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The Regulation of Autoimmunity Genes in Pancreatic β-Cells by NF-κB

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THE REGULATION OF AUTOIMMUNITY GENES IN PANCREATIC β-CELLS BY NF-κB

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease where the immune system targets the pancreatic islet β-cells, which are responsible for insulin production and secretion, leading to a state of insulin insufficiency. Many of the molecular details of disease onset and progression remain poorly understood, including key aspects of cytokine signaling. Therefore, we have examined some of the consequences of inflammatory cytokine signaling as a critical module relevant to T1D. Using interleukin-1 (IL-1) signaling as a well-defined paradigm for inflammation, we investigated key components of this signaling pathway in pancreatic β-cells. We identified ICAM-1, one of the proteins expressed in response to IL-1, as an early response gene in pancreatic β-cells. This is significant because ICAM-1 has an important role in the development of autoimmunity that leads to T1D. First, we review the literature on ICAM-1 in autoimmune diabetes. We then examine the transcriptional mechanisms of *Icam1* expression in the β-cell, with a focus on rapid protein-DNA interactions as well as epigenetic events within the first few hours of cytokine stimulation. We found that IL-1β activates the NF-κB pathway to upregulate the production of ICAM-1 in the β-cell via binding to specific genomic regulatory sites within its promoter. These discoveries were made using *in vitro* models, *ex vivo* rodent and human islets, and autoimmune mouse models. Moreover, the expression of additional IL-1β-inducible genes were investigated, with a focus on syntaxin 11 (Stx11), another gene rapidly upregulated in pancreatic β-cells. Stx11, though understood in other physiological contexts, has no known function in β-cells. The biology of Stx11 to traffic secretory granules in other cell types has major implications for production and release of small proteins from β-cells; islet β-cells are known to produce and secrete insulin but are also capable of producing chemokines, which are small secreted proteins that regulate immune cell trafficking. Collectively, the findings presented herein represent novel molecular mechanisms that define how islet β-cells respond to cytokine signals by altering their genetic program through interaction with the immune system.

Chapter 1. Introduction

The regulation of blood glucose within safe levels is effected by a range of competing and complementary endocrine signals. Energy expenditure by all cells of the body depletes blood glucose, so a suite of hormones such as glucagon, epinephrine, and cortisol can stimulate the release of more sugars into the blood to match this demand. Conversely, the disposal of the post-prandial influx of blood glucose also requires endocrine input. Unlike the task of raising blood sugar levels, signaling its absorption is entirely dependent on the hormone insulin; furthermore, the task of secreting insulin falls squarely on one cell type, the pancreatic β-cell. That the β-cell is the only cell which produces this hormone means insulin is a particularly vulnerable point in metabolic regulation. Chronic elevation in glucose levels caused by dysfunctional insulin signaling can lead to the condition of diabetes.

Diabetes is a worldwide health threat. About 8.8% of the global adult population has the disease, and populations on every continent are projected to see an increase in incidence by 2035; in the US alone, 34% of the population is believed to have prediabetes or diabetes [1-3]. The two major forms of the disease are Type 1 and Type 2 Diabetes (T1D and T2D). In T1D, the loss of insulin secretion is caused by a spontaneous autoimmune attack on the pancreatic β-cells. Pathology typically strikes patients during their youth, so it was once known as Juvenile Diabetes. The other major form, T2D, results from insulin resistance leading to steadily increasing insulin demand and, ultimately, loss of β-cell mass due to their inability to meet this demand. The most common risk factor for T2D is obesity. Diabetes that is not properly managed can lead to negative health consequences such as renal damage, retinal pathologies, vascular complications, and systemic inflammation [2].

Although the initial triggers of T1D and T2D are different, inflammation plays a central role in both forms of the disease. Islet inflammation in T1D is caused by the cell-mediated autoimmune attack on the β-cells themselves [4], while in T2D it arises from stimuli released by overworked or dying β-cells [5]. In both cases, proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ within the islets result in a number of autoimmune defects, such as increased nitric oxide production [6], β-cell dedifferentiation [7] and recruitment of immune cells like macrophages and T cells [8, 9]. Since dyslipidemia is already associated with obesity and T2D, another risk factor for those patients is the direct inflammatory effects of fatty acids on β-cells [10]. And, because chronic hyperglycemia results in systemic inflammation in both forms of diabetes, a further complication is the fact that this inflammation can further reduce tissue insulin sensitivity [11].

A central player that causes many of these inflammatory effects is the NF-κB transcriptional pathway. Originally identified as “nuclear factor of the κB light chain in B cells”, NF-κB is a dimer of transcription factors that controls a wide swath of genes primarily involved in innate immunity, but it also plays a role in guiding other central processes such as adaptive immunity, proliferation, differentiation, and apoptosis [12, 13]. NF-κB responds to a diverse array of stimuli, including various cytokines such as TNF-α and IL-1, ligands for pattern recognition receptors (PRRs), and the T- and B-cell receptors [14]. The NF-κB complex itself can be a homodimer or a heterodimer of any of the five Rel transcription factors. The most common complex is a dimer of p65:p50 subunits, with p65 being the most active and plentiful member of the family, but most possible combinations of the subunits have been observed [13]. Under the canonical pathway, NF-κB is sequestered in the cytosol by the inhibitory protein IκB;

upon stimulation of the cell with an NF-κB ligand, IκB is phosphorylated and then degraded, liberating NF-κB to enter the nucleus. It then binds to genomic κB sites, at which it can either activate or repress gene expression [13]. This is effected through the recruitment of the general transcription factors, local changes in histone modifications, and activation of transcription by RNA Polymerase II [15-18].

Further regulation of p65 is achieved through post-translational modification: much (though not all) of p65's ability to induce transcription is via CBP/p300 recruitment to nearby chromatin, which requires phosphorylation at the S276 residue of p65 [19]. More nuanced regulation can be accomplished by modification at other residues. For example, phosphorylation at S536 seems to downregulate its activity in some contexts [20], upregulate its transactivation abilities in other contexts [21], or accelerate its proteasomal turnover in certain cell types [22]. Another mechanism of regulation is methylation of certain lysine residues [23]. Differentially modified p65 has been shown to direct NF-κB-mediated transcription to certain subsets of genes under control of the pathway [24, 25]. Since NF-κB controls such a wide range of genes with many functions, post-translational modification of the p65 protein provides a method for cells to tune this transcriptional response to their needs. Excellent reviews of the dazzling complexity of NF-κB and the many proteins involved have been written by Ghosh [12] and Baltimore [13], both early pioneers in research on the pathway.

Common cytokines in the islet inflammatory milieu include IL-1, TNF-α, and IFN-γ, with the former two being potent NF-κB ligands [26]. NF-κB activation in the β-cells by these cytokines has important consequences on the islets. A major result is the recruitment of immune cells: NF-κB-mediated secretion of chemokines CXCL1 and CXCL2 by the β-cells leads to the entry of more macrophages into the islet [27], while CXCR3-binding chemokines (CXCL9, CXCL10, and CXCL11) draw in T cells [28]. Other chemokines secreted by β-cells exposed to inflammatory signals include CCL2 and CCL20, both of which are potent chemoattractants for several types of immune cells [29, 30]. Obesity is included as a risk factor which results in chemokine secretion. Once the leukocytes have entered the islet, they secrete even more cytokines, impair islet function, and can eventually kill the β-cells [9, 31, 32]. Recruitment of T cells has a particularly grievous effect on the islet, as their secretion of IFN-γ provides a synergistic signal for the expression of many of the offending inflammatory genes [6, 30]. Further consequences of NF-κB activation in the β-cells include the production of nitric oxide [6] and the expression of immunoadhesins such as ICAM-1 [33]. In short, runaway expression of NF-κB in the β-cells, if unresolved, results in a cascade of events that ultimately leads to their demise.

A particular outcome of IL-1 signaling we would like to highlight is the role of ICAM-1 in the development of islet autoimmunity. This protein serves as a ligand for a number of adhesion reactions primarily involved in immune function; it binds several partners, though its main ligand is lymphocyte function-associated antigen 1 (LFA-1) [34-36]. In the last decades of the 20th century, a number of groups investigated ICAM-1 and its role in the development of autoimmune diabetes. A consistent finding was that disruption of the ICAM-1-LFA-1 interaction by various methods such as neutralizing antibodies or peptide blockade could reduce or protect mice from autoimmune diabetes [33, 37-40]. Strikingly, mere disruption of the *Icam1* gene on the genetic background of the Non-Obese Diabetic (NOD) mouse, the gold standard mouse model for autoimmune diabetes, was sufficient to completely protect these mice from diabetes [41, 42]. A separate, parallel strand of investigation was the use of similar anti-ICAM-1 antibodies, oligonucleotides, and gene disruption models in the study of islet tissue

transplantation. Similar results were observed: the ICAM-1-LFA-1 interaction is an important element in the rejection or tolerance of syngeneic, allogeneic, and xenogeneic islet grafts [43-49]. In both the autoimmune research and the transplantation research, experiments involving various cell transfer or donor/recipient configurations were implemented in an attempt to elucidate the mechanism of protection. Our appraisal of the ICAM-1 literature relevant to autoimmune form(s) of diabetes is documented in Chapter 2 of this document. It appears that the critical ICAM-1-LFA-1 interactions inhibited by these forms of blockade are the following: leukocyte-endothelial binding during extravasation, priming of naïve T-cells by APCs in the islet lymph nodes, and β-cell interactions with autoreactive T cells during the final stages of cell-mediated immunity. The literature implicates that the endothelial-immune cell interaction is dispensable for this process due to pathway redundancy, while the latter two processes both seem to be crucial events for both autoimmunity and graft rejection. A rational future approach is the use of tissue-specific knockout models to answer outstanding questions in the field.

The expression of *Icam1* as an NF-κB target gene has been well-documented in various cell types [50-52]. Given that NF-κB ligands are abundant in the inflamed islet milieu, it is reasonable to suspect that *Icam1* is expressed by different cell types within the islet. Early work indicated the presence of ICAM-1 on islet-infiltrating immune cells and islet endothelial cells in diabetic NOD mice, and in other early experiments it was found to be inducible on isolated islets, though ICAM-1 was not observed *in vivo* on β-cells of diabetic mice at this time [37, 53]. Later research has made it abundantly clear that ICAM-1 is also expressed on the β-cells of diabetic mice [33, 54]. In Chapter 3 of this document, we investigate the expression of *Icam1* using both *in vitro* and *in vivo* models and demonstrate the following: 1) *Icam1* expression is increased in rodent and human β-cells *ex vivo* in response to cytokines, 2) the ICAM-1 protein is present on β-cells of diabetic NOD mice *in vivo* at a greater magnitude than non-diabetic littermates, and 3) *Icam1* expression requires and is primarily mediated by NF-κB. Furthermore, we use molecular approaches such as site-directed mutagenesis and chromatin immunoprecipitation to further elucidate the epigenetic events responsible for causing *Icam1* expression in β-cells.

Because autoimmunity in T1D is associated with reductions in insulin secretion, we have used unbiased RNA-sequencing approaches to identify additional target genes regulated by IL-1 in β-cells. One of these targets that may influence insulin trafficking, secretion, or both is Syntaxin 11 (*Stx11*). Stx11 and the other syntaxins belong to a class of proteins called SNAREs; SNARE (soluble N-ethylmaleimide-sensitive factor-attachment receptor) proteins play a primary role in membrane fusion events such as Golgi processing, exocytosis, and synaptic activity [55, 56]. Certain SNARE proteins, especially Stx1a and Stx4, are well-known to be important for both insulin secretion and insulin action [57]. Stx11 participates in Golgi function in Hela cells [58], secretion of granules from platelets [59], and T lymphocyte cytotoxic activity [60]. However, to our knowledge, no group has published work describing a role for Stx11 in pancreatic β-cells. Thus in Chapter 4 of this document we investigate the regulation of *Stx11* in the β-cell by NF-κB and IL-1, hypothesize on roles implicated by the expression data, and describe future plans and tools for investigating the function of Stx11 in β-cell molecular transport and membrane fusion.

Chapter 2. Islet β-Cell Intercellular Adhesion Molecule-1: Integrating Immune Responses That Influence Autoimmunity

2.1. Introduction

Type 1 diabetes (T1D) is generally referred to as an autoimmune disease, and the presence of autoantibodies has often been used to distinguish this form of diabetes from Type 2 diabetes (T2D). Because autoimmune diseases in general have as a typical feature the presence of immune cells that target a self-tissue, leading to dysfunction of the host tissue, there is major interest in understanding the mechanistic underpinnings that lead to initiation and progression of the disease(s). From the T1D perspective, there has never been a single trigger identified to explain onset in susceptible individuals. What is generally agreed upon is that the number of auto-antibodies present is an indicator of greater disease risk [61], that insulin in circulation is reduced to a level that requires exogenous sources (e.g., from injections of recombinant insulin, secretion from transplanted islets, etc.), and that immune cell infiltration into pancreatic tissue is a feature of the disease [62]. The extent to which immune cell infiltration (often referred to as insulitis) occurs is variable and the number of leukocytes required to state that insulitis is present has been recommended [63].

Cytokines, secreted from immune cells, are also likely to be involved in both physiology and pathophysiology of the autoimmune disease process. From the standpoint of leukocyte recruitment and activation, there are specialized proteins, termed chemotactic cytokines (aka chemokines), which promote immune cell recruitment towards a site of inflammation [64]. In addition, there are various adhesion molecules present on antigen-presenting cells, endothelial cells, and target tissues that normally facilitate physiological immune cell function but become dysregulated in conditions of autoimmunity. One such adhesion molecule is the intercellular adhesion molecule-1 (ICAM-1), which is the focus of this chapter. We outline its importance in various models of autoimmunity to gain greater insights into mechanisms that are relevant to T1D and eventual development of effective therapeutics.

ICAM-1 is a 7-exon gene that is strongly regulated by cytokine signals [51, 65-67]. The gene encodes a glycoprotein that can be expressed on the cell surface as well as in secreted form [68, 69]. ICAM-2, encoded by a separate gene is typically not inducible but is also an LFA-1 ligand [70]. The function of the soluble version of the ICAM-1 protein (sICAM-1) is not completely understood. sICAM-1 levels are elevated in the circulation of Type 1 diabetic patients and people with genetic risk for T1D [71, 72]. Stimulation of T cells *in vitro* was greatly inhibited when combined with sICAM-1 or chimeric immunoglobulin fused to sICAM-1 [73]. These functions of membrane-bound ICAM-1 versus sICAM-1 may be competitive to limit overactive signaling through LFA-1.

LFA-1 is a heterodimer of CD11a and CD18 proteins that function together as a β2 integrin. It was first discovered in mice [74, 75] and subsequently in humans [76]. LFA-1 is present on most, if not all, T cells and facilitates interaction with antigen-presenting cells (APCs) [35]. Modulation of LFA-1 components regulates both inside-out signaling and cell spreading ability [77]. In addition, the LFA-1/ICAM-1 interaction controls the ability of T cells to expand in response to T cell receptor (TCR) activation, such as during exposure to viral infection. Thus, LFA-1/ICAM-1 interactions provide increased stability for cell-cell contacts (adhesion effects) and convey additional (co-stimulatory) signals that help modulate the correct T cell effector

responses. Collectively, these interactions assist with appropriate refining of the physiological immune response.

Chemokines promote recruitment of immune cells to a site of inflammation [64, 78] while adhesion molecules, such as ICAM-1 facilitate cell-cell contact as well as providing a secondary (co-stimulatory) signal for immune cell activation. With this in mind, it is perhaps not surprising that ICAM-1 is a central component of both healthy immunity as well as a promising target to control autoimmunity. Below we discuss several different scenarios by which ICAM-1 function is disrupted either by systemic interventions or by genetic approaches. Each of these model systems provides insights into the function of the ICAM-1 protein and its roles in the autoimmunity associated with onset of T1D.

2.2. ICAM-1 in Autoimmunity and T1D: Blockade-Based Strategies

The Non-Obese Diabetic (NOD) mouse is the gold standard rodent model for pre-clinical studies designed to understand various aspects of autoimmunity relevant to T1D [42]. Similar to human disease, the onset of T1D is spontaneous, proceeds with the presence of specific immune populations (e.g., macrophages, T cells, etc.), and requires insulin as a life-saving measure after diabetes onset. CD4+ and CD8+ T-lymphocytes, as well as macrophages, are important for the overall development of disease [79, 80]. In addition, diabetes can be accelerated in NOD mice by administration of the drug cyclophosphamide [81]. Because ICAM-1 is viewed as an important component of antigen presentation, its expression was predicted to be an important part of the autoimmune process. Early studies have examined that notion using a variety of pre-clinical model systems.

Monoclonal antibodies targeting ICAM-1 or LFA-1 reduced diabetes onset when injected into five-week-old NOD mice. This age was chosen because insulitis was either not present at all or very limited in terms of immune cell infiltration. Onset of hyperglycemia was monitored until the mice were 30 weeks of age [37]. There was a noticeable reduction in immune cell accumulation near and within islets as a partial explanation for the reduced onset of disease. Remarkably, this strategy did not block cyclophosphamide-induced onset of hyperglycemia [37].

The use of both ICAM-1 and LFA-1 antibodies in combination provides complete protection against diabetes onset compared to each intervention individually. Similar to the above study, injections into young (2-4 weeks of age) female NOD mice afforded stable therapeutic benefit persisting months after treatment [38]. In addition, adoptive transfer of splenocytes from 22-week-old NOD that had been injected with antibodies against LFA-1 and ICAM-1 were not able to produce diabetes in the NOD-SCID mice recipients [38]. Furthermore, administration of cyclophosphamide does not promote diabetes onset in mice that were previously exposed to the monoclonal antibodies targeting LFA-1/ICAM-1. This is in contrast to targeting just CD4+ cells, where protection against diabetes is reversed by cyclophosphamide administration [82].

Intravenous administration of an adenovirus encoding soluble ICAM-1/Ig fusion proteins to NOD mice provides long-term protection against invasive insulitis and onset of diabetes without suppressing immune function [83]. This intervention also induced lasting remission (>6 months) in 50% of mice with overt diabetes. However, the exact mechanism of how this protection is achieved is unknown. In contrast to previous studies showing the requirement for LFA-1/ICAM-1 interaction to prime effector cells, this strategy may function by blocking T cell adhesion to prevent or reduce ongoing islet β-cell destruction. There is no doubt that administration of sICAM-1 had a protective effect against diabetes onset *in vivo* in NOD mice [84]. In addition to reducing

islet destruction, the sICAM-1 and sICAM-1-Ig therapy reduced the expression of TH1 type cytokines within the islet. NOD mice given a plasmid that encodes sICAM-1 increased the ratio of TH2/TH1 cytokine production, reduced islet infiltration, and less islet destruction [85]. This outcome points to an immunosuppressive effect of sICAM-1, leading a possible scenario whereby sICAM-1 induction is a protective mechanism against autoimmunity. However, more work is needed to understand the induction mechanisms, source tissue(s), and consequences of sICAM-1 at various states of disease progression to gain a greater understanding about the autoimmunity mechanisms relevant to T1D.

Administration of soluble LFA-1 and ICAM-1 peptides, which block the membrane-associated ICAM-1 protein from binding its cognate receptor, reduced immune cell infiltration and preserved islet integrity in NOD mice. This intervention started with mice at 13 weeks of age; the caveat is that only 25% of the peptide-treated mice examined for infiltration were diabetic (blood glucose > 250mg/dL) compared to 60% of the saline treated animals. [40]. The effect of the soluble LFA-1 and ICAM-1 peptides is also present during transfer of NOD splenocytes to nondiabetic mice. This model provides rapid (1-2 weeks) infiltration of the islets by untreated T cells and macrophages, leading to rapid loss of glycemic control via islet destruction [86]. However, administration of ICAM-1 and LFA-1 antibodies to non-diabetic NOD mice is sufficient to completely protect these nondiabetic recipients from onset of diabetes induced by transfer of islet-derived mononuclear cells or splenocyte transfer from diabetic NOD donors [33, 37-39]. This protection correlated with mild insulitis or no detectable immune cell infiltration in recipient mice [38].

Blockade of ICAM-1 and LFA-1 can also protect against cyclophosphamide-induced diabetes in NOD mice, but this effect is dependent on the order of administration: when 10-week-old NOD mice were given cyclophosphamide and then subsequently anti-LFA-1 and ICAM-1 antibodies, there was no protection from diabetes. Whether this indicates an effect of disrupting ICAM-1/LFA-1 interactions on immune cell trafficking or T_{REG} ability to suppress pro-inflammatory responses is not clear [37]. On the other hand, NOD mice injected with a combination of LFA-1 and ICAM-1 monoclonal antibodies at 2 weeks of age were completely protected from diabetes when treated with cyclophosphamide at 12 weeks old [38]. Additionally, disruption of the *Icam1* gene on the NOD background reduced insulitis severity in cyclophosphamide-injected mice [41]. C57BL/6 mice deficient for the *Icam1* gene are not protected from immune cell infiltration or diabetes induced by multiple low doses of streptozotocin (STZ) [87]. On the other hand, antibody-mediated blockade of LFA-1 and ICAM-1 in combination, (but not separately) reduced insulitis and hyperglycemia in STZ-treated mice [88, 89]. We note that chemically induced (e.g., STZ) vs. spontaneous autoimmunity observed in NOD mice likely are very different mechanisms which respond differently to blocking LFA-ICAM-1 interventions.

2.3. Genetic Deletion of ICAM-1 in NOD Mice

With the systemic antibody- and peptide-based strategies targeting ICAM-1 proving effective in reducing diabetes onset in NOD mice, studying ICAM-1 in NOD mice with gene disruption soon followed. The Non-Obese Diabetic (NOD) mouse is the gold standard rodent model to study aspects of Type 1 Diabetes relevant to humans [90]. T1D in humans and NOD mice clearly requires interactions between discrete populations of immune cells [91]. For example, dendritic cells, macrophages, and T lymphocytes are critical for disease progression [92]. In efforts

to understand how immune cells and β-cells interact leading to onset and progression of T1D, transgenic mice with whole-body deletion of the *Icam1* gene were generated. NOD mice with genetic deletion of the *Icam1* gene were completely protected from diabetes [41]. Importantly, NOD mice with whole body *Icam1* deletion only display mild insulitis when compared with wild-type NOD mice. Remarkably, this reduction in insulitis also extends to NOD mice injected with cyclophosphamide. One possible explanation for the reductions in diabetes is decreased IFNγ expression in pancreatic tissue of the NOD *Icam1*$^{-/-}$ mice compared to *Icam1*$^{+/+}$ mice. This decrease in IFNγ is congruent with reductions in immune cells that produce cytokines (e.g., CD3+ cells). It is notable that mice heterozygous for ICAM-1 deletion exhibited an intermediate phenotype (delayed onset relative to *Icam1*$^{+/+}$ mice, $p = 0.09$) suggesting gene dosage is critical for determining disease onset.

2.4. Mechanism of Protection

Disrupting the ICAM-1/LFA-1 interaction with a combined anti-ICAM-1 and anti-LFA-1 antibody strategy early in life (2-4 weeks old) can render the splenocytes from 22-week-old NOD mice unable to transfer diabetes to NOD-SCID mice [38, 39]. This points to a possible modifiable phase in the early development of autoimmunity, wherein the ICAM-1/LFA-1 pathway is an important mediator. The observation that preventing the ICAM-1/LFA-1 interaction early in life appears sufficient to spare the pancreatic islets from the full effect of the cytotoxic lymphocytic response might be one key to understanding the autoimmunity leading to T1D. This pathway might also reveal potential avenues of treatment once the molecular details of immune cell priming and effector function have been completely delineated. For example, during the presentation of islet autoantigen(s) to naïve autoreactive T cells, the ICAM-1 and LFA-1 interaction could be one critical factor that tips the balance toward loss of self-tolerance. Many of the early studies into the role of the ICAM-1/LFA-1 pathway test the premise that autoreactive T cells were the key. Once these membrane proteins are blocked, the autoreactive T cell could enter a state of anergy and remain dormant or eliminated through physiological cellular death mechanisms. For example, splenic T cells isolated from NOD mice treated with ICAM1/LFA-1 mAbs were just as proliferative in response to the islet autoantigen GAD65 as controls [38]. Another group found no depletion of T cell numbers in the spleen or *ex vivo* proliferative responses from mice who were treated with the mAbs versus control mice [88]. However, other work shows that splenocytes from NOD mice treated with ICAM1/LFA-1 peptides [40] or with an *Icam1* genetic deletion [93] did not proliferate in response to islet antigen *ex vivo*.

It is possible that a population of suppressor T cells is induced during the tolerizing phase promoted by anti-ICAM-1/LFA-1 intervention. Supporting this notion, systemic administration of the T_{REG} cytokine IL-10 reduces insulitis and hyperglycemia in the NOD mouse [94]. In addition, the compartment of exposure may be an important factor with IL-10, as transgenic production of the cytokine by the β-cell actually accelerated diabetes unless paired with *Icam1* gene deficiency [93]. Moriyama *et al.* argue against an important role of suppressor T cells, since mixing the splenocytes of anti-ICAM-1/LFA-1 treated mice with splenocytes of acutely diabetic NODs and transferring them into nondiabetic recipients only slightly delayed diabetes onset [38]. One group attempted to expand the T_{REG} population with IL-2 while simultaneously suppressing effector T cell and NK cell proliferation with anti-LFA-1 antibody. Although the T_{REG} population was indeed expanded and even reduced islet destruction by autoreactive T cells in transfer experiments, this treatment did not provide persistent protection because of the robust expansion of effector cells in

the pancreatic lymph node [95]. Thus, a balance between T_{REG} control of inflammation and expansion of self-reactive effector T cells is constantly in flux and may be why onset of T1D is heterogeneous in both mice and humans.

2.5. Which Cellular Interaction Is Critical?

Endothelial Cells

Due to the role of ICAM-1 in extravasation of immune cells from the blood vessels, it is possible that this protein's expression in endothelial cells is a critical element of autoimmune-mediated loss of tissue function. Indeed, ICAM-1 is easily visible on islet endothelium during autoimmune diabetes progression [37, 96]. However, in a number of diabetes models, the inhibition of ICAM-1 does not actually prevent extravasation of immune cells to the pancreatic islets. Mice with a whole-body genetic deficiency in the *Icam1* gene who are treated with STZ show no reduction in islet immune infiltration compared to wild-type mice [87]. The sICAM-1/Ig treatment used by Bertry-Coussot *et al* also demonstrates no inhibition of diabetes in their NOD*scid* transfer experiments [83]. One group found slowed, but not inhibited, entrance of 3A9 T cells into the islets of IP-HEL mice, and they observed a similar result with BDC2.5 T cells injected into irradiated NOD mice with *Icam1* genetic disruption [97]. Redundancy within the range of pathways involved in extravasation means that other membrane receptors could mediate the trafficking process in the absence of ICAM-1. Antibodies against VLA-4/VCAM-1 can more effectively inhibit extravasation into the islet tissue of NOD mice, but this therapy only delays the onset of diabetes until the treatment is removed [98]. This is in stark contrast to blockade of the ICAM-1/LFA-1 pathway, where treatment during a critical tolerizing phase is necessary, and tolerance remains after removal of the blockade. The study of Fabien *et al* showed that blockade of L-selectin more effectively prevented islet infiltration than LFA-1 mAbs, despite the latter having a more beneficial effect in preserving islet function [99]. Thus, even if ICAM-1 contributes to autoimmune diabetes through its role in extravasation, the redundancy of transendothelial migration pathways indicates that trafficking of immune cells may not be the major benefit of ICAM-1 blockade to prevent losses in function and mass of islet β-cells.

Stimulation of Naïve T Cells

Immune invasion of the autoimmune-prone NOD pancreas involves the presence of macrophages and dendritic cells, and ICAM-1 is readily detectable on the surface of these cells [86, 96, 100]. Due to the role of ICAM-1 in providing a second signal for T cell activation, it is conceivable that professional APCs within the islet bring β-cell-specific antigens back to the pancreatic lymph node and stimulate naïve T cells there. In addition, it is also possible that T cells recognize antigen on the β-cell surface and use ICAM-1 on β-cells as the secondary signal for activation during autoimmunity. Prior to hyperglycemia, the islets of NOD mice are enriched with CD103+ dendritic cells, which are highly efficient at cross-presenting MHC-I bound by peptide to CD8+ T cells; the presence of a dendritic cell that presents antigen efficiently to CD4+ and CD8+ T cells enables islet destruction to proceed [101]. Thus, the presence or absence of ICAM-1 on that APC could be a critical element in the induction of autoimmunity. In addition, specific polymorphisms associated with T1D risk among humans are related to other genes involved in costimulation and the immune synapse, including the genes encoding CTLA4, PD-1, IL-2RA, and

PTPN22 [102]. The mechanistic work probing a role for ICAM-1 during antigen presentation during islet autoimmunity makes a strong case for it being a vital piece.

The work of Moriyama *et al*. using NOD mice showed that a two-week treatment of ICAM-1/LFA-1 mAbs early in life protected mice from disease [38]. It is plausible that interactions blocked by these antibodies include the initial antigen presentation events that precede the immune infiltration of the islets. Furthermore, donor T cells that could only recognize transgenic β-cell-specific antigens when these were presented by donor APCs revealed that antigen presentation from APC only to T cell was sufficient to cause disease [100]. Another group found that anti-LFA-1 treatment reduced proliferation of transferred autoreactive T cells within the pancreatic lymph nodes of NOD mice [95]. In a creative experiment described by Sarvetnick *et al.*, CD4+ T cells specific for OVA peptide were primed *in vitro* by Drosophila APCs, either in the presence or absence of ICAM-1 on the APC surface. These T cells were then transferred into mice who expressed the OVA peptide under the control of the rat insulin promoter. Although both groups of T cells (those primed by APCs with or without ICAM-1) infiltrated the islets of their respective hosts, *only the cells stimulated in the presence of ICAM-1 led to islet destruction* [103]. In an MLR experiment with splenocytes from mice that had received islet transplants, APCs from ICAM-1 knockout mice caused a decreased proliferation response [104]. All of this evidence points to ICAM-1 on the surface of the APC as a vital element in autoimmunity.

Modulating Effector T Cell Function

Another function of the ICAM-1/LFA-1 pathway is to strengthen the immune interaction specifically between the cytotoxic lymphocyte and the somatic cell targeted for killing as the former engages in its effector function. Although the immunological synapse between target cell and antigen specific T cell lasts a shorter period of time than the one between APCs and naïve T cells, strengthening it nonetheless enhances effector functions [35, 105]. Treatment with a mAb against CD8 was sufficient to prevent insulitis and diabetes in NOD mice, pointing to the necessity of CD8+ CTLs in the disease process [39]. Hyperexpression of MHC I could be seen on endocrine cells of the diabetic NOD pancreas [86], and its expression was increased by cytokines *in vitro* [106]. Isolated islets and β-cells in culture have little to no expression of ICAM-1 in the basal state, but they readily express the gene when exposed to IL-1β, IFN-γ, and TNF-α [33, 53, 96, 106]. Whether the ICAM-1 protein is present on β-cells in the disease state *in vivo* was debated: some labs have observed its presence [31], while others have not [37]. Yagi *et al.* demonstrated that ICAM-1 is indeed expressed on the beta cells of diabetic NOD mice when examined under immunoelectron microscopy [33]. Their images also indicate LFA-1 on mononuclear cells which contact the beta cells. With the more advanced imaging techniques available at present, in combination with co-localization antibody strategies, it is clear that beta cells of diabetic mice, and more specifically within inflamed islets, express ICAM-1 [33, 54].

Does β-cell surface expression of ICAM-1 enhance immune cell-mediated killing? Due to the transient nature of this interaction and the lack of tissue-specific knockout models to directly address the role of ICAM-1, it has been difficult to investigate the tissue-specific importance of ICAM-1 for autoimmune disease. However, strategies using *ex vivo* approaches include isolation and expansion of CTLs that had invaded NOD islets. These CTLs were then incubated with NOD-derived β-cells and demonstrated significant cytotoxic activity; this cytotoxic activity was amplified by pretreatment of the β-cells with cytokines, and it was reduced in a concentration-dependent manner by mAbs against LFA-1 and ICAM-1 [33]. Thus, it is reasonable to speculate

that ICAM-1 is normally induced on the β-cell in response to viral infection; this surface expression would serve to alert the immune system that a virus is present and needs to be cleared. Although isolated islets and cultured beta cells typically do not express appreciable amounts of ICAM-1 in the basal condition, infection by SV40 was found sufficient to induce its expression in the absence of cytokine [106]. On the other hand, reovirus infection did not alter ICAM-1 abundance on islet beta-cells, indicating that ICAM-1 induction is likely to be signal-specific [53].

Chapter 3. ICAM-1 Abundance is Increased in Pancreatic Islets of Hyperglycemic Female NOD Mice and is Rapidly Upregulated by NF-κB in Pancreatic β-Cells

3.1. Introduction

Type 1 diabetes (T1D) is classified as an organ-specific autoimmune disease that arises when blood glucose control can no longer be adequately maintained. While the initial triggering event(s) is currently unknown, major outcomes of this disease are the onset of hyperglycemia secondary to decreases in circulating insulin due to loss of β-cell mass, insulin secretion, or both [62]. There is a longstanding view that immune cell targeting of the pancreatic β-cells within the islets of Langerhans is a critical component of the disease process [107-109]. Pro-inflammatory cytokines, such as IL-1β and IFN-γ, have been viewed as part of this process for decades [26, 110, 111]. The ability of pro-inflammatory cytokines to promote changes in β-cell gene transcription is likely to be a critical part of the disease component at least in part by regulating the production and secretion of chemokines [78, 112, 113]. Once secreted, chemokines influence immune cell trafficking to sites of inflammation [64, 114].

Pancreatic β-cells exhibit rapid alterations in gene expression patterns upon exposure to IL-1β, IFN-γ, or both cytokines in combination [115-119]. This process involves the NF-κB pathway, with rapid nuclear entry by RelA/p65, occupancy of κB elements within promoter regions of the chemokine genes, and histone chemical modifications [115-121]. The robust transcriptional response to cytokines promotes β-cells to produce and secrete large amounts of chemokine proteins concomitantly with reductions in insulin secretion [116]. The actions of the NF-κB pathway are often augmented by signaling through IFN-γ receptor and activation of STAT1 [78, 112].

Once immune cells arrive within a site of inflammation, often having been recruited by chemokine gradients and primed by exposure to antigen(s), there is interaction between the discrete types of immune cells as well as communication with the inflamed tissue [91, 122]. Part of this interactive process includes the increased abundance of cell surface proteins that allow for adhesion. One such example is the intercellular adhesion molecule-1 (ICAM-1), a transmembrane domain containing protein that interacts with specialized integrin proteins typically present on immune cells [67, 123]. One such specialized set of proteins makes up the lymphocyte function-associated antigen-1 (LFA-1, aka $\alpha_L\beta_2$), a heterodimer composed of CD11a and CD18 [124]. Upon activation, LFA-1 interacts primarily with ICAM-1, but can also interact with other members of the ICAM family [125]. Thus, fine tuning an immune response is balanced by a number of important events, including cytokine and chemokine production, ICAM-1 upregulation, and the ICAM-1/LFA-1 interaction. The direct involvement of ICAM-1 in organ-specific autoimmunity and rejection of grafted tissue has been demonstrated in various studies [37, 41, 46, 93, 126].

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In this study, we have investigated the signal-specific induction of ICAM-1 in pancreatic β-cells. Using bulk RNA-sequencing (RNA-Seq), we found that the 832/13 rat β-cell line and human islets exposed to IL-1β each displayed strikingly enhanced *Icam1* transcript levels over untreated cells. In addition, we observed that the *Icam1* gene is rapidly induced in β-cell lines as well as isolated mouse, rat, and human islets in response to interleukin-1β. Upon further investigation of the molecular determinants underlying these responses, we found that RNA polymerase II and the RelA/p65 subunit of NF-κB occupied genomic regions controlling *Icam1* in a temporal manner consistent with the first appearance of transcript over baseline levels. These transcriptional data are supported by two key *in vivo* approaches: 1) ICAM-1 protein is enhanced in pancreatic islets four hours after systemic injection of IL-1β, 2) Female NOD mice display more *Icam1* expression in islet β-cells when compared with male NOD mice, and 3) female NOD mice display elevations in ICAM-1 protein days after becoming hyperglycemic when compared with age-matched normoglycemic controls. Collectively, these genetic and molecular approaches offer novel insights into a key gene regulating immune system function and thus add to the existing molecular framework explaining events critical for onset and progression of T1D.

3.2. Materials and Methods

Cell Culture, Adenoviruses, and Reagents

Culture and passage of the 832/13 rat insulinoma cell line has been described [127]. Cell lines were confirmed to be free of mycoplasma contamination using the Lonza MycoAlert Mycoplasma Detection Kit. Recombinant adenoviruses expressing GFP, p65, $p65^{S276A}$, β-galactosidase (βGAL), CA (constitutively active) IKKβ (S177E/S181E) and IκBα super-repressor (S32A/S36A) have been described [117, 128]. TPCA was from Bio-Techne (Minneapolis, MN, USA). Recombinant IL-1β and IFN-γ were from Peprotech (Cranbury, NJ, USA).

Experimental Animals, Islet Isolation, Pancreas Histology, and Human Islets

Seven-week-old male and female NOD (stock # 001976), and male C57BL/6J (stock # 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Seven-week-old male Wistar rats (Strain #003) were purchased from Charles River (Wilmington, MA). Various ages of mice with the following conditional alleles were all from the Jackson Laboratory: IL-1R floxed (Jax #: 028398), RelA (p65) floxed (Jax #: 024342), and Pdx1-cre (Jax #: 014647). All animals were multi-housed with a 12-h light/dark cycle, and were allowed to acclimate to the facility for a minimum of one week prior to beginning experimentation. All animals had *ad libitum* access to food and drink prior to isolation of islets or pancreata for fixation. Female NOD mice were checked two-three times weekly for the presence of hyperglycemia ($\geq$ 250 mg/dL) and were collected within two days of two consecutive hyperglycemic values. An age-matched normoglycemic control was also collected at the same time. C57BL/6J mice were injected with either saline or IL-1β (1 μg/kg body weight) and four hours later pancreatic tissue was collected for fixation in neutral buffered formalin. Procedures for sectioning and staining of pancreatic tissue have been reported [129, 130]. The primary antibodies used were: STAT-1, Cell Signaling #14994, (1:400); ICAM-1, LSBio, LS-313412, (1:200); glucagon, eBioscience #14-9743-82, (1:1500); insulin, BioRad #5330-0104G, (1:1000). The secondary antibodies used for fluorescence imaging were from Jackson ImmunoResearch: Donkey anti-Guinea Pig Alexa Fluor

488, #706-545-148, (1:300) and Donkey anti-Rabbit Alexa Fluor 594, #711-586-152, (1:300). For the chromogenic stains, we used the Leica Biosystems ChromoPlex 1 Dual Detection kit, DS9665 which provided red plus brown detection. For comparisons of tissues from 8 and 12 week old C57BL/6J and NOD female mice, we used a Nanozoomer HT slide scanner equipped with a triple bandpass filter set for DAPI/FITC/TxRed fluorescence detection and a 20x /0.8NA objective. For the comparison of the normoglycemic versus hyperglycemic tissues from 18 week old female NOD mice, we used a Leica DM6000 microscope with 40x /0.95 NA objective. Our techniques for isolation of both mouse and rat islets have been described in detail previously [116, 120, 129]. Human islets were obtained from Lonza (Clonetics™ Fresh Human Pancreatic Islets) with donor information described previously [129]. All animal procedures were approved by the respective Pennington Biomedical Research Center or University of Tennessee Medical Center Institutional Care and Use Committees.

Identification of Predicted κB Genomic Regions and Construction of Luciferase Plasmids

Six putative NF-κB elements were identified in the -3kb region upstream of the rat *Icam1* gene using the JASPAR web-based promoter analysis tool [131] and confirmed using PROMO, a separate web-based program [132]. Of the six sites identified by each of these computer-based approaches, four *Icam1* promoter sequences were found to be conserved between rat, mouse, and human. These four sites were given the identifiers κB 1-4 relative to the transcriptional start site and were carried forward for further analysis. Using genomic DNA from rat 832/13 cells, 2.9 kb of the rat *Icam1* promoter sequence was amplified with AccuPrime *Pfx* SuperMix (Invitrogen) and *Icam1* 3kb cloning primers (Supplementary Table 1). This amplicon was digested with *SacI* and *HindIII* and inserted into pNL1.2 (Promega) at the multiple cloning site. The same reverse primer was used with the *Icam1* 1kb cloning primer to amplify 1.1 kb of the rat *Icam1* promoter. This amplicon was digested with *HindIII* and inserted into pNL1.2 at the multiple cloning site. Mutations were generated in the 2.9 kb pNL1.2 construct by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Agilent). Primers for mutant sequences can be found in Supplemental Table 1. Each construct and successful site-directed mutagenesis event were confirmed by sequencing at the Pennington Biomedical Research Center Genomics Core Facility.

Transient Transfections and Luciferase Assays

For luciferase assays, 832/13 cells were grown to 75% confluence in 24 well plates. Luciferase reporter plasmids and siRNA duplexes were transfected into cells using TransFectin Lipid Reagent (Bio-Rad) according to the manufacturer's instructions. 24 hours (h) post-transfection, cells were treated as indicated in the respective figure legends. Cells were lysed in 50 μL Nano-Glo Luciferase Assay Reagent (Promega) for 10 minutes (min) with rocking at room temperature. Luminescence was measured with a Glo-Max Multi+ Luminometer (Promega). Silencer Select siRNA duplexes (Invitrogen, Waltham, MA) used in this study are as follows: negative control siScramble (AM4611), sip65 (siRNA ID no. s159516) and sip50 (siRNA ID no. s135617).

RNA extraction, cDNA synthesis, and Gene Expression Analysis

Our procedures and reagents for isolation of RNA from cell lines and islets, cDNA synthesis, and transcript analysis by real-time PCR have all been reported [129, 133]. PCR analysis was conducted using a Bio-Rad CFX 1000 thermal cycler. Primer sequences are listed in Supplementary Table 1.

Serial Analysis of Gene Expression (SAGE), Gene Expression Data Analysis, and Pathway Enrichment Analysis

SAGE**:** RNA content and quality (260/280 ratio range 1.9 - 2.1) were assessed using a Nanodrop 1000 and then used to perform the SAGE analysis [134, 135]. Briefly, gene expression profiling was performed by expression tag sequencing (SAGE) on an AB SOLiD 5500XL next-generation sequencing instrument using reagent kits from the manufacturer (Applied Biosystems, Foster City, CA). Sequence reads were aligned to human (hg38) or rat (rn6) reference RefSeq transcripts, respectively, via SOLiDSAGE (Applied Biosystems). Only uniquely mapped sequence reads were counted to generate the expression count level for each respective RefSeq gene. Expression levels of genes of interest were confirmed by RT-PCR using methods described above. The SAGE dataset has been uploaded to the Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/) using Accession # GSE124166.

Gene expression data analysis: Differential analysis of RNA read count data was performed using DESeq2 v1.4.5 software [136], which models read counts as a negative binomial distribution and uses an empirical Bayes shrinkage-based method to estimate priors for signal dispersion and fold-changes, and to calculate posterior estimates of these parameters. Gene expression signals were logarithmically transformed (to base 2) for all downstream analyses (the lowest expression value being set to 1 for this purpose). Gene expression based principal components analysis using JMP Genomics v6.0 was carried out as a quality control measure in the human and rat studies with two outlier samples detected in the human study, which were removed from further analysis (PCA shown in Supplementary Figure 1).

Pathway enrichment analysis**:** Pathway enrichment was conducted via the competitive gene-scoring based gene set enrichment analysis tool (GSEA) [137]. GSEA was performed by first ranking the expression of all genes in the untreated and IL-1β treated islets and beta cells via the signal-to-noise ratio (SNR) metric, and then employing a weighted Kolmogorov-Smirnov test to determine if the gene SNRs deviate significantly from a uniform distribution in *a priori* defined gene-sets (pathways) obtained from Wikipathways (www.wikipathways.org). Human and rat gene expression data was analyzed against human and rat specific pathway lists, respectively. Statistical significance for the observed enrichment was ascertained by permutation testing over size-matched gene-sets. Significant gene-sets were selected by control of the false discovery rate, FDR at $\leq$5% [138]. The per-sample expression profiles of genes contributing to core enrichment of the significant pathways were visualized via row-normalized blue-red heatmaps with blue representing lower, and red representing higher gene expression levels.

Preparation of Whole Cell Extracts and Immunoblotting

832/13 cells were seeded in 6 well plates and treated as indicated in figure legends. Cells were then lysed in 100 µl M-PER lysis reagent (ThermoFisher Scientific) supplemented with Halt Protease Inhibitor Cocktail (ThermoFisher Scientific). Whole cell lysates quantified using a BCA

assay (ThermoFisher Scientific). Denaturation of samples and immunoblotting conditions have been described in detail [130, 139]. Antibodies used are listed in Supplementary Table 2.

Chromatin Immunoprecipitation

Preparation: Cells were grown to 75% confluence in 10 cm dishes. Cells were serum starved in RPMI for 1 h before treatment with cytokine. After treatment, the cells were washed twice with 5 mL PBS and then crosslinked with ChIP Crosslink Gold (Diagenode) in PBS for 30 min at room temperature according to the manufacturer's instructions. The plates were washed twice with 5 mL PBS again and then crosslinked in 1% methanol-free formaldehyde in 4.5 mL PBS for 10 min at room temperature. The crosslinking reaction was quenched with glycine at a final concentration of 125 mM for 5 min. The plates were washed twice with ice-cold PBS and cells scraped into 1 mL PBS with Halt Protease Inhibitor Cocktail (Thermo Scientific). Cells were pelleted by spinning 2 min 4ºC at 5000 x g. Pellets were resuspended in 950 µL lysis buffer (1% SDS, 0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 0.5 mM DTT, pH = 8.0) and incubated 30 min at 4ºC with rotation. Lysates were divided into 3 x 300 µL aliquots and dispensed into polystyrene sonication tubes (Evergreen Scientific). Chromatin was sheared to an average fragment size of 200-250 bp by sonicating 12 cycles (30 sec ON/30 sec OFF) in a Bioruptor Pico (Diagenode). Chromatin was cleared by centrifugation at 12,000 x g for 10 mins at 4ºC. Fragment size was confirmed by addition of 1 µL of sheared chromatin to 19 µL of TE, incubation for 30 mins with RNase A (Thermo Scientific) at 37ºC, addition of proteinase K, incubation for 1 h at 65ºC, and running for 30 min on a 1.5% agarose gel. Samples were diluted by adding 100 µL of lysate to 900 µL of Dilution Buffer (1.1% Triton X-100, 0.01% SDS, 17 mM Tris, 1.2 mM EDTA, 167 mM NaCl, pH = 8) and 2 µL of protease inhibitors. Diluted material was precleared overnight with 5 µL Protein G Dynabeads (Thermo Scientific) by incubation at 4ºC with rotation. Input samples were prepared by adding 2 µL lysate to 150 µL ChIP Elution Buffer (1% SDS, 50 mM Tris, 10 mM EDTA). These 2% input samples were set aside at 4ºC overnight.

Immunoprecipitation: 5 µL of Dynabeads were prepared for each IP in the following manner. Beads were incubated in PBS-TWEEN with 1 µg antibody for 30 min with rotation at room temperature. Beads were then blocked by incubation in 1% BSA in PBS for 30 minutes at room temperature. After preclearing beads were removed from IP reactions, 5 µL of antibody-bound immunoprecipitation beads were added to each reaction and rotated at 4ºC for 1 h. IP reactions were then transferred to clean microfuge tubes and washed with the following buffers: twice with Low Salt Wash (0.1% SDS, 1% Triton X-100, 0.05% TWEEN, 20 mM Tris, 2 mM EDTA, 150 mM NaCl), twice with High Salt Wash (0.1% SDS, 1% Triton X-100, 20 mM Tris, 2 mM EDTA, 500 mM NaCl), twice with LiCl Wash (1% NP-40, 1% sodium deoxycholate, 0.25 mM LiCl, 10 mM Tris, 1 mM EDTA), and once with TE (10 mM Tris, 1 mM EDTA). All washes were 5 min with rotation at 4ºC except the TE wash, which was performed at room temperature. Beads were then resuspended in 150 µL ChIP Elution Buffer and, along with input samples, were incubated at 65ºC for 30 min with shaking in a thermomixer. After elution, supernatants were transferred to clean microcentrifuge tubes for overnight decrosslinking with Proteinase K and 200 mM NaCl at 65ºC. DNA was extracted from ChIP samples and inputs using GenCatch Advanced

PCR Extraction Kit (Epoch Life Science). Primer sequences are available in Supplementary Table 1. Antibodies used are listed in Supplementary Table 2.

Statistical analysis

Statistical analysis was conducted using Prism version 9.3 (GraphPad). One or two-way ANOVA analyses were used to calculate the *p*-values indicated in the figure legends.

3.3. Results

RNA-Seq reveals the gene encoding Icam1 was rapidly upregulated in response to IL-1β in human islets and the 832/13 rat β-cell line.

Human islets and the 832/13 rat β-cell line were exposed to the cytokine IL-1β for 3 h or left untreated. Using an adjusted *p*-value of $p < 0.1$ and absolute fold-change of ≥ 1.5 for differential gene expression in both human and rat-derived cells, the top 50 responsive genes under these conditions were analyzed and shown in heatmap format for human islets (Figure 1A) and rat 832/13 cells (Figure 1B). Among the highest responding genes in human islets were various

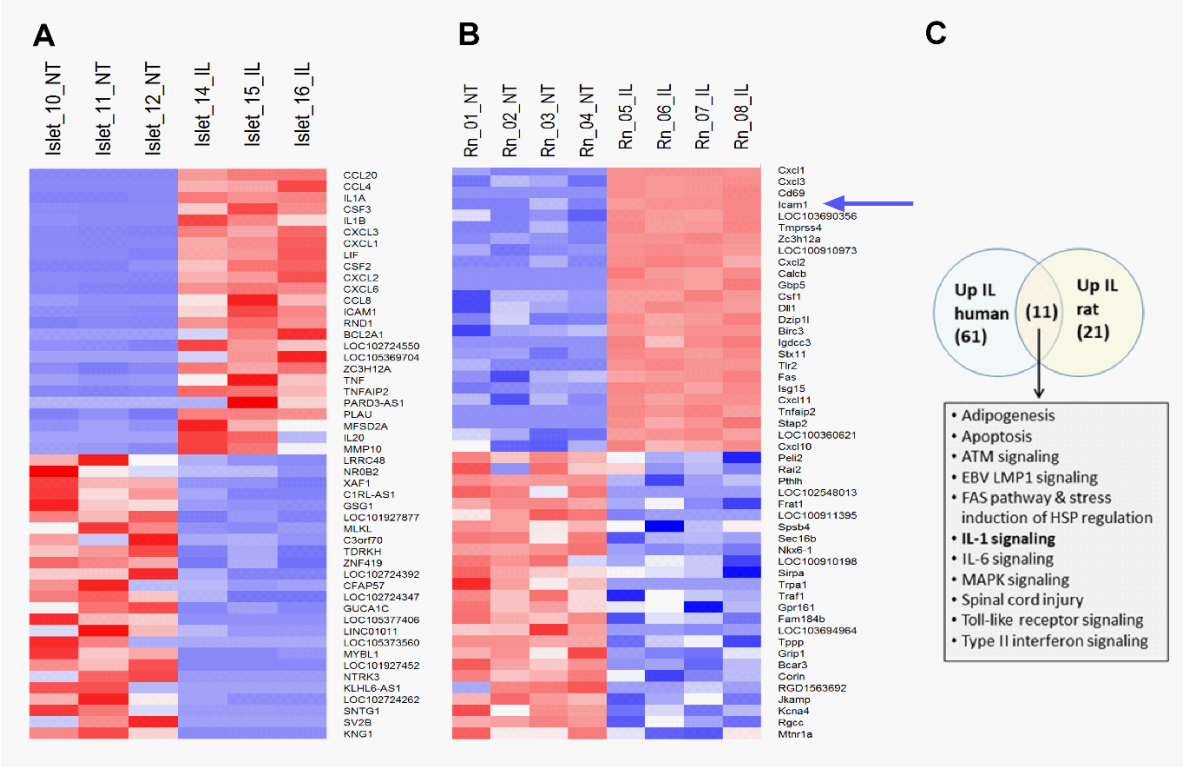

Figure 3.1. RNA-Seq reveals the gene encoding *Icam1* was rapidly upregulated in response to IL-1β in human islets and the 832/13 rat β-cell line. Clustered heatmap of the top 50 most up-regulated and down-regulated genes in human islets (**A**) and 832/13 rat insulinoma cells (**B**) exposed to IL-1β for 3 h (selected by magnitude of fold-change). Treatment conditions across replicates are indicated at the top of the heatmap and genes are indicated in rows. Gene expression signals were row-normalized with higher gene expression is expressed in shades of red and lower expression in shades of blue. The blue arrow indicates the *Icam1* gene. **C.** Pathway enrichment analysis of human islets and 832/13 rat β-cells as determined via GSEA. The top enriched pathways from each study (FDR≤5%) were compared via a Venn analysis. Numbers in parentheses refer to the number of significantly enriched *pathways* in each set. Pathways identified in common in both species are listed in bulleted format. NT = no treatment; IL = IL-1β- treated.

chemokines (e.g., CXCL1, CXCL2, CCL20, etc.) and cytokines (e.g., TNF-α). Similar upregulation of chemokine and inflammatory genes were seen in 832/13 cells (e.g. Cxcl1, Cxcl3, and Tlr2). This finding using unbiased bulk RNA-Sequencing is consistent with gene expression observations from our group [78, 116, 117, 120, 121] and others [113]. From this analysis, we

identified *Icam1* as one of the most highly responsive genes (Figure 1A and 1B). Pathway enrichment analysis GSEA was consistent with IL-1β signaling (Figure 1C). Further analysis of the IL-1β signaling pathway showed 61 genes upregulated in human islets, 21 in 832/13 cells, and 11 of those genes are upregulated in both species (Figure 1C Venn diagram). There was also a high significant overlap (overlap p-value<2.2e-16 by Fisher's exact test) between significantly differentially expressed genes from both human islet and rat cell lines (283 human genes and 554 rat genes at an absolute fold-change $\geq$2, adjusted p-value $\leq$ 0.1; not shown). The PCA analyses for these samples are provided in Supplementary Figure 1.

Expression of Icam1 is inducible ex vivo in mouse, rat, and human islets and in vivo in both C57BL/6J and NOD mice.

Based on the RNA-Seq results shown in Figure 1, we next collected RNA from human islets after being left untreated or exposed to 10 ng/mL IL-1β for 1, 2, or 3 h and found that *Icam1* was markedly elevated by 3 h (Figure 2A). This finding was also reproduced in cultured rat islets (Figure 2B). We next cultured mouse islets in 10 ng/mL IL-1β for 3 h and found that *Icam1* was also induced by exposure to this cytokine (Figure 2C). After these observations across multiple species, we next tested the hypothesis that islet ICAM-1 protein would be inducible in response to a systemic cytokine signal; this was accomplished with intraperitoneal injection of recombinant mouse IL-1β (1 μg/kg body weight), which is a dose used previously for physiological outcomes [140]. Male C57BL/6J mice injected i.p. with IL-1β displayed robust staining for ICAM-1 (Figure 2D; shown in pink in the right hand panel). We interpret this data as clear evidence that the ICAM-1 protein was markedly and rapidly upregulated in pancreatic islets *in vivo* after systemic delivery of IL-1β, which is consistent with the *ex vivo* results using cultured mouse, rat, and human islets. Moreover, using an established mouse model of autoimmunity and T1D, we found that female NOD mice display more expression of *Icam1* relative to male NOD mice (Figure 2E). In addition, when female NOD mice convert to hyperglycemia (>250 mg/dL), *Icam1* expression is elevated when compared with age-matched normoglycemic female NOD mice (Figure 2F). This phenotype is also observed at the protein level in pancreatic tissue isolated from normoglycemic relative to hyperglycemic female NOD mice (Figure 3) Importantly, we note that a portion of the immune cells surrounding the islets and also infiltrating the islets are ICAM-1 positive. In addition, the β-cells also display increased ICAM-1 protein in hyperglycemic NOD mice (Figure 3). At NOD mice aged eight and twelve weeks, only a subpopulation of the immune cells are positive for ICAM-1 (Supplementary Figure 2). Using C57BL/6J mice as a model that does not display autoimmunity or hyperglycemia, we note the tissue-resident immune cells are not likely to express ICAM-1 at levels observed in the NOD mice (Supplementary Figure 2).

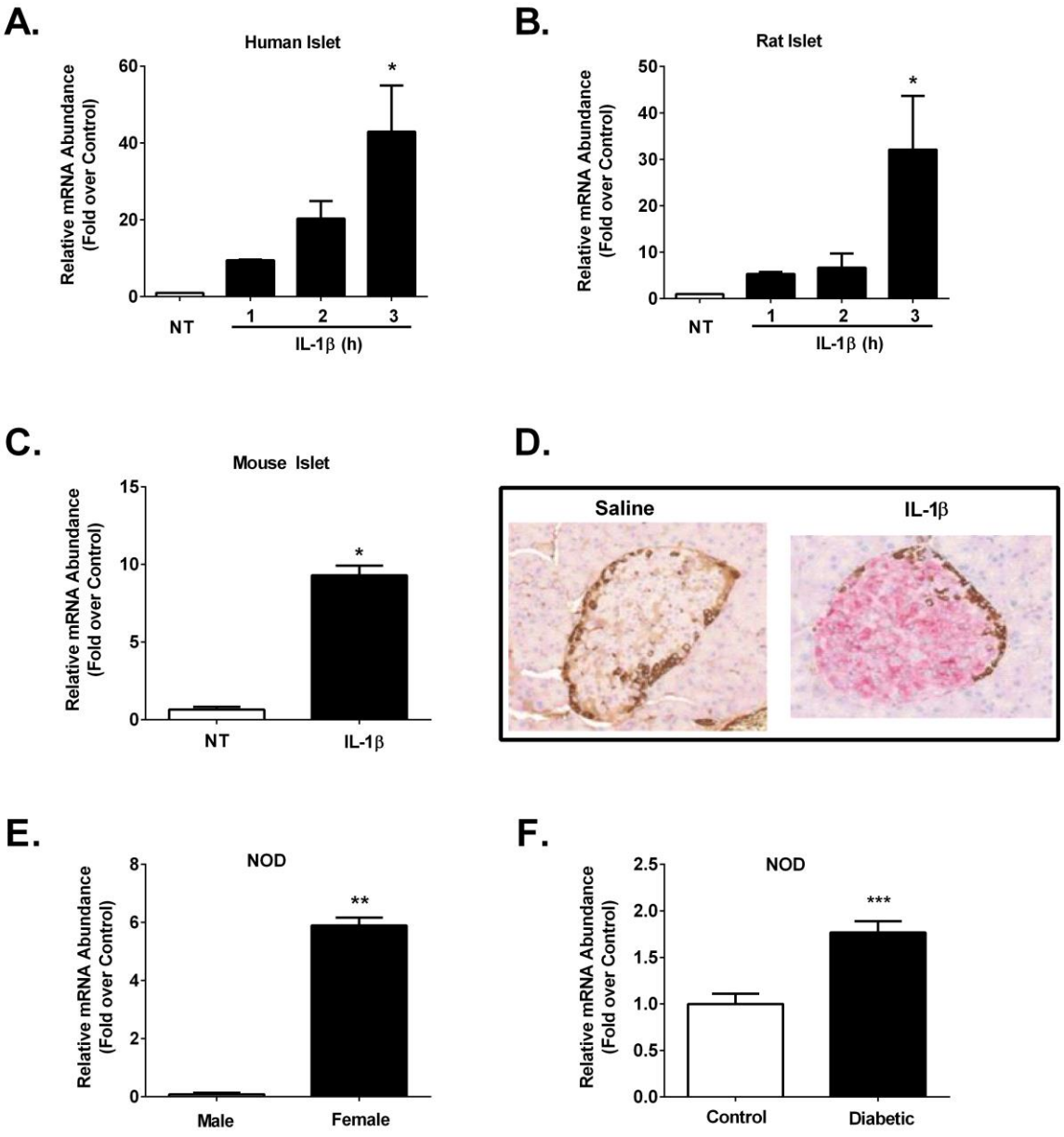

Figure 3.2. Expression of *Icam1* is inducible *ex vivo* in mouse, rat, and human islets and *in vivo* in both C57BL/6J and NOD mice. **A.** Human islets (n=3) were untreated (NT) or stimulated with 10 ng/mL IL-1β for the indicated times. **B.** Rat islets (n=3) were NT or stimulated with 10 ng/mL IL-1β for the indicated times. **C.** Mouse islets (n=3) were NT or stimulated for 3 h with 10 ng/mL IL-1β. **D.** Formalin-fixed paraffin-embedded (FFPE) pancreatic tissue was sectioned and stained for ICAM-1 (pink) and glucagon (brown) in 12 week old male C57BL/6J mice injected with either saline control or IL-1β (1 μg/kg body weight). **E.** *Icam1* mRNA abundance in islets isolated from 12 week old normoglycemic male (n=3) and female (n=7) NOD mice. **F.** *Icam1* expression levels in islets isolated from 16 week old female control (n=10) or hyperglycemic (n=9) NOD mice. The transcript data (**A-C, E-F**) are shown as means ± SEM. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. p values are vs. respective NT controls.

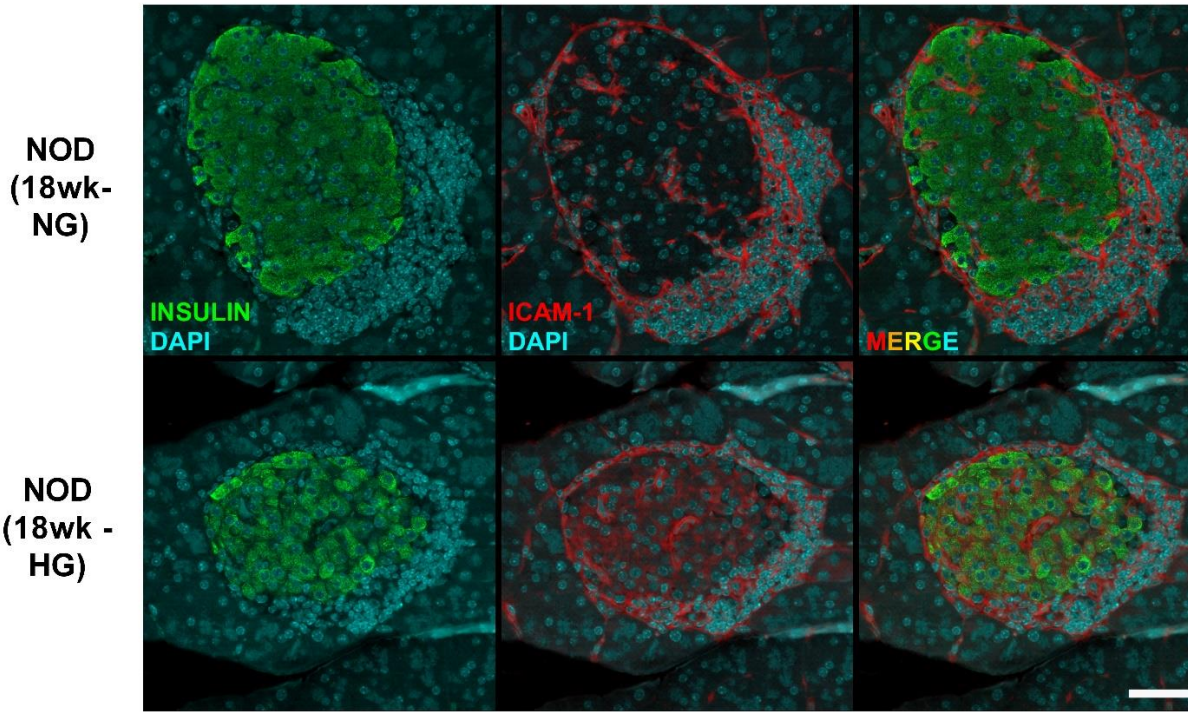

Figure 3.3. Hyperglyemic female NOD mice display ICAM-1 expression in pancreatic β-cells. Pancreatic tissue from female NOD mice were collected at 18 weeks of age after two consecutive measurements of fed blood glucose values > 250 mg/dL (HG). Age matched normoglycemic controls (NG) were also collected at the same necropsy. Formalin-fixed paraffin-embedded pancreatic tissue was sectioned and stained using antibodies against insulin (shown in green) and ICAM-1 (shown in red) and imaged using a 40x objective. The scale bar represents 50 microns.

IL-1β increases Icam1 gene promoter activity, mRNA content, and protein abundance in 832/13 cultured rat β-cells.

832/13 cells were exposed to IL-1β for time points ranging from 5 min to 24 h, with maximal expression occurring around 2-4 h (Figure 4A), consistent with elevations at 3 h observed in the RNA-Seq presented in Figure 1. A concentration response demonstrated that the *Icam1* gene responds to very small amounts of IL-1β, with as low as 0.01 ng/mL providing 13.6-fold increase in mRNA abundance (Figure 4B). We next investigated the role of interferon-gamma (IFN-γ) to potentiate the response of the *Icam1* gene to IL-1β. We found that the addition of IFN-γ increased the expression of *Icam1* by 1.63-fold over the IL-1β response (Figure 4C), although this observation did not reach the threshold for statistical significance used in this study. Using luciferase reporters with either -3kb or -1kb regions of the proximal gene promoter, we observed analogous responsiveness to cytokines as the endogenous gene, with the -3kb region, containing greater quantities of key NF-κB and GAS genomic regions, required for the greater response to

cytokines. In addition, ICAM-1 protein abundance was also elevated in response to IL-1β and IL-1β plus IFN-γ (Figure 4E). Secretion of ICAM-1 was significantly increased in 832/13 cells following a 6 h exposure to IL-1β (data not shown). The iNOS protein is shown as a known control for cytokine responsiveness [118, 141], while β-actin serves as a control for protein loading. Taken together, these results are consistent with the *Icam1* gene responding to β-cell exposure to IL-1β.

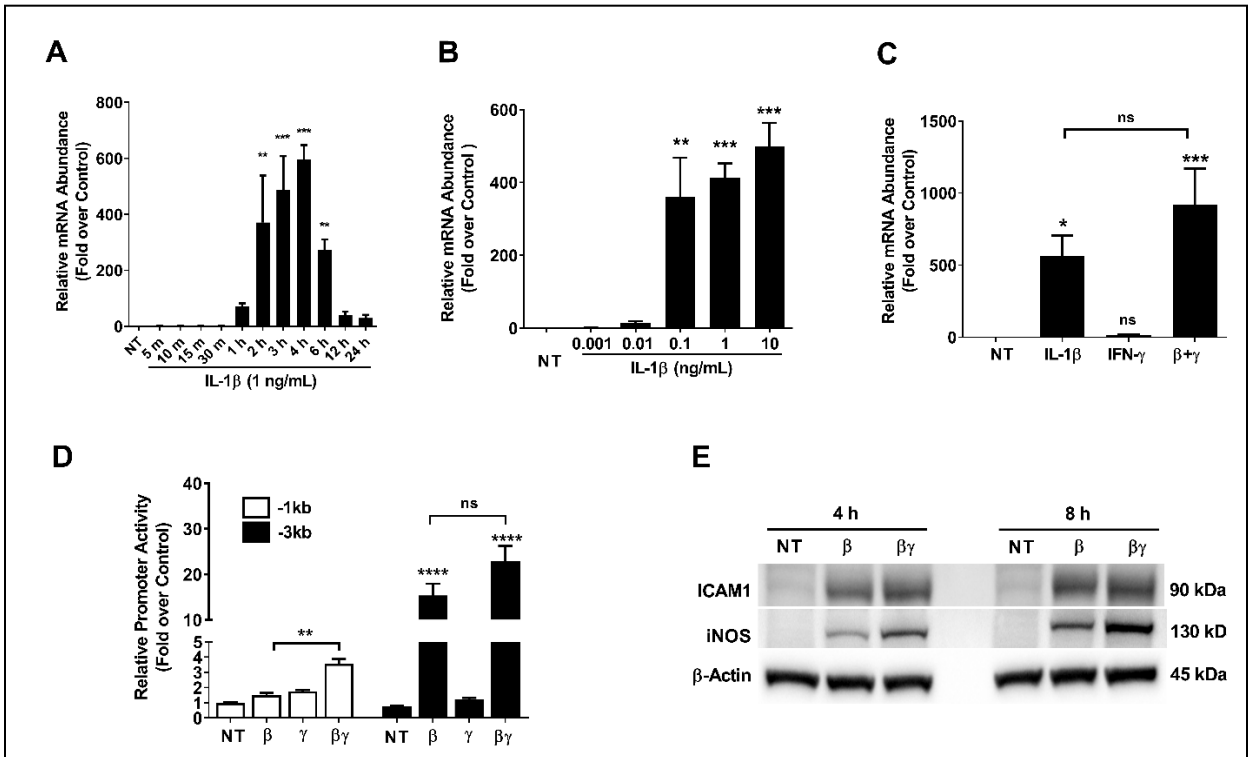

Figure 3.4. IL-1β increases *Icam1* gene promoter activity, mRNA content, and protein abundance in 832/13 cultured rat β-cells. **A.** 832/13 insulinoma cells were not treated (NT) or stimulated with 1 ng/mL IL-1β for the indicated times. **B.** 832/13 cells were either untreated (NT) or treated with the indicated concentrations of IL-1β for 3 h. **C.** 832/13 cells were treated with 1 ng/mL IL-1β, 100 U/mL IFN-γ, or a combination of both cytokines for 3 h. **D.** 832/13 cells were transfected with *Icam1* promoter luciferase constructs containing either -1kb or -3kb of the *Icam1* gene promoter. 24 h post-transfection, cells were either untreated or stimulated with 1 ng/mL IL-1β, 100 U/mL IFN-γ, or a combination of both cytokines for 4 h. Data are normalized to the respective NT control for each construct (-1kb and -3kb). **E.** Cells were untreated or treated with 1 ng/mL IL-1β or a combination of both IL-1β and 100 U/mL IFN-γ for either 4 h or 8 h. β-actin is shown as a control for protein loading. An image representative of two independent experiments is shown. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *p* values are vs. respective NT controls unless indicated otherwise in figures. ns = not significant.

Interleukin-1 receptor activation uses the NF-κB pathway to support enhanced Icam1 mRNA synthesis.

Islet β-cells display highly enriched expression of the interleukin-1 receptor (IL-1R) relative to other tissues [10, 142]. Using islets isolated from mice with pancreatic islet deletion of the IL-1R, we observed a 49% reduction in *Icam1* gene expression in response to IL-1β relative to control islets (Figure 5A). We next used TPCA, an inhibitor of the IKK complex downstream of the IL-1R, which reduced the ability of IL-1β to support *Icam1* gene expression (Figure 5B). As a complementary approach, we overexpressed a constitutively-active form of IKKβ (CA IKKβ), known to drive NF-κB response genes in β-cells and other tissues [128, 143], coupled with inhibition of NF-κB by a mutated form of the IκBα protein [termed the super-repressor; refs. [128, 144]]. We found that IKKβ alone drove a near 100-fold increase in expression of the *Icam1* gene and that the $I\kappa B\alpha^{SR}$ largely suppressed this response (Figure 5C; white bars). In response to IL-1β, CA IKKβ overexpression was redundant, which we interpret to indicate that IL-1R activation is maximal (Figure 5C; black bars). The $I\kappa B\alpha^{SR}$ dose-dependently reduced the expression of the *Icam1* gene in response to IL-1β even in the presence of CA IKKβ (Figure 5C; black bars).
We next used islets isolated from mice with pancreatic islet deletion of the p65 subunit of NF-κB, which is the major transcriptional subunit associated with activation of this pathway. The conditional alleles for p65 also include a mechanism for activating GFP expression using cre-mediated recombination [145]. Indeed, we saw that as p65 abundance decreased, GFP protein increased (Figure 5D; inset). In addition, IL-1β was unable to induce expression of the *Icam1* gene in islets from the $p65^{pdx1-/-}$ mice (Figure 5D). In 832/13 cells, siRNA-mediated silencing of p65 and p50 subunits of NF-κB also reduced the expression of the *Icam1* gene in response to IL-1β (Figure 5E). Because of the clear dependence of the *Icam1* gene on NF-κB, we next investigated whether increasing the abundance of p65 activated *Icam1* promoter activity to the level induced by exposure to IL-1β. Using a plasmid to deliver increasing expression of p65, we found that promoter luciferase activity was enhanced in a concentration-dependent manner (Figure 5F). We note that a plasmid expressing GFP was used to ensure that all cells received the same concentration of transfected DNA.

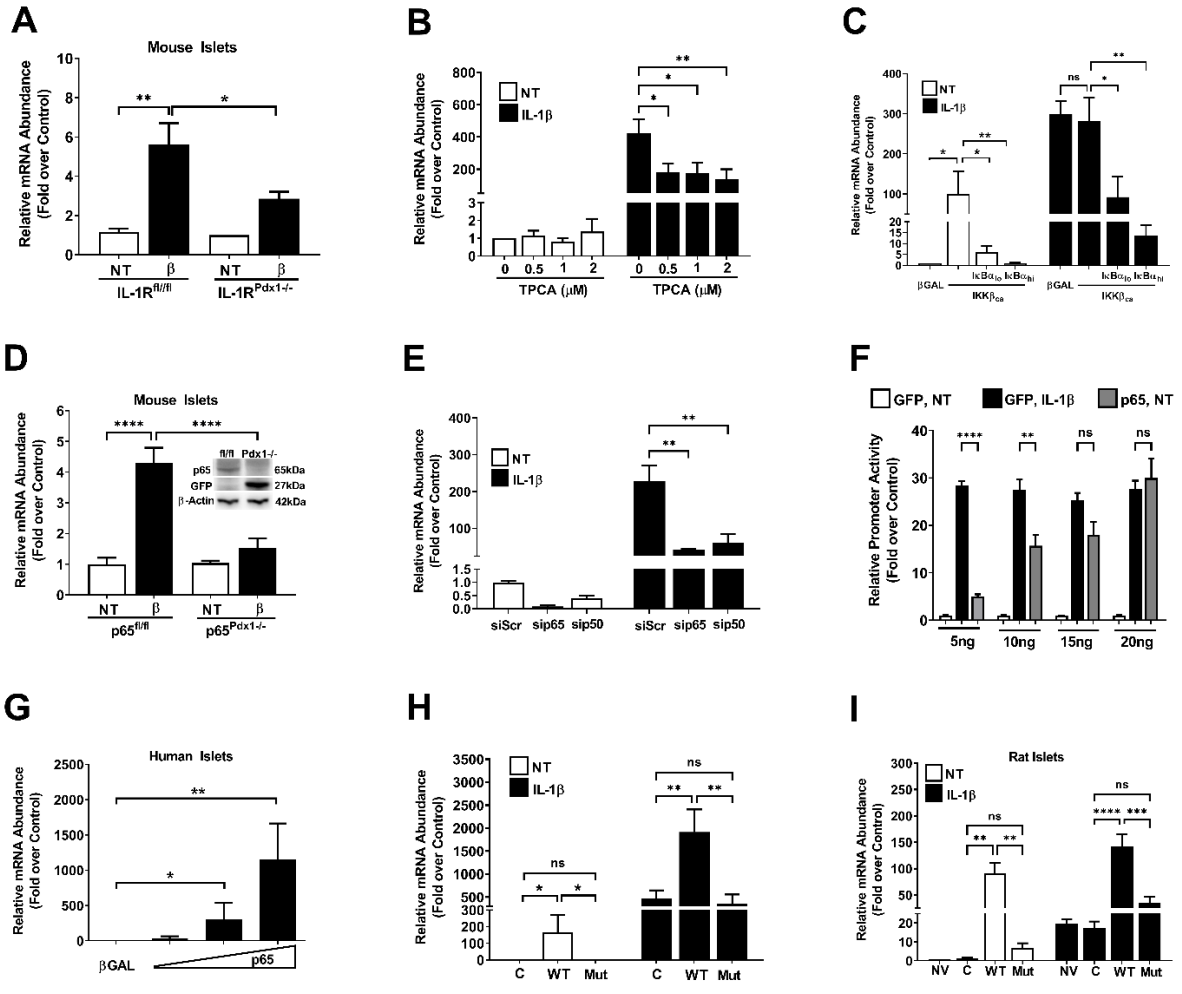

Figure 3.5. Interleukin-1 receptor activation uses the NF-κB pathway to support enhanced *Icam1* mRNA synthesis. **A.** Islets isolated from either control mice (IL-1R$^{\mathbf{fl/fl}}$), or mice with a pancreas-specific deletion of the IL-1R (IL-1R$^{\mathbf{Pdx1\text{-}/\text{-}}}$) were either untreated (NT) or stimulated with 10 ng/mL IL-1β for 3 h (n=5-8 per group). **B.** 832/13 cells were pre-treated with the indicated concentrations of TPCA for 1 h, followed by 3 h treatment with 1 ng/mL IL-1β. **C.** 832/13 cells were transduced with either a βGal control adenovirus or adenoviruses overexpressing either caIKKβ alone or a combination of the caIKKβ and IκBα$^{\mathbf{SR}}$. At 18 h post-transduction, cell were either left untreated (white bars) or exposed to 1 ng/mL IL-1β for 3 h (black bars). **D.** Islets isolated from either control mice (p65$^{\mathbf{fl/fl}}$), or mice with a pancreas-specific deletion of p65 (p65$^{\mathbf{Pdx1\text{-}/\text{-}}}$), were either NT or exposed to 10 ng/mL IL-1β for 3 h (n=6 per group). Inset shows an immunoblot for p65 and GFP with beta-actin as a loading control. **E.** 832/13 cells were transfected with siRNA duplexes targeting either the p65 or p50 subunits of NF-κB. At 18 h after transfection, the cells were either left untreated (white bars) or exposed to 1 ng/mL IL-1β for 3 h (black bars). **F.** 832/13 cells were transfected with 5 ng per well of the -3kB *Icam1* promoter-luciferase vector in combination with GFP (control) or the indicated concentrations of p65 plasmid. At 24 h post-transfection, cells were either NT or

exposed to 1 ng/mL for 4 h. **G.** Human islets were transduced with adenovirus expressing either βGal or p65. At 24 h post-transduction, islets were harvested for RNA extraction. **H.** 832/13 cells were transduced with adenoviruses expressing either βGal, p65, or $p65^{S276A}$. At 18 h after viral administration, cells were either NT (white bars) or exposed to 1 ng/mL IL-1β (black bars). **I.** Isolated rat islets were untreated or transduced with adenoviruses expressing either βGal, p65, or $p65^{S276A}$. At 18 h after viral administration, islets were either NT (white bars) or exposed to 10 ng/mL IL-1β (black bars).

Similar to what we observed with cultured 832/13 β-cells, we found that human islets transduced with a recombinant adenovirus expressing p65 also demonstrated a dose-dependent increase in the expression of the *Icam1* gene (Figure 5G). We next expressed either wild-type p65 or p65 with a Ser276Ala mutation, a site which regulates transcriptional activity of this NF-κB subunit [19, 146]. We found that in 832/13 cells, similar to human islets, p65 drove increased expression of the *Icam1* gene in the absence of IL-1β while the $p65^{S276A}$ did not have this ability (Figure 5H; white bars). Moreover, overexpression of p65 augmented expression of the *Icam1* gene in response to IL-1β while expressing the $p65^{S276A}$ construct did not (Figure 5H; black bars). Similar results were obtained using isolated rat islets (Figure 5I). Taken together, it is clear that p65 is necessary for the IL-1β induction of the *Icam1* gene and that p65 overexpression alone (in the absence of IL-1β) was sufficient to drive increased *Icam1* transcript abundance.

Site-directed mutation analyses within the Icam1 gene promoter reveal key genomic elements controlling transcriptional responses.

In silico analyses revealed the presence of several putative response elements, which included four κB elements and one gamma activated sequence (GAS; Figure 6A). These promoter sequences are conserved across mouse, rat, and human genomic DNA. Using a promoter construct with only the GAS element mutated (to prevent STAT1 binding), we found that there was a 25.2% decrease in IL-1β driven promoter activity and a 56.1% decrease in the IL-1β plus IFN-γ response (Figure 6B). This is consistent with other IL-1β responsive genes in β-cells that use STAT1 as an accessory factor to support the IL-1β response as well as signaling input from IFN-γ [117, 118]. Because of the clear dependence on the IKK/NF-κB pathway to support *Icam1* expression in response to IL-1β, we made mutations in each of the predicted κB elements within the *Icam1* gene promoter (noted κB1 – κB4 in Figure 6A). Mutations in the κB1 element reduced IL-1β-mediated activation of the *Icam1* promoter by 85.9% (Figure 6C), while mutations to the κB2 element were less severe, producing only a 34.2% decrease (Figure 6D). Moving further distal from the transcriptional start site, mutations in the κB3 element also strongly reduced promoter activity in response to IL-1β (Figure 6E) while κB4 mutations were more modest (Figure 6F). Collectively, we interpret this data to indicate that multiple κB elements within the *Icam1* gene promoter support transcriptional responses after exposure to IL-1β.

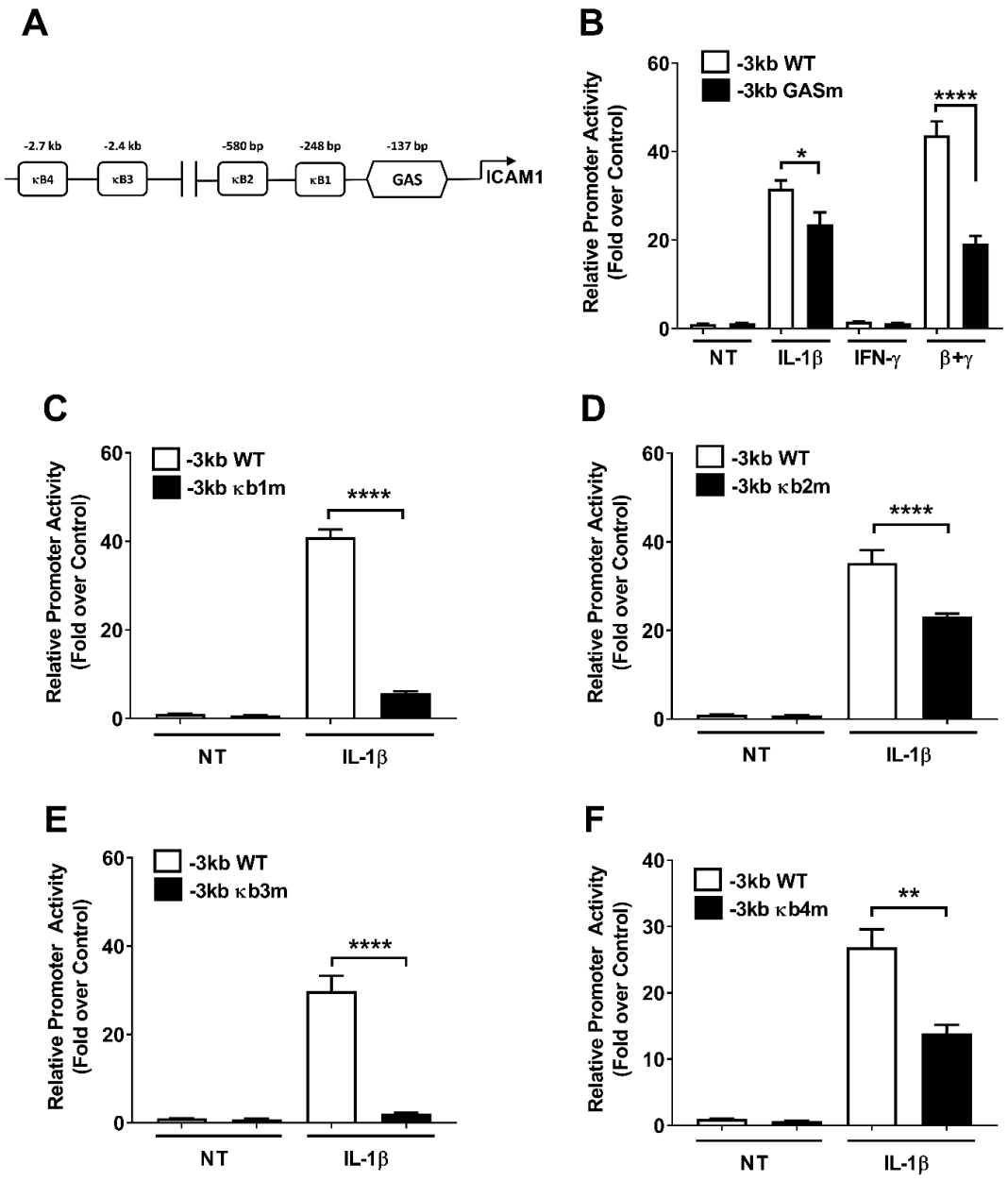

Figure 3.6. Site-directed mutation analyses within the *Icam1* gene promoter reveal key genomic elements controlling transcriptional responses. **A.** Schematic indicating the four computer software predicted NF-κB sites and one GAS element within the -3kb promoter region of the *Icam1* gene. **B-F.** Promoter luciferase data obtained using either wild-type (WT) or promoter constructs with each individual predicted genomic element mutated. 832/13 cells were transfected with promoter luciferase plasmids. At 24 h post-transfection, the cells were either untreated (NT) or exposed to 1 ng/mL IL-1β, 100 U/mL IFN-γ, or both cytokines for 4 h. Data are shown as means of the luciferase signal normalized to the NT control for each group. Error bars represent standard error of the means. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

IL-1β induces recruitment of the NF-κB p65 subunit to the Icam1 gene promoter regions containing functional κB elements.

Mutations within the κB1 region of the *Icam1* gene promoter strongly reduced transcriptional activity in response to IL-1β (Figure 6C and 6E). We next used chromatin immunoprecipitation (ChIP) assays to determine occupancy of p65 at each of the kB elements within the *Icam1* gene; the regions analyzed by ChIP are shown schematically in Figure 7A. Congruent with this finding, we observed occupancy of the κB1 site by p65 after exposure to IL-1β (Figure 7B) with a detectable trend for increased binding within 15 min that increased to 9.2- and 13.2-fold over IgG controls at 30 and 60 min after IL-1β exposure (Figure 7B). The binding of p65 at the κB2 site did not reach statistical significance (Figure 7C). We found that p65 occupancy at the κB3 site was also robust at 30 and 60 min after IL-1β exposure (Figure 7D), while binding at the κB4 site was considerably less proficient (Figure 7E).

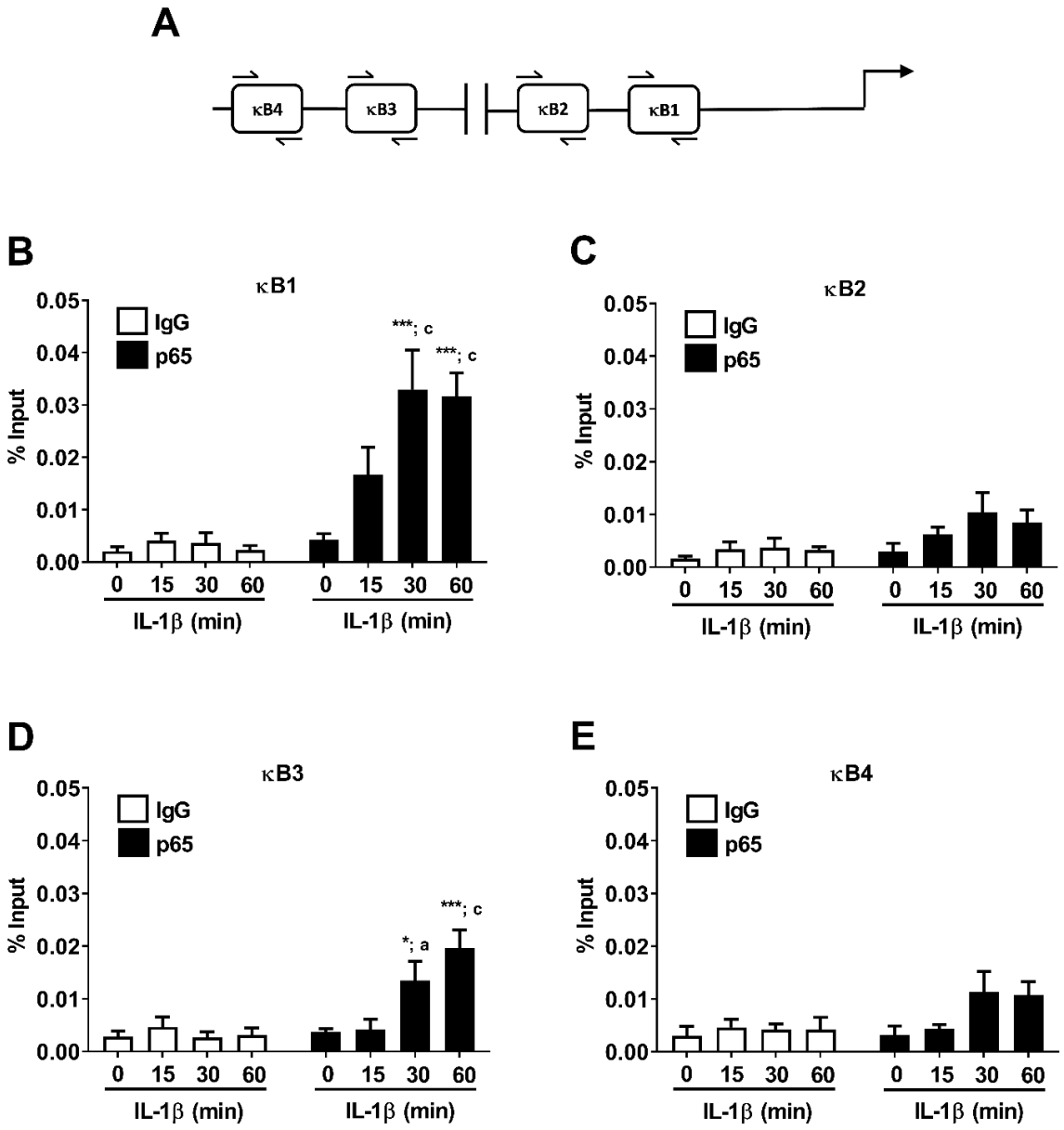

Figure 3.7. IL-1β induces recruitment of the NF-κB p65 subunit to the *Icam1* gene promoter regions containing functional κB elements. **A.** Schematic indicating the four NF-κB sites investigated using ChIP assays. **B-E.** 832/13 cells were untreated (0 min) or stimulated with 1 ng/mL IL-1β for the indicated times. ChIP assays were performed to determine p65 promoter occupancy at the indicated sites. Rabbit IgG was used as a negative control for the immunoprecipitation. Data is represented as percent of input, with 4-5 replicates of each condition. * $p < 0.05$ vs. no IL-1β treatment for p65 antibody (black bar), *** $p < 0.001$ vs. no IL-1β treatment for p65 antibody (black bar), [a] $p < 0.05$ vs. respective IL-1β treatment for IgG antibody (white bar), [c] $p < 0.001$ vs. respective IL-1β treatment for IgG antibody (white bar).

IL-1β promotes phosphorylation of carboxy-terminal domain regions of RNA polymerase II associated with the Icam1 gene promoter and coding regions.

RNA polymerase II displays a large tail region within the C-terminus of the protein that is subjected to phosphorylation during the initiation and elongation stages of transcription [147]. The genomic regions analyzed by ChIP are shown schematically in Figure 8A. We found that phosphorylation of Ser5, a marker of transcription initiation, was enhanced at the transcriptional start site of the *Icam1* gene promoter after exposure to IL-1β (Figure 8B; green bars).

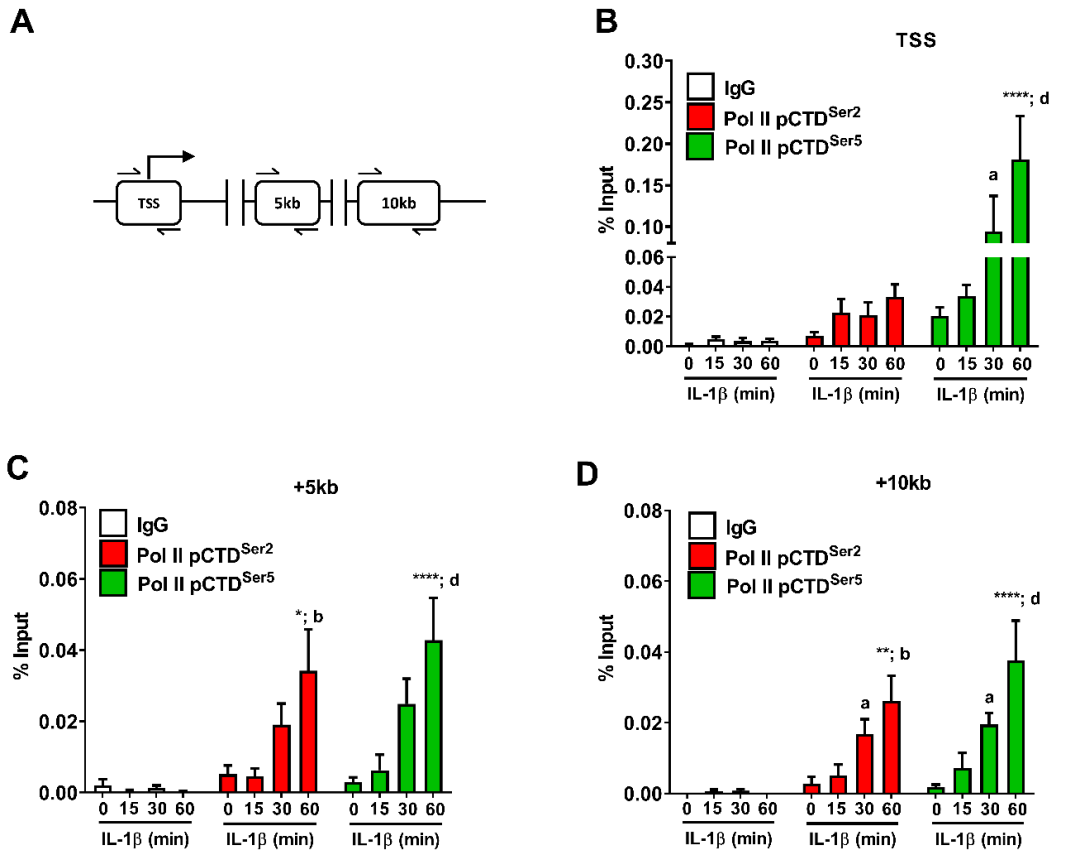

Figure 3.8. IL-1β promotes phosphorylation of carboxy-terminal domain regions of RNA polymerase II associated with the *Icam1* gene promoter and coding regions. **A.** Schematic indicating the transcriptional start site (TSS) and two coding regions investigated using ChIP assays. **B-D.** 832/13 cells were untreated (0 min) or stimulated with 1 ng/mL IL-1β for the indicated times. ChIP assays were conducted to investigate RNA Polymerase II (phospho-S5 or phospho S-2) occupancy for the indicated sites. Rabbit IgG was used as a negative control for the immunoprecipitation. Data is represented as percent of input, with 4 replicates of each condition. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$, [a] $p < 0.05$, [b] $p < 0.01$, [d] $p < 0.0001$. Letters compare to same IL-1β treatment time for IgG antibody; asterisks compare to 0 min time-point of respective antibody.

Phosphorylation of Ser2, a marker associated with transcriptional elongation (i.e., moving of the RNA Pol II through the coding region), was not enriched at the transcriptional start site, as would be expected (Figure 8B; red bars). Indeed, after 60 min of IL-1β exposure, there was an 85.5-fold increase in Ser2 phosphorylation over IgG control at the +5kB region (downstream of the transcriptional start site; Figure 8C; red bars), which was maintained out to the +10kB region (Figure 8D; red bars). The overall signal strength for Pol II Ser5, while still present over baseline, was reduced in magnitude at the +5kB and +10kB genomic regions (Figures 8C and 8D; green bars). This is consistent with observations at other cytokine responsive genes in pancreatic β-cells [117] and signal-specific gene regulation in other model systems [148].

Histone chemical modifications associated with the Icam1 gene are regulated in response to IL-1β.

Signal-specific gene transcription is often associated with alterations in chemical modifications to histones. Modifications of histone H3 at lysine 4 (H3K4) are often found within gene promoters and are typically well conserved across species [149]. Here we investigated changes in both mono- (H3K4me1) and tri-methylation (H3K4me3) of H3K4 as well as acetylation of H3K4 (H3K4ac) in the *Icam1* gene in response to IL-1β (regions shown schematically in Figure 9A). We found that the H3K3me1 and H3K4me3 signals were not above what was observed for the IgG controls in the genomic region containing the κB3 element while the H3K4ac modification was responsive to IL-1β in this region (Figure 9B; blue bars). The κB1 element displayed robust H3K4me3 signal, which decreased in response to IL-1β (Figure 9C; green bars) while the H3K4ac signal in the region containing κB1 remained similar to that seen in κB3 containing portion of the *Icam1* gene promoter (Figure 9C; blue bars). The genomic regions containing the *Icam1* transcriptional start site displayed strong enrichment for H3K4me3 and this chemical signature was not affected by IL-1β exposure (Figure 9D; green bars). H3K4 acetylation was enriched over baseline and increased significantly in response to IL-1β (Figure 9D; blue bars). When examining the region of the *Icam1* gene 5kb downstream of the transcriptional start site (+5kb), we note that H3K4 acetylation was present in the baseline state (no IL-1β) and stayed enriched during IL-1β exposure (Figure 9E; blue bars). A schematic representation showing p65 occupancy at the genomic regions of greatest occupancy (κB1 and κB3) with associated histone markings associated with the *Icam1* gene is given in Figure 10.

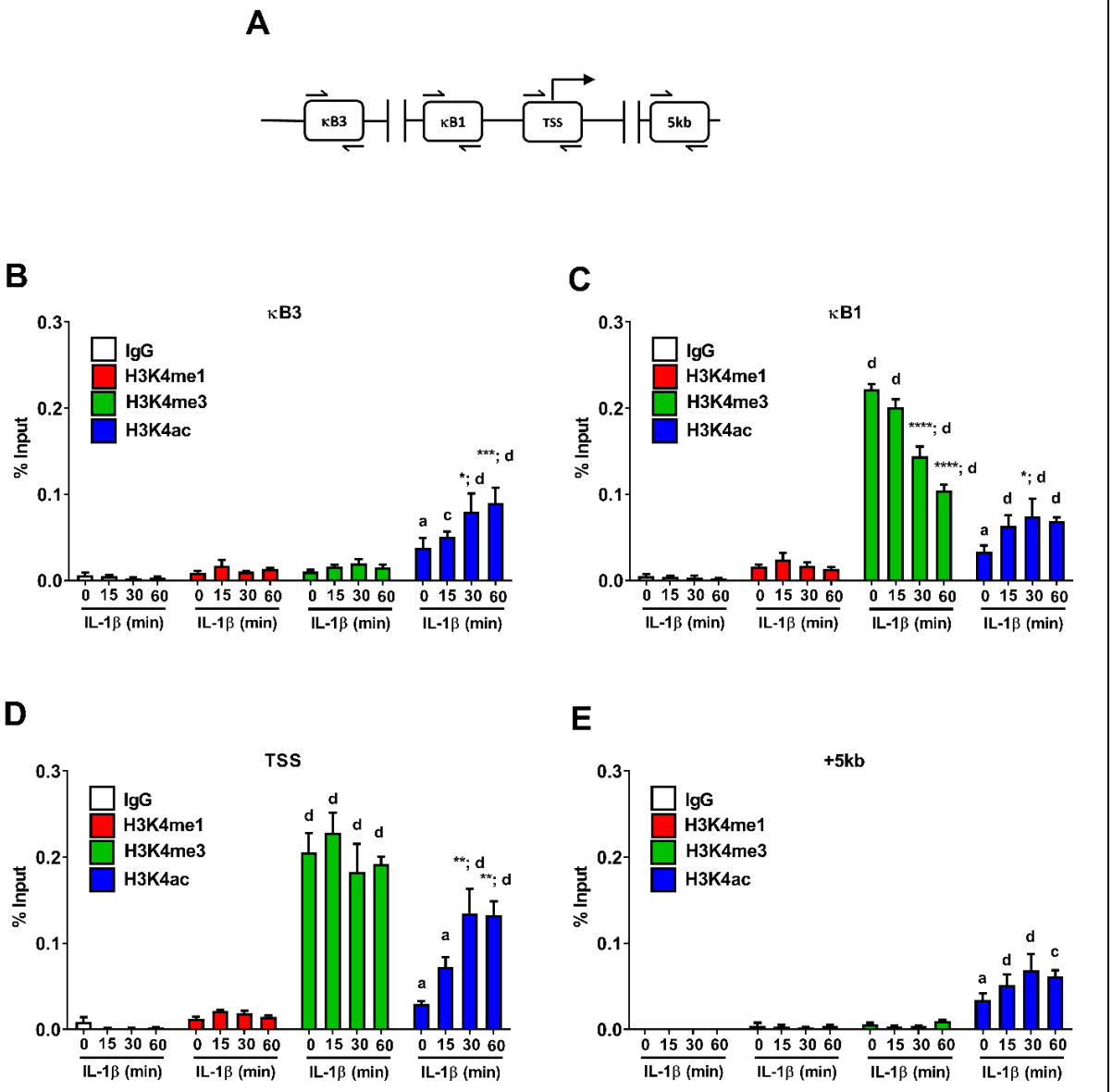

Figure 3.9. Histone chemical modifications associated with the *Icam1* gene are regulated in response to IL-1β. **A.** Schematic indicating the sites investigated for binding events using ChIP assays. **B-E.** 832/13 cells were untreated (0 min) or stimulated with 1 ng/mL IL-1β for the indicated times. ChIP assays were used to determine histone chemical modification (H3K4me1, H3K4me3, or H3K4ac) at the indicated sites. Rabbit IgG was used as a negative control. Data is represented as percent of input, with 3-4 replicates of each condition. Letters compare same IL-1β treatment time for IgG antibody; asterisks compare to 0 min time-point of respective antibody. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. [a] $p < 0.05$, [c] $p < 0.001$, [d] $p < 0.0001$.

3.4. Discussion

The *Icam1* gene encodes a membrane-associated protein that contributes to various immune system functions, including trafficking, docking, and signaling-based alterations in leukocyte activity [69, 125]. For example, T-cells express the integrin LFA-1, which is composed of CD11a and CD18, and interacts with ICAM proteins, particularly ICAM-1 [123]. The activities of both CD4 and CD8 T-cells are influenced through this LFA-1/ICAM-1 interaction [150, 151]. Consequently, ICAM-1 is a critical contributor to a multitude of immune cell activities and is thus important for physiological responses. Dysregulation of ICAM-1 expression or the ICAM-1 / LFA-1 interactions also can become pathological and contribute to autoimmune and other diseases. One such autoimmune disease clearly influenced by ICAM-1 is Type 1 diabetes [41].

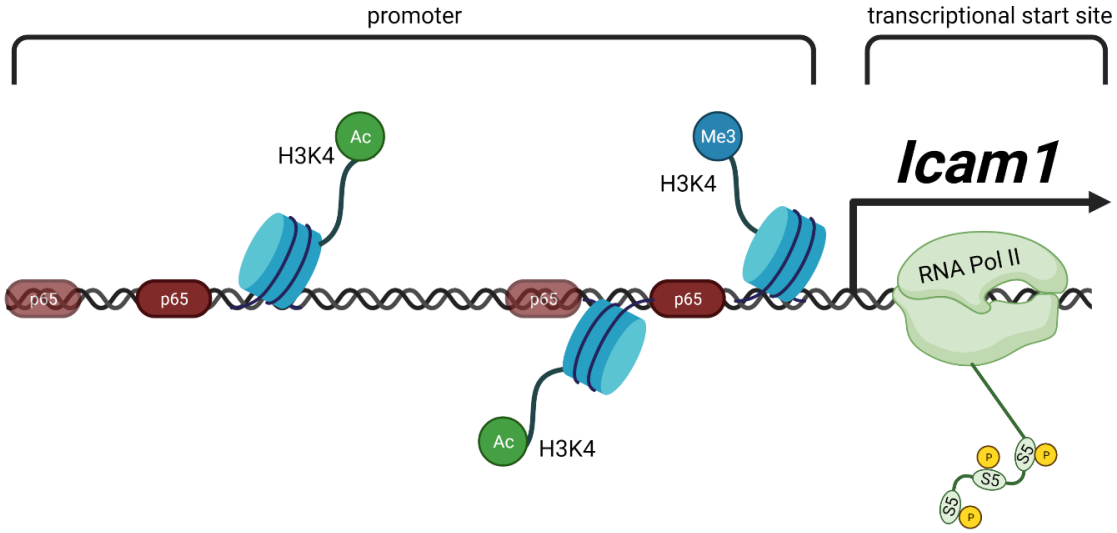

Figure 3.10. Schematic representation of the *Icam1* transcriptional response to IL-1β. In response to IL-1R activation by IL-1β, β-cells increase the abundance of *Icam1* mRNA. This transcriptional activity requires recruitment of the NF-κB p65 transcriptional subunit, histone chemical modifications, and occupancy of RNA polymerase II at sites within the proximal *Icam1* gene promoter. Figure created using BioRender.com.

Many cell types, including pancreatic β-cells, express *Icam1*; the *Icam1* gene is highly responsive to cytokines, with positive regulation by interleukin-1β and interferon-gamma [67, 106]. However, many studies investigate the regulation of the *Icam1* gene after 18-24 hours of

cellular exposure to an inflammatory stimulus, such as a pro-inflammatory cytokine. Because we are interested in early genetic reprogramming events that are likely to change the status of pancreatic β-cells and their interaction with the immune system, we investigated β-cell exposure to IL-1β at much earlier time points. We started with a bulk RNA-seq approach using human islets and 832/13 rat insulinoma cells (Figure 1) and found that *Icam1* was an early response gene in both human islets and rat insulinoma cells. Consequently, we report several key discoveries about the early regulation of the *Icam1* gene in pancreatic β-cells and also show that the abundance of the ICAM-1 protein is enhanced by both systemic inflammatory signals and in situations of organ-specific autoimmunity.

We first identified *Icam1* as highly and rapidly responsive (within 3 hours) to the cytokine interleukin-1β in both rat β-cell line 832/13 and in cultured human islets (Figure 1). Further studies using mouse, rat, and human islets indeed showed that the *Icam1* gene was also an early response gene across species after β-cell exposure to IL-1β (Figure 2A, 2B, and 2C). These increases in gene activity in culture can also be recapitulated *in vivo* using C57BL/6J mice injected with IL-1β, where ICAM-1 protein abundance was greater in pancreatic tissue four hours after cytokine injection (Figure 2D). Further supporting a role for ICAM-1 in situations of autoimmunity, we also found that islets isolated from female NOD mice have increased *Icam1* gene expression relative to male mice (Figure 2E). Moreover, hyperglycemic female NOD mice show elevated *Icam1* transcripts (Figure 2F) and protein abundance (Figure 3) relative to their age-matched normoglycemic counterparts. Collectively, this data helps to partially explain the sexual dimorphism in NOD mice (females have much higher incidence of disease) and supports the notion that ICAM-1 is a critical contributor to the autoimmune responses that lead to Type 1 diabetes [41]. We note that both immune cells and islet β-cells are capable of expressing ICAM-1 in the NOD mouse (Figure 3 and Supplementary Figure 2). This is likely to be an important component of the disease phenotype that will be explored in further detail in future studies.

While it is clear that ICAM-1 participates in the severity of insulitis and eventual onset of diabetes in NOD mice [41], the genetic control of this gene in pancreatic β-cells is incompletely understood. Using a multitude of genetic and molecular approaches, our novel data now reveals that expression of the *Icam1* gene is clearly dependent on the NF-κB pathway. Tracing the NF-κB pathway from cell membrane to nucleus demonstrates a reliance on IL-1R signaling to support IL-1β induced-expression (Figure 5A). Pharmacological inhibition of IKK activity reduces IL-1β-mediated *Icam1* expression (Figure 5B), while overexpression of constitutively-active IKKβ enhances expression of the *Icam1* gene (Figure 5C). In addition, genetic reduction of the p65 subunit of NF-κB reduces expression of the *Icam1* gene during exposure to IL-1β (Figure 5D). Furthermore, siRNA-mediated silencing of p65 in 832/13 cells also reduced the ability of IL-1β to induce expression of the *Icam1* gene (Figure 5E). We further found that the *Icam1* gene promoter (Figure 5F) and human *Icam1* gene are sensitive to increases in p65 abundance (Figure 5G). Moreover, eliminating the phosphoacceptor site at position 276 within the p65 subunit also reduced the ability of the *Icam1* gene to respond to increases in p65 abundance (Figure 5H and 5I; white bars) as well as to IL-1β (Figure 5H and 5I; black bars). These observations are also consistent with elevated *Icam1* expression in NOD mice, a model of autoimmunity, and in a separate model of systemic cytokine signaling (using i.p. injection of IL-1β) which each revealed increases in *Icam1* expression in the pancreatic islets (Figure 2D, Figure 2E, Figure 2F, and Figure 3).

We next sought to understand the molecular details underlying genomic control of the *Icam1* gene in response to IL-1β. Computer-based prediction software identified several possible κB elements and one GAS element in the -3kb region of the gene promoter (shown schematically in Figure 6A). Site-directed mutagenesis revealed that the κB elements designated κB1 and κB3 contributed strongly to the ability of IL-1β to activate this gene promoter (Figures 6C and 6E). These data are consistent with the observed occupancy of p65 at genomic regions containing these sites (Figures 7B and 7D). Moreover, total RNA polymerase II is recruited to the gene promoter in response to IL-1β prior to the first appearance of transcript (data not shown). In addition, RNA polymerase II phosphorylated at Ser5 within the carboxy terminal domain was present at the transcriptional start site within 30 and 60 minutes after cellular exposure to IL-1β (Figure 8B). This observation is consistent with initiation of gene transcription [148] and with specific appearance of *Icam1* mRNA at 2-4 hours after exposure to IL-1β (Figure 4A). Phosphorylation of RNA Pol II at Ser5 increased within the coding region of *Icam1* consistent with elongation of transcription (Figures 8C and 8D). We interpret this data to indicate that *Icam1* is an important early responsive gene that is transcriptionally activated in β-cells after exposure to IL-1β.

The increase in transcription of the *Icam1* gene also correlated with changes in specific histone chemical modifications. Acetylation of histone H3 at lysine 4 (H3K4) increased in the genomic region containing the κB3 element in response to IL-1β (Figure 9B). This change in chemical modification could be to facilitate p65 binding, or alternatively, may occur in response to p65 occupancy and associated assembly of a multi-regulatory transcriptional complex. We further found that the region containing the κB1 element demonstrated greater H3K4 trimethylation (H3K3me3) at baseline, which was reduced in response to IL-1β (Figure 9C). The genomic region containing the transcriptional start site retained H3K4me3 markings during all times IL-1β was present (Figure 9D). We suspect these markings indicate an active gene promoter and that perhaps the biological significance of the reduction in H3K4me3 at the κB1 site indicates RNA Pol II clearance. This could be consistent with H3K4me4 markings retained at the transcriptional start site for anchoring another round of TFIID for re-initiation of successive rounds of transcription [152]. Collectively, p65 occupancy, histone chemical modifications, and recruitment of RNA Pol II occur in response to IL-1β to enhance *Icam1* transcription in β-cells (Figure 10).

Taken together with existing knowledge that genes encoding chemokines, such as CCL2, CXCL1, CXCL2, CXCL10, and CCL20, are also highly responsive to IL-1β in pancreatic β-cells [113, 117, 119, 120, 128], it is likely that this coordinated program of gene expression (i.e., chemokines plus *Icam1*) will both recruit and retain immune cells in close proximity to islet β-cells. The dysregulation of this genetic control may lead to overactive chemokine production, enhanced ICAM-1 abundance, and the insulitis observed in T1D. Even if insulitis is mild, as may be true for many cases of T1D in humans [153], the combination of elevated chemokines and ICAM-1 could influence severe targeting of β-cells by antigen-primed leukocytes that leads to reduced insulin secretion and eventual decreases in total numbers of β-cells. Thus, strategies aimed at a greater understanding of the molecular events that lead to dysregulated crosstalk between β-cells and immune cells will provide greater understanding of the T1D disease process.

Chapter 4. IL-1β Induces Rapid Transcription of the *Stx11* Gene in β-Cells via NF-κB

4.1. Introduction

A defining feature of eukaryotic cells is their ability to segregate biological activities within membrane-bound compartments. Cellular materials can be trafficked between the different organelle compartments via specialized proteins that assist with the movement of vesicles. One class of proteins, called Soluble *N*-ethylmaleimide-Sensitive Factor Attachment Protein Receptors, or SNAREs, is well-known to provide support for movement of proteins contained within vesicles. The most common categorization of SNARE assembly is that of t-SNAREs and v-SNAREs: two proteins on the target membrane contribute three alpha helices to the complex, and one protein on the vesicle-associated membrane contributes the fourth alpha helix [55]. Once brought into close contact, the helix domains zip together into a tight, thermodynamically-favored four-helix bundle, allowing the two membrane compartments to join together [154]. This process is vital for many cellular tasks such as lysosomal degradation [155], exocytosis [156], and neuronal activity [157]. Other proteins coordinate with the SNARE complex: NSF and SNAP work to disassemble the helical bundle, SM proteins act as clasps that regulate the binding events, and synaptotagmin operates as a calcium sensor for SNARE activity [55]. Although humans have a greater number of SNARE genes than the unicellular yeast *S. cerevisiae*, the core proteins and features remain largely unchanged between the two species, indicating that this pathway is highly conserved among eukaryotes [158].

The typical SNARE complex comprises syntaxin and SNAP-23/25 acting as t-SNAREs contributing one and two helices, respectively, and synaptobrevin (VAMP) acting as a v-SNARE contributing the fourth [154]. The Sec1/Munc18 (SM) proteins regulate this complex, particularly through action on syntaxin [159]. Diversity of these components is a mechanism by which cells can specialize the various membrane fusion tasks. In β-cells, there are already established roles for certain SNARE proteins in glucose stimulate insulin secretion (GSIS), and this regulated secretion of secretory granules (SGs) proceeds in two phases. First phase secretion of pre-docked SGs is largely mediated by a SNARE complex of Stx1a, SNAP-25, and VAMP-2, regulated by SM protein Munc18a [160, 161]. Newcomer granules (i.e. not pre-docked) in both first- and second-phase insulin secretion form a different complex of Stx3, SNAP-25, and VAMP-8, regulated by Munc18b [160]. Stx1a has also been found to be involved in replenishment of SGs during second-phase secretion [162], and its expression is reduced in T2D patients [163]. Recent research has implicated other proteins in the process of insulin secretion, such as Stx4 [164], Munc18c [165], and tomosyn [166]. Much of the research on SNAREs and insulin secretion is ongoing, so further mechanisms and proteins involved likely remain to be discovered.

One SNARE protein whose function in the β-cell remains unknown is Syntaxin 11. Unlike the rest of the known syntaxins, Stx11 does not possess a hydrophobic transmembrane domain [56, 167]; rather, its membrane association appears to be partially dependent on the acylation of its cysteine-rich C-terminus [168]. The role of Stx11 in several cell types has already been established. For example, Stx11 aids in the exocytosis of lytic granules in T cells [169] and is required for platelet secretion [59]. Further contexts in which Stx11 has been observed include the Golgi network of Hela cells [58], GH-containing granules in pituitary cells [170], and the plasma membrane of human macrophages [171]. Mutations in *STX11* and *STXBP2* (encoding its

regulatory protein Munc18b) are known to cause familial hemophagocytic lymphohistiocytosis (FHL) type 4 and 5, respectively [172, 173].

Using bulk RNA-Seq, we initially identified strong upregulation of the *Stx11* gene in response to IL-1β in both rat and human β-cells. Considering the vital importance of NF-κB signaling in islet inflammation, Stx11 may be a crucial element in understanding islet β-cell responses to cytokine exposure. In this chapter we demonstrate that IL-1-stimulated *Stx11* gene expression in β-cells is indeed regulated by NF-κB, and we identify relevant p65 binding sites within the *Stx11* promoter. Furthermore, we show that, among many proteins known to interact with Stx11 and the SNARE proteins involved in insulin secretion, *Stx11* is the only gene whose expression is upregulated by IL-1 signaling.

4.2. Materials and Methods

Cell Culture, Adenoviruses, and Reagents

Culture and passage of the 832/13 rat insulinoma cell line has been described [127]. All cytokine treatments occurred in serum-free RPMI 1640 (Gibco). Cell lines were confirmed to be free of mycoplasma contamination using the Lonza MycoAlert Mycoplasma Detection Kit. Recombinant IL-1α, IL-1β, TNF-α, and IFN-γ were from PeproTech (Cranbury, NJ). Inhibitor of p38 is SB202190 (Tocris), which inhibits the α and β isoforms of p38 MAP kinase.

Promoter analysis and construction of luciferase plasmids

Four putative NF-κB genomic binding elements were detected in the 3 kb upstream of the *Stx11* transcriptional start site using JASPAR's web-based *in silico* promoter analysis tool [131]. DNA fragments containing either the 1 kb or 3 kb upstream promoter region of the rat *Stx11* gene were amplified. Each fragment was then digested with *XhoI* and *HindIII* Fast Digest restriction enzymes (Thermo Fisher) and cloned into luciferase reporter pNL1.2 (Promega). Mutations were generated in both reporter plasmids by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). Each construct was verified by sequencing at the Pennington Biomedical Research Center Genomics Core Facility.

Transient Transfections and Luciferase Assays

832/13 cells were grown to 75% confluence in 24-well plates. Luciferase reporter plasmids were transfected into cells using TransFectin Lipid Reagent (Bio-Rad # 1703351) according to the manufacturer's instructions. 16 h post-transfection, cells were treated as indicated in the respective figure legends. Cells were lysed in 50 uL Nano-Glo Luciferase Assay Reagent (Promega) for 10 min with rocking at room temperature. Luminescence was measured with a Glo-Max Multi+ Luminometer (Promega).

RNA Extraction, cDNA Synthesis, and Gene Expression Analysis

Our procedures and reagents for isolation of RNA from cell lines and islets, cDNA synthesis, and transcript analysis by real-time PCR have all been reported [129, 133]. PCR analysis was conducted using a Bio-Rad CFX 1000 thermal cycler.

Chromatin Immunoprecipitation

Cells were grown to 75% confluence in 10 cm dishes and serum starved in RPMI for 1 h before treatment with cytokine. After treatments, cells were washed in PBS and then crosslinked in ChIP Crosslink Gold (Diagenode) for 30 minutes with rocking. Cells were washed and then crosslinked again in 1% formaldehyde in PBS for 10 min, followed by quenching with glycine at 125 mM for 5 min. Cells were scraped in PBS with Halt Protease Inhibitor Cocktail (Thermo Scientific) and pelleted with centrifugation. Pellets were resuspended in 950 μL lysis buffer (1% SDS, 0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 0.5 mM DTT, pH = 8.0) and lysed for 30 min at 4ºC with rotation. Lysates were transferred to polystyrene tubes (Evergreen Scientific) and sonicated to an average fragment size of 175 bp in in a Bioruptor Pico with 10 cycles of sonication. Sonicated lysates were cleared by centrifugation, and fragment size was confirmed by agarose gel electrophoresis. To prepare for IP, 100 μL of lysate and 900 μL of Dilution Buffer (1.1% Triton X-100, 0.01% SDS, 17 mM Tris, 1.2 mM EDTA, 167 mM NaCl, pH = 8) were combined for each IP reaction. These were rotated overnight each with 5 μL Protein G Dynabeads (Thermo Scientific) at 4ºC for preclearing. For each IP, 5 μL of Dynabeads were bound to 1 μg antibody for 30 min in PBS-TWEEN, followed by 30 min of blocking in 10% BSA in PBS. Antibody-Dynabead complexes were then added to diluted lysates and immunoprecipitated for 1 h. IP reactions were then transferred to new microfuge tubes and sequentially washed with the following buffers: twice with Low Salt Wash (0.1% SDS, 1% Triton X-100, 0.05% TWEEN, 20 mM Tris, 2 mM EDTA, 150 mM NaCl), twice with High Salt Wash (0.1% SDS, 1% Triton X-100, 20 mM Tris, 2 mM EDTA, 500 mM NaCl), twice with LiCl Wash (1% NP-40, 1% sodium deoxycholate, 0.25 mM LiCl, 10 mM Tris, 1 mM EDTA), and once with TE (10 mM Tris, 1 mM EDTA). Beads were then resuspended in 150 μL ChIP Elution Buffer and, along with input samples, were incubated at 65ºC for 30 min with shaking in a thermomixer. After elution, supernatants were transferred to clean microcentrifuge tubes for overnight decrosslinking with Proteinase K and 200 mM NaCl at 65ºC. DNA was extracted from ChIP samples and inputs using GenCatch Advanced PCR Extraction Kit (Epoch Life Science). The authors may be contacted if a more detailed protocol of this procedure is desired.

Statistical analysis

Statistical analysis was conducted using Prism version 9.3 (GraphPad). One or two-way ANOVA analyses were used to calculate the *p*-values indicated in the figure legends.

4.3. Results

Stx11 *mRNA expression is inducible by IL-1β*

Using the 832/13 rat insulinoma cell line as a differentiated, highly glucose-sensitive cell culture model for islet β-cells, IL-1β exposure at 1 ng/mL was sufficient to drive 27.8-fold expression of *Stx11* by 1 h, with peak transcript of 46.2-fold observable at 2 h (Fig 1A). Exposure of 832/13 cells to 40ng/mL of TNF-α, another known NF-κB ligand, was also capable of driving 11.9-fold transcript accumulation at 3 h of treatment (Fig 1B). To test the hypothesis that induction of the *Stx11* gene by IL-1β is potentiated by the additional presence of 100 U/mL of IFN-γ, 832/13 cells were stimulated by both cytokines for 3 h; the results indicate that no augmented expression is brought about by IFN-γ (Fig 1C). This is important because many IL-1β responsive genes in β-cells are augmented in the presence of IFN-γ [6, 141, 174].

We then examined the expression of the *Stx11* gene and a variety of other SNARE and SNARE-associated genes in the presence of low or high glucose. As shown in Fig 1D, *Stx11*

Table 4.1. Cycle threshold (Ct) values for select SNARE and SNARE-associated genes. 832/13 cells at 100% confluence were exposed to RPMI with 10% FBS at 5 mM glucose overnight. Cells were then incubated for 3 h in HBSS in glucose concentrations of either 3 or 15 mM, and either not treated (NT) or stimulated with 1 ng/mL IL-1β.

gene	3 mM NT	3 mM IL-1β	15 mM NT
Rs9	24.01	23.80	23.88
cyclophilin	20.27	20.65	20.42
Stx11	34.93	29.87	34.22
Stx1a	30.00	29.88	29.70
Stx3	30.07	29.51	29.73
Stx4	26.01	25.79	25.81
Syt4	29.67	29.56	29.08
Syt5	29.36	29.34	28.58
Syt7	32.00	31.91	31.45
Stxbp1	27.27	27.37	27.09
Stxbp2	30.59	30.68	30.41
Stxbp3	27.49	27.75	27.57
Stxbp5	36.63	36.14	37.07
Syp	27.88	27.62	27.41
Vamp2	29.17	29.09	28.35
Vamp3	27.57	27.40	27.52
Vamp4	32.53	32.27	32.87
Vamp7	[N/A]	[N/A]	[N/A]
Vamp8	24.45	24.17	24.30
Snap23	29.18	29.03	29.41
Snap25	29.69	29.62	29.74
Snap29	30.91	30.82	30.45

gene expression was not different between 832/13 cells treated in 3 mM glucose and cells in 15 mM glucose. Similarly, glucose concentration did not alter the IL-1β-stimulated expression (Fig 1D). Fig 1E shows the fold response compared to IL-1β of selected SNARE and SNARE-related genes in low glucose. Interestingly, *Stx11* was the only gene whose expression increased upon IL-1β exposure, at 29.2-fold of the untreated control. Despite the reliable transcriptional induction of *Stx11* mRNA by cytokines, Western blot of IL-1β-treated cells indicates that total Stx11 protein levels remain unchanged after 6 h (Fig 1F). Table 1 shows cycle threshold values of all the genes included in the above analysis, indicating that most genes had basal expression that was not changed by cellular exposure to IL-1β.

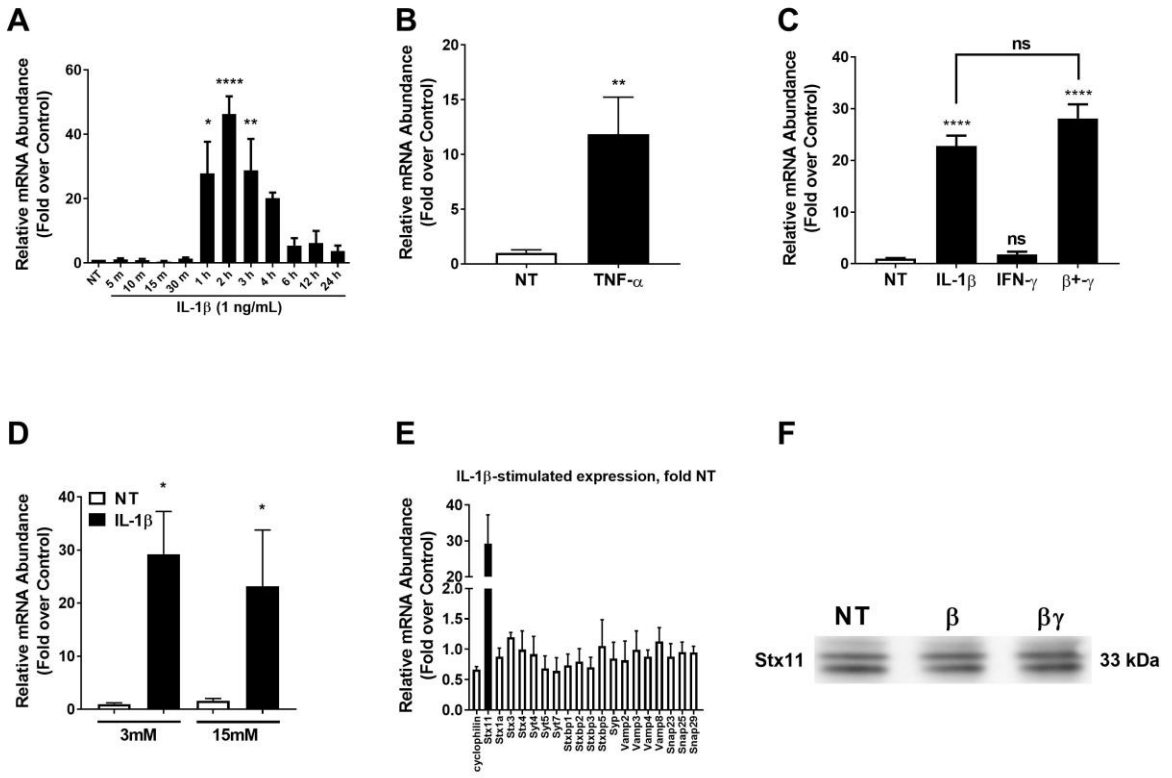

Figure 4.1. *Stx11* mRNA expression is inducible by IL-1β. (A) 832/13 cells were either not treated (NT) or stimulated with 1 ng/mL IL-1β for the indicated times. (B) 832/13 cells were either NT or stimulated with 40 ng/mL TNF-α for 3 h. (C) 832/13 cells were treated with 1 ng/mL IL-1β, 100 U/mL IFN-γ, or a combination of both cytokines for 3 h. (D) 832/13 cells were exposed to HBSS with glucose at the indicated concentrations with or without 1 ng/mL IL-1β for 3 h. (E) Depicts the IL-1β response divided by the NT response of each gene from the 3 mM glucose treatments in Fig 1D. (F) Cells were NT or exposed to either 1ng/mL IL-1β alone or in combination with IFN-γ for 6 h. *$p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns = not significant. p values are versus respective NT controls unless otherwise indicated in the figure.

Luciferase-promoter construct responses mirror RNA expression data, and Stx11 *expression is partially dependent on NF-κB p65*

The 3 kilobase genomic region upstream of the rat *Stx11* transcriptional start site was cloned into a luciferase reporter plasmid. This plasmid was transfected overnight into 832/13 cells, and on the following day the cells were exposed to cytokines for 4 h. As shown in Fig 2A, luciferase activity increased 53.4-fold in response to IL-1β; no potentiation was observed in the presence of IFN-γ. This transcriptional response was just as potent with 1ng/mL of either IL-1α or IL-1β (Fig 2B). 0.1 ng/mL, a suboptimal dose of IL-1β, increases luciferase activity by 14.5-fold over control, which is 66% less than the saturating dose (Fig. 2C). Furthermore, Stx11 promoter activity is partially sensitive to p38 MAPK inhibition when IL-1β is at a saturating dose.

To test the hypothesis that *Stx11* expression can be driven by the presence of p65 alone without cytokine stimulation, we measured both RNA abundance and luciferase activity after overexpressing wild-type p65. Fig 3A compares *Stx11* mRNA expression between cells transduced with adenoviruses encoding either FLAG-tagged p65 or β-galactosidase as a control. In this context, p65 overexpression without cytokine promoted a trend in mRNA accumulation, though the 5.61-fold response at the highest dose of p65 virus was still much lower in magnitude than that of the IL-1β-treated cells. p65 overexpression on the reporter plasmids increased the luciferase activity, though the response differed depending on which sections of the region upstream of *Stx11* were present (Fig 3B and C). Using the 1 kb promoter (see Fig 4A for schematic), 150 ng of p65 plasmid provided a luciferase response 11.8-fold of the GFP control, though this response was still significantly lower than that of the cytokine-treated activity. On

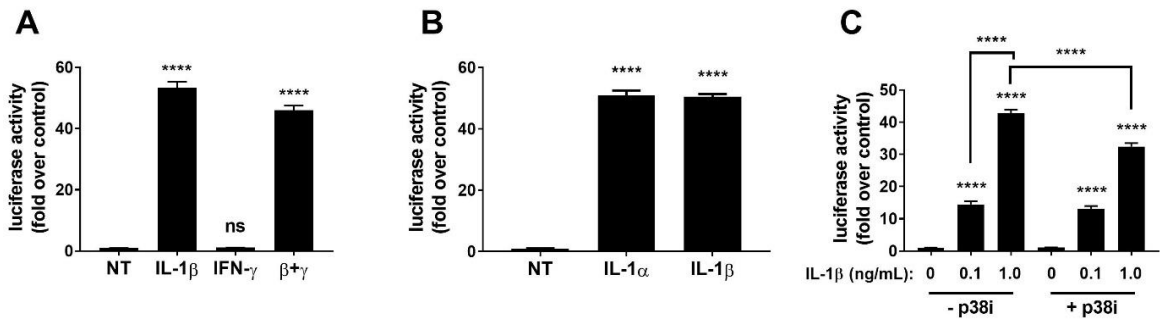

Figure 4.2. Luciferase-promoter construct responses mirror RNA expression data. In A-C, 832/13 cells were transfected overnight with a luciferase reporter plasmid containing the 3 kilobase promoter region of rat *Stx11*. (A) Cells were either not treated (NT) or stimulated with 1 ng/mL IL-1β, 100 U/mL IFN-γ, or a combination of both for 4 h. (B) Cells were NT or exposed to 1 ng/mL of either isoform of IL-1 for 4 h. (C) Cells were pretreated for 1 h with 10 uM of p38i in serum-free RPMI, media was removed, and then they were treated with the indicated doses of IL-1β for 4 h. **** $p < 0.0001$, ns = not significant. p values are versus respective NT controls unless otherwise indicated in the figure.

the other hand, the 3 kb promoter plasmid with the same dose of p65 gave a response of 24.9-fold over control, just as responsive as the IL-1β-treated cells.

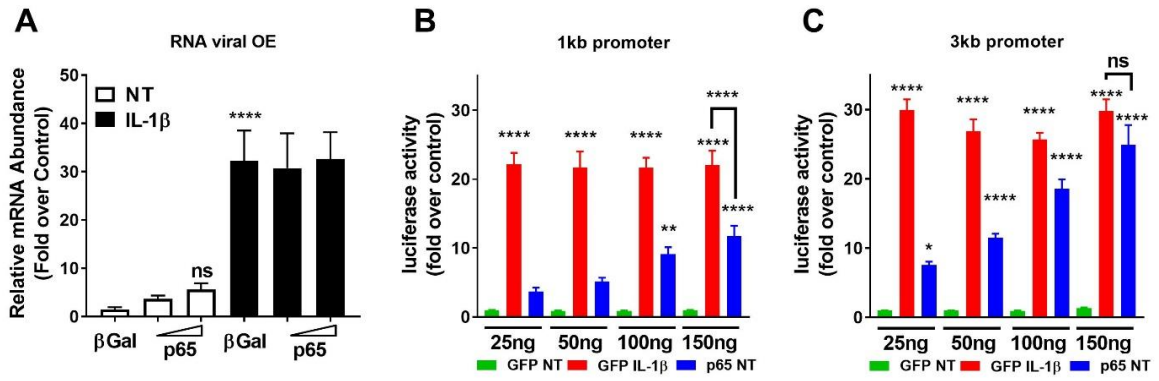

Figure 4.3. *Stx11* expression is partially dependent on p65. (A) 832/13 cells were transduced with adenovirus encoding either β-galactosidase or increasing doses of FLAG-tagged p65 overnight. Cells were then either not treated (NT) or exposed to 1 ng/mL IL-1β for 3 h. (B and C) Cells were transfected overnight with the 1 or 3 kb *Stx11* luciferase promoter plasmid along with the indicated doses of GFP or p65 expression plasmid. Then cells were NT or exposed to 1 ng/mL IL-1β for 4 h. *p < 0.05, ** p < 0.01, **** p < 0.0001. p values are versus respective NT controls unless otherwise indicated in the figure.

The response of the Stx11 *gene to IL-1β is dependent on NF-κB binding sites in the proximal promoter region*

Using JASPAR, *in silico* analysis allowed us to identify four predicted NF-κB binding sites within the 3 kb rat *Stx11* promoter region (see Methods for details). We termed these K3, K2, K1b, and K1a for the purpose of our study; the two K1 sites are separated by only 3 base pairs. A graphic depicting the two promoter constructs and the putative sites contained in each can be seen in Fig 4A. Due to the close proximity of K1a and K1b, we utilized site-directed mutagenesis at each site to eliminate their homology to consensus NF-κB sites. Mutating either site on either the 1 kb or the 3 kb promoter was sufficient to completely abolish promoter sensitivity to IL-1β (Fig 4B and C). To test the hypothesis that NF-κB binds these sites of interest, we utilized chromatin immunoprecipitation (ChIP) to investigate p65 binding. No significant enrichment of p65 was observed at the K3 element, but K2 and K1 did reveal 7.97-fold and 4.96-fold p65 enrichment by 60 minutes of IL-1β treatment, respectively (Fig 4D-F).

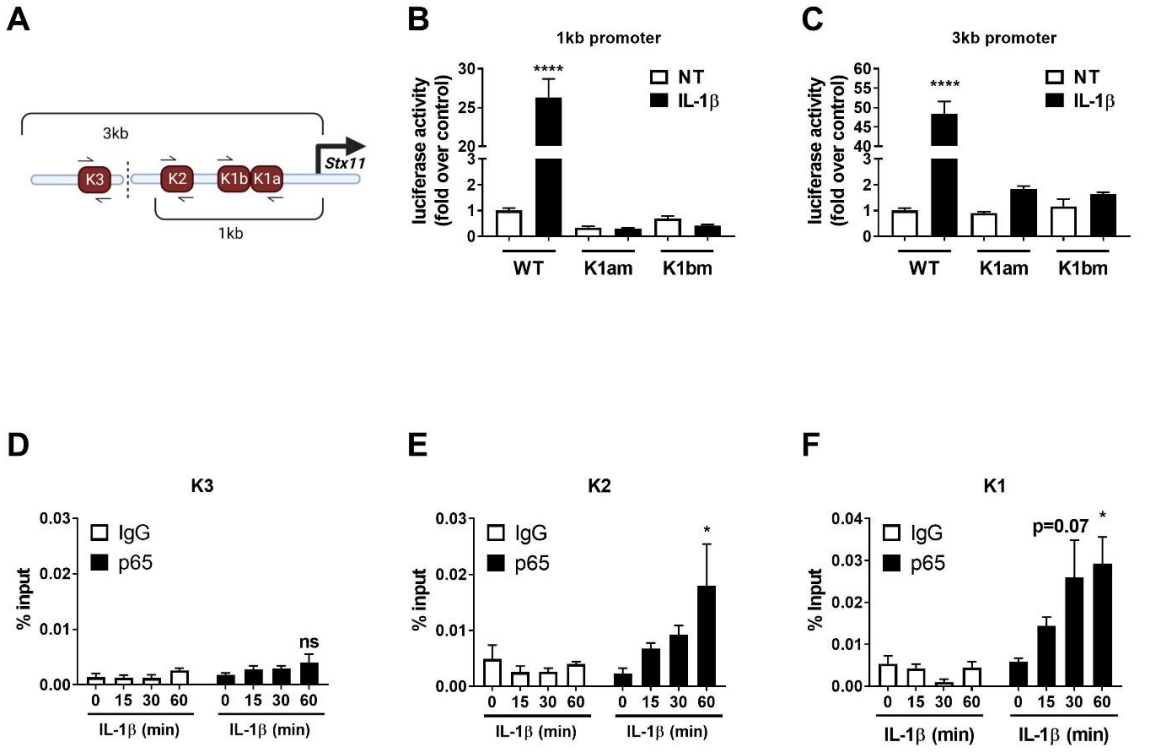

Figure 4.4. The response of *Stx11* to IL-1β is dependent on NF-κB binding sites in the immediate promoter region. (A) Schematic depicting the two promoter luciferase constructs cloned and the putative NF-κB binding sites in each. Also indicated are the sites contained within the ChIP PCR amplicons. (B and C) 832/13 cells were transfected with either wild-type (WT) or mutant *Stx11* promoter plasmids overnight followed by no treatment (NT) or 1 ng/mL IL-1β for 4 h. (D-F) Cells were either untreated (0 min) or stimulated with IL-1β for the indicated times. ChIP assays were performed to determine p65 occupancy at putative NF-κB binding sites. Rabbit IgG was used as a negative control for the immunoprecipitation. Data is represented as the percentage of input with four replicates per condition. *$p < 0.05$, **** $p < 0.0001$, ns = not significant. p values are versus respective NT controls unless otherwise indicated in the figure.

RNA Pol II phosphorylated at the C-terminal domain is enriched at the Stx11 *promoter and coding region upon IL-1β stimulation*

During the initiation and elongation phases of transcription, the C-terminus of RNA Polymerase II experiences phosphorylation [147]. We examined the enrichment of RNA Pol II phosphorylated at Ser2 and Ser5 of this domain at the *Stx11* gene locus after IL-1β treatment. Fig 5A shows that, compared to the untreated control, there was nearly a 10-fold enrichment of phospho-Ser5 at the *Stx11* transcriptional start site after 60 min of IL-1β stimulation. This modification is associated with RNA Pol II in the initiation phase of transcription [147]. In contrast, there was no significant enrichment of the phospho-Ser2 modification, which is

associated with elongation. Within the coding region of the gene, significant enrichment of RNA Pol II phospho-Ser5 begins at 15 minutes at 5 kb downstream of the TSS and 30 minutes at 10 kb (Fig 5B and C). The phospho-Ser2 modification is enriched at these sites slightly later, at 30 and 60 minutes, respectively. One point to note is that, as a percentage of input, the magnitude of phospho-Ser5 occupancy in the gene body is much lower than at the transcriptional start site.

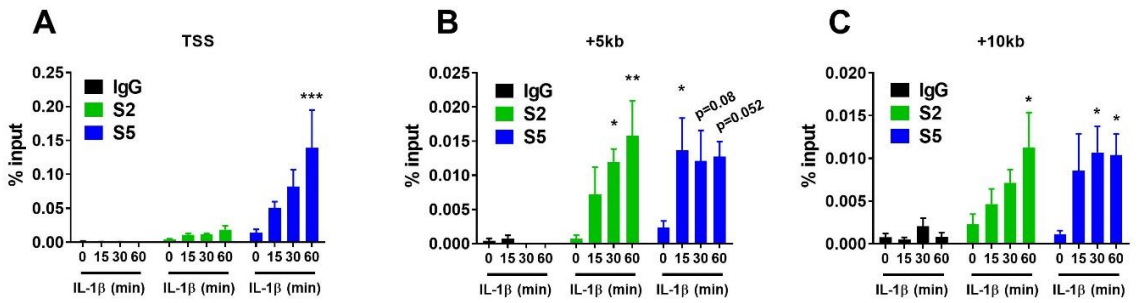

Figure 5. RNA Pol II phosphorylated at the C-terminal domain is enriched at the *Stx11* promoter and coding region upon IL-1β stimulation. Cells were either untreated (0 min) or stimulated with IL-1β for the indicated times. ChIP assays were performed to determine phospho-RNA Pol II occupancy at the TSS or gene body. Rabbit IgG was used as a negative control for the immunoprecipitation. Data is represented as the percentage of input with four replicates per condition. *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant. p values are versus respective NT controls unless otherwise indicated in the figure.

4.4. Discussion

SNARE proteins direct membrane fusion events within the cell. The trafficking of cellular functions within membrane-bound compartments is not only fundamental to eukaryote biology, but it is especially of interest in secretory cells such as the pancreatic β-cell. Prior work has demonstrated the essential role of a number of SNARE proteins during GSIS, particularly that of Stx1a, SNAP-25, and VAMP-2 [57]. One SNARE that has received little attention in the β-cell is Syntaxin 11. We demonstrate that *Stx11* is expressed in the β-cell both in the basal state and upon IL-1 stimulation.

A hallmark of autoimmune diabetes is the non-resolving inflammation of β-cells by chronic exposure to inflammatory cytokines such as IL-1, causing them to upregulate many genes that lead to further islet inflammation. One outcome is the upregulation of nitric oxide production, leading to reduced insulin secretion [175-177]. Another major defect observed is the secretion of chemokines that attract immune cells such as T cells, macrophages, and neutrophils into the islet [78, 113, 178]. Our observation that *Stx11* is rapidly upregulated in β-cells in response to IL-1 suggests that it may be another factor regulated during islet β-cell exposure to IL-1β. The induction of *Stx11* by IL-1 is mediated largely by NF-κB; this observation is characteristic of many of the inflammatory genes observed in islet β-cells mentioned above. Stimulation with IL-1 alone appears to be sufficient to reach the maximum level of Stx11 gene

expression over baseline among the cytokines we tested. This result contrasts with data from similar rapid response genes in β-cells, such as *iNOS* [6], *Icam1* [54], and some chemokines [28], which all experience further augmented expression when IFN-γ is present with IL-1β. Interestingly, although we did not observe an effect on *Stx11* expression by IFN-γ either alone or in concert with IL-1, other groups have found that *Stx11* can be induced in macrophages after stimulation with only IFN-γ [171, 179]. This contrast points to the complexity of cell- and context-specific gene regulation. We did detect a STAT1 binding site in our *in silico* promoter analysis, so this site may be relevant in the IFN-γ response seen in macrophages. It is also possible that STAT1 bound to this site serves as a key accessory factor to support the IL-1β response, as has been shown for β-cell expression of the *iNOS* [6] and the *CXCL1* and *CXCL2* [27] genes.

Many studies of cytokine-sensitive genes used exposure times of 16-24 h before examining epigenetic effects. Since we are interested in the rapid genetic reprogramming events in the β-cell during cytokine exposure, we examined much earlier time points. Our results point to a model wherein p65 binds to NF-κB sites in the immediate *Stx11* promoter region within 30 minutes of IL-1 stimulation (Fig 4E and F), followed by appearance of transcript within an hour (Fig 1A). Although the luciferase data in Fig 3C suggests that p65 administration alone may be able to induce *Stx11* expression, the RNA data in Fig 3A may be suggestive of additional factors necessary to support transcriptional changes at the endogenous genetic locus. One possible scenario is that an additional element within the IL-1 signaling cascade is necessary to provide the full transcriptional response. An example of this is provided by the p38 inhibitor data in Fig 2C; it is possible that the activation of the MAP kinase pathway by IL-1 contributes to the maximal gene response.

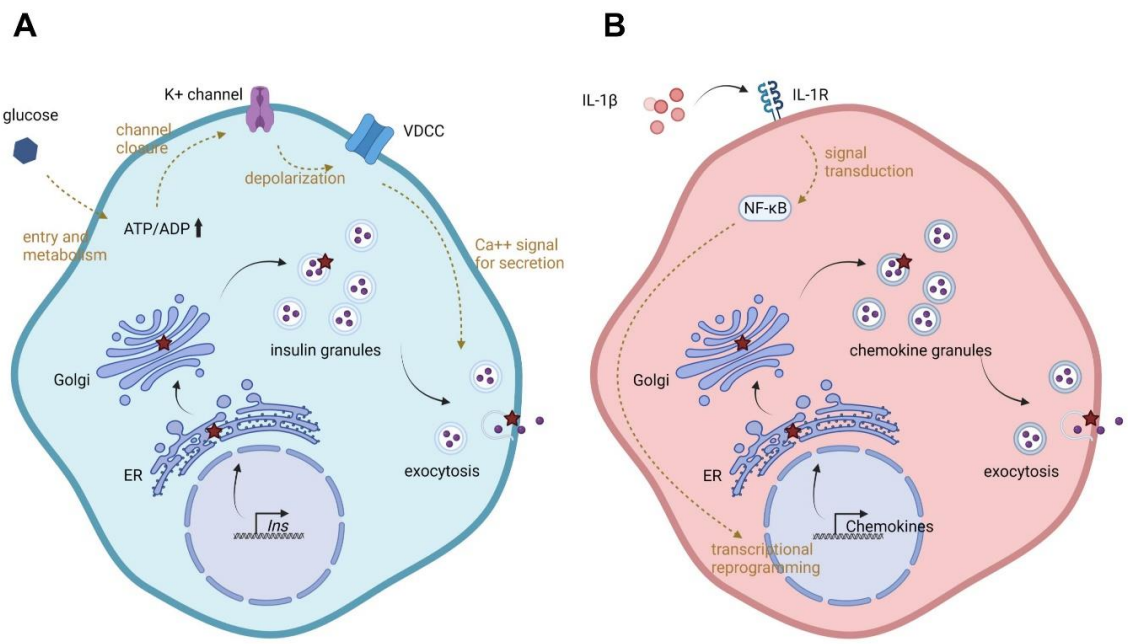

Figure 4.6. Membrane trafficking events in two scenarios of β-cell protein secretion. (A) Simplified graphic depicting the transcription, translation, packaging, and secretion of insulin in response to elevated glucose levels. Red stars indicate plausible sites of Stx11 action. (B) Simplified graphic depicting the transcription, translation, packaging, and secretion of chemokines in response to NF-κB activation. Red stars indicate plausible sites of Stx11 action. Figure created using BioRender.com.

An important question that remains unanswered is the role of Stx11 in the β-cell. We deem it important to examine the transcriptional regulation of the gene in order to provide clues towards its function. As demonstrated in Table 1, *Stx11* has basal expression in the β-cell that is augmented by IL-1 stimulation. Its expression did not change in response to heightened glucose concentrations (Fig 1D); however, other SNARE genes that are known to participate in this process (such as *Stx1a* and *Snap25*) also did not display upregulation in the same condition. Thus, we do not think this result precludes the possibility that Stx11 may participate in GSIS. On the other hand, among a wide selection of SNARE genes and genes encoding SNARE regulatory proteins, *Stx11* was the only gene that displayed any change in expression upon stimulation with IL-1 (Fig 1E). Some of the genes included in this analysis are *Stxbp2* (encoding Munc18b), *Vamp2*, *Vamp8*, and *Snap23*, all of which are known to interact with Stx11 in other cell types [58-60, 180, 181]. Assuming that one or more of these gene products cooperates with Stx11 in its functional activity in the β-cell, it is interesting that still none are observed to have altered expression upon IL-1 signaling.

The movement of immature translated insulin from the ER to the Golgi, the packaging of insulin into granules within the Golgi, the docking of mature secretory granules at the membrane, and the exocytosis of insulin are all membrane trafficking events that may provide a role for Stx11. Similar membrane activities in chemokine production could involve Stx11 as well. An illustration depicting both scenarios is provided in Fig 6. Given that *Stx11* is expressed in both

the basal state and upon IL-1 stimulation, these two scenarios may not be mutually exclusive. One putative model is that Stx11 serves a certain vesicle trafficking role in the basal state, but when the β-cell receives the signal to produce chemokines, Stx11 can be upregulated to take on this additional secretory burden. Another puzzling feature of this gene is that, despite increases in *Stx11* mRNA levels, our Western blot data shows no increase in the quantity of protein (Fig 1F). We have observed similar results in immunofluorescence imaging performed on 832/13 cells treated with cytokine (data not shown). A possible explanation is that the Stx11 protein is expended in some way during cytokine treatment, perhaps by being secreted with granules; under this scenario, the transcriptional upregulation of the gene could be explained as a method for the cell to replenish Stx11 proteins lost in the secretory process.

NF-κB activity is a central component of islet inflammation. Much work has been done to elucidate the consequences of overactive NF-κB signaling in the β-cell; less work has focused on the physiological roles for NF-κB signaling. Two such examples are work showing that GSIS actually requires NF-κB [182] and IL-1β improves GSIS in mice [183]. Whether Stx11 contributes to this NF-κB -dependent improvement of β-cell function remains to be seen, though it is a plausible mechanism. We believe that studying Stx11 in the islet is important to coming to a more complete understanding of diabetes, and that examining its expression in response to IL-1 is a first step towards this goal.

Chapter 5. Summary and Future Directions

Inflammation is a major driving force in the development of diabetes, especially in T1D. The existing literature supports this conclusion in a variety of contexts [4, 5, 184]. The work in this dissertation summarizes the present research on one of the outcomes of this inflammation (ICAM-1) and delves into the transcriptional regulation of two of the genes that are induced by NF-κB (*Icam1* and *Stx11*).

It is clear from the present literature that ICAM-1 is one of that many factors that lead to the development of autoimmunity in T1D. What has not yet been demonstrated clearly is which function and context of ICAM-1 signaling precipitates the autoimmune attack. We describe three possible cellular interactions in Chapter 2 of this document: extravasation of leukocytes into the tissue, initial stimulation of naïve autoreactive T cells, and β-cell-T cell interactions during cell-mediated killing (Fig 1). These scenarios are not mutually exclusive, and at present we find the latter two to be plausible. However, much of this work was performed before Cre/lox technology became widespread in biomedical research, so the tissue-specific knockout models that would adequately answer these questions were not present. To clarify the role of ICAM-1 in the development of islet autoimmunity, we propose the creation of an *Icam1* floxed mouse. This mouse could be backcrossed onto the NOD strain and combined with Cre driver lines for endothelial cells, innate immune cells, and β-cells to further address this topic.

The present work indicates that Stx11 expression, unlike that of other SNAREs in the β-cell, is upregulated by IL-1 using NF-κB signaling. Work remains to be done to determine its

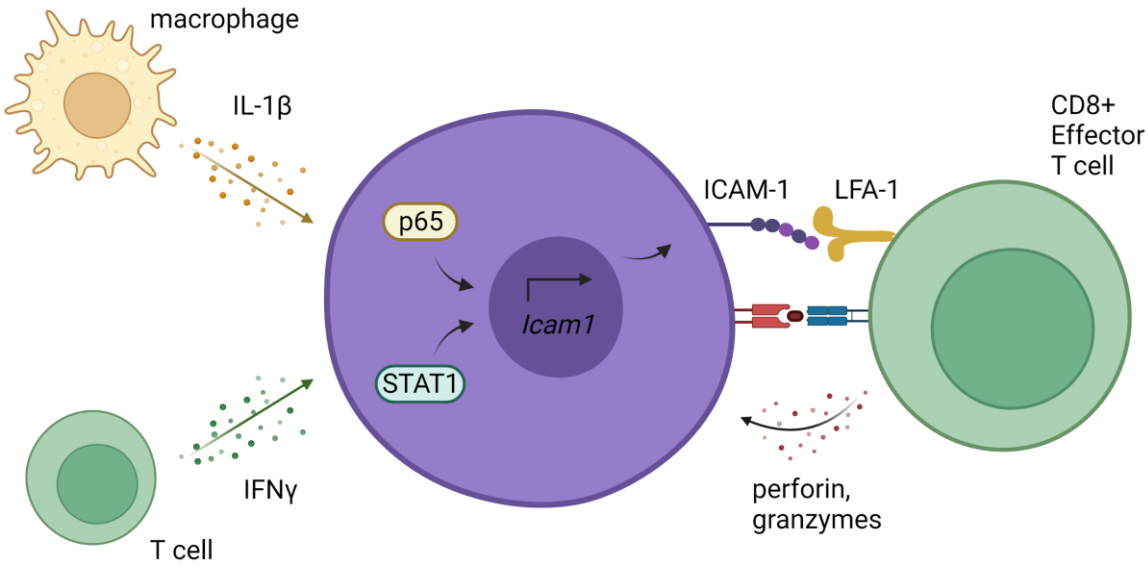

Figure 5.1. Expression and role of ICAM-1 during cell-mediated immunity in autoimmune diabetes. This schematic depicts the major cell types that secrete cytokines to induce ICAM-1 expression on β-cells. ICAM-1 binds LFA-1 on T cells to enhance cytotoxic activity. Figure created using BioRender.com.

function in this cell type. We have prepared a number of tools and reagents in order to study this. First, we have developed an adenovirus encoding wild-type *Stx11*, which allows us to overexpress it *in vitro* in experiments. This can be used standalone in ELISAs measuring GSIS and chemokine secretion to determine whether overexpression can augment either process; or the virus could be combined with siRNA oligos knocking out endogenous *Stx11* expression, with viral overexpression used in an attempt to rescue secretory processes. Furthermore, we have also prepared two mutant Stx11 adenoviruses containing the R4A and L58P amino acid substitutions, which are known mutations that cause FHL4 in humans due to abolished binding to Munc18b [185]. These mutant forms of Stx11 can be used to test the hypotheses that 1) Stx11 binds Munc18b in the β-cells, and 2) that such an interaction is necessary for Stx11 function. Finally, we have also prepared an adenovirus encoding a GFP-tagged Stx11 using the pMVP/pMAGIC cloning platform [186]. Using this fusion protein, we can engage in live-cell imaging of 832/13 cells exposed to glucose or IL-1 to track Stx11 movement throughout the cell, gaining a more thorough understanding of the protein's cellular localization.

A key, and reasonable, assumption in studying islet inflammation is that it is a mostly pathological process. However, evidence is growing that not all inflammation in the islet is inherently damaging. First, one must consider the sensitivity of the islet to IL-1: the β-cell has the highest expression of the IL-1 receptor (IL-1R) relative to any cell in the body [10], and IL-1R is the most abundant receptor on the surface of the β-cell [187]. Additionally, ChIP-seq has shown strong binding within the *Il1r1* locus for Pdx1, Nkx6.1, and NeuroD1, all β-cell transcription factors [187]. When inflammatory signaling in the β-cell is considered as a merely pathological process, it seems that evolution has doomed the islet for autoimmunity. On the contrary, NF-κB has actually been shown to be necessary for insulin secretion [182]. During GSIS, macrophage-derived IL-1β enhances insulin secretion [183], and mice with pancreatic deletion of the IL-1R have worsened glucose tolerance [188]. Macrophages may be critical for the compensatory β-cell proliferation observed in the early stages of T2D [189].

The seemingly contradictory evidence between physiological and pathophysiological consequences of inflammation in the β-cell can be reframed as such: NF-κB signaling is a normal process augmenting GSIS and compensatory hypertrophy, but unresolved inflammation can lead to islet autoimmunity and β-cell dysfunction. Macrophages can be viewed in this light as regulators of islet function and maintenance. It is plausible, then, to hypothesize that ICAM-1 on the β-cell may be a mechanism to facilitate crosstalk between the two cell types, allowing them to exchange signals regarding β-cell health and proliferation. Furthermore, it stands to reason that Stx11 upregulation may be a critical component of understanding IL-1 augmentation of GSIS. In this light, autoimmunity in the β-cell more closely resembles physiological inflammation that has not been appropriately tempered, and thus it develops into a full-blown immune attack on the β-cells.

Diabetes represents a significant threat to global human health. The economic and personal burden on patients is enormous, and the epidemiology indicates that both forms of the disease are rising in prevalence across the world [1, 2]. We believe that a more complete understanding of islet inflammation will pave the way for future treatments and cures to reduce this burden on those suffering from diabetes. The research contained in this dissertation represents efforts made to elucidate the mechanisms of NF-κB signaling in the β-cell and some of the resulting consequences.

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Vita

Thomas Malone Martin, born in Baton Rouge, LA, graduated from Louisiana State University in 2016 with a B.S. in Microbiology. He began working as a Research Associate in the lab of Dr. Jason Collier, who heads the Department of Islet Biology and Inflammation at Pennington Biomedical Research Center. Soon after joining, Thomas decided to pursue his Ph D. in the lab studying autoimmunity in β-cells. Starting in Fall 2017, his research has remained focused on epigenetic events in cytokine stimulation. After completion of his Ph.D., Thomas plans to continue his career as a postdoctoral fellow at Pennington Biomedical.