NF-κB and STAT1 control CXCL1 and CXCL2 Gene Transcription

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Abstract

Diabetes mellitus results from immune-cell invasion into pancreatic islets of Langerhans, eventually leading to selective destruction of the insulin-producing β-cells. How this process is initiated is not well understood. In this study, we investigated the regulation of the CXCL1 and CXCL2 genes, which encode proteins that promote migration of CXCR2+ cells, such as neutrophils, towards secreting tissue. Herein, we found that IL-1β markedly enhanced the expression of the CXCL1 and CXCL2 genes in rat islets and β-cell lines which resulted in increased secretion of each of these proteins. CXCL1 and CXCL2 also stimulated the expression of specific integrin proteins on the surface of human neutrophils. Mutation of a consensus NF-κB genomic sequence present in both gene promoters reduced the ability of IL-1β to promote transcription. In addition, IL-1β induced binding of the p65 and p50 subunits of NF-κB to these consensus κB regulatory elements as well as to additional κB sites located near the core promoter regions of each gene. Additionally, serine phosphorylated STAT1 bound to the promoters of the CXCL1 and CXCL2 genes. We further found that IL-1β induced specific post-translational modifications to histone H3 in a time frame congruent with transcription factor binding and transcript accumulation. We conclude that IL-1β-mediated regulation of the CXCL1 and CXCL2 genes in pancreatic β-cells requires stimulus-induced changes in histone chemical modifications, recruitment of the NF-κB and STAT1 transcription factors to genomic regulatory sequences within the proximal gene promoters and increases in phosphorylated forms of RNA polymerase II.
Introduction

Autoimmune diseases are often categorized as systemic, such as rheumatoid arthritis, or organ-specific, such as Type 1 diabetes mellitus [T1DM; ref (49)]. Both types of autoimmune diseases arise through inappropriate immune cell targeting of a self tissue. In the case of T1DM, leukocyte infiltration into the pancreatic islets culminates with the destruction of the insulin-producing β-cells (42). This loss in functional islet β-cell mass leads to the clinical symptoms associated with T1DM. The eradication of islet β-cells requires multiple immune cell types, with T cells and macrophages being among the most well known contributors to T1DM (13, 46, 57). The accumulation of these immune cells in pancreatic islets is referred to as inflammatory insulitis and is a hallmark of T1DM (13-14, 29, 45) and is beginning to be recognized in lipid overload and T2DM (25-26).

A logical basis for initiating and/or maintaining leukocyte infiltration into pancreatic islets is the production and secretion, by β-cells themselves, of chemotactic proteins that control recruitment of immune cells into the islet tissue (7, 18, 65). Indeed, studies using transgenic mice reveal that monocytes and macrophages are recruited into pancreatic islets by β−cell release of the chemokine CCL2 (50), while CXCL10 secretion from insulin-producing cells promotes T cell infiltration (60). Macrophages and various T cell populations are known to participate in autoimmune-mediated β-cell destruction in both rodents and humans (9, 13, 28, 46). However, how this process is initially instigated is not well understood.

It has only recently become apparent that the interplay of other immune cells, including B cells, dendritic cells and neutrophils, is also a critical component of the initiation of autoimmune-mediated β-cell destruction (22). Neutrophils are highly abundant in the circulation and rapidly respond to inflammatory stimuli to provide host defense against many microorganisms (53). In addition to their role in maintenance of host homeostasis, neutrophils also contribute to the development of many different autoimmune diseases (54). Similar to macrophages and T cells, neutrophils can be recruited to sites of inflammation by specific chemokines (2).

Neutrophils express the CXCR1 and CXCR2 chemokine receptors. CXCR2 is activated by a variety of chemokine ligands, including CXCL1 and CXCL2 (3). The CXCL1 and CXCL2 genes are expressed in both rodent and human islets in response to pro-inflammatory cytokines (18, 65). Moreover, these two chemokines are strongly linked to a variety of autoimmune diseases, including T1DM (72). Indeed, CXCL1 levels are elevated in the blood of both rodents and humans with T1DM (71) and in humans with type 2 diabetes mellitus (64). In addition, pharmacological inhibition or genetic deletion of the CXCR2 receptor improves islet graft survival and function (10). However, the regulation of the CXCL1 and CXCL2 genes in pancreatic β-cells by inflammatory signals, such as IL-1β, is not well understood.

IL-1β induces a variety of signaling pathways linked to inflammatory responses (31) and directly contributes to β-cell death and dysfunction (57). IL-1β induced activation of the NF-κB pathway is one of the central signaling cascades leading to the alterations in gene transcription that promotes islet inflammation. NF-κB transcriptional regulatory proteins include p65/RelA, RelB, c-Rel, p50, and 52 (56). This family of transcription factors forms homo- or hetero-dimers to alter gene expression patterns,
many of which are associated with inflammatory processes (69). Therefore, while IL-1β and NF-κB strongly correlate with a distinct variety of autoimmune diseases through systems biology analyses (72), the molecular mechanisms underlying regulation of genes controlling inflammatory processes have not been fully delineated. Herein, we report that IL-1β requires NF-κB and STAT1 for transcriptional regulation of the CXCL1 and CXCL2 genes and that specific post-translational modifications to histone proteins are associated with IL-1β-mediated alterations in gene transcription patterns.
Materials and Methods

Cell Culture, Islet Isolation and Reagents

The 832/13 rat insulinoma cell line was cultured as previously described (36). Isolation of islets from Wistar rats has also been described (12). IL-1β was purchased from Thermo Fisher Scientific (Waltham, MA) and IFN-γ was from Shenandoah Biotechnology Inc. (Warwick, PA). TPCA was from Tocris Bioscience (Ellisville, MO). Recombinant adenoviruses expressing β-galactosidase (35), 5x NF-κB-luciferase (7), and IκBα S32A/S36A (40) are described elsewhere. The adenovirus encoding IκKβ S177E/S181E was a generous gift from Dr. Haiyan Xu (Brown University).

RNA isolation, cDNA synthesis and real-time RT-PCR

RNA was isolated from cell lines using Isol-RNA Lysis Reagent (5 Prime Inc., Gaithersburg, MD), and from rat islets using the RNeasy kit (QIAGEN). cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific). iTAQ Universal SYBR Green Supermix (Bio-Rad) was used for real-time RT-PCR analysis. Analysis of real-time PCR data was done using the comparative cycle threshold (Ct) method also commonly referred to as the ΔΔCt method (67). Primers used to detect transcript levels of RS9, CXCL1 and CXCL2 were designed using Primer3Plus software and are available upon request.

Plasmid Construction.

The CXCL1 promoter-reporter luciferase plasmid was constructed by PCR amplifying -1.5kb of the CXCL1 promoter using rat genomic DNA as a template, AmpliTaq Gold 360 DNA polymerase (Life Technologies Co.) and the following primer pair: (F) 5’ CAGAGGGAGGCCCATCAT 3’ and (R) 5’ GGAGTGTGGCTGGAGTCT 3’. The resulting amplicon was inserted into the TOPO TA cloning vector (Life Technologies Co.) and then subcloned into pGL3 Basic using the SacI and XhoI sites in both vectors. The CXCL2 -1.5- luciferase reporter plasmid was constructed by similar methods using the following primer pair: (F) 5’ CAAAACCCAGGGTGAGAACT 3’ and (R) 5’ CTTGAAGTCAACCCCTTGGTAGG 3’.

Site-directed Mutagenesis

The QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to incorporate mutations within the conserved NF-κB response elements of the CXCL1 and CXCL2 -1.5kb- luciferase plasmids. The following primer pairs were used to incorporate these mutations:

CXCL1: (F) 5’ GGAGTTTGGAGTTCTGGAacctCCCGAGTTCAAAAGCAAG3’ and (R) 5’ CTTTGGCTTTTTAAACTCGGGGagggTTCGAGAACTCCCCAACTCC 3’.

CXCL2: (F) 5’ CACTGGACCTCGGAacccTCCCGAATTTCACAG 3’ and (R) 5’ CTGTGAAATTCGGGAaggTTCCGAGGCTCCAGTG 3’.

Mutations were confirmed by DNA sequencing at the Molecular Biology Resource Facility at the University of Tennessee, Knoxville.

Transfection and Luciferase Assays
Transfection of plasmid and siRNA duplexes into 832/13 cells, harvesting of cellular lysates, luciferase reporter activity determination and measurement of total protein content were all described previously (7, 11). Silencer Select siRNA duplexes (Life Technologies Co.) used in this study are as follows: negative control siScramble (M4611), sip65 (siRNA ID #:s159516 and s159517) and sip50 (siRNA ID#: s135617). For the CRE reporter assay, 2x10^5 293HEK cells stably expressing wild-type CXCR2 were plated per well in a 24 well dish. 24hr later cells were transfected with 375ng 4XCRE plasmid (Biomyx, San Diego, CA) with 1.5 μl Lipofectamine 2000 (Life Technologies). After 3 h post-transfection, media was remove and replaced with 1mL fresh media. After an overnight incubation with plasmid, cells were serum starved for 3-4 h prior to exposure to chemokines. Cells were stimulated for 1 h then exposed to different concentrations of Pichia-isolated CXCL1 with or without 1μM forskolin for 4 h as indicated in the figure legends. Cells were harvested and luciferase assays performed according to the manufacturer’s instructions (Promega). The protein concentration was determined with BCA (Thermo Scientific) and used to normalize each well to total protein.

ELISA

Secretion of CXCL1 and CXCL2 into the culture media was detected using Quantikine kits (R & D Systems, Inc., Minneapolis, MN) according to the manufacturer’s protocol. CXCL1 and CXCL2 release into the media was normalized to total protein to account for any potential variability in cell number.

Neutrophil Migration

Peripheral blood neutrophils (PBN) were isolated from EDTA-treated blood from healthy human volunteers using dextran sedimentation and density gradient centrifugation as previously described (48). Erythrocytes were lysed with hypotonic lysis in 0.2% NaCl. PBN were resuspended in HBSS with 0.1% BSA and 10 mM HEPES. 20 μl of 10nM CXCL1 or 1:10 dilution of β-cell supernatants from IL-1β treated or untreated cells were loaded into the lower well of the 96 well modified Boyden chamber (Neuroprobe, Gaithersburg, USA) and fitted with a 5μm filter. PBN were labeled with 1ng/ml CalceinAM (Invitrogen, Carlsbad, CA) and 400nM of SB225002 (Millipore) or DMSO control for 0.5 h on a rotating wheel at 37°C. Cells were washed with PBS and resuspended to 1×10^6 cells/ml in HBSS with SB225002 (400nM) or DMSO control. 30 μl of the cell suspension was added to the upper well. The cells were then incubated for 1.5 h at 37 °C. Migration of PBN was measured with fluorescent plate reader (Synergy 2, Biotek, USA) using Ex/Em wavelength 495/515. The fluorescence from buffer only (no chemokine) control wells was the background migration. The use of human subjects for neutrophil isolation has been approved by the University of Tennessee Institutional Review Board (IRB# 6476B).

Integrin Expression

1x10^6 PBNs, isolated as described above, were stained with varying concentrations of CXCL1, CXCL2 or both. After 2hr incubation, cells were stained for integrin expression using 1:6 dilution of directly conjugated antibodies [CD11a-PerCP, CD11b-APC, CD11c-PE (Biolegend) ref (52)]. After 2hr incubation, cells were washed and fixed with paraformaldehyde for 10min. The abundance of integrin expression was analyzed via flowcytometry (BD FACsCalibur) followed by FlowJo analysis (Tree Star).
Chromatin Immunoprecipitation

ChIP assays and analysis of downstream data were performed as described previously (6). Antibodies used for immunoprecipitation of acetylated histone H3 (K9 and K14) and H4 (K5, K8, K12, and K16), methylated histone H3 (K4, 9 and 27), acetylated histone H3 (K14) were from Millipore while p65 and p50 were from Santa Cruz Biotechnology. Normal rabbit and mouse serum (control IgG) were from Sigma Aldrich. Primers used for detection of recovered DNA were designed using Primer3Plus software and are available upon request.

Protein Isolation and Immunoblot Analysis

Cellular lysates were harvested using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) supplemented with a cocktail of protease and phosphatase inhibitors (Thermo Scientific). Proteins were quantified using a BCA assay (Thermo Scientific) and immunoblotting was performed as previously described (11). Antibodies used for detection of proteins were from the following manufacturers: Santa Cruz Biotechnologies (PO4-IκBα and IκBα), Cell Signaling (PO4-Y701 STAT1) and Sigma Aldrich (β-Actin).

Statistics

Statistical analysis was performed on the data using a one-way ANOVA followed by Tukey’s post-hoc correction. Statistical significance is reported in the individual figure legends with confidence intervals denoted. All calculations were performed using GraphPad Prism 5 software.
**Results**

**IL-1β Increases the Expression of the CXCL1 and CXCL2 Genes in rat islets and β-cell lines.**

CXCL1 and CXCL2 levels are elevated in human islets exposed to cytokines (18, 65) and in individuals with diabetes mellitus (34, 68). Thus, we investigated the signals that increase the expression of the CXCL1 and CXCL2 genes using rat islets and β-cell lines. We found that 1 ng/mL IL-1β induced the first appearance of transcript encoding the CXCL1 and CXCL2 genes over baseline within 30 min in 832/13 rat insulinoma cells (Figure 1A & B). Expression of the CXCL1 gene peaked at 1h, while maximal expression of the CXCL2 gene was at 3h (Figure 1A & B). Furthermore, the response of these genes to IL-1β is not sensitive to cycloheximide, indicating that new protein synthesis is most likely not required for signal-induced transcription of these two genes (data not shown). A similar response was obtained in the INS-1E rat insulinoma cell line (not shown). We have previously reported that 1ng/mL IL-1β is required to drive maximal expression of the COX2 and CCL2 genes (4, 7). In addition, 1ng/mL IL-1β is also the saturation point for 832/13 cell death (11). However, the CXCL1 and CXCL2 genes respond to a 10-fold lower dose of IL-1β, displaying robust and maximal gene expression at 0.1ng/mL of this cytokine (Figure 1C & D). In addition, IL-1β also increased CXCL1 and CXCL2 mRNA levels in isolated rat islets (Figure 1E & F), results which are consistent with those reported in human islets (65).

To examine the transcriptional responses of each gene, we cloned 1.5kb of the genomic DNA sequence upstream of the transcriptional start site, which corresponds to the proximal gene promoters of the CXCL1 and CXCL2 genes. Transfection of the CXCL1 promoter-luciferase reporter construct into 832/13 cells revealed 6.7-fold response to 1ng/mL IL-1β with no further potentiation by IFN-γ (Figure 1G). The activity of the CXCL2 gene promoter was induced 10.3-fold upon stimulation with 1ng/mL IL-1β (Figure 1H). These results are also consistent with the CXCL1 and CXCL2 transcript profiling obtained using mouse and human islets (18).

We next used ELISA to determine the quantity of CXCL1 and CXCL2 protein being secreted in response to IL-1β, IFN-γ, or a combination of these cytokines. The secretion of CXCL1 was robust and rapid after cellular incubation with 1ng/mL IL-1β, with a 28-fold increase in secreted protein within 3h (Figure 2A). In addition, CXCL2 protein release was induced 132-fold by IL-1β at 3h and rose to 464-fold at 6h (Figure 2B). These results are consistent with the kinetics of transcript accumulation shown in Figures 1A and 1B. We further note that IFN-γ did not augment the synthesis or secretion of either CXCL1 or CXCL2. Thus, IL-1β appears to be the predominant signal controlling synthesis and secretion of CXCL1 and CXCL2 in pancreatic β-cells.

Because the pancreatic β-cell produces a variety of chemokines in response to IL-1β (7, 65), we used recombinant CXCL1 or CXCL2 to test the efficiency of these chemokines either in isolation or in combination with each other on PBN function. PBNs express integrins CD11a (LFA-1), CD11b (Mac-1), and CD11c (ITGAX, alphaX) for attaching to the endothelial surface prior to extravasation out of the blood stream (78). Integrins are members of a superfamily of heterodimers that are responsible for cellular adhesion to the extracellular matrix. Either CXCL1 or CXCL2 alone were sufficient to induce neutrophil surface expression of integrins CD11b and CD11c, but not CD11a (Figure 2C and data not
shown). Combining these chemokines in differing ratios of CXCL1/CXCL2 (1nM/10nM, 10nM/10nM, 10nM/1nM) did not further enhance integrin surface expression over either chemokine alone (Figure 2D and data not shown). We further found that human blood neutrophils migrate in response to either CXCL1 or CXCL2 recombinant proteins in a dose-dependent manner (data not shown). However, interfering with the activity of CXCR2 using the allosteric inhibitor SB225002 markedly reduced the migration of neutrophils in response to recombinant CXCL1 (72%) and in response to β-cell supernatants (41% for control and 33% for IL-1β exposure, respectively; Figure 2E) Taking the data from Figures 1 and 2 together, we conclude that IL-1β promotes synthesis and secretion of the CXCL1 and CXCL2 genes in pancreatic β-cells, and these proteins enhance the expression of integrins at the neutrophil cell surface as well as promote cell migration through CXCR2.

NF-κB subunits p65 and p50 and a consensus κB sequence are required for transcription of the CXCL1 and CXCL2 genes in response to IL-1β. To identify components required for signal integration at the CXCL1 and CXCL2 gene promoters, we first targeted NF-κB signaling using the IκBα super-repressor (SR), a protein with mutations that render it resistant to stimulus-induced degradation (40). Overexpression of the IκBαSR in 832/13 cells blocked IL-1β-mediated transcriptional activity of the CXCL1 gene promoter (Figure 3A). We detected a 53%, 61%, and 75% decrease in promoter activity with increasing doses of the IκBαSR. In addition, we noted a 54%, 73%, and 74% decrease in the ability of IL-1β to drive CXCL2 transcriptional activity in the presence of the IκBαSR (Figure 3B). This was also true for the endogenous genes, as the CXCL1 and CXCL2 transcripts were decreased 81% and 82%, respectively, in the presence of the IκBαSR (Figures 3C &D). The diminution in gene expression and promoter activity also corresponds to a 76% and 65% decrease in secreted CXCL1 and CXCL2 proteins (Figure 3E & F).

Because the experimental approach with IκBαSR indicated that NF-κB proteins are critical for expression of each gene, we used transcription factor search algorithms to predict known regulatory response elements within the CXCL1 and CXCL2 gene promoters. We observed several predicted κB sequences for each gene and discovered a conserved consensus κB site with an identical sequence in both CXCL1 and CXCL2 promoters at positions -641 (CXCL1) and -640 (CXCL2) [relative to the transcriptional start site; results shown in Table 1]. Mutating this consensus κB sequence decreased CXCL1 (40%) and CXCL2 (53%) gene transcription in response to IL-1β (Figure 4A & B).

Since heterodimers of p65/p50 are considered the major dimer species controlling signal-specific transcriptional activation (32), we next used siRNA-directed suppression of the p65 and p50 NF-κB subunits. Upon duplex transfection, p65 transcript levels were decreased by 71%, resulting in a marked loss in p65 protein abundance (not shown). This maneuver resulted in a 73% decrease in CXCL1 mRNA abundance (Figure 4C). In addition, the CXCL2 gene was decreased by 83% and 80%, using two different siRNA sequences targeting different exons of the p65 transcript (Figure 4D). Similarly, siRNA-mediated suppression of p50 also decreased the IL-1β-induced expression of the CXCL1 and CXCL2 genes by 42% and 38%, respectively (Figures 4E & F). Thus, IL-1β controls the expression of the CXCL1 and CXCL2 genes using the p65 and p50 subunits of NF-κB and a conserved κB binding site located in their proximal gene promoter regions.
The inhibitor of κB kinase β (IκKβ) is necessary and sufficient for activation of the CXCL1 and CXCL2 genes. The IκKβ is a major convergence point for regulation of NF-κB signaling (32, 38) and also modulates the viability of pancreatic β-cells upon exposure to IL-1β (11). To investigate whether IκKβ is involved in mediating the increase in expression of the CXCL1 and CXCL2 genes in response to IL-1β, we used the small molecule inhibitor 2-[(Aminocarbonyl)amino]-5-(4-fluor-ophenyl)-3-thiophenecarboxamide (TPCA) to inhibit IκKβ (59). We observed that the IL-1β-mediated enhancement in transcriptional activity at the CXCL1 and CXCL2 gene promoters was decreased (35% and 32%, respectively) with the addition of TPCA (Figure 5A & B). Additionally, IL-1β induced steady-state mRNA accumulation of the CXCL1 gene was diminished 47%, 52%, and 61% in the presence of TPCA (Figure 5C), while CXCL2 transcript was reduced by 64%, 67%, and 77% under the same conditions (Figure 5D). These results indicate that IκKβ is necessary for IL-1β signaling-induced increases in the expression of each of these chemokine genes.

Alternatively, the p38 mitogen-activated protein kinase (MAPK) is not involved in induction of either the CXCL1 or CXCL2 genes in response to IL-1β (Figure 5E & F). Using three different pyridinyl imidazole based inhibitors, which effectively reduce the expression of the CCL2 gene upon exposure to IL-1β [ref (7) and data not shown], we saw no loss in transcript abundance.

To determine whether or not IκKβ was sufficient for induction of the CXCL1 or CXCL2 genes, we overexpressed a constitutively-active version of the kinase (CA IκKβ). This adenoviral construct expresses IκKβ with S177E/S181E substitutions to create a constitutively-active kinase (19, 51). We detected dose-dependent expression of the mutant protein, which corresponded with degradation of IκBα protein (Figure 6A), a known target of IκKβ (38). Expression of CA IκKβ was also capable of driving transcription from a multimerized NF-κB promoter luciferase reporter gene in a dose-dependent fashion (Figure 6B).

We next examined whether expression of CA IκKβ was capable of increasing transcription from luciferase reporter constructs driven specifically by the CXCL1 or CXCL2 proximal gene promoters. These experiments were performed in the absence of cytokines. Indeed, we detected 2.3-, 2.7-, and 8.8-fold enhancements in promoter activity on the CXCL1 reporter gene (Figure 6C). Moreover, the CXCL2 reporter gene displayed 2.7-, 3.0-, and 8.0-fold increases in promoter activity in the presence of the CA IκKβ (Figure 6D). The CA IκKβ also increased the abundance of the endogenous CXCL1 and CXCL2 transcripts; this expression was completely blocked by the IκBαSR (Figure 6E & F), which confirms that the gene expression promoted by CA IκKβ is through downstream NF-κB proteins. In addition, CA IκKβ further enhances the IL-1β-induced increases in CXCL1 and CXCL2 mRNA (data not shown). Therefore, we conclude that IκKβ participates in the regulation of the CXCL1 and CXCL2 genes and is both necessary and sufficient to promote expression of these two genes.

The NF-κB subunits p65 and p50 are recruited to κB regulatory elements within the CXCL1 and CXCL2 gene promoters in an IL-1β-dependent manner. Mutational analysis of the CXCL1 and CXCL2 gene promoters, as well as siRNA-mediated suppression of the p65 and p50 subunits, revealed a role for NF-κB in the IL-1β-dependent upregulation of the CXCL1 and CXCL2 genes (Figure 4). Thus,
we next examined occupancy of the p65 and p50 subunits at conserved proximal and core promoter NF-
κB binding sites, respectively, for each gene. We found a significant increase in p65 promoter
occupancy at the common consensus κB site for both genes in response to IL-1β (Figure 7A&B). In
addition, there was an enhancement in p65 association with κB sequences near the core promoter for
both genes after exposure to IL-1β (Figure 7C&D). Moreover, there was an IL-1β-mediated increase in
occupancy of p50 near the core promoters of both the CXCL1 and CXCL2 genes (Figures 7E&F). Thus,
IL-1β promotes association of key regulatory factors with the appropriate response elements driving
transcription of the CXCL1 and CXCL2 genes.

Consistent with the binding of NF-κB subunits to κB response elements, IL-1β signaling to IκBα is
rapid, as we detected phosphorylation of the protein within 5min of IL-1β exposure and observed
degradation after 15min of cytokine stimulus (Figure 8A). The response is also stimulus-specific since
IFN-γ, which rapidly promotes phosphorylation of STAT1 at Tyr701, does not induce phosphorylation
or degradation of IκBα (Figure 8A and data not shown). We next loaded 50% less protein to fully
visualize IκBα degradation, at which point we observed full turnover of protein within 15min with
noticeable re-synthesis detectable by 60min (Figure 8B). This observation fits with increased binding of
p65 at gene promoters 15 and 30 min after exposure to IL-1β (Figure 7).

We next examined how overexpression of the IκBαSR protein impacted binding of p65 and p50 after an
IL-1β stimulus. There was a 48.3% decrease in p65 occupancy at the core κB sites within the CXCL1
gene promoter in the presence of the adenoviral-delivered IκBαSR (Figure 8C). Note that there is no
change in the control condition, indicating stimulus-specific binding in response to IL-1β and a
reduction by overexpression of the IκBαSR protein. There was also a 40.5% decrease in p50 association
at the CXCL1 κB sequence in the presence of the IκBαSR protein (Figure 8D). Binding of p65 and p50
at the CXCL2 gene promoter was also reduced by 41% and 39%, respectively, by overexpression of
IκBαSR (Figures 8E & F). Thus, we conclude that signal-specific association of the NF-κB subunits p65
and p50 at precise gene promoter response elements matches the timing of degradation of IκBα.

STAT1 is an accessory factor for the IL-1β-mediated expression of the CXCL1 and CXCL2 genes.
In addition to κB response elements, in silico analysis of the genomic regions upstream of the CXCL1
and CXCL2 coding regions reveal a number of sites potentially recognized by STAT1 (Table 2).
Therefore, we used siRNA-directed suppression of STAT1 to determine whether the IL-1β response to
either CXCL1 or CXCL2 was impacted. We detected a 63% decrease in STAT1 mRNA by siRNA
duplex transfection (Figure 9A), which produced a 50% decrease in STAT1 protein (Figure 9B). The
 diminution in STAT1 reduced the ability of IL-1β to enhance transcript levels of both CXCL1 and
CXCL2 (Figures 9C and 9D; left panels). We next examined whether specific phosphoacceptor sites at
Tyr701 and Ser727, which are known to control STAT1 activity (77), interfered with the IL-1β response
of each gene. Using adenoviral overexpression of STAT1 with Y701F/S727A mutations, we observed a
decrease in the accumulation of CXCL1 and CXCL2 transcripts in 832/13 cells (Figures 9C and 9D;
middle panels) and in isolated rat islets (Figures 9C and 9D; right panels).
Using chromatin immunoprecipitation, we detected occupancy of phosphorylated STAT1<sup>Ser727</sup> in the distal promoter region of the CXCL1 gene, which also contains the consensus κB response element (Figure 9E; white bars). However, this STAT1<sup>Ser727</sup> binding is not enhanced by the addition of IL-1β (Figure 9E; white bars). By contrast, the binding of STAT1<sup>Ser727</sup> near the core promoter is increased by 4.25-fold after IL-1β exposure (Figure 9E; gray bars). We did not detect binding of STAT1 in the coding region of the CXCL1 gene, thus indicating stimulus-specific occupancy of this transcription factor within the proximal core promoter (Figure 9E; black bars).

Upon examining STAT1<sup>Ser727</sup> occupancy at the CXCL2 gene, we observed a 2.49-fold increase at the region near the core promoter in response to IL-1β (Figure 9F; gray bars). There was no discernible increase in STAT1<sup>Ser727</sup> binding near the consensus κB site within the CXCL2 gene promoter (Figure 9F; white bars) as well as no detectable binding in the coding region (Figure 9F; black bars). Because IFN-γ promotes phosphorylation of STAT1 at Tyr701 in pancreatic β-cells (5, 8, 33), we specifically examined whether tyrosine phosphorylated STAT1 was present at the CXCL1 and CXCL2 gene promoters. We found that IFN-γ stimulates a 5.09-fold increase in STAT1<sup>Tyr701</sup> occupancy at the core promoter region of the CXCL1 gene (Figure 9G; gray bars). This fits with the CXCL1 core promoter containing a gamma-activated sequence (GAS; Table 2). However, the increase in STAT1<sup>Tyr701</sup> binding at this genomic regulatory site does not induce transcription of the gene or secretion of protein (Figures 1 and 2A & B). By contrast, the CXCL2 gene, which does not contain a matching GAS element within its core promoter, does not result in STAT1<sup>Tyr701</sup> occupancy in response to IFN-γ at any regulatory regions examined (Figure 9H). Thus, STAT1 serves as a potential accessory factor for the IL-1β-mediated increases in CXCL1 and CXCL2 gene transcription.

**IL-1β promotes changes in histone modifications at regions associated with transcriptional regulation of the CXCL1 and CXCL2 genes.** There are many alterations in chromatin that occur during transcription, including signal induced changes in post-translational modifications (43). Using ChIP assays, we found that IL-1β promotes changes in overall acetylation of histone H3 at both the CXCL1 and CXCL2 gene promoters (data not shown). Upon examining more precise sites of modification that correlate with regulation of gene transcription, we first found that methylation of H3K4 increased over 2-fold after exposure to IL-1β at both the CXCL1 and CXCL2 genes (Figure 10A & B). Furthermore, methylation of H3K9 decreased 65% and 63%, respectively, after a 15 and 30 min stimulus with IL-1β (Figure 10C). There was a similar 63% and 74% decrease in methylation of the H3K9 at the CXCL2 gene (Figure 10D). Upon examining the acetylation of Lys14 of histone H3, we observed more than a 2-fold increase at both the CXCL1 and CXCL2 genes after IL-1β exposure (Figure 10E & F). Additionally, methylation of histone H3 at Lys27 decreased by 43% and 48% at the CXCL1 gene (Figure 10G), while methylation of this residue decreased by 41% and 57% at the CXCL2 gene (Figure 10H). Note that all of these changes occurred in an IL-1β dependent manner. We conclude that the regulation of CXCL1 and CXCL2 gene transcription by IL-1β is associated with key modifications to histones within the promoter regions of each of these genes.

**RNA polymerase II phosphorylation at the core promoter and coding regions of the CXCL1 and CXCL2 genes changes upon exposure to IL-1β.** Phosphorylation of RNA polymerase II (pol II) at the
Carboxy terminal domain (CTD) regulates both transcription and RNA processing (37). Because CXCL1 and CXCL2 are induced by IL-1β, we examined whether RNA Pol II phosphorylation status was changed concurrently. RNA Pol II phosphorylation at Ser5 increased by 3.71-, 4.98-, and 2.70-fold at 15, 30, and 60 min, respectively, at the CXCL1 core promoter region (Figure 11A). This timing is consistent with the first appearance of CXCL1 transcript upon exposure to IL-1β (Figure 1). Similar results were obtained at the core promoter region in the CXCL2 gene (Figure 11B). In addition, phosphorylation of Ser 2, which is associated with elongation, increased by 2.63- and 2.39-fold at 30 and 60 min, respectively, in the coding region of CXCL1 after IL-1β exposure (Figure 11C). Within the CXCL2 coding region, we detected 3.11-fold and 2.65-fold increases in Ser 2 phosphorylation, respectively, after a 30 and 60 min stimulus with IL-1β (Figure 11D). Thus, phosphorylation of RNA Pol II is altered in response to IL-1β in a manner consistent with transcription factor binding, histone chemical modifications, and timing of gene transcription.
**Discussion**

The autoimmune-mediated elimination of pancreatic β-cells is a complex process that requires intricate interactions between diverse types of immune cells (46). While the initiating event in the organ-specific autoimmunity that leads to T1DM has not been identified, it is clear that chemokines are critical signals for immune cell recruitment into pancreatic islets (28, 50, 60). Chemokines are small secreted proteins (≈8-10kDa) that are not typically stored in individual cells, but rather are synthesized and secreted in response to a specific stimulus. In this study, we examined the transcriptional control of two chemokine genes, CXCL1 and CXCL2, in response to IL-1β in pancreatic β-cells.

IL-1β is one of the principal cytokines involved in β-cell death and dysfunction during development of T1DM (47, 57) and is a key contributor to onset of Type 2 diabetes mellitus [T2DM; (23, 30)]. The chemokines CXCL1 and CXCL2 are synthesized and secreted in response to IL-1β in pancreatic β-cells [Figures 1 and 2; and ref (18)]. Serum CXCL1 levels are also higher in individuals with T1DM (71) and T2DM (64). Moreover, a recent systems biology study showed an association of both CXCL1 and CXCL2 with various autoimmune diseases, including T1DM (72). Thus, transcriptional regulation of the CXCL1 and CXCL2 genes by IL-1β is likely to represent a key critical component underlying β-cell inflammation.

Chromatin remodeling is one of the central events associated with transcriptional regulation. There are many post-translational modifications to histone proteins that either alter the milieu of regulatory factors promoting the transcriptional activation or repression of specific genes and/or that provide docking sites for additional proteins participating in the regulatory process (43). To our knowledge, our results are the first to demonstrate histone chemical modifications at the CXCL1 and CXCL2 gene promoters in response to IL-1β in the pancreatic β-cell. Indeed, sites associated with transcriptional activation, such as methylation at H3K4, increased with IL-1β exposure (Figure 10A & B), while sites associated with transcriptional repression, such as methylation at H3K9, decreased with IL-1β exposure (Figure 10C & D). Overall, the histone chemical modifications detected at the CXCL1 and CXCL2 genes are consistent with signal-specific activation of gene transcription by IL-1β. These data provide heretofore undescribed regulatory information regarding control of these key chemokine genes under inflammatory conditions in pancreatic β-cells.

CXCL1 and CXCL2 are capable of attracting CXCR2+ cells, such as neutrophils, to sites of inflammation (63). Neutrophils participate in the initiation of the autoimmune-mediated process that results in a decrease in functional β-cell mass (22). However, how different leukocytes collaborate to initiate the autoimmune process is not well understood. Initiation of autoimmunity and/or continuation of the auto-inflammatory response that leads to β-cell destruction may require the synthesis and secretion of chemokines, such as CXCL1 and CXCL2, directly from the islet β-cells. These chemoattractant proteins recruit cells that begin (or maintain) the inflammatory process, eventually leading to the onset and progression of autoimmunity (22, 46). Indeed, both rodent and human β-cells make and secrete CXCL1 and CXCL2 [see refs (18, 65) and Figures 1 and 2 of this study] which bind to CXCR2 to initiate neutrophil activation and migration [(55) and data not shown]. While very effective...
against recombinant CXCL1 alone, the allosteric inhibitor of CXCR2 does not entirely block the migratory response to β-cell supernatants (Figure 2E). We interpret this data to indicate that there may be additional chemokines capable of activating or recruiting neutrophils independently of CXCR2.

Neutrophils are involved in initiating autoimmune-mediated β-cell destruction (22, 46) and β-cells are killed in response to pro-inflammatory cytokine exposure (11-12, 27, 70). Islet β-cell death occurs with release of the damage-associated molecular pattern (DAMP) molecules, such as HMGB1(70). We posit that release of HMGB1 (and potentially other DAMPs) triggers neutrophil activation and recruitment of additional immune cells to the site of dead and dying β-cells. The amalgamation of chemokines secreted from inflamed tissue (e.g., islets exposed to IL-1β) in combination with DAMPs, such as HMGB1, may synergize to enhance leukocyte chemoattraction in addition to effects on immune cell function (66, 81). Because of the many diverse leukocyte populations that participate in autoimmune-mediated β-cell destruction (46, 57), there may be additive or synergistic effects between multiple chemokines and/or DAMPs on immune cell recruitment, activation, and release of cytotoxic molecules. Along these lines, viral chemokines increase cell surface expression of CD11b and CD11c on PBNs, which in turn can affect down stream activation signals (52). CXCL8 increases the expression of integrins, including CD11b/CD18, on PBNs (20). Herein, we report that exposure to either CXCL1 or CXCL2 alone (Fig. 2C) increased CD11b and CD11c expression in human PBNs without altering CD11a expression, while the combination of these chemokines was redundant (Fig. 2D) Thus, there are no additional combinatorial or priming effects of CXCL1/CXCL2 on integrin expression at the concentrations tested in this study.

The evidence for the importance of CXCL1 and CXCL2 in diabetes continues to grow. For example, there is a mild decrease in circulating PBNs that precedes the onset of T1DM (74). Although this initially seems to contradict the role of neutrophils in T1DM, the authors also reported an increase in neutrophils in the pancreas. An explanation for the loss of PBNs in circulation is that they extravasated from the blood stream into the pancreas in response to elevated synthesis and secretion of chemoattractants, such as CXCL1 and CXCL2. In T2DM, the associated lipid overload induces tissues dysfunction and promotes M1 macrophage activation in pancreatic islets (58, 73); the expression of the CXCL1 and CCL2 genes are linked to this phenotype (25). In addition, CXCL1 and CCL2 expression are increased in the islets of genetic models of obesity and diabetes, such as the db/db mouse (25). Thus, islet inflammation, a key factor associated with losses in functional β-cell mass in both T1DM and T2DM (24, 26, 57), may be initiated and/or exacerbated by locally high chemokine gene expression and secretion (e.g., such as within pancreatic islets). Expression of these chemokines is driven largely by activation of the NF-κB signaling pathway in response to IL-1β [refs. (5, 7, 57) and present study]. This may explain why sequestering IL-1β, using cytokine trap strategies, promotes improvements in β-cell function (75) and survival of transplanted islets (62), but does not ameliorate peripheral insulin resistance (75).

One commonality between all the chemokine genes induced by IL-1β in the pancreatic β-cell studied thus far is their regulation by NF-κB proteins, in which the p65 subunit plays a critical role. Indeed, a key finding in the present study is that the CXCL1 and CXCL2 genes both have a consensus NF-κB
response element within their proximal gene promoters, at an almost identical genomic positioning (Table 1). p65/RelA, one of the major transcriptional activators of the NF-κB family of proteins, occupies these sites in response to IL-1β (Figure 7A&B). Additional κB sequences are also present in the promoters of the CXCL1 and CXCL2 genes, including near the transcriptional start site (Table 1).

Thus, the data presented in this study provides molecular evidence supporting the systems biology approach that indicated a strong link between IL-1/NF-κB and CXCL1/2 in autoimmune diseases (72). Our data also explain why small molecule inhibition of the CXCR1 and CXCR2 receptors prolongs the survival of transplanted pancreatic islets (10).

An additional novel observation in our study was the identification of serine phosphorylated STAT1 as a key accessory factor for the IL-1β-mediated transcriptional induction of the CXCL1 and CXCL2 genes (Figure 9). While IFN-γ can promote STAT1 phosphorylation at Tyr701 (Figure 8A) and association with genomic regions of these genes (Figure 9), it does not promote synthesis of transcript (Figures 1E & F) or secretion of chemokine proteins (Figures 2A & B). IL-1β promotes p65 association with the CXCL1 and CXCL2 gene promoters (Figures 7 & 8) which induces robust transcript production (Figure 1) and secretion of chemokine protein (Figures 2A & B). We speculate that pre-loading of STAT1 phosphorylated at Ser727 onto genomic regions in the proximal gene promoters might serve to prime the CXCL1 and CXCL2 genes for the major stimulus that enhances transcription (e.g., IL-1β). In support of this notion, we discovered that there are several “GAS-like” sites distributed throughout the proximal promoters of these two chemokine genes (Table 2). We speculate that while each of these sites individually may be relatively low affinity binding sites (compared to a consensus GAS), they collectively provide support for STAT1 occupancy prior to an IL-1β stimulus.

IL-1β increases transcription in a time frame that is congruent with alterations in histone chemical modifications (Figure 10) and RNA polymerase II phosphorylation at both the core promoter and coding regions (Figure 11). Furthermore, our results using STAT1 deletion or removal of phosphoacceptor sites reveal a decrease in the ability of pancreatic β-cells to induce chemokine gene transcription (Figure 9). These observations provide a novel molecular explanation for a prior study showing that STAT1 deletion prevents insulitis in NOD mice (41). Accordingly, interfering with STAT1 abundance or activity diminishes production of the chemoattractant signals that promote immune cell invasion into islets. In addition, diminishing STAT1 activity also impacts the IL-1β-mediated expression of the inducible nitric oxide synthase (iNOS) gene (8). The expression of iNOS and subsequent production of nitric oxide is strongly associated with losses in functional β-cell mass (15-17, 70). Thus, STAT1 is a major contributor to the β-cell inflammation that is associated with islet β-cell destruction.

Overall, chemokines impact a variety of human diseases (39, 76). Therefore, a full understanding of the transcriptional processes controlling their expression is critical for development of novel therapeutic approaches to treat or prevent many human diseases, including diabetes and cancer. For example, the inflammatory responses induced by chemotherapeutic drugs also promote NF-κB activation, which in turn, activates chemokine gene transcription (44, 61). Along these lines, increased expression of the CXCL1 and CXCL2 genes in breast cancer cells promotes tumor survival in metastatic regions (1). Interference with CXCR2 signaling enhances the efficacy of chemotherapy, implicating CXCR2 in
metastasis, and demonstrating another key role for chemokines in human disease. In addition, CXCL1 and CXCL2 are elevated in tumorigenic melanocytes, allowing these cells to form tumors in immune-compromised mice (79); normal melanocytes do not express these chemokines (21, 80).

Finally, we also demonstrated herein that IKKβ plays a key role in regulating the expression of the CXCL1 and CXCL2 genes in β-cells (Figures 5 & 6), matching the NF-κB driven expression of chemokines in human melanoma cells (80). TNF-α, IL-1β, or other inflammatory stimuli capable of activating IKKβ would thus have the potential to drive CXCL1 and CXCL2 expression in a variety of tissues. This finding has major implications for both autoimmune diseases and the upregulation of NF-κB responsive genes after chemotherapeutic treatments in specific cancers. It therefore establishes IKKβ as an attractive target with broad therapeutic potential. Furthermore, a combination therapy of CXCR2 interference with discrete approaches that dampen NF-κB activation (e.g., IKKβ inhibition) may be one way to either enhance therapeutic efficacy of existing treatments and/or to circumvent side effects caused by high doses of individual salutary approaches. Still more appealing may be the targeting of specific transcription factor complexes or enhanceosomes, which would allow for cell-specific, signal-specific, and perhaps even gene-specific therapeutic interventions. Such approaches await full characterization of the transcriptional mechanisms controlling precise regulatory networks involved in inflammatory processes.
Acknowledgements

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References


Figure Legends

Figure 1. Cytokine-mediated activation of the CXCL1 and CXCL2 genes in the 832/13 rat β-cell line and isolated rat islets. A, B. 832/13 cells were untreated (NT) or treated with 1 ng/mL IL-1β for the indicated times. C, D. 832/13 cells were untreated (NT) or treated with increasing concentrations of IL-1β for 3 h. E, F. Rat islets were either untreated (NT) or treated with 10 ng/mL IL-1β, 100 U/mL IFN-γ or both cytokines for 6 h. A–F. CXCL1 (A, C, E) and CXCL2 (B, D, F) mRNA levels were measured and normalized to those of the housekeeping gene, Ribosomal S9 (RS9). *p<0.05 vs. NT (A, C, D, F), **p<0.01 vs. NT (A, B, E), ***p<0.001 vs. NT (A, C), n.s. = not significant vs. NT (E, F). G, H. 832/13 cells were transfected with 1.5 kb of the CXCL1 (G) or CXCL2 (H) promoter upstream of the transcriptional start site fused to a luciferase reporter. 24 h post-transfection cells were untreated (NT) or stimulated for 4 h with 1 ng/mL IL-1β or IL-1β plus 100 U/mL IFN-γ. Relative promoter luciferase activity is shown. **p<0.01 vs. NT (G, H). Data are represented as means ± SEM from 3-4 individual experiments.

Figure 2. IL-1β promotes release of the CXCL1 and CXCL2 chemokines and activates PBN for integrin expression and migration via CXCR2. A, B. 832/13 cells were untreated (NT) or treated with 1 ng/mL IL-1β, 100 U/mL IFN-γ or the combination of both cytokines for 0, 3, 6 and 12 h. CXCL1 (A) and CXCL2 (B) release into the culture media was measured by ELISA and normalized to protein content via BCA assay. Values are presented as means ± SEM from 3 individual experiments. PBN were unstimulated (PBS control; red lines) or exposed to either CXCL1 (blue) or CXCL2 (green) at 100nM and the level of expression of intergrins CD11a, C11b, or C11c measured by flow cytometry (C). PBNs were unstimulated (red) or incubated with a combination of CXCL1/CXCL2 at (1nM/10nM; green lines) or CXCL1/CXCL2 at 10nM/1nM (blue lines) and the levels of integrin expression were measured (D). Plots are representative experiments from 3 replicates. PBNs were exposed to human CXCL1 (10nM) or 1:10 dilutions of supernatants from control 832/13 (Con) or IL-β exposed (IL-1β) in
the presence of CXCR2 inhibitor, SB225002 (400nM), in a chemotaxis assay (E). Bars are the average
decrease in migration in the presence of the CXCR2 inhibitor relative to control with no inhibitor. Each
bar is the average of 4-6 replicates +/- standard deviation from six biological replicates.

Figure 3. Overexpression of a mutant IκBα protein blocks cytokine-mediated activation and
secretion of both CXCL1 and CXCL2. A, B. 832/13 cells were transfected with luciferase reporter
plasmids containing -1.5kb of the CXCL1 (A) and CXCL2 (B) promoters. 4 h post-transfection
adenoviruses expressing either βGAL or IκBα<sup>SR</sup> (SR= superrepressor which denotes the S32A/S36A
mutations) were transduced and cultured overnight. Cells were then untreated or stimulated with 1
ng/mL IL-1β for 4 h. Relative promoter luciferase activity normalized to total protein is shown.
**p<0.01 vs. βGAL. C, D. 832/13 cells were treated with the indicated adenoviruses for 12 h then
stimulated with 1 ng/mL IL-1β for 6 h. Relative mRNA abundance of CXCL1 and CXCL2 was
normalized to RS9. **p<0.01 vs. βGAL (C, D). E, F. 832/13 cells were treated with the indicated
adenoviruses. 12 h post- adenoviral transduction, the cells were treated with IL-1β for an additional 12
h. CXCL1 (E) and CXCL2 (F) release into the media was measured by ELISA and normalized to
protein content via BCA assay. **p<0.01 (E), *p<0.05 (F). Data are shown as means ± SEM from 3
individual experiments.

Figure 4. NF-κB subunits p65 and p50 are required for cytokine-dependent activation of the
CXCL1 and CXCL2 genes. A, B. 832/13 cells were transfected with luciferase reporter constructs
containing -1.5kb of the CXCL1 (A) or CXCL2 (B) promoters (WT) or similar length constructs
wherein the upstream conserved NF-κB response elements were mutated (NF-κBm; as shown in
schematic). 24 h post-transfection, the cells were stimulated with 1 ng/mL IL-1β for 4 h and luciferase
activity quantified. *p<0.05 vs. WT (A, B). C- F. 832/13 cells were transfected with duplexes against
p65 (C, D) or p50 (E, F) using a scrambled siRNA sequence duplex as a control. 48 h post- transfection
cells were treated for 6 h with 1 ng/mL IL-1β. Relative CXCL1 and CXCL2 mRNA levels were normalized to RS9. **p<0.01 vs siScramble (C, D), *p<0.05 vs siScramble (E, F). Data are expressed as means ± SEM from 3 independent experiments.

**Figure 5. IκKβ inhibition impairs the IL-1β-mediated induction of the CXCL1 and CXCL2 genes.**

* A, B. 832/13 cells were transfected with luciferase reporter constructs containing -1.5kb of the CXCL1 (A) or CXCL2 (B) promoters. The following day cells were pre-treated for 1 h with 2 µM TPCA, then stimulated with 1 ng/mL IL-1β for 4 h. Cells were lysed and CXCL1 and CXCL2 promoter luciferase activity was quantified. #p<0.1 vs DMSO (A, B). C, D. 832/13 cells were pre-treated for 1 h with 0.5, 1 or 2 µM TPCA, followed by a 6 h stimulation with 1 ng/mL IL-1β. Relative CXCL1 and CXCL2 mRNA abundance was normalized to RS9. **p<0.01 vs DMSO (C, D). E, F. 832/13 cells were pre-treated for 1 h with 10 µM of three p38 inhibitors (SB202190, SB203580 and SB239063), followed by a 6 h incubation with 1 ng/mL IL-1β. Relative mRNA abundance of CXCL1 and CXCL2 was calculated. n.s. = not significant vs. DMSO (E, F). Data shown represent means ± SEM from 3 individual experiments.

**Figure 6. IκKβ drives expression of the CXCL1 and CXCL2 genes.** A. 832/13 cells were transduced with either βGAL or five increasing concentrations of IκKβ S177E/S181E (CA IκKβ) overnight. Whole cell lysates were harvested and immunoblot analysis was performed using antibodies directed against IκKβ, IκBa and β-Actin. B. Cells were transfected with 5X NF-κB-Luc and subsequently transduced with the indicated adenoviruses (the same concentrations as used in Figure 6A); relative promoter luciferase activity was measured. **p<0.01 vs βGAL, ***p<0.001 vs βGAL. C, D. 832/13 cells were transfected with -1.5kb- luciferase plasmids (C= CXCL1, D= CXCL2), and 4 h post-transfection cells were stimulated with adenoviruses expressing βGAL or three increasing doses of CA IκKβ (the doses shown correspond to the three highest doses seen in the immunoblot) for a further 12 h. At the end of the
12 h, cells were lysed and CXCL1 and CXCL2 promoter activity was quantified. *p<0.05 vs βGAL, **p<0.01 vs βGAL. E, F. 832/13 cells were treated with adenoviruses expressing CA IκKβ and either βGAL or two increasing concentrations of IκBα^SR (IκBα). CXCL1 (E) and CXCL2 (F) mRNA levels were measured and normalized to those of RS9. **p<0.01 vs βGAL. Data shown represent means ± SEM from 3 independent experiments. The immunoblot in A. is representative of two independent experiments.

Figure 7. **p<0.01 vs NT (A, B, F), *p<0.05 vs NT (A-F), #p<0.1 vs. NT (B). Data in E-F represent means ± SEM from 4 individual experiments.

Figure 8. NF-κB subunits are recruited to κB genomic response elements within the CXCL1 and CXCL2 gene promoters in a signal-dependent and IκBα-sensitive manner. A. 832/13 cells were stimulated with 1 ng/mL IL-1β for 5, 10 and 15 min or 100 U/mL IFN-γ for 5 and 10 min. Whole cell lysates (40 μg) were immunoblotted for PO4-IκBα, total IκBα, PO4-STAT1 and β-Actin (as the loading control). B. 832/13 cells were treated for 15, 30 and 60 min with 1 ng/mL IL-1β. Whole cell lysates (20 μg) were blotted for abundance of total IκBα with β-Actin serving as the loading control. C-F. 832/13 cells were cultured with recombinant adenoviruses that express either βGAL or IκBα^SR for 12 h. Cells were then stimulated for 15 min with 1 ng/mL IL-1β. ChIP assays were performed with antibodies that immunoprecipitated p65 (C, E) and p50 (D, F). Proximal (C-F) NF-κB elements in the CXCL1 and
CXCL2 core promoters were targeted for real-time PCR amplification. **p<0.01 vs βGAL + IL-1β.

Data in C-F represent means ± SEM from 3 individual experiments. The immunoblots shown in A. and B. are representative of two independent experiments.

**Figure 9. STAT1 is a key accessory factor for IL-1β-mediated gene transcription.** A, B. 832/13 cells were transfected with an siRNA duplex targeting STAT1 or a scrambled sequence (siScramble) as a control. mRNA (A) and protein (B) levels of STAT1 were determined 48 h post-transfection. In (B), 832/13 cells were treated for 15 m with either 1 ng/mL IL-1β or 100 U/mL IFN-γ 48 h after siRNA transfection (B). Blots are from whole cell lysates using β-Actin as the loading control (B). C, D. 832/13 cells were transfected with an siSTAT1 duplex. Following a 48 h incubation, cells were stimulated for 6 h with 1 ng/mL IL-1β for 6 h (left panels). 832/13 cells were treated with adenoviruses expressing two increasing concentrations of STAT1 DM (double mutant; Y701F/S727A) for 24 h, followed by a 6 h stimulation with 1 ng/mL IL-1β (middle panels). Rat islets were transduced with an adenoviruses expressing either βGAL or STAT1 Y701F/S727A for 24 h, then treated for a further 6 h with 10 ng/mL IL-β and 100 U/mL IFN-γ (right panels). CXCL1 (C) and CXCL2 (D) mRNA levels were measured.

*p<0.05 vs. siScramble (C, D), **p<0.01 vs. siScramble (A), #p<0.1 vs. βGAL (D), *p<0.05 vs. βGAL (C, D), **p<0.01 vs. βGAL (C, D). E, F. 832/13 cells were induced with 1 ng/mL IL-1β for 15 m. ChIP assays were performed with antibodies that immunoprecipitate STAT1 PO4-S727 and IgG as a negative control. G, H. 832/13 cells were stimulated for 15 m with 100 U/mL IFN-γ. ChIP assays were employed to immunoprecipitate STAT1 PO4-Y701 with IgG as the negative control. Primer sets used for real-time PCR are indicated for CXCL1 (left panels) and CXCL2 (right panels). n.s. = not significant vs. respective control (E, F, G, H), *p<0.05 vs. control (F), ***p<0.001 vs. control (E, G). All experiments are expressed as means ± SEM from 3-4 individual experiments. The immunoblot shown in B. was performed on two separate occasions.
Figure 10. IL-1β promotes specific changes in histone acetylation and methylation at regions associated with transcriptional regulation of the CXCL1 and CXCL2 genes. A-H. 832/13 cells were untreated (NT) or stimulated with 1 ng/mL IL-1β for 15 or 30 min. ChIP assays were performed using antibodies that immunoprecipitate methylated histone H3 (A-B: lysine 4; C-D: lysine 9, G-H: lysine 27) and acetylated histone H3 (E-F: lysine 14). The core promoter region of the CXCL1 and CXCL2 genes was amplified by real-time PCR. *p<0.05 vs NT, **p<0.01 vs NT. ChIP assays are represented as means ± SEM from 4 individual experiments.

Figure 11. IL-1β induces signal-specific phosphorylation of RNA Pol II on the CXCL1 and CXCL2 promoters. A-D. 832/13 cells were untreated (NT) or stimulated with 1 ng/mL IL-1β for either 15, 30 or 60 min. ChIP assays using antisera to immunoprecipitate Pol II CTD PO4-Serine 5 (A, B) or Pol II CTD PO4-Serine 2 (C, D). The core promoter region and a segment of the coding region, downstream of the transcriptional start site, corresponding to the CXCL1 (left panels) and CXCL2 (right panels) genes were targeted for real-time PCR amplification. *p<0.05 vs NT, **p<0.01 vs NT. ChIP assays are expressed as means ± SEM from 3-4 individual experiments.
Table Legends

Table 1. Predicted NF-κB sequences in the rat CXCL1 and CXCL2 proximal gene promoters. For each gene, TFSEARCH (v1.3) was used to analyze approximately 1.5kb of the upstream promoter region (relative to the transcriptional start site) for recognized κB genomic regulatory sequences. Predicted κB sequences for each gene are shown in the left (CXCL1) and middle (CXCL2) columns, with a comparison of relative positioning of each detected κB site shown in the far right column. The bolded sequence is a consensus κB response element detected in both genes at almost identical genomic positions. nd = no comparable site detected by the software algorithm within that region.

Consensus κB sequence: GGRNYYYCC, where R = purine; N = any nucleotide; Y = pyrimidine.

Table 2. Potential STAT1 binding sites within the CXCL1 and CXCL2 gene promoters. In silico examination of the 1.5kb regions controlling the proximal gene promoters for CXCL1 and CXCL2 reveals several regulatory sequences that are similar to a consensus GAS element. The putative STAT1 elements shown are either different by one nucleotide (nt) from a consensus GAS element or have a 2nt spacer (NN) instead of a 3nt spacer (NNN). One element in the CXCL1 gene promoter is a composite GAS/NF-κB sequence with demonstrated stimulus-induced STAT1 occupancy as shown in Figure 9G.
Table 1

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Consensus κB element: GGRRNNYYCC, R = purine; Y = pyrimidine; N = any nucleotide.
**Table 2**

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* Part of a Composite Element

Consensus GAS: TTCNNNGAA, where N = any nucleotide.