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Differential usage of NF-κB activating signals by IL-1β and TNF-α in pancreatic beta cells

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ABSTRACT

The cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α induce β-cell death in type 1 diabetes via NF-κB activation. IL-1β induces a more marked NF-κB activation than TNF-α, with higher expression of genes involved in β-cell dysfunction and death. We show here a differential usage of the IKK complex by IL-1β and TNF-α in β-cells. While TNF-α uses IKK complexes containing both IKKα and IKKβ, IL-1β induces complexes with IKKα only; this effect is achieved by induction of IKKβ degradation via the proteasome. Both IKKγ and activation of the TRAF6-TAK1-JNK pathway are involved in IL-1β-induced IKKβ degradation.

1. Introduction

Type 1 Diabetes mellitus (T1D) is an autoimmune disease characterized by a selective destruction of the insulin producing β-cells [1]. During the inflammatory process known as insulitis, pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ are secreted by immune cells invading the islets and contribute for β-cell dysfunction and apoptosis [1]. Activation of the transcription factor NF-κB is necessary for cytokine-induced β-cell dead [2–6], which is surprising, since in other cell types NF-κB activation has mostly an anti-apoptotic role [7,8]. This key transcription factor controls, directly or indirectly (mainly via NO production), the expression of several genes and transcription factors in β-cells. These genes are involved in the regulation of survival/apoptosis, function and immune system cells attraction [9–11].

NF-κB is composed by a family of five members that can form homo or heterodimers, depending on the cell type and stimulus [12–14]. These dimers are normally kept inactive in the cytoplasm by their binding to the inhibitory κB (IκB) proteins [14]. Binding of IL-1β or TNF-α to their specific receptors recruits scaffold proteins and activates a cascade of kinases and ligases leading to the IκB kinase (IKK) complex activation. This complex induces IκB phosphorylation leading to its degradation, allowing NF-κB translocation to the nucleus [14]. NF-κB transcriptional activity can be also regulated by post-translational mechanisms such as phosphorylation of the p65 subunit by the mitogen-activated protein kinases (MAPKs), IKK complex, AKT (or protein kinase B) and others [14,15]. Many NF-κB-activating signalling cascades converge to the IKK complex whose activation therefore has a key role in cell biology [16,17]. This complex is formed by three proteins: IKKα, IKKβ and IKKγ (NEMO) [14,17]. The kinase subunits IKKα and IKKβ share a similar structural identity, including a N-terminal catalytic domain, a central dimerization leucine zipper and a C-terminal helice-loop-helice domain [14,17]. They are associated as homo- or heterodimer to the regulatory IKKγ subunit [14,17]. In spite of their high similarity, IKKα and IKKβ differ on their function and activation depending on the type of stimulus [18–21].

Due to its pro-apoptotic and inflammatory effects, it is important to better understand the specific characteristics of NF-κB activation in β-cells. We have previously shown that cytokine-induced

Abbreviations: KD, knock down; IKK, IκB kinase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; JNK, jun N-terminal kinase

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NF-κB activation in β-cells differ from other cell types by its intensity and duration [7]. Furthermore, IL-1β induced NF-κB activation has a more important pro-apoptotic effect than TNF-α [5,7]. This seems to be related to the stronger intensity of NF-κB induction by IL-1β and of preferential activation of kinases that modulate NF-κB and other genes, including the IKK complex and ERK [5,7]. In order to unravel the signal transduction involved in these differences, we presently studied the specific characteristics of IKK activation by IL-1β or TNF-α in β-cells.

2. Materials and methods

2.1. Cell culture and treatment

Insulin-producing INS-1E cells [22], a kind gift from Prof. C. Wolheim (Centre Médical Universitaire, Geneva, Switzerland), and rat fibroblasts 208F cells [European Collection Cell Cultures (ECACC), Salisbury, UK] were cultured as previously described [7]. Key findings were confirmed in primary FACS-purified rat β-cells, isolated as previously described [23,24]. Adult Wistar rats (Charles River Laboratories Belgium, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care; the Ethical Committee for Animal Experiments of the ULB approved the experimental protocol. Cells were exposed to recombinant human IL-1β (100 U/ml, a kind gift from Dr. C.W. Reinolds, National Cancer Institute, Bethesda, MD-USA) or recombinant murine TNF-α (1000U/ml, Innogenetics, Gent-Belgium); these concentrations were selected based on previous dose–response experiments [11,25]. The IKK inhibitors BMS-345541 (Sigma) (15–100 μM), inhibitor IV (Calbiochem) (25 μM) and JNK inhibitor SP600125 (Sigma) (10 μM) were used prior (2–3 h) to cytokine treatment.

2.2. Immunofluorescence

Cells were plated in poly-lysine coated cover-slips, and after treatment fixed with 4% paraformaldehyde and permeabilized with 70% acetone +30% methanol. Incubation with anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA-USA) or anti-hemaglutinin (HA) (Roche Diagnostics, Mannheim-Germany) and secondary antibody anti-rabbit IgG conjugated with FITC (Jackson ImmunoResearch, Westgrove, PA-USA) done as previously described [5].

2.3. RNA interference

Small interfering RNA (siRNA) against IKKα, IKKβ, IKKγ, TRAF6 and TAK1 Silencer® Select Pre-designed siRNA (Ambion, Austin – TX, USA) and Stealth RNAi™ siRNA (Invitrogen, Carlsbad – CA, USA) were used to knock down expression of respective genes. Allstars Negative Control siRNA (Qiagen, Venlo, Netherlands) was used as a negative control. siRNA transfection was done as previously described [26].

2.4. Western blot assay

Total or immunoprecipitated protein extracts were obtained from cells after exposure to treatment and/or transfection with specific siRNA [5,7]. Proteins were fractioned in a 10% SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with anti-IKKα, IKK-β, IKKγ, P-IκBα (Santa Cruz, Biotechnology, Santa Cruz – CA, USA), TRAF6, TAK1, total JNK, P-JNK, anti-polyubiquitin and anti-α-tubulin (Cell Signaling, Beverly, MA) antibodies, followed by incubation with a secondary horseradish peroxidase-labeled anti-IgG (Santa Cruz Biotechnology or Cell Signaling). The quantification of the specific bands was done by the Scion Image (Scion Corporation, Frederick, MD). Values were corrected by values obtained for α-tubulin (housekeeping protein).

2.5. IKK kinase assay

Protein extracts were obtained as described [5]. An equal amount of protein was incubated for 2 h at 4 °C with either anti-IKKγ (Santa Cruz Biotechnology) or anti-HA (Roche Diagnostics, Mannheim-Germany; negative control), followed by 2 h incubation with protein A-sepharose™CL-4B beads (GE Healthcare, Uppsala; Sweden). Beads were washed and incubated at 30 °C in kinase buffer with GST-IκBα recombinant protein and 5 mM ATP [5]. Western blot was performed as described above.

3. Results

3.1. Differential usage of IKK complex subunits by IL-1β and TNF-α in pancreatic β-cells

To prevent NF-κB activation in β-cells we used an IKK inhibitor (BMS-345541 – BMS) with specificity for IKKβ containing IKK complexes [27]. Pre-treatment with BMS inhibited TNF-α-induced NF-κB activation (≃80%) as measured by p65 (NF-κB subunit) migration to the nucleus in INS-1E cells (Fig. 1A and Supplementary Fig. 1S A) and primary rat β-cells (Fig. 1B). BMS, however, did not prevent IL-1β-induced NF-κB activation (Fig. 1A, B and Supplementary Fig. 1S A). An IKK activation assay confirmed that BMS inhibits TNF-α- but not IL-1β-induced NF-κB activation (Fig. 1C). Subsequent experiments indicated that BMS failure to prevent IL-1β-induced NF-κB activation was observed at different IL-1β concentrations (from 5 to 100 U/ml) (Supplementary Fig. 2S A), BMS concentrations (40–80 μM) or time of pre-treatment (from 3 to 16 h) (Supplementary Fig. 2S B). The use of another IKK inhibitor targeting IKKβ containing IKK complexes (inhibitor IV), showed again a higher efficiency against TNF-α than IL-1β (Supplementary Fig. 1S B and C).

3.2. IL-1β induces IKKβ degradation in pancreatic β-cells

We next analyzed the composition of the IKK complex in INS-1E cells after exposure to IL-1β or TNF-α for 10–90 min, since NF-κB activation by these cytokines starts between 10–30 min [5,7]. Analysis of the IKK complex showed that IL-1β, but not TNF-α induced IKKβ disappearance without changing IKKα expression (Fig. 2A). Neither IL-1β nor TNF-α modified IKKα or IKKβ levels in rat fibroblast cells (208F) (Supplementary Fig. 3S A), suggesting a preferential effect on β-cells.

IL-1β-induced IKKβ degradation was already observed after 10 min and lasted up to 2 h (Fig. 2B and Supplementary Fig. 3S B). On the other hand, TNF-α did not trigger IKKβ degradation at any of the time points studied (Supplementary Fig. 3S B). IL-1β-induced IKKβ degradation was confirmed in FACS-purified β-cells (Supplementary Fig. 3S C). IKKβ degradation is dependent on proteasome activity, since MG132 (a proteasome activity blocker) prevented it (Fig. 2C).

3.3. IL-1β induced IKKβ degradation relies on IKKγ

The use of specific siRNAs against the components of the IKK complex (α, β and γ) indicated that IL-1β does not induce IKKβ degradation following knock down (KD) of IKKγ (Fig. 3). On the other hand, IKKα KD did not prevent IL-1β induced IKKβ degradation (Fig. 3). The specificity of each siRNA was confirmed by its ability to KD only the specific IKK targeted (Fig. 3A and Supplementary Fig. 4S). Use of a second set of siRNAs against these three subunits confirmed the observations described above (data not shown).

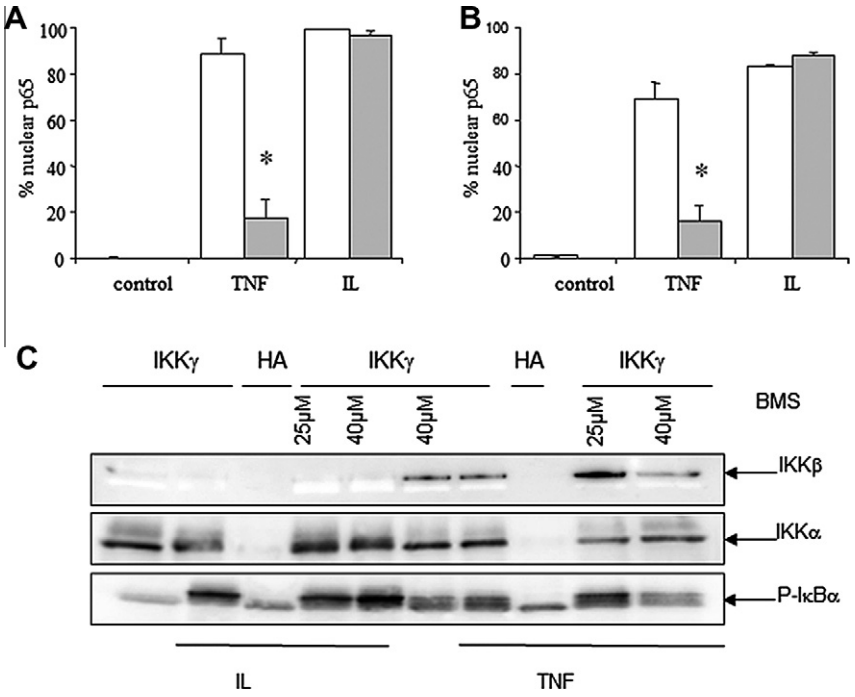

Fig. 1. BMS-345541 inhibits TNF-α- but not IL-1β-induced IKK activation. (A–B): analysis of IL-1β or TNF-α-induced NF-κB (p65 subunit) nuclear localization in INS-1E cells (A) and rat primary β-cells (B) in the presence (grey bars) or absence (white bars) of 3 h pre-treatment with IKK inhibitor BMS-245541 (BMS, 50 μM). Cells were exposed to IL-1β (IL), TNF-α (TNF) or left untreated (control) for 30 min. Results are the mean ± SEM of 4 independent experiments. *$P < 0.05$ BMS treated cells vs. respective controls; paired *t*-test. (C): IKK activity assay; INS-1E cells were pre-treated as described in A–B with 25 or 50 μM of BMS and then exposed to IL-1β, TNFα or left untreated (control) for 30 min. Cells were lysed and the IKK complex was immunoprecipitated (IP) with anti-IKKγ antibody. IKK activation was measured by its capacity to phosphorylate the substrate glutathione-*S*-transferase (GST)-IκBα. Western blot for IKKα showed similar IP of the IKK complex. Anti-haemagglutinin (HA) antibody was used as a negative control for IP. The figure is representative of three similar experiments.

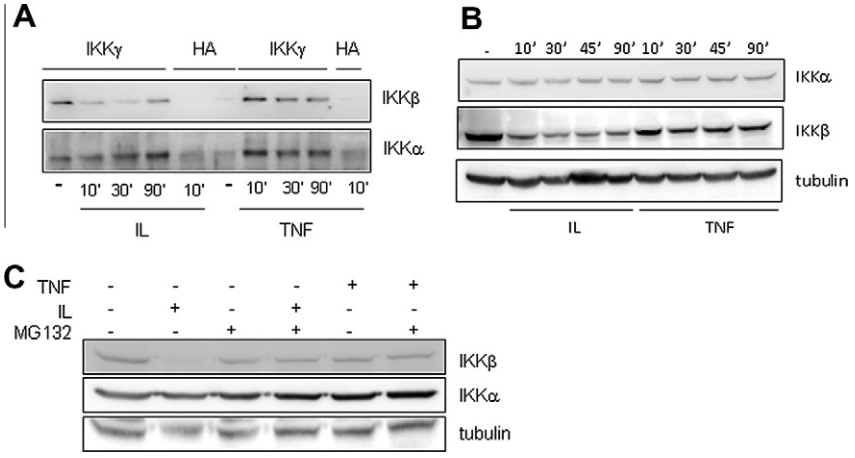

Fig. 2. IL-1β, but not TNF-α, induces IKKβ degradation in β-cells. (A–B): INS-1E cells exposed to IL-1β (IL) or TNF-α (TNF) or left untreated (control) for the time indicated in the figure. (A): cells were lysed and the IKK complex was immunoprecipitated (IP) with anti-IKKγ antibody and Western blot for IKKα and IKKβ performed as described in Methods. Anti-haemagglutinin (HA) antibody was used as a negative control for IP. (B): the protein cell lysate was directly used in Western blot assays for IKKα and IKKβ; tubulin was analyzed to confirm similar protein loading. (C): INS-1E cells were pre-treated or not with the proteasome inhibitor MG132 (2 μM) for 2 h, and then exposed to IL-1β (IL) or TNF-α (TNF) or left untreated (control) for 15 min. Cells were lysed and used in Western blot assay for IKKα and IKKβ; tubulin was used to confirm similar protein loading. Figures shown are representative of three to four experiments.

3.4. IL-1β induced IKKβ degradation uses the TRAF6-, TAK1- and JNK-dependent signalling pathway

We next investigated the role of TNF receptor associated factor (TRAF)-6, an adaptor protein involved in IL-1β- but not TNF-α-induced NF-κB activation [14,28] in IKKβ degradation. A specific siRNA against TRAF6 decreased by >60% its expression and partially prevented IKKβ degradation induced by IL-1β (Fig. 4). Knocking-down transforming growth factor (TGF)-β-activated kinase 1 (TAK1), the kinase recruited by TRAF6 for the activation of IKK complex [14,29], was not as efficient as observed for TRAF6 (less than 30%), but this was sufficient to partially prevent IKKβ degradation induced by IL-1β (Fig. 4). Beside IKK activation, TRAF6 also induces Jun-N-terminal Kinase (JNK) via TAK1 [29,30]; in line with this, TRAF6 and TAK1 KD decreased JNK activation (Fig. 4). Of note, IL-1β induced higher activation of JNK than TNF-α in β-cells

(Supplementary Fig. 5S A); this stimulatory effect of IL-1β on JNK started already at 5 min of treatment, with further increase by 10 min (Supplementary Fig. 5S B and C). To determine if JNK activation plays a role in IKKβ degradation, we used the JNK inhibitor SP600125 (SP). SP inhibited JNK activation by ⩾80%, as measured by its phosphorylation. JNK inhibition partially prevented IL-1β-induced IKKβ degradation (Fig. 4), an effect similar to the one observed with a combination of TAK1 KD and JNK inhibition. This suggests that these two proteins are part of the same signalling pathway involved in IKKβ degradation. Use of a second set of siRNAs confirmed the observations described above (data not shown).

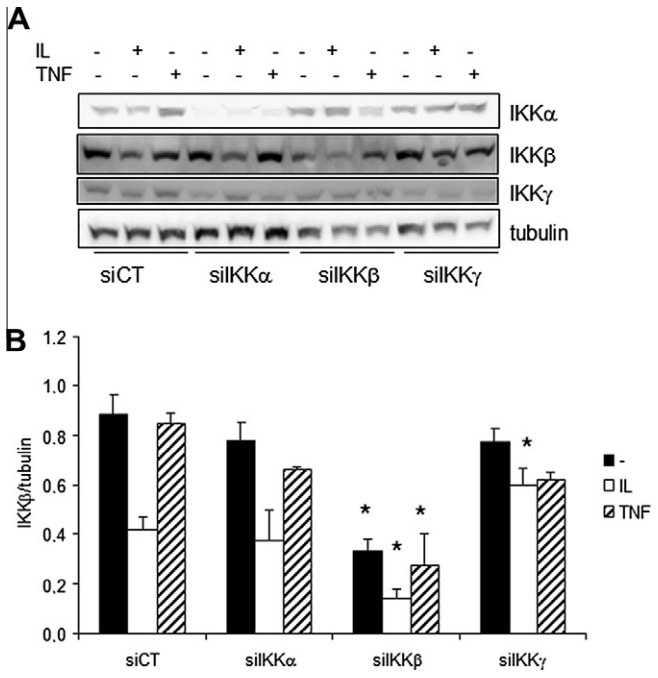

Fig. 3. IKKβ degradation following inhibition of different components of the IKK complex. INS-1E cells were transfected with siRNAs specific for IKKα, IKKβ or IKKγ subunits, or a control siRNA (siCT). Three days after transfection cells were treated either with IL-1β or TNF-α (as indicated in the figure) for 30 min. Western blots for IKKα, IKKβ, IKKγ and tubulin were then performed. (A): representative figure of 5–8 independent experiments. (B): quantification of the results. $^{*}P \leqslant 0.05$ cells transfected with siIKK vs. siCT under the same treatment conditions.

4. Discussion

The main findings of the present study, taken together with our previous observations [5,7], are summarized in Fig. 5. We have previously shown that IL-1β induces an earlier and stronger activation of ERK and of the IKK complex, as compared to TNF-α, leading to a more intense and protracted NF-κB activation and consequent expression of downstream genes involved in β-cell dysfunction and death. Furthermore, IL-1β, but not TNF-α, alone induce INS-1E cell dead [7]. We presently show that there is a differential usage of IKK complexes following β-cell exposure to IL-1β or TNF-α. IL-1β induces degradation of IKKβ via the proteasome, leading to a preferential use of IKK complexes containing only the IKKα subunit. This degradation is dependent, at least in part, on the presence of the IKKγ subunit and activation of the TRAF6 signalling pathway (Fig. 5). Of note, TRAF6 is involved in the activation of NF-κB by IL-1β but not by TNF-α [14,17], which may contribute to the different effects of the two cytokines on IKK. Activation of JNK via TAK1 is also important for IL-1β-induced IKKβ degradation, suggesting a new effect of this kinase, previously shown to contribute for cytokine-induced β-cell apoptosis [10,31].

The IKKβ subunit was described as indispensable for NF-κB activation by different pro-inflammatory cytokines [32,33]. Due to the importance of NF-κB activation in auto-immune diseases [33], such as T1D, many "specific" inhibitors for IKK complexes possessing the IKKβ subunit were developed [27,33]. Recently, however, it was shown that while TNF-α-induced NF-κB activation is indeed

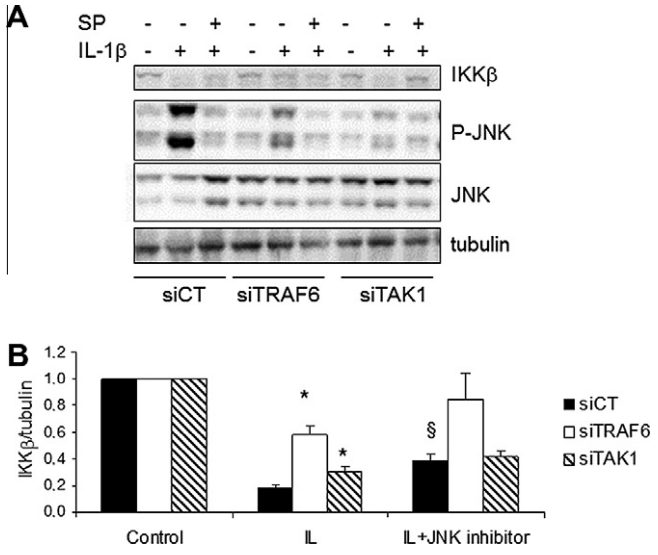

Fig. 4. Involvement of TRAF6, TAK1 and JNK activation in IL-1β-induced IKKβ degradation in β-cells. INS-1E cells were transfected with siRNA specific for TRAF6 (siTRAF6) and TAK1 (siTAK1), or a control siRNA (siCT). Three days after transfection the cells were pre-treated or not with the JNK inhibitor (SP600125) and then left untreated (control) or treated with IL-1β for 30 min, as indicated in the figure. Western blots for IKKβ, P-JNK and tubulin were then performed. (A): representative figure of 4–8 independent experiments is shown. (B): quantification of the results. Data are means ± SEM of 4–8 experiments. $^{*}P \leqslant 0.05$ cells transfected with siCT vs. siTRAF6 or TAK1 with similar treatment. $^{\S}P \leqslant 0.005$ IL-1β treated cells vs IL-1β + JNK inhibitor transfected with the same siRNA.

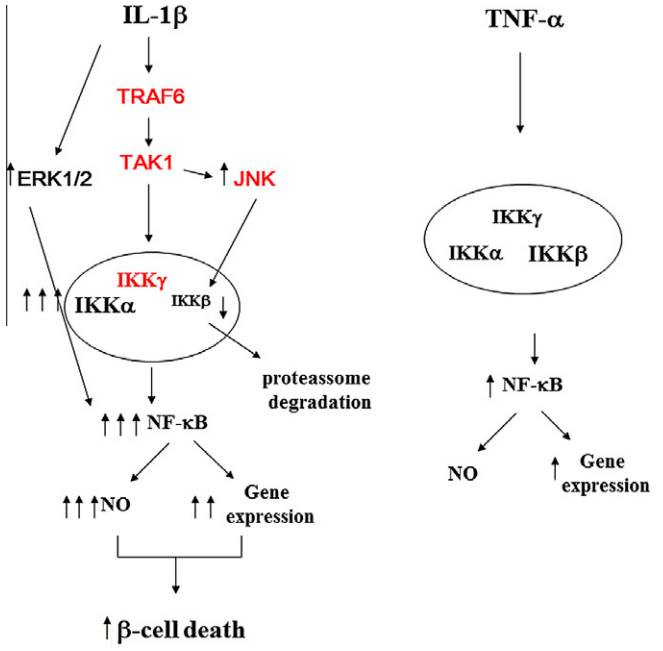

Fig. 5. Proposed model for the differential IKK activation by IL-1β and TNF-α in β-cells. In red are the proteins putatively involved in IKKβ degradation induced by IL-1β.

dependent of IKKβ, IL-1β is able to induce NF-κB via IKKβ or IKKα [20]. The heterodimers of IKKα and IKKβ, in association with IKKγ, are the most common IKK complexes observed in cells containing the three isoforms [14,17,34]. We presently describe, however, that β-cells treated with IL-1β have preferentially IKKα homodimers in association with IKKγ (Fig. 2A). This is probably secondary to IL-1β-induced IKKβ degradation. This effect seems to be specific for β-cells, in which NF-κB activation is pro-apoptotic, since it is not observed in rat fibroblasts (Supplementary Fig. 3S A). Of note, NF-κB activation in rat fibroblasts is anti-apoptotic, and TNF-α induces stronger activation of this transcription factor as compared to IL-1β [5], emphasizing the context- and cell-dependent regulation of NF-κB and downstream genes.

IKKα and IKKβ share high structural similarity, but they play different roles in the regulation of NF-κB translocation to the nucleus [17] and its post-translational modulation [16]. IKKα regulates both NF-κB transcriptional activity and the regulation of transcriptional co-activators, co-repressors and histones [35–40]. On the other hand, IKKβ contains an ubiquitin-like domain which seems to be important for its activation and degradation [41]. In fact, IKKβ degradation may be an important mechanism to attenuate NF-κB activation in other cell types, and disruption of this mechanism favors neoplasias [42]. Thus, the presently observed preferential usage of IKK complexes containing only the IKKα subunit following IL-1β exposure might contribute to the more intense NF-κB activation and expression of downstream genes observed in β-cells [5,7]. Of note, we have previously shown that neither IL-1β nor TNF-α induce the non-canonical (IKKα-dependent) NF-κB pathway [7].

Our data suggest that IKKβ degradation requires the presence of IKKγ. IKKγ has no kinase activity, but it allows association between the IKK complex and regulatory proteins that modulate IKK activation/repression [14,28]. Additional experiments are required to clarify how IKKγ contributes for the presently observed IL-1β-induced IKKβ degradation.

Different pro-inflammatory cytokines may contribute to insulitis and β-cell apoptosis during development of T1D [1,43]. We presently show that IL-1β and TNF-α utilize different "strategies" for NF-κB activation in pancreatic β-cells, including usage of different members of the IKK complex. Since NF-κB is a key transcription factor for insulitis and apoptosis [1,44], this novel information open interesting possibilities for a context-dependent modulation of the transcription factor to protect β-cells in early T1D.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.021.

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