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Transcription of the gene encoding TNF- α is increased by IL-1 β in rat and human islets and β -cell lines

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Abstract

Synthesis and secretion of immunomodulatory proteins, such as cytokines and chemokines, controls the inflammatory response within pancreatic islets. When this inflammation does not resolve, destruction of pancreatic islet β -cells leads to diabetes mellitus. Production of the soluble mediators of inflammation, such as TNF- α and IL-1 β , from resident and invading immune cells, as well as directly from islet β -cells, is also associated with suboptimal islet transplantation outcomes. In this study, we found that IL-1 β induces rapid increases in TNF- α mRNA in rat and human islets and the 832/13 clonal β -cell line. The surge in transcription of the TNF- α gene required the inhibitor of kappa B kinase beta (IKK β), the p65 subunit of the NF- κ B and a signal-specific recruitment of RNA polymerase II to the gene promoter. Of note was the increased intracellular production of TNF- α protein in a manner consistent with mRNA accumulation in response to IL-1 β , but no detectable secretion of TNF- α into the media. Additionally, TNF- α specifically induces expression of CD11b, but not CD11c, on neutrophils, which could contribute to the inflammatory milieu and diabetes progression. We conclude that activation of the NF- κ B pathway in pancreatic β -cells leads to rapid intracellular production of the pro-inflammatory TNF- α protein through a combination of specific histone covalent modifications and NF- κ B signaling pathways.

Keywords

cytokine; diabetes mellitus; inflammation; islet; NF- κ B; transcription

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1. Introduction

Tumor necrosis factor- α (TNF- α) is a soluble protein linked with many human pathologies, including autoimmune diseases [1], obesity-associated insulin resistance [2, 3], cardiovascular complications [4, 5], and cancer [6]. It was first discovered over 30 years ago as an immune-cell derived molecule with ability to cause necrosis of tumors [7]. The TNF family currently consists of many different ligands with multiple corresponding types of receptors [8]. Most of the biological effects of TNF- α are mediated through the appropriate associated receptor, which is linked to activation of the NF- κ B pathway [9, 10].

The NF- κ B transcriptional regulators include p65 (RelA), RelB, c-Rel, p50, and p52. These proteins control numerous cellular processes, including proliferation, inflammation, and immune cell responses through signal-induced homo- and hetero-dimerization that lead to specific alterations in gene transcription [10]. In pancreatic β -cells, the activation of NF- κ B by cytokines, such as IL-1, leads to increased production of nitric oxide [11-13], synthesis and secretion of various chemokines [14-17], losses in insulin secretion [18, 19] and diminished viability [20-22]. These cytokine-driven processes are thought to be a major cause of autoimmune-mediated (T1DM) diabetes mellitus [11, 23] and may also be involved with development of Type 2 diabetes mellitus (T2DM; ref [24, 25]).

Due to inflammatory processes underlying development of both T1DM and T2DM, IL-1 and TNF have been targeted individually as possible clinical treatments for treat diabetes in experimental rodent models, including non-obese diabetic (NOD) mice and Goto-Kakizaki (GK) non-obese type II diabetic rats [26-28]. In the NOD mouse, TNF- α can reduce the incidence of diabetes [29] or accelerate the development of diabetes [30, 31], depending on timing and modulatory strategy undertaken. TNF- α production directly from pancreatic islets of neonatal mice enhances diabetes development by promoting islet inflammation [31]. Moreover, therapies reducing IL-1 or IL-1 action in obese humans, or in individuals with overt diabetes, have revealed improved β -cell function with no parallel amelioration of peripheral insulin resistance [32-34].

Because of the prominent role of IL-1 β in both major forms of diabetes as well as the apparently dichotomous role of TNF- α in modulating β -cell death and dysfunction leading to diabetes onset, we investigated the regulation of the TNF- α gene in pancreatic β -cells exposed to IL-1 β . Several novel observations emerged: 1) Expression of the TNF- α gene is markedly upregulated in rat and human islets and β -cell lines in response to IL-1 β exposure. 2) TNF- α induces Cd11b, but not Cd11c, expression in bone-marrow derived neutrophils. 3) The I κ K β protein is involved in mediating the intracellular response to IL-1 β . 4) The p65 protein binds to κ B response elements in the proximal TNF- α gene promoter. 5) The timing of TNF- α transcript accumulation is congruent with rapid signal-induced alterations in methylation status of histone H3 at K4 and K9 and recruitment of RNA polymerase II to the TNF- α promoter region.

2. Materials and Methods

2.1. Adenoviral vectors, cell culture, isolation of islets and reagents

Selection process and culture of 832/13 rat insulinoma cells has been described [35]. Isolation of rat islets has also been previously described [21]. Human islets were obtained commercially from Lonza (Clonetics™ Fresh Human Pancreatic Islets) on two separate occasions. Human islets used herein were from two different donors, with each shipment of islets handpicked into duplicates for each biological replicate used for cytokine exposure. The construction and use of adenoviruses encoding β -Galactosidase [36] and $I\kappa B\alpha^{SR}$ [37] have been previously documented. All reagents were from Fisher-Scientific unless otherwise noted.

2.2 Chromatin immunoprecipitation (ChIP) assays

832/13 cells were grown to confluence in 10cm dishes, treated as indicated in the figure legends, and harvested for ChIP as outlined in detail previously [12]. Antibodies used to immunoprecipitate anti-trimethyl-Histone H3 (Lysine 4 and 9) and RNA Polymerase II were from Millipore, while p65 was from Santa Cruz Biotechnology. Primers used to amplify regions of interest within promoter and coding regions are available upon request.

2.3 Isolation of total RNA, synthesis of cDNA, and detection of transcription by real-time PCR

832/13 cells, rat islets, and human islets were cultured in 12 well plates prior to and during exposure to cytokines. RNA isolation was carried out using Tri-Reagent for 832/13 cells and RNeasy kits (Qiagen) for rat and human islets. All procedures have been described [38]. Primers for detection of transcripts were designed using Primer3 Plus software and sequences are available upon request.

2.4 siRNA duplex transfection and immunoblot analysis

All siRNA duplexes were obtained via Ambion (Life Technologies) and transfected into 832/13 cells using Dharmafect I (Dharmacon) as recommended by the transfection reagent protocol and previous published methods [12, 16]. siRNA-mediated suppression of target genes was monitored using reverse transcription followed by real-time PCR analysis and immunoblotting using our previously described methodologies [12, 16].

2.5 Bone marrow neutrophil integrin expression following exposure to TNF- α

Four to six Balb/c mice were euthanized with isoflurane and femurs were flushed with PBS. After washing in PBS, erythrocytes were lysed with ammonium-chloride-potassium (ACK) lysis buffer and passed through a 70 μ m cell strainer (BD falcon). Cells were layered on top of a percoll gradient (58%) centrifuged for 30min at 1250 \times g. The bone marrow neutrophil pellet was resuspended in PBS and counted. 10⁶ neutrophils were placed into a snap cap tube and TNF- α was added to a final concentration of 1ng/ml followed by incubation at 37°C for 30min. After incubation, the cells were stained with anti-murine CD11b-PE and CD11c-APC (Biolegends) and analyzed on a BD LSRIL. Data were analyzed using Flojo software (ver. 10.0.7r2).

2.5 ELISA and Statistical Analysis

832/13 cells were grown in 12-well plates, treated as indicated in the figures and associated legends. The cell culture media was supplemented with 0.3% BSA and collected at the indicated time points to analyze for secreted proteins. The cells were then lysed in M-PER (Thermo-Scientific) to analyze for intracellular TNF- α protein content. The TNF- α ELISA kit was from Life Technologies and was used exactly as recommended by the manufacturer to detect TNF- α protein in both supernatant samples and directly from lysed cell material. All ELISA data were normalized to total protein to account for any differences in cell number. All statistics were calculated using GraphPad Prism 6.0 software.

3. Results

3.1 Interleukin-1 β rapidly increases TNF- α production in pancreatic β -cells

Alterations in TNF- α levels in serum of humans and rodents correlate with insulinitis and development of T1DM [39-41]. Therefore, we examined the regulation of the gene encoding TNF- α using rat and human islets and β -cell lines. In 832/13 rat insulinoma cells, we discovered that TNF- α transcript levels were increased as early as one hour and peaked at three hours in response to 1ng/mL IL-1 β (Figure 1A). A similar pattern was observed in isolated rat islets (Figure 1B). In addition, the presence of IFN- γ potentiated the response to IL-1 β by 42% in rat islets (Figure 1C) and by 93%, 45%, and 38% at one, two, and three hours, respectively, in 832/13 cells (Figure 1D). Comparable results were also obtained using clonal INS-1E cells (not shown).

To investigate whether the IFN- γ -mediated potentiation of the IL-1 β response was due to stabilization of the mRNA, we monitored transcript abundance in the presence and absence of IFN- γ following a 3h pre-exposure to IL-1 β . We found that IFN- γ was unable to induce a significant increase in stability of the TNF- α mRNA after removal of the IL-1 β stimulus (Figure 1E). The transcript encoding TNF- α degraded rapidly after removal of the IL-1 β stimulus (Figure 1E), indicating that regulation of the TNF- α gene is most likely at the transcriptional level. We note that results similar to those shown in Figure 1E were also obtained following a 6h pre-exposure to IL-1 β (not shown). Finally, we found that IL-1 β also induced a 9-fold increase in TNF- α mRNA in human islets (Figure 1F).

Because TNF- α transcript levels accumulate rapidly after IL-1 β exposure, we next measured TNF- α content and secretion. To our surprise, TNF- α was not released from the cells under any condition examined (Figure 2A). We then measured TNF- α content within the cell and found that TNF- α protein accumulated within three hours after cells were exposed to IL-1 β with no early potentiation by IFN- γ (Figure 2B). By contrast, the increase in TNF- α content was potentiated by IFN- γ at the 6 hour time point (Figure 2B). The decrease in TNF- α transcript by 12 hours (Figure 1A) is consistent with the detectable decline in protein by 12 hours (Figure 2B).

If TNF- α is released from the dying β -cells, the first response would ostensibly be circulating neutrophils. These neutrophils would serve to amplify the inflammatory response, contributing to T1DM. Using the mouse model, we examined the effect of TNF- α on bone marrow-derived (BM) neutrophils. Up regulation of integrin expression on

neutrophils can increase the tight attachment of the neutrophil to vascular endothelial cells and subsequent extravasation to a site of inflammation. To examine whether TNF- α alters integrin expression, BM neutrophils were exposed to TNF- α . This resulted in a marked shift in expression of the integrin, CD11b (Mac-1) [mean fluorescence intensity (MFI): TNF (6244) vs CON: (2554)] (see Fig. 2C), while there was little to no change in CD11c expression (Fig. 2D). This observation demonstrates that upregulation of integrins in response to TNF- α is specific and not a result of generalized augmentation of all integrins.

3.2 Interference with I κ K β decreases IL-1 β -mediated induction of TNF- α mRNA abundance

We have reported previously that the chemokine CCL2 requires p38 MAPK and I κ K β for synthesis and secretion in response to IL-1 β [17]. Since TNF- α is also a soluble secreted mediator of inflammation, we tested whether the expression of the TNF- α gene also involves similar signaling pathway activation. First, we used 2-[(Aminocarbonyl)amino]-5-(4-fluor-ophenyl)-3-thiophenecarboxamide (TPCA) at doses that protect β -cells against cytokine-mediated cell death [20] and inhibit expression of the CCL2 gene [17]. We discovered that TPCA blunted the ability of IL-1 β to increase TNF- α mRNA accumulation (Figure 3A). However, inhibiting p38 MAPK with a variety of pyridinyl imidazole based compounds, which effectively inhibit expression of the CCL2 gene [17] did not block the increase in TNF- α mRNA by IL-1 β (not shown). Furthermore, siRNA-mediated suppression of the I κ K β mRNA (Figure 3B) and protein (Figure 3C) confirmed the results seen with TPCA, i.e., diminished I κ K β abundance is associated with a reduction in the IL-1 β -driven accumulation of TNF- α transcripts (Figure 3D).

3.3 NF- κ B subunit RelA/p65 is required for the IL-1 β -mediated induction of the TNF- α gene

IL-1 β activates the NF- κ B pathway and promotes the expression of a variety of pro-inflammatory genes within pancreatic β -cells [10, 12, 16, 38, 42, 43]. Thus, we next examined whether or not the prototypical heterodimer of NF- κ B, consisting of p65/p50, was involved in the IL-1 β -mediated induction of the TNF- α gene. Using the I κ B α super-repressor (I κ B α ^{SR}), a non-degradable form of the inhibitor of NF- κ B transcription factor proteins [37], we found that the abundance of this ectopically-expressed regulatory protein is maintained in the presence of IL-1 β , while the endogenous protein is degraded (Figure 4A). TNF- α mRNA accumulation was decreased by 82% in the presence of I κ B α ^{SR} relative to the β -Gal control after exposure to IL-1 β (Figure 4B).

To confirm the I κ B α ^{SR} results, we next used siRNA duplexes to reduce either p65 or p50 abundance. Transfection of siRNA duplexes that each target a distinct exon with the p65 mRNA sequence decrease p65 protein levels (duplex #1; Figure 4C) and blocked the IL-1 β -mediated increase in TNF- α transcript accumulation (by 89% and 83%, respectively; Figure 4D). By contrast, targeting the p50 subunit of NF- κ B, also using siRNA transfection, did not interfere with IL-1 β stimulated increases in TNF- α transcript levels (data not shown). Thus, the p65 subunit appears to be the major factor controlling expression of the TNF- α gene in response to IL-1 β .

3.4 p65 binding, histone chemical modifications, and RNA polymerase II recruitment to the TNF- α gene promoter are IL-1 β stimulated events

Because the p65 subunit of NF- κ B is required for expression of the TNF- α gene in response to IL-1 β (Figure 4), we examined its occupancy on the proximal gene promoter. *In silico* analysis of 1.2kb of the TNF- α proximal gene promoter revealed a κ B response element approximately 500bp upstream of the transcriptional start site (Figure 5A). Using primers spanning this region of the promoter to amplify DNA recovered after chromatin immunoprecipitation, we detected 6.43-, 7.74-, and 5.10- fold increases in p65 occupancy after a 15, 30, and 60min exposure to IL-1 β , respectively (Figure 5B). No binding over baseline was observed in the absence of IL-1 β , indicating a stimulus-specific occupancy of p65 within the proximal TNF- α gene promoter. In addition, there was no detectable binding of p65 within the coding region (shown as arrow-indicated amplicon on the right hand side of Figure 5A), indicating specific occupancy at genomic regions containing κ B sites.

Moreover, we found that occupancy of the RNA polymerase II was increased on the promoter as well as on the coding region (Figure 5C) of the TNF- α gene, congruent with active transcription. In addition, methylation of histone H3 lysine 4 (H3K4), a well-known marker of gene activation [44], was enhanced in response to IL-1 β (Figure 5D). By contrast, methylation of histone H3 at lysine 9 (H3K9), a modification associated with gene repression, was decreased after exposure to IL-1 β (Figure 5E). No corresponding changes were detected at genes unresponsive to IL-1 β (not shown). We also observed an increase in phosphorylation of the RNA Pol II carboxy terminal domain (CTD) at sites associated with promoter clearance (Ser5; Figure 5F) and movement along the coding region (Ser2; Figure 5G). The time frame of p65 occupancy (Figure 5A) and histone chemical modifications induced by IL-1 β coincided with an increase in total and phosphorylated RNA polymerase II recruitment to the promoter and coding regions of the TNF- α gene. These results are consistent with robust appearance of transcript within 1 hour after IL-1 β exposure (Figure 1A).

4. Discussion

Immune cell infiltration and islet inflammation are key features of both T1DM and T2DM [11, 23, 45, 46]. A major component of the inflammatory process leading to β -cell destruction is exposure to pro-inflammatory cytokines, such as TNF- α and IL-1 β . Cytokine trap strategies blocking IL-1 β action improve islet transplantation in rodents [47] while IL-1 receptor antagonism improves β -cell function in humans [33, 34]. In addition, TNF- α is markedly elevated in islets from humans with T2DM and correlates with poor islet function [48].

TNF- α can either prevent or enhance T1DM, depending on the model system used. For example, systemic administration of recombinant TNF- α decreases insulinitis [49] and diabetes [28] in NOD mice. In addition, transgenic production of TNF- α directly from islet β -cells does not prevent insulinitis, but does prevent autoimmune-mediated diabetes [29]. This contrasts with the toxic effects on TNF- α on islets in culture [50]. Thus, it is critical to understand the signal-driven production of TNF- α because of its unmistakably complex role in immune-mediated β -cell destruction.

We found that TNF- α is regulated transcriptionally by IL-1 β in rat and human islets and the 832/13 rat β -cell line (Figure 1). These results are consistent with a previous report using mouse β -cells [51] and also extend those earlier findings by identifying key signaling events associated with increases in TNF- α transcription. For example, the I κ K β protein, a critical signaling node in cellular inflammatory responses, is involved in both systemic and organ-specific inflammatory diseases, making it a target for drug discovery [52]. Herein, we identify I κ K β as a critical component of the signal-specific induction of TNF- α gene transcription (Figure 3).

In addition, the p65 subunit of NF- κ B, a strong transcriptional activator downstream of I κ K β , is required for TNF- α expression after β -cell exposure to IL-1 β (Figure 4). p65 controls the expression of a number of genes involved in islet inflammation, including COX2, iNOS, CXCL1, CXCL2, CXCL10, and CCL2 [12, 14, 16, 17, 38]. Similar to the complex role TNF- α plays in islet inflammation [39], CCL2 can also promote either insulinitis or diabetes, or both, on genetic backgrounds that are not associated with autoimmunity [53, 54]. Alternatively, CCL2 produced directly from β -cells prevents diabetes in autoimmune-predisposed settings, such as the NOD mouse [55]. Thus, specific genes directly regulated by NF- κ B proteins, such as CCL2 and TNF- α , can have diametrically opposing effects on disease development, which may depend on timing and quantity of expression as well as genetic context. This explanation fits with the promising results of anti-TNF- α therapy in diabetic children [56] and adults with metabolic syndrome [57] and also with the negative outcomes observed during other situations of TNF- α modulation [58].

Remarkably, the IL-1 β -mediated production of TNF- α transcript and protein were not coupled to release of TNF- α from the cell (Figure 2). Although we and others have shown that several chemokine proteins are synthesized and secreted upon synthesis in the pancreatic β -cell (e.g., CXCL1 and CXCL2 [14, 15], CXCL10 [16, 59], and CCL2 [17]), we speculate that TNF- α either requires signals in addition to IL-1 β (and γ -IFN) for release from the cell or may only be released upon β -cell necrosis. For the latter possibility, a similar situation occurs where the immunological adjuvant protein HMGB1 is released during cytokine-mediated necrotic β -cell death, but not during bona fide apoptosis [22].

Since TNF- α specifically modulates surface expression of the CD11b integrin on neutrophils (Figure 2C), it is possible that a spillage of TNF- α from dying β -cells alter neutrophil function and migration, initiating the development or progression to diabetes. In addition, macrophage-derived TNF- α could also modulate neutrophil function, consistent with the immune cell crosstalk known to occur during T1DM [23]. Finally, TNF- α also contributes to diabetes development via islet antigen-specific Th17 cells [60], further increasing the complexity associated with leukocyte-mediated β -cell destruction. Conceptually, these possibilities fit with islet inflammation being associated with altered immune cell responses leading to β -cell destruction during progression to diabetes [11, 23, 45].

Thus, many challenges remain before the efficacy of single or combination immunomodulatory strategies are proven completely effective for treatment of inflammation-associated diabetes in humans. Part of the remaining puzzle is the lack of comprehensive

mechanistic information regarding expression of subsets of genes and associated gene networks regulated by cytokines and other signaling molecules. Understanding the signal-specific, tissue-specific, and gene-specific regulatory mechanisms will enable the development of more selective therapeutic strategies to target immune-cell/ β -cell crosstalk.

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Highlights

- TNF- α mRNA and protein are increased by IL-1 β in pancreatic β -cells
- Induction of TNF- α requires I κ K β , a component of the NF- κ B signaling pathway
- p65, a NF- κ B transcriptional subunit, is recruited to the TNF- α gene promoter
- Methylation status of histone H3 at the TNF- α gene is altered in response to IL-1 β
- RNA polymerase II is recruited to the TNF- α gene promoter in response to IL-1 β

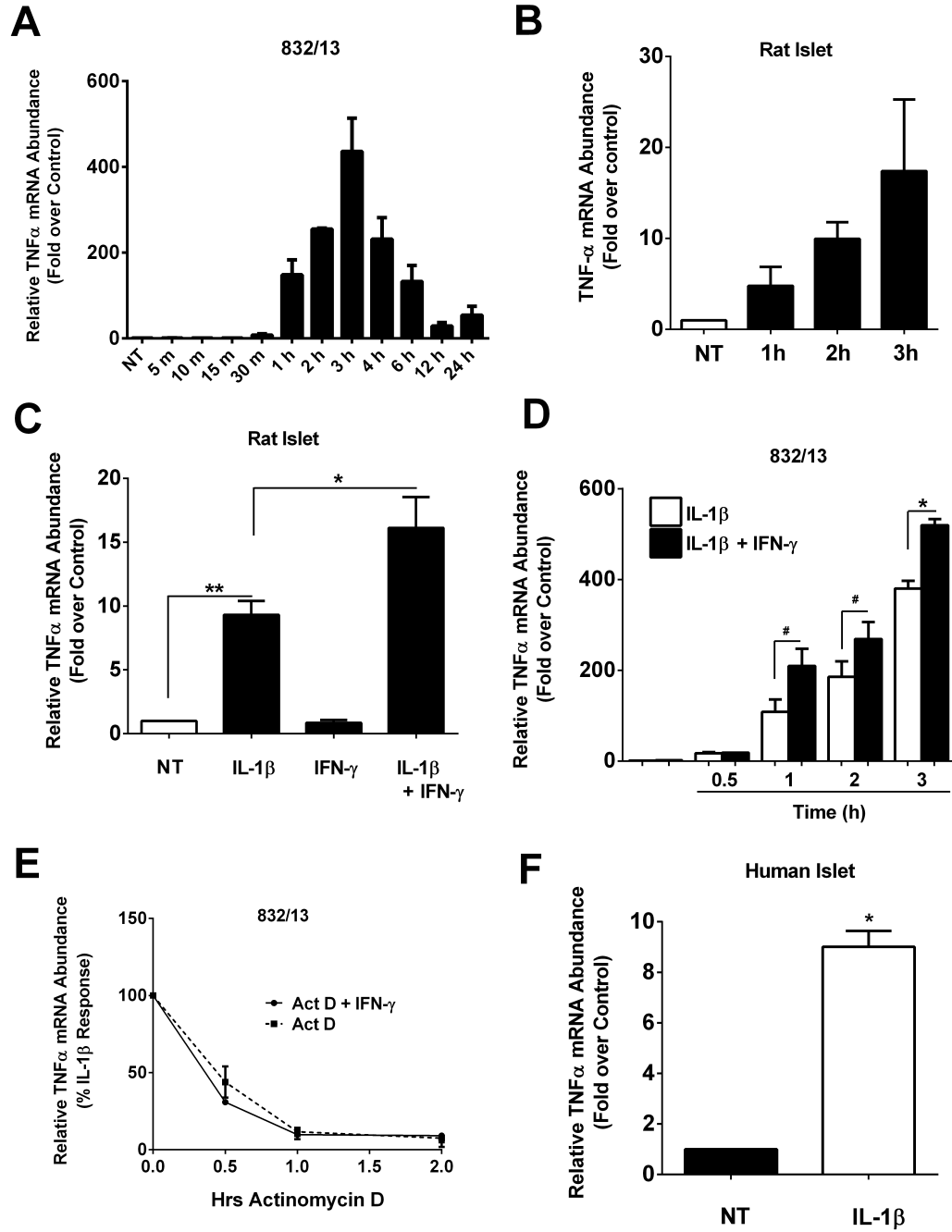


Figure 1. Cytokine-mediated induction of TNF α mRNA in rat and human islets and a β -cell line
 A. 832/13 cells were untreated (NT) or treated with 1 ng/mL IL-1 β for the indicated times.
 B. Isolated rat islets were untreated (NT) or treated with 10 ng/mL IL-1 β for the 1, 2 or 3h.
 C. Isolated rat islets were treated for 6 h with 10 ng/mL IL-1 β , 100 U/mL IFN- γ or both cytokines in combination. **, $P < 0.01$, *, $P < 0.05$. D. 832/13 cells were treated with either 1 ng/mL IL-1 β or IL-1 β + 100 U/mL IFN- γ for 0.5, 1, 2 or 3 h. *, $P < 0.05$, #, $P < 0.1$. E. Following a 3 h stimulation with 1 ng/mL IL-1 β (pre-exposure response induce by IL-1 β is set at 100%), 832/13 cells were exposed to Actinomycin D (to block transcription) in the

presence or absence of 100 U/mL IFN- γ . Total RNA was isolated at 0, 0.5, 1 and 2 h. F. Human islets were untreated (NT) or stimulated with IL-1 β for 3 h. *, $P < 0.05$. A-F. TNF α transcript abundance was normalized to the housekeeping gene Ribosomal S9 (RS9). Data are shown as means \pm SEM from three individual experiments.

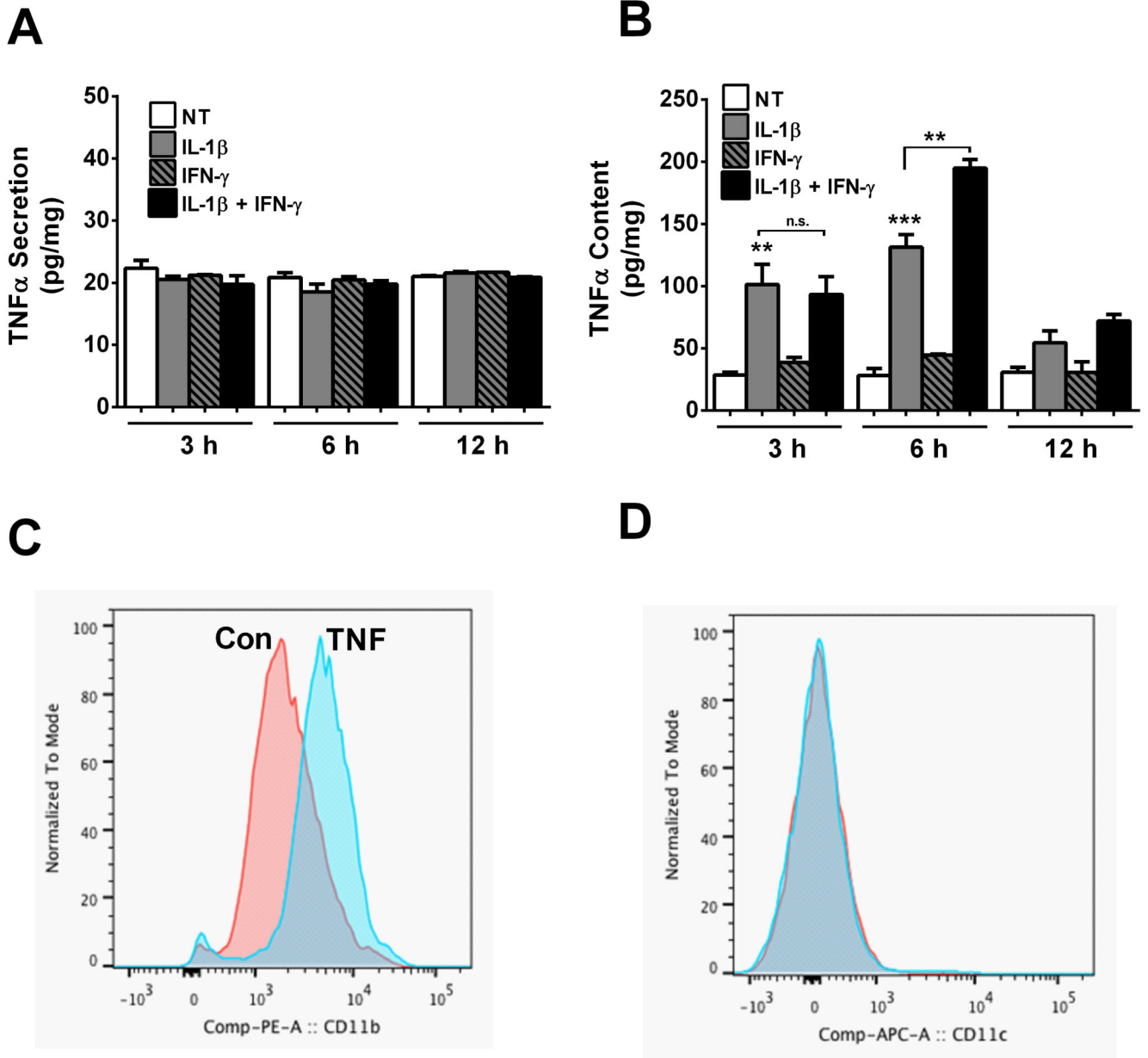


Figure 2. Cytokines induce production of TNF α in 832/13 cells and TNF- α enhances surface expression of Cd11b, but not Cd11c, in bone-marrow derived neutrophils

A, B. 832/13 cells were treated with IL-1 β (1 ng/mL), IFN- γ (100 U/mL) or the combination for 3, 6 or 12 h. TNF α secretion into the media (A) and cellular TNF α content (B) were quantified by ELISA and normalized to total protein. ***, $P < 0.001$ vs. NT, **, $P < 0.01$ (grey bars vs. NT), n.s. = not significant. ELISA assays were performed on three separate occasions. Data are expressed as means \pm SEM. C,D. Isolated murine bone marrow neutrophils were exposed to 1ng/ml of TNF α for 30min (TNF) or media alone (Con). Cells were stained with antibodies to CD11c APC or CD11b PE and analyzed by flowcytometry. Results shown are representative of three biological replicates.

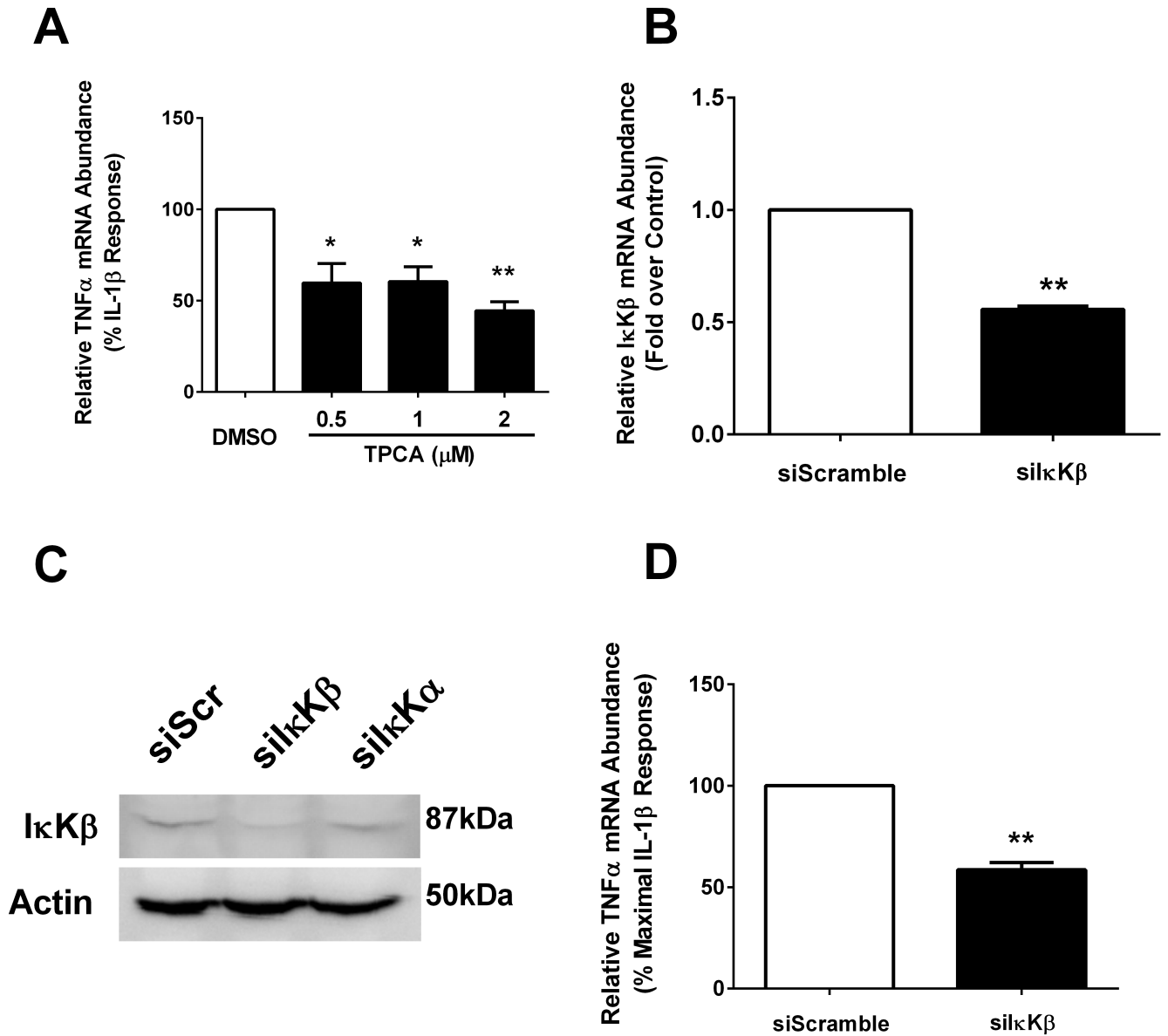


Figure 3. IL-1 β -dependent stimulation of TNF α requires I κ K β

A. 832/13 cells were pre-treated for 1 h with either DMSO (vehicle control) or the indicated concentrations of the I κ K β inhibitor TPCA. Cells were subsequently treated for 3 h with 1 ng/mL IL-1 β . Relative TNF α mRNA abundance was normalized to RS9. **, $P < 0.01$ vs. DMSO, *, $P < 0.01$ vs. DMSO. B. 832/13 cells were transfected with siRNA duplexes targeting either a scrambled control sequence (siScramble) or I κ K β (siI κ K β). After 48 h exposure to siRNA, cells were harvested and mRNA levels of I κ K β were quantified. **, $P < 0.01$. C. 832/13 cells were transfected with siScramble (siScr), siI κ K β or siI κ K α . After 48 h culture with the indicated siRNA duplexes, cells were harvested and an immunoblot performed to determine the cellular abundance of I κ K β ; Actin was used as the loading control. The immunoblot shown is representative of two independent experiments. D. 832/13 cells were transfected with siScramble and siI κ K β duplexes. After 48 h, cells

were stimulated with 1 ng/mL IL-1 β for 3 h. TNF α mRNA level was quantified and normalized to RS9. **, $P < 0.01$. For mRNA experiments, three individual replicates were generated and expressed as means \pm SEM.

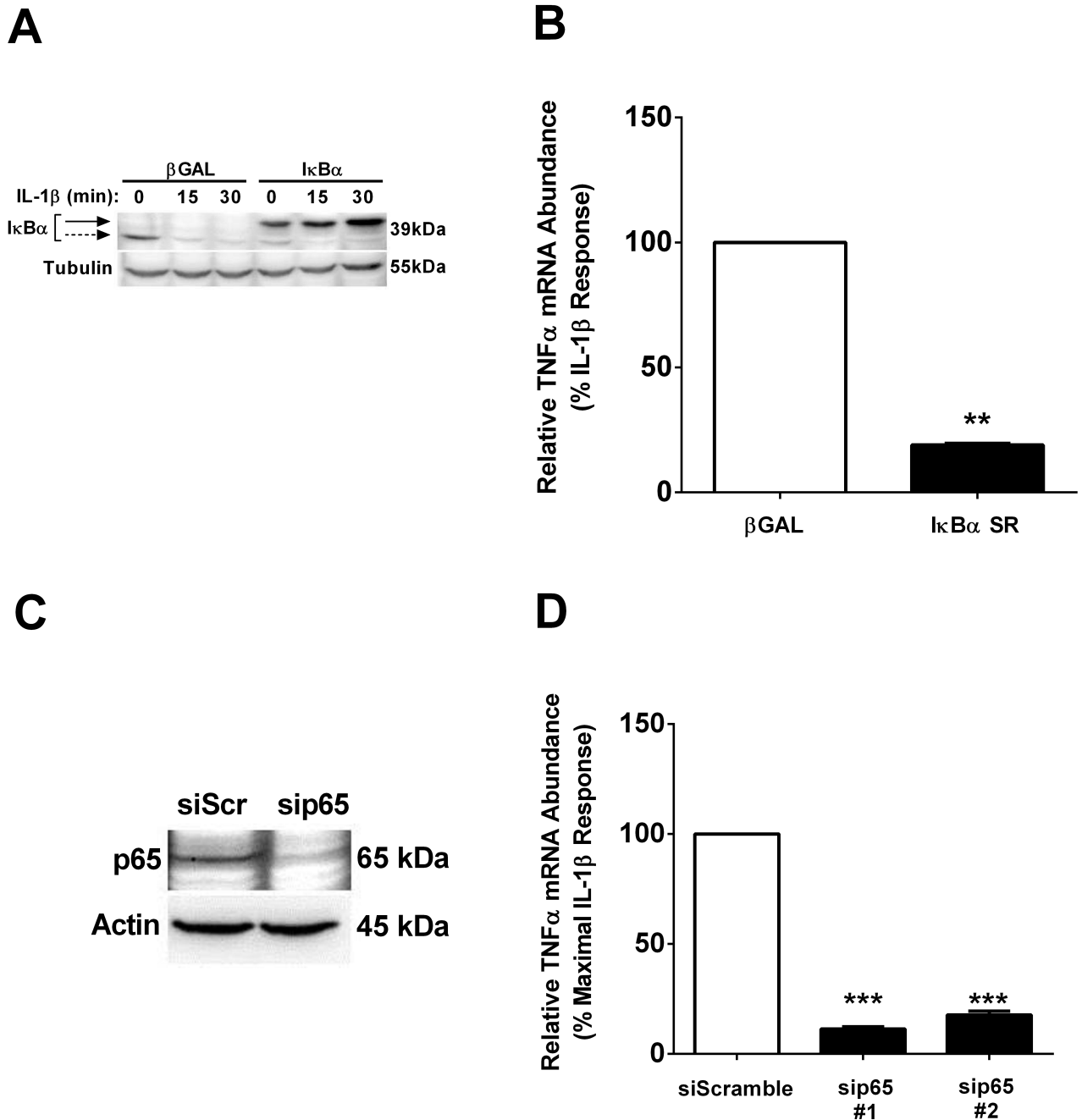


Figure 4. NF- κ B subunit p65, but not p50, is required for IL-1 β -dependent activation of the TNF α gene

A. 832/13 cells were transduced with adenoviruses overexpressing β Galactosidase (β GAL) or I κ B α Superrepressor (SR). Following a 24 h transduction with the indicated adenoviruses, cells were stimulated for 15 or 30 min with 1 ng/mL IL-1 β . An immunoblot was performed using whole cell lysates and antibodies against I κ B α using actin as the loading control. B. 832/13 cells were transduced with adenoviruses overexpressing β GAL or I κ B α SR. Following a 24 h exposure to adenoviruses, cells were stimulated for 3 h with 1 ng/mL IL-1 β . **, $P < 0.01$. C, D. 832/13 cells were transfected with siRNA targeted to p65

and incubated for 48 h. C. 48 h post-transfection, whole cell lysates were blotted to determine abundance of p65. Actin served as the loading control. D. After 48 h exposure to siRNA cells were stimulated for 3 h with 1 ng/mL IL-1 β . ***, $P < 0.001$, n.s. = not significant. B-D. mRNA levels of TNF α were measured and normalized to RS9. Immunoblots were performed on two separate occasions and a representative image is shown. Data are shown as means \pm SEM from three independent experiments.

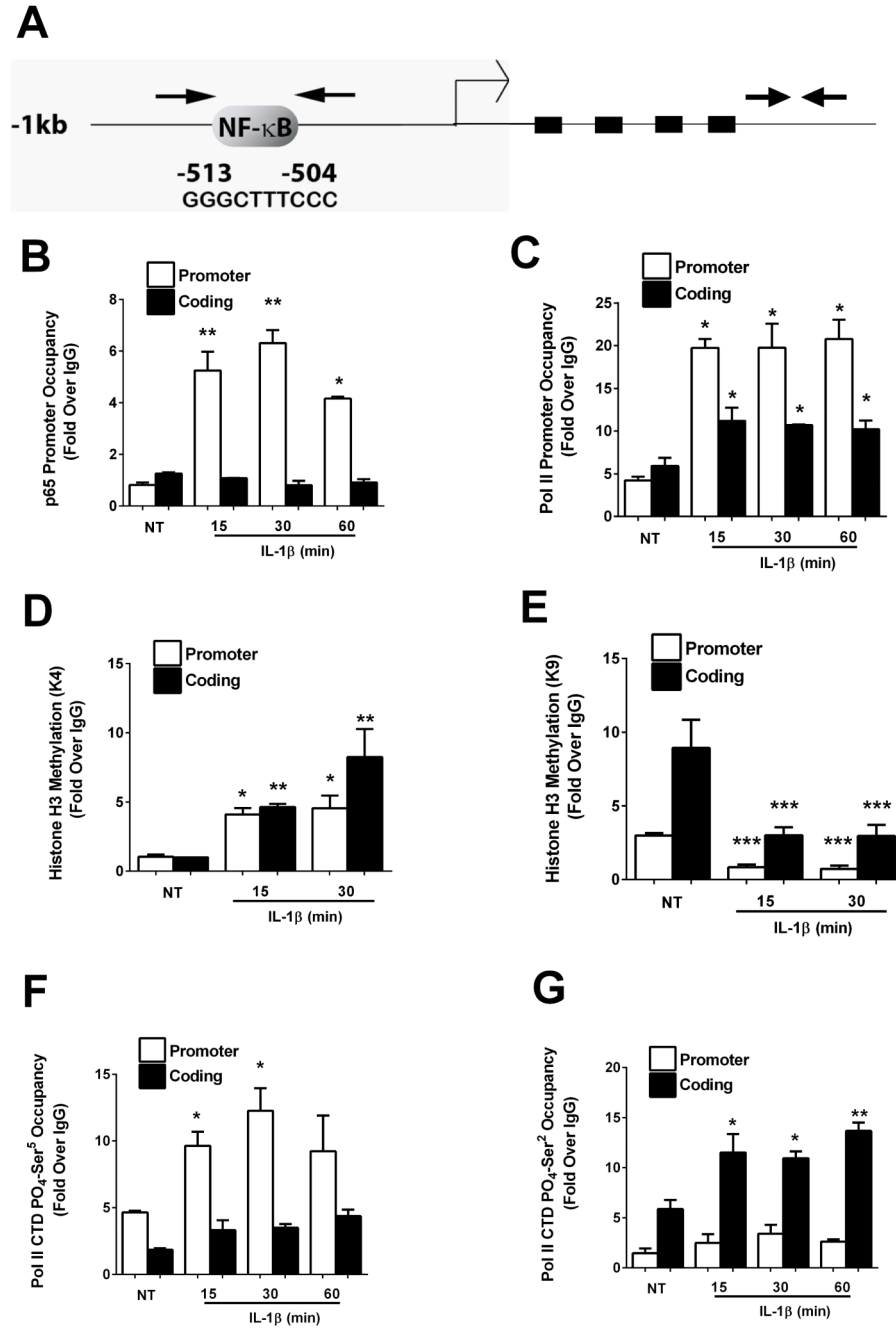


Figure 5. IL-1 β recruits p65 to the TNF α promoter and promotes changes in histone methylation and RNA Pol II phosphorylation

A. Schematic representation of the TNF α promoter and coding regions. Arrows indicate regions amplified by PCR using recovered DNA as a template. B-G. 832/13 cells were treated with 1 ng/mL IL-1 β for the indicated times. ChIP assays were performed using antisera to, methylated histone H3 (lysine 4; B and lysine 9; C), p65 (D), total Pol II (E) and Pol II CTD-phospho Serine 5 (F) and Serine 2 (G) on both the TNF α promoter and coding

regions. ***, $P < 0.001$ vs. NT, **, $P < 0.01$ vs. NT, *, $P < 0.05$ vs. NT. ChIP signal is shown relative to IgG control as the means \pm SEM from 3-4 individual experiments.