			
	-15 to +130	AGGGAAACGAATGCCTGATT	GCAGGAAACACACTTCACCA

FIG E1. Sequence and positional mapping of the promoter-specific primers used in ChIP analysis.

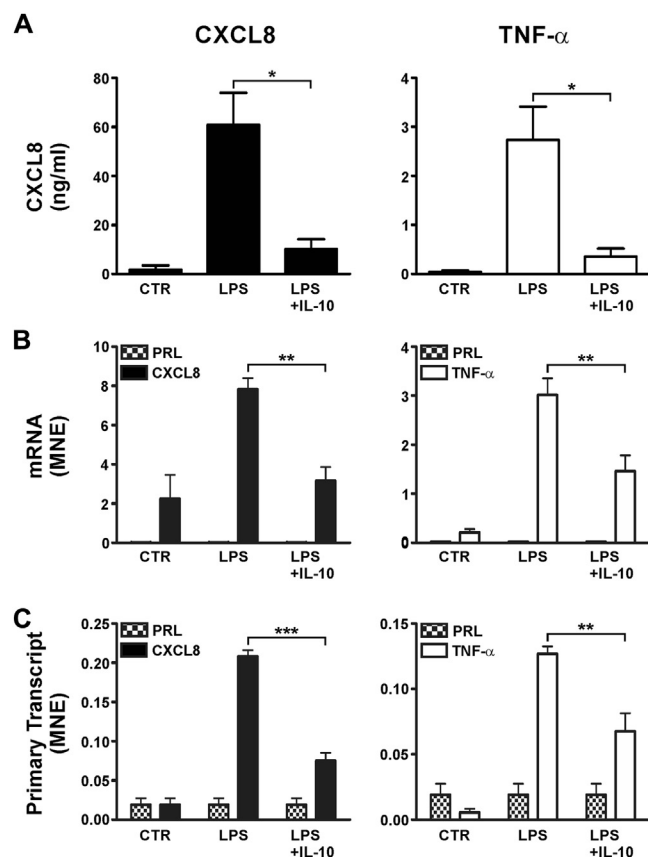


FIG E2. IL-10 inhibits LPS-induced CXCL8 and TNF- α protein secretion, mRNA, and primary transcript expression. **A**, The amount of antigenic CXCL8 was quantified by means of ELISA in cell-free supernatants collected after 4 hours from monocytes stimulated as indicated. **B** and **C**, Total RNA was extracted, and CXCL8 and TNF- α mRNA levels (Fig E2, **B**) and primary transcript (PT) expression (Fig E2, **C**) were determined by means of qPCR in monocytes cultured with LPS or LPS plus IL-10 for 1 hour. Data are displayed as means $\pm$ SEMs ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$.

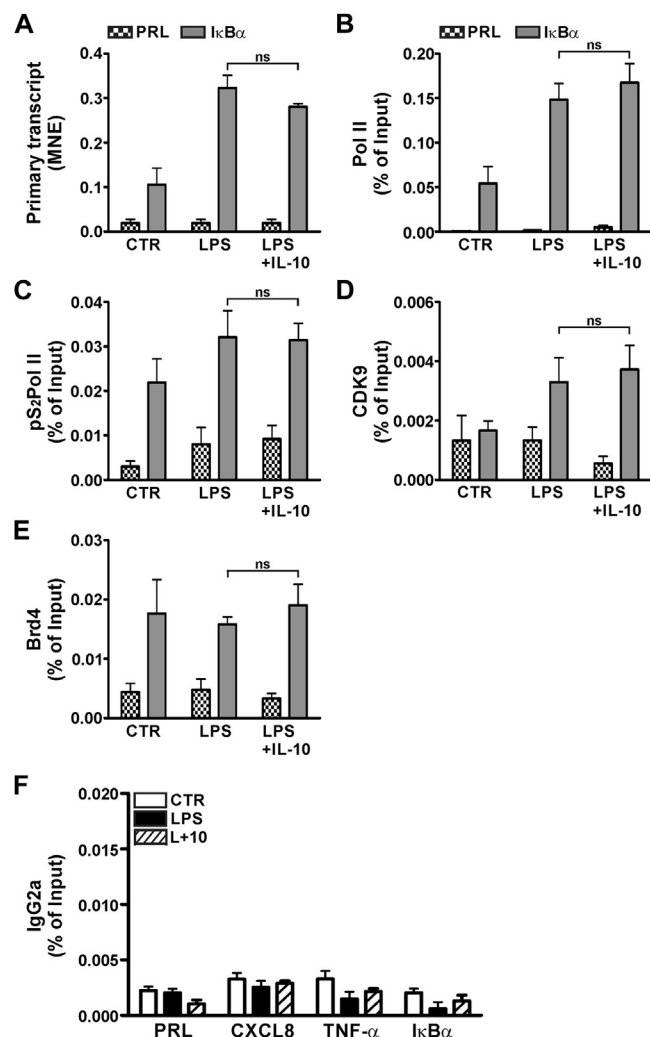


FIG E3. IL-10 does not inhibit IκBα transcript elongation in LPS-stimulated monocytes. **A**, IκBα and PRL primary transcript expression was analyzed by means of qPCR. **B-E**, ChIP analysis with anti-Pol II (Fig E3, **B**), anti-pS2Pol II (Fig E3, **C**), anti-CDK9 (Fig E3, **D**), and anti-Brd4 (Fig E3, **E**) antibodies was performed. Data from qPCR using promoter-specific primers amplifying the IκBα and PRL promoters are expressed as percentage over input DNA. **F**, Enrichment of PRL, CXCL8, TNF-α, and IκBα promoters was analyzed by using qPCR in a ChIP assay performed with IgG2a isotype control antibody (n = 3). CTR, Control; ns, not significant.

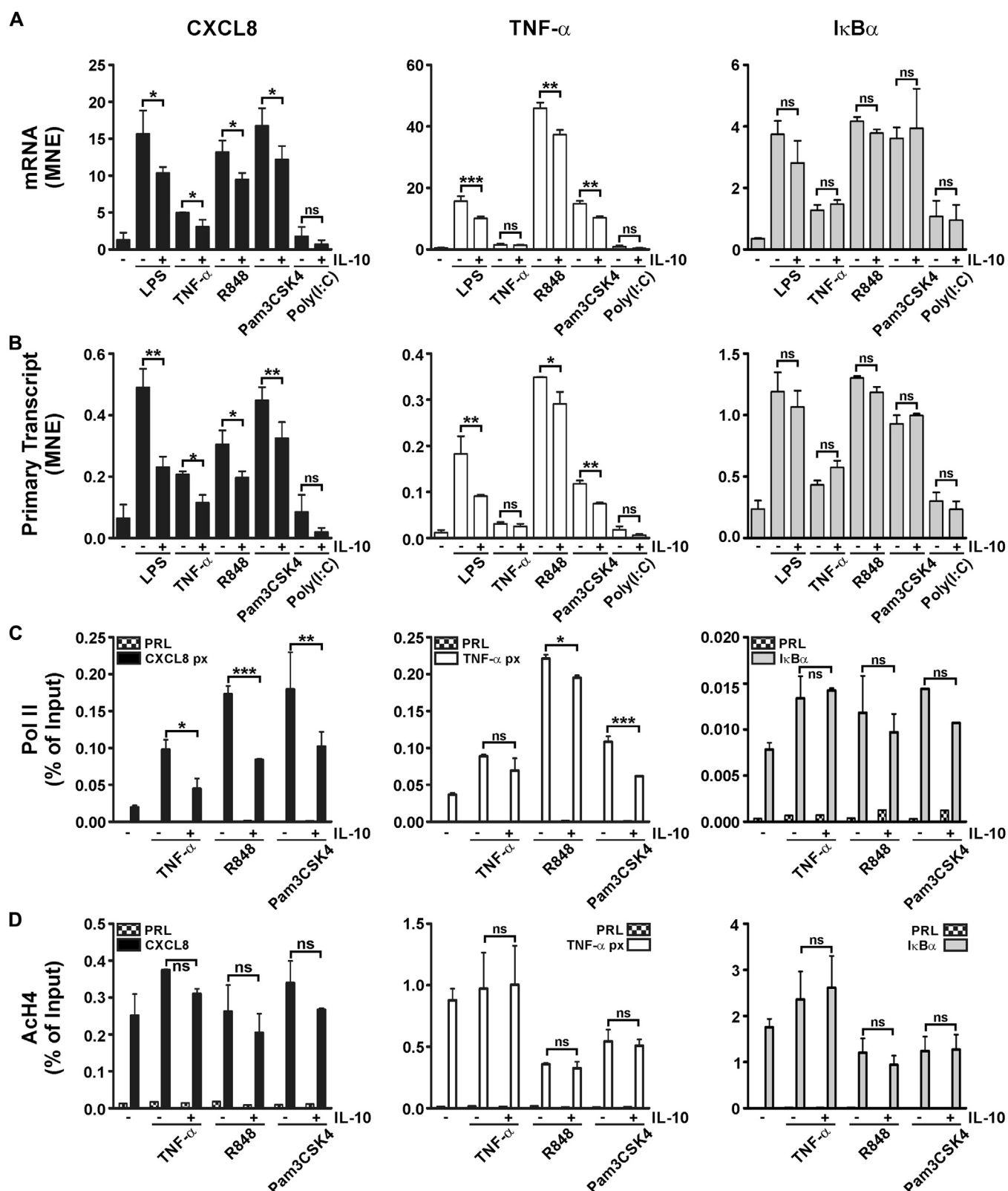


FIG E4. IL-10 inhibits Pol II recruitment to the CXCL8 and TNF- α promoters in monocytes stimulated with TNF- α , R848, and Pam3Cys. Monocytes were stimulated with LPS, TNF- α , R848, Pam3Cys, and polyinosinic:polycytidylic acid in the presence or absence of IL-10 for 1 hour. **A** and **B**, The level of expression CXCL8, TNF- α , and I κ B α mRNA (Fig E4, A) and primary transcript (PT; Fig E4, B) were analyzed by using qPCR. **C** and **D**, In parallel, ChIP analysis with anti-Pol II (Fig E4, C) and anti-ACh4 (Fig E4, D) antibodies was performed. Data are displayed as means $\pm$ SEMs ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

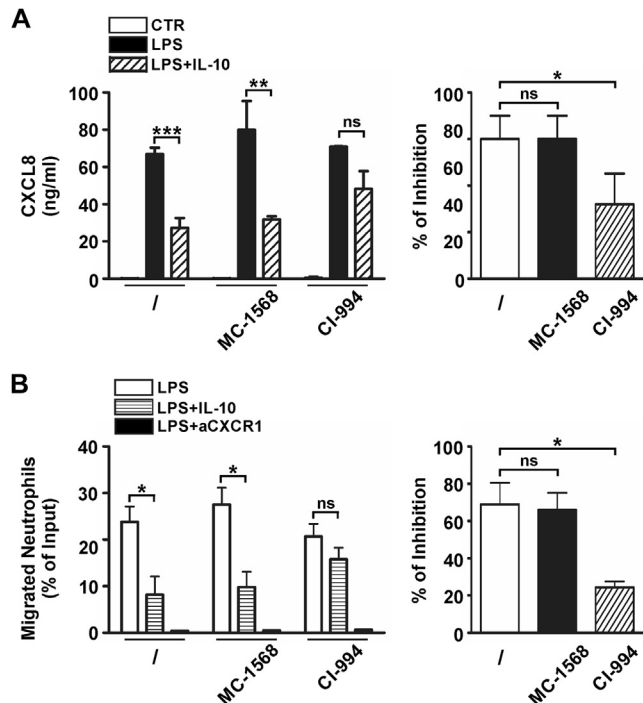


FIG E5. IL-10 does not inhibit LPS-induced release of biologically active CXCL8 in the presence of type I HDAC inhibitors. Monocytes were cultured in the presence or absence of 10 μ mol/L CI-994 or MC-1568 for 45 minutes and subsequently stimulated with LPS or LPS plus IL-10 for 4 hours. **A**, Cell-free supernatants were collected, and the amount of antigenic CXCL8 was quantified by means of ELISA. **B**, Biologic activity of CXCL8 release in each condition was assessed in chemotactic assays, as described in the [Methods](#) section in this article's Online Repository. Data are displayed as means $\pm$ SEMs ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$. CTR, Control; ns, not significant.

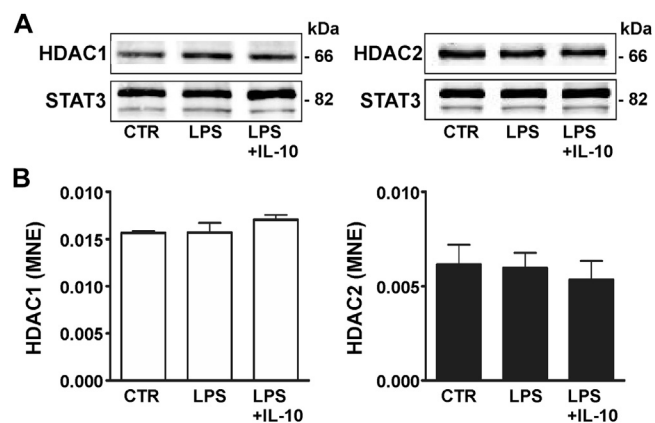


FIG E6. IL-10 and LPS do not modify HDAC1 and HDAC2 expression in monocytes. **A**, Whole-cell lysate was loaded on gels, and immunoblots were probed with anti-HDAC1, anti-HDAC2, and anti-STAT3 antibodies, followed by incubation with Alexa Fluor 680-labeled goat anti-rabbit antibodies. One experiment representative of 3 is shown. **B**, Total RNA was extracted, and HDAC1 and HDAC2 mRNA levels were evaluated by using qPCR ($n = 3$). CTR, Control; MNE, mean normalized expression.

TABLE E1. Baseline characteristics of the subjects enrolled in the study

	Patients with COPD (n = 6)	Patients with ARF (n = 4)	Healthy control subjects (n = 6)
Sex (M/F)	4/2	3/1	3/3
Mean age (y)	69 ± 4.25	52 ± 13.48	75 ± 1.30
FEV ₁ /FVC ratio	<70%		
Corticosteroid dose	80 mg IV	/	/
Oxygen	Yes	Yes	/
Neutrophils (10 ⁹ /L)	12.03 ± 3.52		4.04 ± 0.23
Lymphocytes (10 ⁹ /L)	0.64 ± 0.13		2.64 ± 0.12
Monocytes (10 ⁹ /L)	0.47 ± 0.08		0.45 ± 0.01
Eosinophils (10 ⁹ /L)	0.01 ± 0.01		0.28 ± 0.05
Basophils (10 ⁹ /L)	0.01 ± 0.00		0.03 ± 0.00

Values are presented as means ± SEs, where shown.

F, Female; FVC, Forced vital capacity; M, male.

TABLE E2. Primers used in RT-qPCR analysis

RT-qPCR target	Sequence	
	Forward primer	Reverse primer
CXCL8 PT	ATTGAGAGTGGACCACACTG	ACTACTGTAATCCTAACACCTG
TNF- α PT	TCAGGATCATCTTCTCGAACC	GAGTCCTTCTCACATTGTCTC
I κ B α PT	GCTCCTCGTGCTTATGTTGTG	GCCCTGGTAGGTAACCTCTGTTG
CXCL8 mRNA	CTGGCCGTGGCTCTCTTG	CTTGGCAAAACTGCACCTT
TNF- α mRNA	GAGCACTGAAAGCATGATCC	CGAGAAGATGATCTGACTGCC
I κ B α mRNA	GCTATTCTCCCTACCAGCTC	CAGTCATCATAGGGCAGCTC
PRL mRNA	TGCTGCTGCTGGTGTCAAAC	GAGGAGAGGTTATGGATGTAG
HDAC1 mRNA	CTGCTCAACTATGGTCTCTAC	GCTGTGGTACTTGGTCATCTC
HDAC2 mRNA	AACCGACAACAGACTGATATGG	CGTAACAGAATCCTGATGCTTC
CCL3 (MIP-1 α) mRNA	AGCCACATTCCGTCACCTG	CGTGTCAGCAGCAAGTGATG
CCL4 (MIP-1 β) mRNA	CGCCTGCTGCTTTTCTTACAC	CAGACTTGCTTGCTTCTTTTGG
CCL20 (MIP-3 α) mRNA	TCCTGGCTGCTTTGATGTCA	CAAAGTTGCTTGCTGCTTCTGA
GAPDH mRNA	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC

PT, Primary transcript.