IL-10 inhibits transcription elongation of the human *TNF* gene in primary macrophages

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IL-10 plays a central nonredundant role in limiting inflammation in vivo. However, the mechanisms involved remain to be resolved. Using primary human macrophages, we found that IL-10 inhibits selected inflammatory genes, primarily at a level of transcription. At the *TNF* gene, this occurs not through an inhibition of RNA polymerase II (Pol II) recruitment and transcription initiation but through a mechanism targeting the stimulation of transcription elongation by cyclin-dependent kinase (CDK) 9. We demonstrated an unanticipated requirement for a region downstream of the *TNF* 3′ untranslated region (UTR) that contains the nuclear factor κB (NF-κB) binding motif (κB4) both for induction of transcription by lipopolysaccharide (LPS) and its inhibition by IL-10. IL-10 not only inhibits the recruitment of RelA to regions containing κB sites at the *TNF* gene but also to those found at other LPS-induced genes. We show that although IL-10 elicits a general block in RelA recruitment to its genomic targets, the gene-specific nature of IL-10's actions are defined through the differential recruitment of CDK9 and the control of transcription elongation. At *TNF*, but not *NFKBIA*, the consequence of RelA recruitment inhibition is a loss of CDK9 recruitment, preventing the stimulation of transcription elongation.

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Abbreviations used: CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CTD, C-terminal domain; Pol II, RNA polymerase II; TSS, transcription start site; UTR, untranslated region.

Dysregulation of the inflammatory response can lead to diseases such as rheumatoid arthritis, Crohn's disease, or septic shock. The cytokine IL-10 plays a role in limiting the magnitude, duration, and detrimental outcome of the inflammatory response (Moore et al., 2001). This was shown both with the generation of IL-10–deficient mice that spontaneously develop inflammatory bowel disease (Kühn et al., 1993) and with the identification of patients with homozygous mutations in the IL-10 receptor subunits who present with early-onset colitis (Glocker et al., 2009).

IL-10 is produced by numerous cells including Th1, Th2, Th17, T reg, $CD8^+$ T cells, B cells, and myeloid cells (Saraiva and O'Garra, 2010). One of IL-10's key functions is the regulation of pathogen-mediated activation of macrophages and dendritic cells. It inhibits the T cell–activating potential of APC by downregulating MHC class II and the expression of costimulatory molecules such as CD80 and CD86 (Buelens et al., 1995). IL-10 also inhibits the expression of chemokines, inflammatory enzymes, and potent proinflammatory cytokines such as TNF, the target for multiple clinical strategies in rheumatoid arthritis and Crohn's disease (Feldmann and Maini, 2003).

Although recent advances have lead to a greater understanding of the regulation of IL-10 production (Saraiva and O'Garra, 2010), the precise mechanism of IL-10–dependent inhibition of TNF is less clear. Activation of STAT3, as a consequence of IL-10 binding to its cell surface receptor (IL-10R1/IL-10R2), is critical for mediating the antiinflammatory response (Takeda et al., 1999; Lang et al., 2002; Yasukawa et al., 2003; Williams et al., 2004a). However, mechanisms after STAT3 activation remain a controversial field. IL-10 has been shown to target both transcriptional (Schottelius et al., 1999; Murray, 2005) and posttranscriptional processes (Kontoyiannis et al., 2001; Denys et al., 2002; Schaljo et al., 2009) in a gene-specific manner (Lang et al., 2002). Many

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 10 2081-2088
www.jem.org/cgi/doi/10.1084/jem.20100414

studies of transcriptional effects have focused on the activity of NF-κB, a dominant transcription factor in the production of inflammatory cytokines (Foxwell et al., 1998; Udalova et al., 1998). However, it remains unclear whether IL-10 inhibits NF-κB activity (Wang et al., 1995; Schottelius et al., 1999; Denys et al., 2002; Murray, 2005; for reviews see Williams et al., 2004b; Grütz, 2005).

The variety of mechanisms proposed may reflect the use of mouse versus human systems or the use of genetically abnormal transformed cell lines, and we have therefore confined our study to the use of primary human macrophages. We have found that inhibition is not only exerted on the process of proinflammatory gene transcription but through a novel mechanism of elongation inhibition, controlled in a gene-specific manner through the actions of cyclin-dependent kinase (CDK) 9. IL-10 robustly inhibits RelA recruitment to κB sites at multiple proinflammatory genes, and this results in an inhibition only at genes that are rapidly induced at a level of elongation by CDK9. These findings shed new light on the gene specific nature of the IL-10 antiinflammatory response.

RESULTS AND DISCUSSION

We initially examined the effects of IL-10 on LPS-induced mRNA expression in primary human macrophages. IL-10 inhibited *TNF* and *IκB-ζ* mRNA production at early and late time points and IL-6, a secondary response gene, at 120 min (Fig. 1 A). IL-10 did not inhibit the production of IκB-α mRNA, which is consistent with the gene-specific effects previously described (Lang et al., 2002; Murray, 2005). A longstanding question has been whether IL-10 inhibits gene expression at the transcriptional (Murray, 2005) or posttranscriptional level (Schaljo et al., 2009), or perhaps both (Denys et al., 2002), in activated macrophages. Chromatin immunoprecipitation (ChIP) was used to measure accumulation of RNA polymerase II (Pol II) at distal regions of genes (Fig. 1 B), which is considered a hallmark of active transcription (Sandoval et al., 2004). LPS induced rapid accumulation of Pol II at a distal region of the *TNF* gene and slower accumulation at a distal region of the *IL6* gene (Fig. 1 C), which is consistent with the identification of these as primary and secondary response genes (Hargreaves et al., 2009; Natoli, 2009; Ramirez-Carrozzi et al., 2009). IL-10 inhibited the accumulation of Pol II in the distal regions of *TNF*, *IL6*, and *NFKBIZ*, but not *NFKBIA* (Fig. 1 D), mirroring its effects on the corresponding mRNAs (Fig. 1 A). Nascent unspliced *TNF* transcripts were also quantified by PCR (Fig. S1 A) and were seen to accumulate with identical kinetics to those of mature *TNF* mRNA in these cells. As estimated by both of these methods, IL-10 decreased transcription of *TNF* (Fig. 1 D and Fig. S1 A). These findings in human macrophages are consistent with previous reports describing IL-10's gene-specific effects on transcription (Lang et al., 2002; Murray, 2005).

Activation of transcription elongation has recently emerged as an important mechanism controlling the rapid induction of proinflammatory genes (Adelman et al., 2009; Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). We therefore investigated whether IL-10 inhibits transcription elongation by comparing Pol II densities at the transcription start site (TSS) to the downstream region of *TNF*. As described by others (Adelman et al., 2009; Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009), Pol II was present at the *TNF* TSS under resting conditions and increased relatively weakly in response to LPS (Fig. 2 A). In contrast, it was scarcely detectable at the downstream region in resting cells and very strongly upregulated by LPS, which is consistent with control of gene expression largely through the release of a block to transcription elongation. IL-10 weakly inhibited the LPS-induced increase of Pol II at the TSS but strongly inhibited the increase of Pol II at the distal region (Fig. 2 A and Fig. S1 B). Phosphorylation of Ser2 of the Pol II C-terminal domain (CTD) by CDK9 occurs during the transition from the initiation of transcription to elongation (Price, 2000). We therefore performed ChIP using a phospho-Ser2–specific antibody. Where IL-10 did not inhibit the recruitment of total Pol II to the start of the *TNF* gene (Fig. 2 A; top left) it completely inhibited the phosphorylation of the Ser2 residue of the Pol II CTD (Fig. 2 A; bottom left). Therefore, at *TNF* the elongating form of Pol II is reduced by IL-10. When we conducted similar analysis at the *NFKBIA* gene, it was noted that neither the elongation of Pol II into the downstream region of the gene nor the phosho-Ser2 form of Pol II was decreased by IL-10 (Fig. 2 B).

We used the CDK9 inhibitor flavopiridol (Chao and Price, 2001) to confirm that the phosphorylation of Ser2 of the Pol II CTD controls elongation at *TNF*. Flavopiridol had negligible effects on total Pol II recruitment to the start of *TNF* in contrast to its inhibition of Pol II Ser2 phosphorylation (Fig. 2 C). Additionally, flavopiridol inhibited the accumulation of total Pol II at the downstream region of the *TNF* gene. These effects mirror IL-10's inhibition of Pol II phospho-Ser2 and elongation into the downstream regions of the gene (Fig. 2 A).

Interestingly, transcription elongation of *NFKBIA* was also sensitive to flavopiridol (Fig. S1 D), indicating that although IL-10 had no effect on elongation of *NFKBIA*, inhibiting the kinase activity of CDK9 with flavopiridol elicited a broader effect. Therefore it is unlikely that IL-10 has inhibitory effects on the kinase activity of CDK9 (as was the case for flavopiridol). We therefore looked at the effect IL-10 had on the recruitment of CDK9 itself to the *TNF* and *NFKBIA* genes (Fig. 2 D). At the start of *TNF*, CDK9 was recruited in a stimulus-dependent manner and this was inhibited by IL-10 (mean 47.2%; SEM 6.5; $n = 4$). In contrast, at the start of *NFKBIA*, CDK9 was seen to be more constitutively associated, inducing only slightly in response to LPS and exhibiting no inhibition upon IL-10 costimulation. Recently, *NFKBIA* was described as a housekeeping primary response gene that is continuously transcribed in macrophages (Hargreaves et al., 2009), perhaps accounting for these differences. These differential effects on CDK9 recruitment between *TNF* and *NFKBIA* may underlie IL-10's gene-specific, as opposed to general, inhibition of transcription elongation.

The current findings contradicted a previous observation from our laboratory, where the TNF 3′ untranslated region (UTR) was required for IL-10 to suppress the TNF reporter gene activity (Denys et al., 2002). This implicated a mechanism

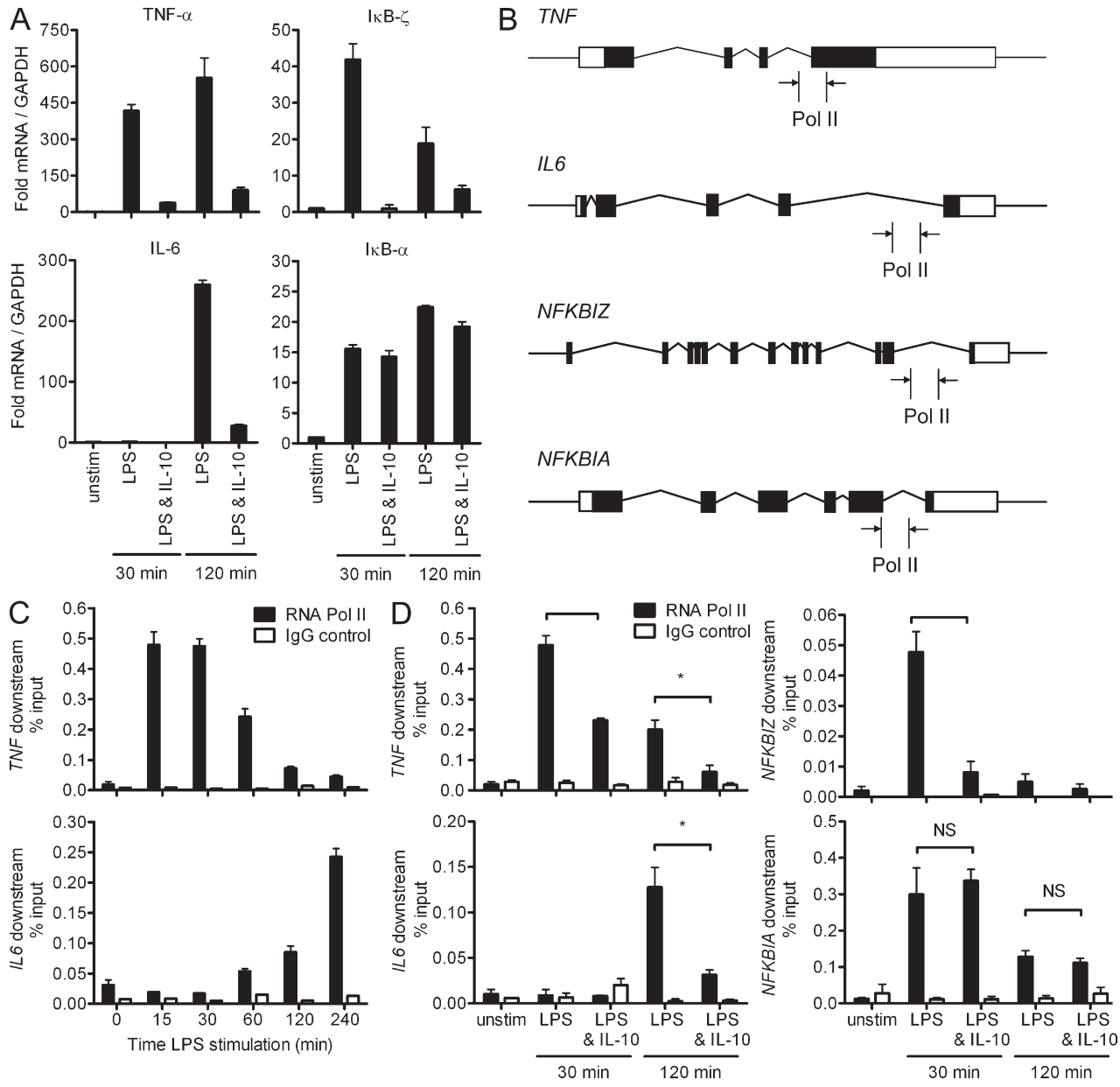

Figure 1. IL-10 inhibits proinflammatory gene transcription. (A) Cells were treated with LPS with or without IL-10 for 30 or 120 min. RNA was isolated and used in quantitative RT-PCR with probes specific for the indicated mRNA. (B) Position of PCR primers for detecting downstream Pol II at the *TNF*, *IL6*, *NFKBIA*, and *NFKBIZ* genes. (C) Cells were stimulated for the indicated times with LPS, followed by ChIP using Pol II antibodies (filled bars) or a rabbit isotype control (open bars) at downstream regions of the *TNF* and *IL6* genes. (D) Cells were treated with LPS with or without IL-10 for 30 or 120 min and Pol II was measured by ChIP at the downstream regions of *TNF*, *IL6*, *NFKBIZ*, and *NFKBIA*. Data in A and C are expressed as mean ± SD of triplicate measurements for a single donor, representative of three independent experiments using different donors. Data in D are pooled data from three donors and are expressed as mean ± SEM. Statistical significance was assessed using the paired Student's *t* test. *, $P < 0.05$; NS (not significant).

of posttranscriptional control; however, on closer inspection, the construct in question, 5′luc3′(1037) (Fig. 3 A), was found to include both the 785-bp TNF 3′UTR and an additional 250 bp of downstream genomic sequence (originally included in the construct for ease of cloning). We hypothesized that this 250-bp region, rather than the 3′UTR, could have mediated the IL-10–dependent inhibition of 5′luc3′(1037). Indeed, when this construct was compared with a 5′luc3′(785) that lacked the additional genomic sequence, IL-10–dependent inhibition was lost (Fig. 3 A and Fig. S2 A). This also resulted in a partial loss of LPS responsiveness, indicating that the 3′ downstream region may contribute to *TNF* transcriptional control.

The downstream 3′ region of the *TNF* gene is known to contain the NF-κB binding site κB4 (Tsytsykova et al., 2007; Krausgruber et al., 2010). Although it is well documented that NF-κB plays a role in *TNF* transcription in macrophages

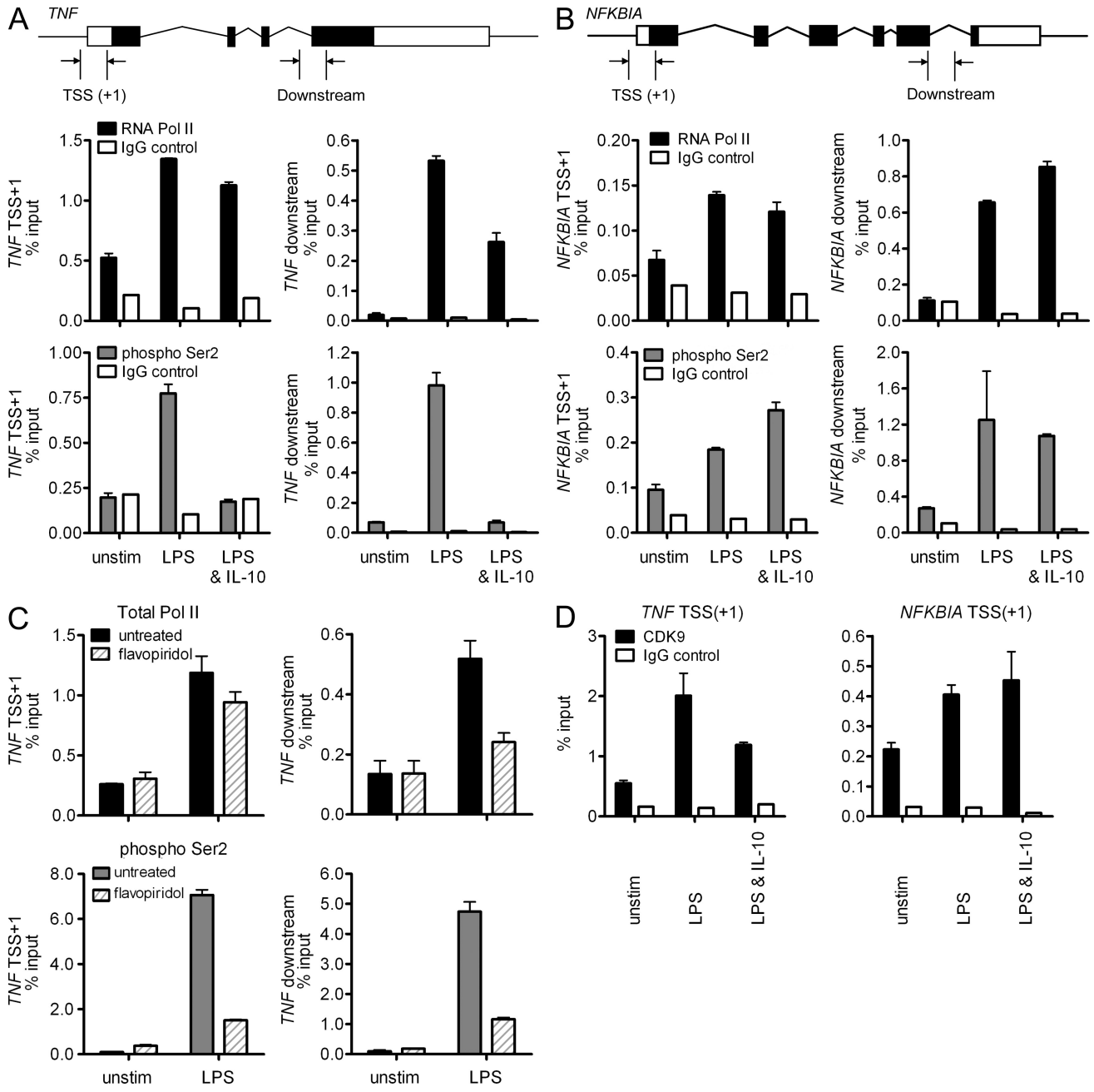

Figure 2. IL-10 inhibits transcription elongation at *TNF*. (A and B) Cells were treated with LPS with or without IL-10 for 30 min followed by ChIP using Pol II, phospho-Ser2–specific, or rabbit isotype control antibodies at the TSS or downstream regions of the *TNF* (A) or *NFKBIA* (B) genes. (C) Cells were left unstimulated or preincubated for 30 min with 500 nM flavopiridol, followed by LPS stimulation for 30 min, and then subjected to ChIP. (D) ChIP assay of CDK9 recruitment to the start of the *TNF* or *NFKBIA* genes upon stimulation with LPS with or without IL-10. Data are expressed as mean ± SD of triplicate measurements for a single donor, representative of three independent experiments using different donors.

(Foxwell et al., 1998; Udalova et al., 2000), previously we and others were unable to demonstrate any IL-10–dependent suppression of NF-κB activity (Denys et al., 2002; Murray, 2005). Therefore, to establish to what extent NF-κB contributes toward TNF transcription, we depleted the levels of RelA protein using a small interfering (si) RNA approach, which was recently optimized for primary human macrophages in our laboratory (Behmoaras et al., 2008; Fig. 3 B). The effect of RelA knockdown was then determined on the LPS induction of TNF (Fig. 3 C), as well as IL-6, IκB-ζ, and IκB-α mRNA (Fig. S2 B), and on the secretion of TNF and IL-6 protein (Fig. S2 C), and it could be seen that their expression was dependent on RelA. This, along with the fact that the IL-10–sensitive downstream 3′ region of the *TNF* gene contains the κB4 binding site, prompted us to explore previously unexamined aspects of NF-κB signaling that IL-10 may regulate. To examine

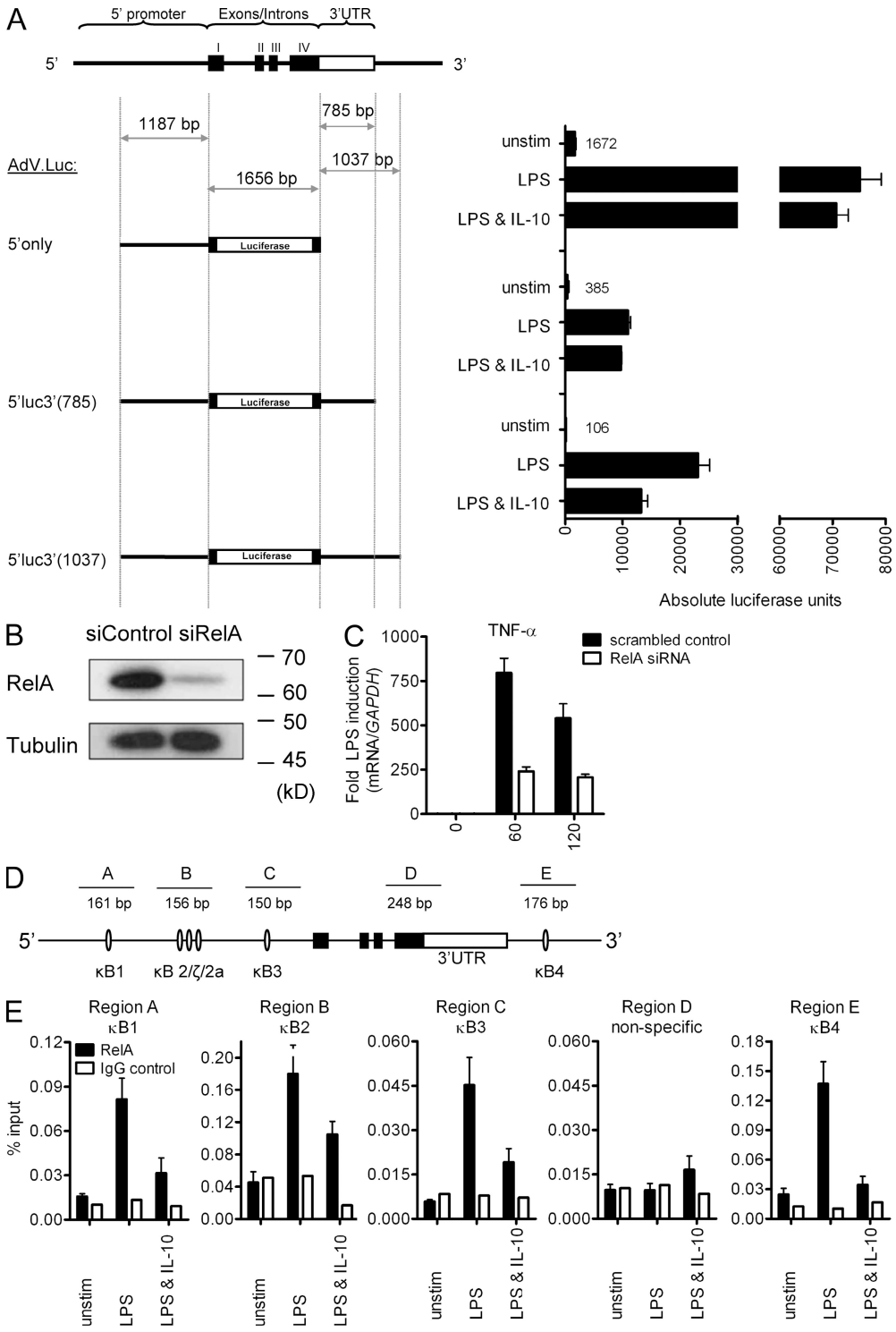

Figure 3. IL-10 inhibits the recruitment of RelA to proinflammatory genes. (A–C) Adenovirus reporters based on the human *TNF* gene were infected into macrophages and stimulated with LPS with or without IL-10 for 4 h, after which luciferase assays were performed. Cells were either transfected with scrambled control oligonucleotides (siControl) or RelA-specific siRNA (siRelA; A), and cytoplasmic extracts were subjected to Western blotting using anti-RelA or tubulin antibodies to evaluate the extent of RelA knockdown (B) or cells were stimulated for the indicated times with LPS and total RNA was isolated and used as template in a quantitative RT-PCR gene expression assay for TNF mRNA (C). (D) Position of primers used in ChIP assays for the detection of RelA recruitment to the human *TNF* gene. NF-κB sites are indicated below the diagram, with the PCR targets represented by black lines with the length of the amplicons indicated above. (E) Cells were stimulated with LPS with or without IL-10 for 60 min, after which ChIP assays were performed using RelA-specific antibodies. Data are expressed as mean ± SD of triplicate measurements for a single donor representative of eight (A) or three (C and E) independent experiments using different donors.

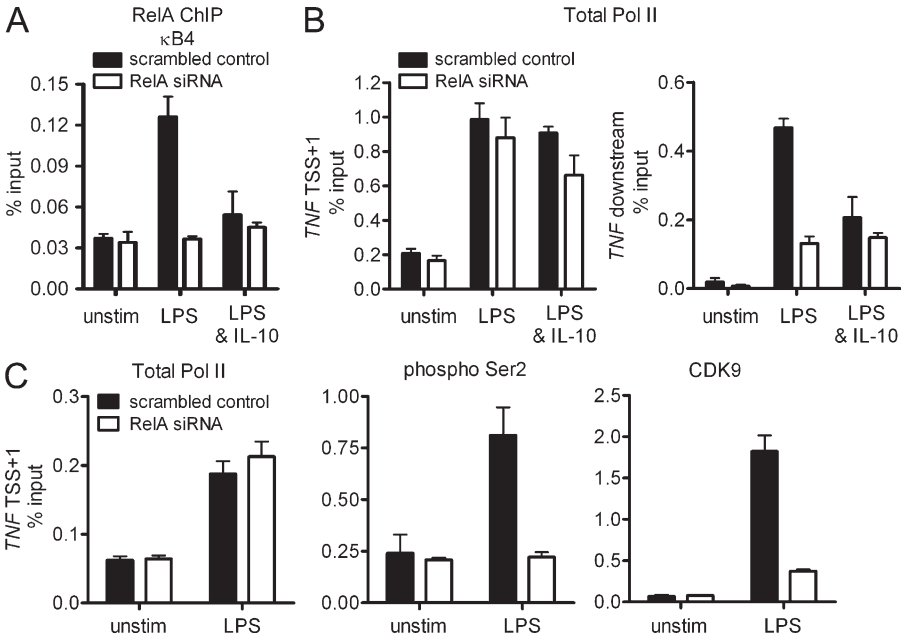

Figure 4. IL-10 inhibition of elongation at *TNF* is RelA dependent. (A and B) Cells were transfected with a siControl or siRelA oligonucleotides. Cells were stimulated with LPS with or without IL-10 for 1 h, after which ChIP was performed using RelA-specific antibodies and primers designed to the κB4 site of *TNF* (A) or Pol II–specific antibodies and primers specific for the *TNF* TSS or downstream regions (B). (C) Cells were treated with or without LPS for 1 h and ChIP was performed using Pol II–specific (left), phosphor-Ser2 (middle), or CDK9 (right) antibodies and primers specific for the *TNF* TSS+1. Data are expressed as mean ± SD of triplicate measurements for a single donor representative of three independent experiments using different donors.

effects on the nuclear translocation of RelA, we compared nuclear versus cytoplasmic protein fractions from macrophages stimulated with LPS or IL-10 and found no major role for IL-10 in preventing RelA nuclear translocation (Fig. S3). The posttranslational modification, by means of LPS-induced phosphorylation of RelA Ser536 (a known mark of RelA function), was also unaffected (Fig. S3). We also rechecked whether IL-10 modulated the LPS-induced degradation and resynthesis of IκB-α in the cytoplasm of human macrophages yet found no effect, which is consistent with previous findings in mouse macrophages (Murray, 2005).

We then went on to examine the actual recruitment of NF-κB to κB binding sites at proinflammatory genes using ChIP. Initially the kinetics of RelA recruitment was assessed in these cells (Fig. S4 A). We observed a time-dependent transient recruitment of RelA to the κB sites of the *TNF* gene peaking at 60 min after LPS stimulation. IL-10 inhibited the recruitment of RelA to all of the regions in the *TNF* gene containing κB sites (Fig. 3 E), including the downstream region containing κB4 (Fig. 3 D). As a control, PCR was performed using primers within the *TNF* introns (Fig. 3 D, region D), where no κB sites were found and no recruitment was seen at this position, confirming the specificity of the assay.

To ascertain whether the IL-10 inhibition of RelA recruitment occurred at additional genes, we performed similar analysis at the κB sites in the *NFKBIA*, *TNFAIP3*, and *CCL20* promoters, to which RelA was similarly inhibited (Fig. S4 B) This also occurred at the *IL6* κB site (albeit with later kinetics of recruitment; Fig. S4 C). It therefore appears that the mechanism elicited by IL-10 is a widespread block in recruitment of RelA to κB sites in the genome.

Numerous studies have shown a role for RelA in the activation of CDK9-mediated control of transcription elongation in alternate systems (Barboric et al., 2001; Huang et al., 2009). Considering that IL-10 inhibits RelA recruitment to the *TNF* gene, we wondered whether depletion of RelA in our experimental system could replicate the effects of IL-10 on transcription elongation. As expected, using an RNAi approach to deplete RelA, after LPS stimulation no RelA was detected to the *TNF* downstream region containing the κB4 site (Fig. 4 A). Concomitantly, Rel A depletion had no significant effect on the recruitment of total Pol II to the start of *TNF*, yet it did result in an inability of LPS to induce its progression down the length of *TNF* (Fig. 4 B). This also coincided with a loss of inducible phosphorylation of Pol II at Ser2, and an inability to recruit CDK9 to the start of *TNF* in RelA-depleted macrophages (Fig. 4 C). These data show that artificial inhibition of RelA recruitment to its target κB sites replicates IL-10's effects on *TNF* transcription elongation.

It is notable that IL-10 also inhibited the recruitment of RelA to the *NFKBIA* κB site, despite the fact that transcription of this gene is spared from inhibition. The fact that neither CDK9 recruitment nor Pol II Ser2 phosphorylation was inhibited by IL-10 at *NFKBIA* suggests that CDK9 recruitment here is independent of RelA. Certain aspects of these data bear resemblance to a study of the gene-specific actions of glucocorticoids on CDK9 recruitment, where inhibition was observed at the gene coding for IL-8 but not at *NFKBIA* (Luecke and Yamamoto, 2005). The up-regulation of IκB-α is part of a negative-feedback loop that limits inflammatory responses; therefore, it is unsurprising that this escapes negative regulation by IL-10 and dexamethasone.

To conclude, we provide novel insights into the mechanisms of the IL-10 antiinflammatory response. We have shown for *TNF* that IL-10 predominantly inhibits transcription through a unique mechanism targeting the rapid and immediate induction of transcription elongation by Pol II. IL-10 achieves this in a gene-specific manner through the inhibition of RelA-mediated recruitment of CDK9 to the *TNF* but not the *NFKBIA* promoter, thus preventing the phosphorylation of RNA Pol II at Ser2. This study, performed entirely in the physiologically relevant primary human macrophage, has

addressed long-standing contradictions within the field. The discovery of a unique negative regulatory checkpoint within the human innate immune system (CDK9 modulation of transcription elongation) has implications for the development of therapeutics that harness the central role that IL-10 plays in the suppression of inflammatory processes in humans.

MATERIALS AND METHODS

Reagents. IL-10 was a gift (Schering Plough), macrophage CSF (M-CSF) was a gift (Pfizer), and LPS was purchased (Enzo Life Sciences, Inc). Flavopiridol (Enzo Life Sciences, Inc.) was used at a 500-nM concentration for 30 min before stimulation with LPS.

Cells. Single-donor platelet phoresis residue packs were purchased from the North London Blood Transfusion Service. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation (specific density, 1.077 g/ml) preceeding T cell/monocyte separation in a JE6 elutriator (Beckman Coulter). T cell purity was assessed by flow cytometry using directly conjugated anti-CD3 (BD), and monocyte purity, assessed using anti-CD45 and anti-CD14 antibodies (Leucogate; BD), was routinely >90%. All media and sera were routinely tested for endotoxin using the Limulus amebocyte lysate test (BioWhittaker; Lonza) and were rejected if the endotoxin concentration exceeded 0.1 U/ml. Macrophages were derived from elutriated monocytes by culturing the cells with M-CSF at 100 ng/ml (Pfizer) in 10% heat-inactivated FCS RPMI 1640 for 3 d.

Antibodies. The anti-IκBα (C-15) antibody, anti–RNA Pol II, and anti-CDK9 (H-169) were purchased from Santa Cruz Biotechnology, Inc. Anti-tubulin was purchased from Sigma-Aldrich. Anti-lamin A/C was purchased from BD. Anti Cox-2 was purchased from Cayman Chemical. The anti-Pol II CTD phosphor-Ser2, rabbit isotype control antibody, and anti-p65 were all purchased from Abcam.

Adenoviral constructs. The *TNF*-luciferase reporters AdV.Luc.5′only and AdV.Luc.3′(1037) were generated as previously described (Denys et al., 2002). The AdV.Luc.3′(785) vector was generated by PCR using the primer pairs 5′-CAGTCTAGAGAGGACGAACATCCAACCTTC-3′ and 5′-CGAGTCGACGCAAACTTTATTTCTCGCCAC-3′. The PCR product was cloned from the pCRII-TOPO TA vector using XbaI and SpeI into the 3′ region of the pAdTrack vector used to make the AdV.Luc.5′only virus. This was used to generate the adenoviral vector by homologous recombination with the pADeasy vector in *Escherichia coli* BJ cells and purified as per previously devised methods (He et al., 1998). Macrophages were infected with virus at a multiplicity of infection of 100 as previously described (Foxwell et al., 1998).

Nuclear extractions and Western blot analysis. After stimulation, cells were scraped into ice-cold PBS and then resuspended in hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 M EDTA, 1 mM $NaV0_3$, 0.5 mM NaF, 1 mM PMSF, protease inhibitor tablet, and 1 mM DTT). Cells were kept on ice for 15 min before the addition of NP-40 to a final concentration of 0.0064%, vortexed for 10 s, and nuclei were harvested by centrifugation (13,000 rpm for 1 min). The supernatant containing the cytosolic extract was kept at −80°C and the nuclear pellet was washed once in hypotonic lysis buffer. Nuclear protein extracts were prepared by incubating the nuclei in hypertonic extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM $NaV0_3$, 0.5 mM NaF, 1 mM PMSF, protease inhibitor tablet [Roche], and 1 mM DTT) for 2 h with constant agitation at 4°C. Postnuclear lysates were then isolated after a 15-min spin at 13,000 rpm and frozen at −80°C. Protein assays was performed using BCA protein assay (Thermo Fischer Scientific) according to manufacturer's instructions. Proteins were resolved by SDS-PAGE and transferred to polyvinyl difluoride membranes (Millipore), which were blocked for 1 h with blocking buffer (5% wt/vol fat-free milk and 0.1% vol/vol Tween-20 in PBS), followed by a 1-h incubation with the antibodies, which were diluted 1:1,000 in blocking buffer. Horseradish peroxidase–conjugated anti–mouse IgG or anti–rabbit IgG (GE Healthcare) were used as secondary antibodies at a dilution of 1:2,000. Bound antibody was detected using the enhanced chemiluminescence kit (GE Healthcare) and visualized using Hyperfilm MP (GE Healthcare).

Quantization of gene expression by real-time PCR. RNA was extracted from macrophages using the Blood RNA extraction kit (QIAGEN), and PCR reactions were performed and measured on a Corbett Rotor-Gene 6000 (QIAGEN) using the Superscript III platinum one-step RT-PCR kit (Invitrogen) and Assay-On-Demand premixed Taqman probe master mixes(Applied Biosystems). The relative gene expression was calculated using the ΔΔCt method with the *GAPDH* gene for normalization of RNA levels. Primary transcript PCR was performed as follows: contaminating genomic DNA was removed from RNA samples using TURBO DNA-free kit (Applied Biosystems). The procedure routinely removed DNA contamination to levels below the detection limits of a standard real-time PCR. Reverse transcription of total RNA was performed at 42°C for 2 h with reagents purchased from Promega, followed by heat inactivation at 65°C for 10 min to inactivate the enzyme. cDNA was then subjected to real-time PCR analysis using SYBR Premix Ex Taq (Lonza). The primers used for measuring primary transcript and mature transcripts were 5′-GCAGTCAGATCATCTTCTCG-3′ and 5′-AGGTACAGGCCCTCTGATGGCAC-3′ and 5′-CCTGCTGCACTTTGGAGTGATCGG-3′ and 5′-GTACAGGCCCTCTGATGGCACCACC-3′, respectively.

ChIP. Macrophages were plated in 10-cm dishes for culture at 8.5×10^5 cells per dish. After stimulation, the cells were fixed for 10 min with formaldehyde (37% stock solution; Sigma-Aldrich) at a concentration of 1% and quenched using 125 mM Tris, pH 7.5. The nuclei were sonicated using conditions optimised for primary human macrophages. Extracts were precleared for 2 h once with 60 µl of a 50% suspension of salmon sperm-saturated protein G (GE Healthcare) and again with another 60 µl of protein G and 2 µg ChIP-grade rabbit isotype control antibody (Abcam). Immunoprecipitations were performed at 4°C overnight. Immunocomplexes were then collected with protein G Sepharose beads for 30 min, rigorously washed, and eluted. Protein DNA cross-links were reversed by heat at 65°C for 4 h, and DNA was purified using the QIAquick PCR Purification kit (QIAGEN) and subjected to real-time PCR analysis using SYBR Premix Ex Taq. Primer pairs used for ChIP in this study were optimised and shown to amplify with similar efficiencies.

Luciferase assays. After stimulation, the amount of GFP fluorescence was measured using a FLUOstar Omega plate reader (BMG Labtech) for normalizing the levels of viral infection. Luciferase activity was measured with a Labsystem luminometer after the addition of 30 µl luciferin (Bright-Glo luciferase assay system; Promega) per well. Levels of luciferase were then normalised to the amounts of GFP.

siRNA. Macrophages were transfected with siRNA (Thermo Fisher Scientific) targeted to either RelA (RelA) or a nonspecific scrambled control (siControl), using methods previously devised and published (Behmoaras et al., 2008).

ELISA. The concentration of TNF and IL-6 in cell culture supernatants was determined by ELISA (BD), according to the manufacturer's instructions. Absorbance was read and analyzed at 450 nm on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromatic) using Ascent software (version 2.4.2).

Online supplemental material. Fig. S1 shows that IL-10 inhibits transcription elongation at *TNF*. Fig. S2 shows that IL-10 requires elements 3′ and downstream of the *TNF* 3′UTR for inhibition. Fig. S3 shows that IL-10 does not inhibit NF-κB activation. Fig. S4 shows that IL-10 inhibits the recruitment of RelA to proinflammatory genes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100414/DC1.

This paper is dedicated to the late Professor Brian Foxwell, who was the driving force behind this work.

We are grateful to Matthew Pierce who critically read this manuscript and we thank Rachel Simmonds for editorial help.

This work was supported by the Arthritis Research Campaign, the Kennedy Institute Trustees, and a Medical Research Council New Investigators Award.

The authors have no conflicting financial interests.

Submitted: 1 March 2010
Accepted: 4 August 2010

REFERENCES

Adelman, K., M.A. Kennedy, S. Nechaev, D.A. Gilchrist, G.W. Muse, Y. Chinenov, and I. Rogatsky. 2009. Immediate mediators of the inflammatory response are poised for gene activation through RNA polymerase II stalling. *Proc. Natl. Acad. Sci. USA*. 106:18207–18212. doi:10.1073/pnas.0910177106

Barboric, M., R.M. Nissen, S. Kanazawa, N. Jabrane-Ferrat, and B.M. Peterlin. 2001. NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. *Mol. Cell*. 8:327–337. doi:10.1016/S1097-2765(01)00314-8

Behmoaras, J., G. Bhangal, J. Smith, K. McDonald, B. Mutch, P.C. Lai, J. Domin, L. Game, A. Salama, B.M. Foxwell, et al. 2008. Jund is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. *Nat. Genet*. 40:553–559. doi:10.1038/ng.137

Buelens, C., F. Willems, A. Delvaux, G. Piérard, J.P. Delville, T. Velu, and M. Goldman. 1995. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur. J. Immunol*. 25:2668–2672. doi:10.1002/eji.1830250940

Chao, S.-H., and D.H. Price. 2001. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. *J. Biol. Chem*. 276:31793–31799. doi:10.1074/jbc.M102306200

Denys, A., I.A. Udalova, C. Smith, L.M. Williams, C.J. Ciesielski, J. Campbell, C. Andrews, D. Kwaitkowski, and B.M. Foxwell. 2002. Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-kappa B in primary human macrophages. *J. Immunol*. 168:4837–4845.

Feldmann, M., and R.N. Maini. 2003. Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat. Med*. 9:1245–1250. doi:10.1038/nm939

Foxwell, B., K. Browne, J. Bondeson, C. Clarke, R. de Martin, F. Brennan, and M. Feldmann. 1998. Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. *Proc. Natl. Acad. Sci. USA*. 95:8211–8215. doi:10.1073/pnas.95.14.8211

Glocker, E.-O., D. Kotlarz, K. Boztug, E.M. Gertz, A.A. Schäffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, et al. 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N. Engl. J. Med*. 361:2033–2045. doi:10.1056/NEJMoa0907206

Grütz, G. 2005. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. *J. Leukoc. Biol*. 77:3–15.

Hargreaves, D.C., T. Horng, and R. Medzhitov. 2009. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell*. 138:129–145. doi:10.1016/j.cell.2009.05.047

He, T.C., A.B. Sparks, C. Rago, H. Hermeking, L. Zawel, L.T. da Costa, P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. *Science*. 281:1509–1512. doi:10.1126/science.281.5382.1509

Huang, B., X.-D. Yang, M.-M. Zhou, K. Ozato, and L.-F. Chen. 2009. Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. *Mol. Cell. Biol*. 29:1375–1387. doi:10.1128/MCB.01365-08

Kontoyiannis, D., A. Kotlyarov, E. Carballo, L. Alexopoulou, P.J. Blackshear, M. Gaestel, R. Davis, R. Flavell, and G. Kollias. 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *EMBO J*. 20:3760–3770. doi:10.1093/emboj/20.14.3760

Krausgruber, T., D. Saliba, G. Ryzhakov, A. Lanfrancotti, K. Blazek, and I.A. Udalova. 2010. IRF5 is required for late-phase TNF secretion by human dendritic cells. *Blood*. 115:4421–4430. doi:10.1182/blood-2010-01-263020

Kühn, R., J. Löhler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 75:263–274. doi:10.1016/0092-8674(93)80068-P

Lang, R., D. Patel, J.J. Morris, R.L. Rutschman, and P.J. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol*. 169:2253–2263.

Luecke, H.F., and K.R. Yamamoto. 2005. The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. *Genes Dev*. 19:1116–1127. doi:10.1101/gad.1297105

Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol*. 19:683–765. doi:10.1146/annurev.immunol.19.1.683

Murray, P.J. 2005. The primary mechanism of the IL-10-regulated anti-inflammatory response is to selectively inhibit transcription. *Proc. Natl. Acad. Sci. USA*. 102:8686–8691. doi:10.1073/pnas.0500419102

Natoli, G. 2009. Control of NF-kappaB-dependent transcriptional responses by chromatin organization. *Cold Spring Harb Perspect Biol*. 1:a000224. doi:10.1101/cshperspect.a000224

Price, D.H. 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol. Cell. Biol*. 20:2629–2634. doi:10.1128/MCB.20.8.2629-2634.2000

Ramirez-Carrozzi, V.R., D. Braas, D.M. Bhatt, C.S. Cheng, C. Hong, K.R. Doty, J.C. Black, A. Hoffmann, M. Carey, and S.T. Smale. 2009. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell*. 138:114–128. doi:10.1016/j.cell.2009.04.020

Sandoval, J., J.L. Rodríguez, G. Tur, G. Serviddio, J. Pereda, A. Boukaba, J. Sastre, L. Torres, L. Franco, and G. López-Rodas. 2004. RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res*. 32:e88. doi:10.1093/nar/gnh091

Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol*. 10:170–181. doi:10.1038/nri2711

Schaljo, B., F. Kratochvill, N. Gratz, I. Sadzak, I. Sauer, M. Hammer, C. Vogl, B. Strobl, M. Müller, P.J. Blackshear, et al. 2009. Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10. *J. Immunol*. 183:1197–1206. doi:10.4049/jimmunol.0803883

Schottelius, A.J., M.W. Mayo, R.B. Sartor, and A.S. Baldwin Jr. 1999. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J. Biol. Chem*. 274:31868–31874. doi:10.1074/jbc.274.45.31868

Takeda, K., B.E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Förster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity*. 10:39–49. doi:10.1016/S1074-7613(00)80005-9

Tsytsykova, A.V., R. Rajsbaum, J.V. Falvo, F. Ligeiro, S.R. Neely, and A.E. Goldfeld. 2007. Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers. *Proc. Natl. Acad. Sci. USA*. 104:16850–16855. doi:10.1073/pnas.0708210104

Udalova, I.A., J.C. Knight, V. Vidal, S.A. Nedospasov, and D. Kwiatkowski. 1998. Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes. *J. Biol. Chem*. 273:21178–21186. doi:10.1074/jbc.273.33.21178

Udalova, I.A., V. Vidal, I.G. Scragg, and D. Kwiatkowski. 2000. Direct evidence for involvement of NF-kappaB in transcriptional activation of tumor necrosis factor by a spirochetal lipoprotein. *Infect. Immun*. 68:5447–5449. doi:10.1128/IAI.68.9.5447-5449.2000

Wang, P., P. Wu, M.I. Siegel, R.W. Egan, and M.M. Billah. 1995. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J. Biol. Chem*. 270:9558–9563. doi:10.1074/jbc.270.16.9558

Williams, L., L. Bradley, A. Smith, and B. Foxwell. 2004a. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J. Immunol*. 172:567–576.

Williams, L.M., G. Ricchetti, U. Sarma, T. Smallie, and B.M. Foxwell. 2004b. Interleukin-10 suppression of myeloid cell activation—a continuing puzzle. *Immunology*. 113:281–292. doi:10.1111/j.1365-2567.2004.01988.x

Yasukawa, H., M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, et al. 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol*. 4:551–556. doi:10.1038/ni938