Unravelling beta cell destruction in type 1 diabetes

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CHAPTER 2

Preproinsulin autoantigen processing by ER aminopeptidase 1 is upregulated during ER stress via IRE1α-mediated miR-17 degradation

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ABSTRACT

The signal peptide of the preproinsulin is a major source for HLA class I autoantigen epitopes implicated in CTL-mediated beta cell destruction in Type 1 Diabetes (T1D). The combined action of signal peptide peptidase and endoplasmic reticulum aminopeptidase 1 (ERAP1) participates to the processing of the signal peptide and to the expression of this epitope at the beta cell surface. The regulatory mechanisms controlling signal peptide trimming and the contribution of the T1D inflammatory milieu on these mechanisms are unknown. We studied the role of inflammation and ER stress on ERAP1 gene expression. Inflammation and ER stress induction in human beta cells lead to increased expression of ERAP1 and to the decreased amounts of miR-17-5p. These results confirm the importance of ER stress in the increased visibility of beta cells to the immune system and position the IRE1α/miR-17 pathway as a central component in beta cell destruction processes. This pathway therefore forms a potential target for the treatment of autoimmune T1D in recent onset patients.

INTRODUCTION

Type 1 diabetes (T1D) results from selective and progressive destruction of insulin-producing cells by autoreactive $CD8^+$ T cells[1,2]. Immunohistochemistry of insulitic pancreases obtained through the Network for Pancreatic Organ Donors with Diabetes program (nPOD)[3] have shown a massive infiltration of immune cells and an increased expression of HLA class I and markers characteristic of a cellular stress response related to endoplasmic reticulum (ER) stress, referred to as the Unfolded Protein Response (UPR)[4-6]. Altogether these results suggest a correlation between ER stress and the increased visibility of beta cells to the immune system[7]. While several proteins have been identified as potential autoreactive T cell targets, evidence from studies in mice and men suggests that insulin itself could be a main autoantigen targeted by infiltrating $CD8^+$ T cells in T1D[8-10]. The posttranslational processing pathway that generates insulin from its precursor molecule preproinsulin (PPI) is well established: the signal peptidase Sec11 cleaves off the signal peptide cotranslationally upon translocation of the protein into the ER via the translocon Sec61[11]. After proinsulin (PI) folding and the formation of three disulfide bonds, proinsulin is transported via the Golgi system into immature secretory vesicles where mature insulin is generated by the action of the prohormone convertases PC1/2. The central region of PI, the C-peptide, is cleaved out and the B- and A-chain located in amino- and carboxy-terminus, respectively remain together by the previously formed disulfide bonds yielding bioactive insulin[11-14]. Accumulating new data highlight the importance of the PPI signal peptide (SP), of 24 amino acids long, as source of insulin derived class I epitopes[15]. Peptide elution experiments performed on HLA-A2 surrogate beta cells led to the identification of prominent HLA class I binders located in the SP domain[16] and $PPI_{(SP15-24)}$-directed CTLs were found to be highly prevalent in recent onset T1D patients[17]. Recently, using cell free translocation assays and CrispR/Cas technology elegantly demonstrated the importance of the combined action of the signal peptide peptidase and ER aminopeptidase 1 (ERAP1) in the trimming of the PPI SP and in the generation of the $PPI_{(SP15-24)}$ epitope[18]. While the epitope length match with the structural characteristic of ERAP1 and the model described previously[19,20], the link between T1D pathophysiology and the immunoreactivity against $PPI_{(SP15-24)}$ remains unclear. Here, we investigated the effect of inflammation and ER stress on ERAP1 gene expression in human

beta cells and show that proinflammatory cytokines as well as chemically induced ER stress modulate ERAP1 expression through induction of miR17-5p degradation. These data establish a direct link between ER stress, and beta cell immunogenicity and demonstrate the importance of the ER sensors in shaping antigenic peptide presentation to CTLs.

RESULTS

In T1D patients, surface presentation of autoantigens in HLA class I and their recognition by $CD8^+$ T cells results in the induction of apoptotic cell-death in β-cells. To quantify CTL-induced cell death, we used a bioluminescent reporter (cFLuc-DEVD) in which luciferase activity is dependent on caspase-3 cleavage[21,22]. To test this construct, we treated HeLa cells stably modified with the cFLuc-DEVD construct, with a potent apoptosis inducer and measured luciferase activity in cell lysates. As anticipated, staurosporin treatment resulted in a strong increase in light emission reflecting the increase caspase 3 activity (Fig. 1A). To determine whether this reporter could be used to monitor T cell-induced cell death, we generated surrogate beta-cells by genetically modifying HEK 293T cFLuc-DEVD cells with a lentiviral vector containing the full-length PPI cDNA driven by a CMV early enhancer and promoter. Transduction of HEK 293T cells resulted in expression of the PPI expression cassette as was evident from qPCR analysis (Fig. 1B). Proinsulin synthesis was verified by western blot analysis (Fig. 1C). Overnight incubation of these surrogate beta cells, with increasing amount of $PPI_{(SP15\text{-}24)}$-specific CTL led to increased light emission reflecting the CTL-induced apoptosis of the target cells. In contrast, luciferase activity was unaffected when unmodified HEK 293T cFLuc-DEVD cells were used as target (Fig. 1D). These data show that ectopic expression of PPI sensitizes HEK 239T cells to $PPI_{(SP15\text{-}24)}$-specific CTL cytolysis and validate the specificity of our assay.

The localization of PPI signal peptide within the ER and the TAP independent routing of the $PPI_{(SP15\text{-}24)}$ point to alternative degradation mechanisms [16]. The presence of three alanine residues at the C-terminal part of the PPI signal peptide represents a high-affinity binding motif for the ERAP1 hydrophobic pocket and the two leucine residues in position 13-14 makes this region a suitable substrate for ERAP1 trimming[19]. To test whether ERAP1 plays a role in the generation of the $PPI_{(SP15\text{-}24)}$ peptide, we transduced surrogate beta reporter

cells with ERAP1-specific shRNA containing lentivirus (MOI=1). Expression of shERAP1 led to 90% reduction of ERAP1 mRNA compared to a non-specific short hairpin (Fig. 2A) without affecting ERAP2 gene expression (data not shown). Of note, the reduced ERAP1 expression in our assay had no impact on insulin gene expression or HLA-ABC surface expression (Fig. 2B, C). Downregulation of ERAP1 reduced $PPI_{(SP15-24)}$-specific CTL-mediated target cell destruction, as a 60% reduction of caspase-3 induced luciferase activity was observed at a 1:10 Target:Effector ratio (Fig. 2D). Taken together, our data confirm the implication of ERAP1 in the maturation of the non-conventional $PPI_{(SP15-24)}$ epitope from human PPI[18].

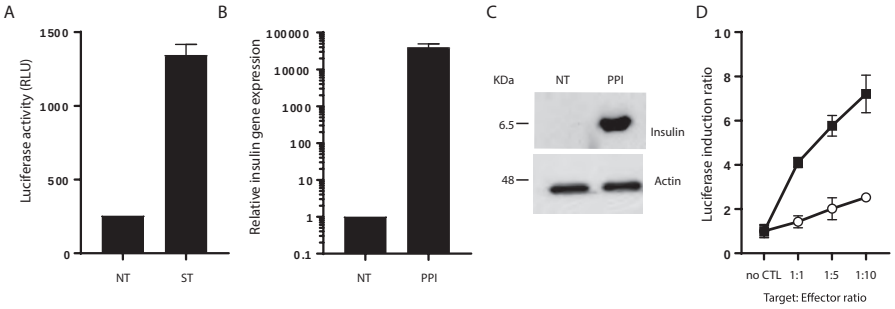

Figure 1: Characterization of caspase 3-inducible reporter and surrogate beta cells. (A) Transduced HeLa cells with cFLuc-DEVD reporter were exposed to 100nM staurosporine (ST) for 1 hr compared to non-treated controls (NT). Results are shown in relative light units (RLU). The results depict the average of 3 experiments. (B) Quantitation of PPI expression in HEK 293T/GFP cells and HEK 293T cells transduced with LV-CMV-PPI-bc-GFP by qPCR (MOI=1). Quantitative PCR analysis has been performed in triplicate and GAPDH corrected. (C) Western blot analysis of PPI expressing cells using an insulin-specific antibody and as control a human actin-specific antibody. (D) HEK293T/GFP cells expressing cFlucDEVD (open symbols) and HEK 293T cells expressing PPI and cFlucDEVD 293T cells (black symbols) were cocultured 16h with increasing numbers of PPI(SP15-24)-directed CTL.

Proinflammatory cytokines play a central role in triggering autoimmunity by inducing ER stress in beta cells[7,23-25]. To evaluate the importance of inflammation-induced ER stress on ERAP1 expression in human beta cells, we treated EndoC-βH1 cells with a mixture of the proinflammatory cytokines IFNγ and Il-1β. ERAP1 was upregulated in the presence of cytokines and this was associated by the upregulation of XBP1s, an important transcription factor induced upon ER stress (Fig. 3A). Similar results were obtained in primary human pancreatic islets (Fig. 3B). Increased ERAP1 gene expression was mirrored by ERAP1 protein levels (Fig. 3C). Activation of the endoribonuclease

IRE1α upon ER stress resulted in the processing of the XBP1 mRNA by and the removal of 26bp to generate an active XBP1s transcription factor. Inhibition of IRE1α by MKC3946, led to reduced XBP1 splicing [26]. Accordingly, co-treatment with MKC3946 dampened cytokine-induced ERAP1 gene expression in EndoC-βH1 cells (Fig. 3D)

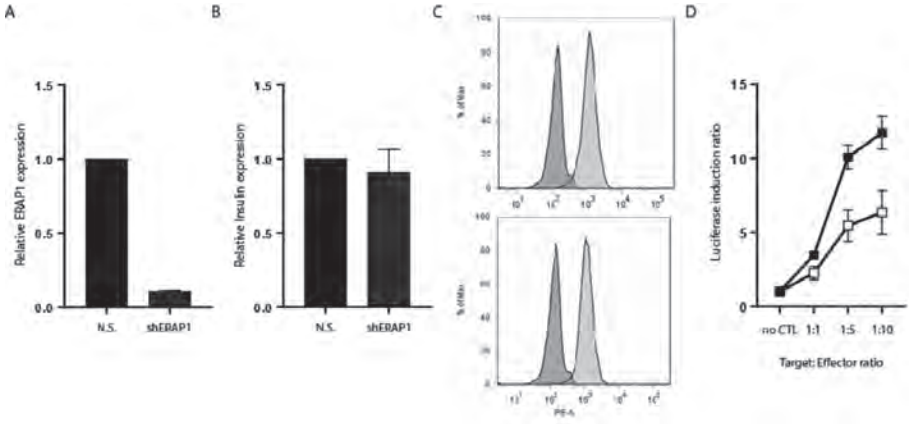

Figure 2: ERAP1 down-regulation by shRNA lentiviruses reduce CTL-mediated killing. A) PPI cFLuc-DEVD HEK 293T cells transduced with lentiviruses containing a non-specific shRNA cassette (N.S.), or a shRNA cassette directed against ERAP1 (shERAP1). Four days post-transduction, cells were analyzed for ERAP1 expression by qPCR. (B) Expression of insulin gene in shERAP1 and non-specific shRNA (N.S.) cells assessed by qPCR. Quantitative PCR analysis has been performed in triplicate and GAPDH corrected. (C) HLA class I surface expression of non-specific shRNA (upper panel) and shERAP1 modified PPI cFLUC-DEVD HEK 293T cells (lower panel). Non stained cells are used as control (dark grey). (D) Killing assay performed on non-specific shRNA (solid squares) and shERAP1 (open squares) expressing PPI cFLUC-DEVD HEK 293T cells after 16 hr co-culture with increasing amounts of PPI(SP15-24) directed CTL. Luciferase activity in absence of CTLs was arbitrary set to 1. The results are shown as fold induction.

IRE1α has been shown to play a critical role in the control of beta cell dysfunction and death via degradation of miR-17-5p and the consequent activation of thioredoxin-interacting protein (TXNIP) [27]. Target sequence analysis of miRNAs expressed in beta cells, revealed 6 potential miRNAs that regulate ERAP1 gene expression[28] (Fig. 4A). All these miRNAs belonged to the miR-17 miRNA family, indicating a conserved miRNA regulatory mechanism (Table 1). In line with these predictions, stimulation of human beta cells with proinflammatory cytokines showed a correlation between increased ERAP1 and XBP1s expression and decreased miR-17 expression as assessed by qPCR (Fig. 4B). Of note, endogenous insulin expression remained unchanged upon cytokine stimulation. To test whether miR-17 has a direct effect on ERAP1, we generated luciferase reporter constructs in which a fragment of the ERAP1

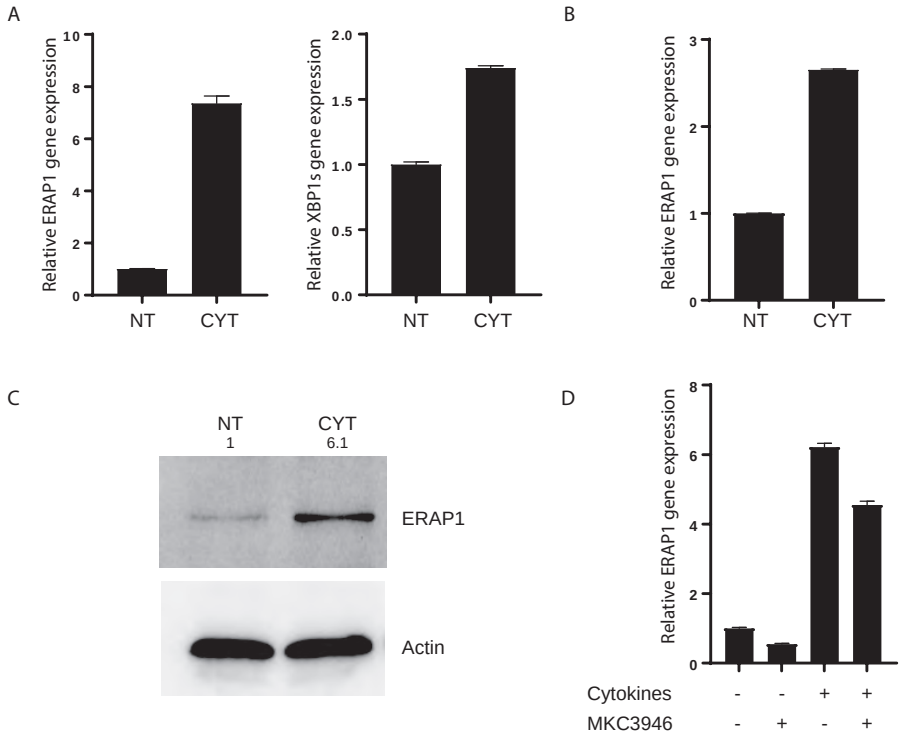

Figure 3: Effect of ER stress induction on ERAP1 expression. A) Relative ERAP1 (left panel) and XBP1s (right panel) expression in EndoC-βH1 cells after 24h treatment with IFNγ and IL-1β (CYT) compared to non-treated cells (NT) analyzed by qPCR. B) Relative ERAP1 expression in primary human pancreatic islets after exposure to proinflammatory cytokines for 24h (CYT) compared to non-treated islets (NT). C) Western blot analysis of EndoC-βH1 lysates for ERAP1 protein expression (upper panel). Actin was used as loading control (lower panel). Quantification has been performed by ImageJ and corrected for actin. D) Relative ERAP1 gene expression in EndoC-βH1 cells after cytokines stimulation in presence or absence of MKC3946 (10uM). Quantitative PCR analysis has been performed in triplicate and GAPDH corrected.

3'UTR containing the potential miR-17 binding site was cloned between the stop codon and the poly-A of the luciferase coding sequence. A UTR mutant in which two mutations were introduced to prevent miR-17 binding was generated and used as control in our assay. Transient expression of miR-17 in HEK 293T luciferase reporter reduced luciferase activity in presence of native UTR sequence, while luciferase activity cells transfected with a mutated reporter UTR sequence remained unchanged compared to control miR-1 (Fig. 5A). These results confirm that miR-17 can directly regulate ERAP1 expression. Supporting these findings, the forced expression of miR17 in human beta cell blunted proinflammatory cytokine-induced upregulation of ERAP1 (Fig. 5B).

Altogether, our data demonstrate a link between proinflammatory cytokine-induced ER stress and ERAP1 via IRE1α-mediated miR-17 suppression in the control of preproinsulin signal peptide trimming, autoantigenic epitope generation, and CTL-mediated target cell destruction.

Table 1: Prediction of ERAP1 targeting miRNAs expressed in human pancreatic beta cells.

miRNA	ERAP1-target motif	Folding energy (Kcal/mol)
Hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG	-16.20
Hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	-16.20
Hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	-16.00
Hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	-16.00
Hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	-14.30
Hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU	-15.50

ERAP1-miRNA motif and the energy folding characteristic of the miR-17 family on the ERAP1 UTR region. Data presented are according to in silico predictions performed on https://cm.jefferson.edu/rna22/Interactive/RNA22Controller.

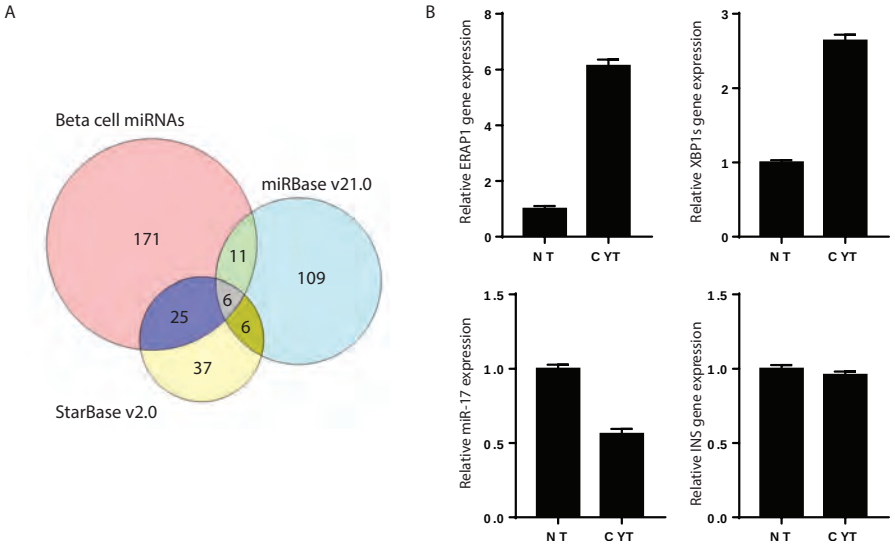

Figure 4: Correlation between ER stress-induced miR-17 and ERAP1 gene expression. A) Venn diagram showing the overlap between miRNAs found in beta cells and predicted ERAP1 targeting miRNAs according to miRbase V21.0 and StarBase V2. B) Relative expression of ERAP1 (top left) XBP-1s (top right), MiR-17 (bottom left) and INS (bottom right) in EndoC-βH1 cells after cytokine stimulation (CYT) compared to non-treated cells (NT). Quantitative PCR analysis has been performed in triplicate. ERAP1, XBP1s, and insulin are GAPDH corrected. MiR-17 expression has been corrected for RNU6.

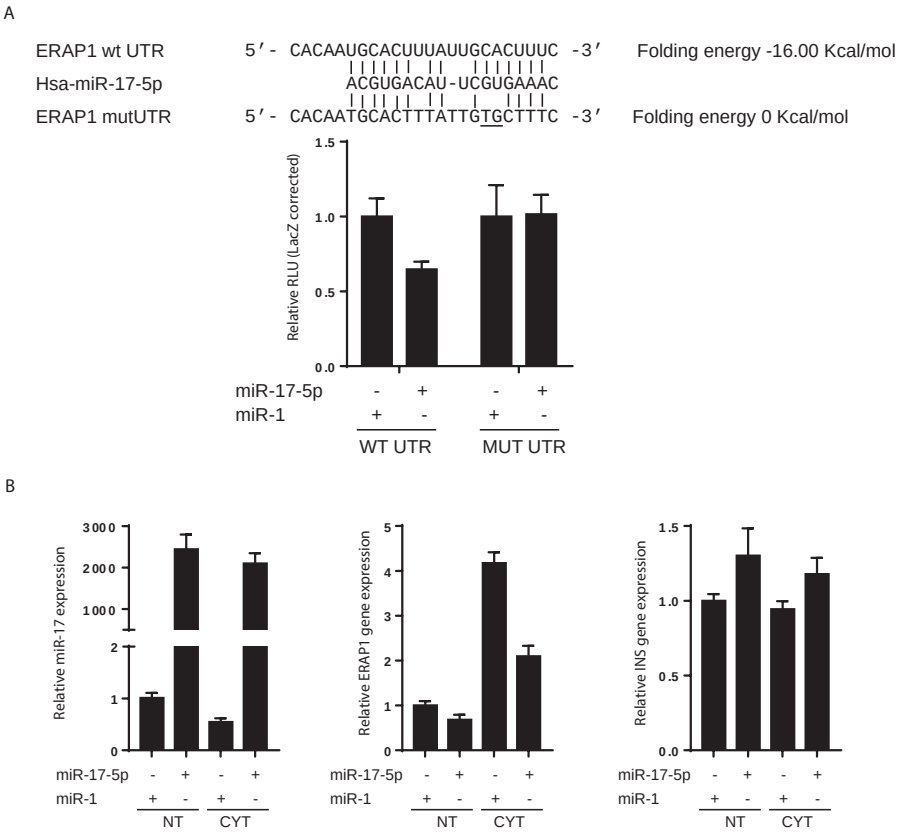

Figure 5: MiR-17 regulates ERAP1 gene expression. A) Schematic representation of the miR-17 targeting sequence in the 3-UTR of ERAP mRNA (wt UTR) and the mutated ERAP1 UTR (mut UTR) in the luciferase reporter constructs with the predicted folding energy (top). Relative luciferase activity after co-transfection of miR-1 (negative control) or miR-17 with ERAP1 wt UTR luciferase construct or ERAP1 mut UTR in 293T cells (bottom). Data are corrected for LacZ activity. B) Relative expression of miR-17 (left panel), ERAP1 (middle panel) and INS (right panel) in EndoC-βH1 following miR-17 or control miR-1 transfection in the presence or absence of cytokine stimulation. Quantitative PCR analysis has been performed in triplicate. ERAP1 and insulin are GAPDH corrected, miR-17 expression has been corrected for RNU6.

DISCUSSION

Here, we demonstrate a new regulatory mechanism connecting ER stress induction and increased beta cell exposure to the immune system.The signal peptide of PPI represents a major source of antigenic peptides derived from insulin gene products [15,16]. Increased ERAP1 expression in isolated human islets and human beta cells upon IFNγ/IL1β stimulation would lead to an increased signal peptide processing and beta cell destruction during inflammation in

T1D patients. While *in vitro* trimming assays have pointed out a peptide length limitation for ERAP1 cleavage (the upper length limit was defined at 16 residues[19]), our data corroborate previous results on the implication of ERAP1 in $PPI_{(SP15-24)}$ processing and presentation [18]. The reduced apoptosis upon ERAP1 knockdown in surrogate beta cells after coculture with $PPI_{(SP15-24)}$-specific CTLs is in line with the reduced expression of T cell activation marker (MIP-1β) observed by Kronenberg-Versteeg *et al*. after targeting ERAP1 by siRNA in their assay [18]. Intriguingly, based on the essential role of peptide trimming for proper presentation of many epitopes, a decrease expression of HLA class I would seem conceivable upon down-regulation of ERAP1. However, transduction of shRNA directed against ERAP1 had no effect on surface expression of HLA. The exact function of ERAP1 in controlling MHC class I expression remains unclear. While ERAP1 knockdown by siRNA in HeLa cells was shown to slightly reduced HLA class I expression, this has not been observed for $H\text{-}2K^b$ surface expression in mice [29,30].

ERAP1 was initially described as IFNγ responsive gene[31]. Yet, cotreatment of proinflammatory cytokines with MKC3946 demonstrates that the IRE1α pathway participates to the regulation of ERAP1 gene expression and reveals a direct link between ER stress and ERAP1 gene expression. Several miRNAs have been implicated in the response to stress [32], among these miR-17 has been shown to be a master regulator of beta cell apoptosis by controlling the thioredoxin-interacting protein (TXNIP)[27,33]. Inhibition of TXNIP prevents diabetes development in mouse models fort T1D and T2D, however the role of TXNIP in beta cells is complex. TXNIP modulates the cellular redox state and participates to the induction of oxidative stress and consequently the induction of apoptosis by activation of caspase 2 and release of cytochrome C, as well as the amplification of insulitis by activating the NLRP3 inflammasome, activation of caspase 1, and maturation of Il1β [34-36]. While TXNIP regulation was demonstrated to be independent of ATF6, PERK and IRE1α were shown to participate to TXNIP transcriptional and post-transcriptional control, respectively [37]. Whether the other arms of the UPR are involved in ERAP1 regulation and expression, as for TXNIP, needs to be determined.

In this study, we confirmed the complexity of antigenic peptide generation originating from signal peptide domain of the human preproinsulin. The implication of the IRE1α/miR-17 pathway in regulating the resident ER protein trimming underscores the key role played by ER stress in the development of autoimmunity. The central role for IRE1α/miR-17 in beta cell dysfunction

and apoptosis via upregulation of TXNIP and autoantigen processing via upregulation of ERAP1 designate IRE1α as an interesting therapeutic target in diabetes. This intervention opportunity is supported by a recent finding of reversed autoimmune diabetes in NOD mice after IRE1α inhibition[38].

MATERIALS AND METHODS

DNA Constructs

pLV-CMV-cFLuc-DEVD was generated by insertion of a PmeI/PmeI fragment from the pcDNA3.1-cFLuc-DEVD[21] into the backbone of pLV-CMV[39] restricted with EcoRV. Preproinsulin cDNA was obtained from reverse transcriptase reaction of total human islets RNA extraction using the following primers: *PPI-Full(Fw)*: ATG GCC CTG TGG ATG CGC CTC CTG CCC; *PPI-Full(Rv)*: GTT GCA GTA GTT CTC CAG CTG GTA GAG GGA GCA. LV-CMV-PPI was generated by insertion of the coding region of the preproinsulin cDNA into pLV-CMV-bcGFP. For miRNA reporter construct the following primers were annealed and cloned into pMIR-REPORT (Thermofisher Scientific) open with PmeI. ERAP UTR Fw: 5'-gta att tga ata tag aca caa tgc act tta ttg cac ttt caa ttc tta taa agc; ERAP UTR Rv 5'-GCT TTA TAA GAA TTG AAA GTG CAA TAA AGT GCA TTG TGT CTA TAT TCA AAT TAC; ERAP UTR mut Fw 5'-gta att tga ata tag aca caa tgc act tta ttg tgc ttt caa ttc tta taa agc; ERAP UTR mut Rv 5'-GCT TTA TAA GAA TTG AAA GCA CAA TAA AGT GCA TTG TGT CTA TAT TCA AAT TAC. The constructs were verified by sequencing.

Cells and reagents

293T (HLA-A2 positive) were grown in high glucose DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (Gibco BRL) and penicillin/streptomycin at 37C, 5%CO2. $PPI_{(SP15-24)}$ directed CTLs were maintained in IMDM supplemented with 10% human serum, IL-2 and IL-15 and restimulated every 14 days with JY cells (pulsed with 10mg/ml PPI15-24 peptide) at 1:5 ratio in presence of IL-2 and IL-15. PPI15-24 was synthesized using solid-phase Fmoc chemistry, analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry for purity and identity. EndoC-βH1 cells, kindly provided by Dr. Raphael Scharfmann (Paris Descartes University, France), were maintained in low glucose DMEM supplemented with 5.5 µg/ml human transferrin, 10 mM nicotinamide, 6.7 ng/ml sodium selenite,

50 μM β-mercaptoethanol, 2% bovine serum albumin fraction V, 100 units/ml Penicillin and 100 μg/ml Streptomycin. Cells were seeded in ECM, fibronectin pre-coated culture plates.

Inflammatory stress was induced by a mixture of 1000 U/ml IFNγ and 2 ng/ml IL1β for 24 h. Staurosporine was used at 100nM for 1h and Thapsigargin was used at 100nM for 24h. MKC3946 inhibitor was used at 10uM for 24h in our assay.

MiRNA transfection

Transient transfection of miRNA mimics were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. EndoC-βH Cells were transfected in a 24-well plate, using a final concentration of 50 nm miRNA precursor, Hsa-miR-17-5p precursor (PM12412, Ambion) or premiR Hsa-miR-1 (Ambion), was used. Experiments were continued 24 h post transfection. For the validation of miRNA targeting sites, 125 ng pMiR-luc-ERAP1-wtUTR or pMIR-luc-ERAP1-mutUTR, 5 ng pLacZ and 50 nM miRNA precursor Hsa-miR-17-5p precursor or premiR Hsa-miR-1 were used per well (96-well plate) to modify 293T cells. Transfections were performed in triplicate and cells were analysed 24 hours post transfection.

Lentiviruses production and transduction

The vectors were produced as described previously[39]. Briefly, the lentiviral backbone containing the gene of interest and the three helper plasmids (encoding HIV-1 gag–pol, HIV-1 rev, and the VSV-G envelope protein) were co-transfected overnight using the calcium phosphate method into 293T cells. The medium was refreshed and viruses were harvested after 48 and 72 h, passed through 0.45-um filters, and stored at -80°C. Virus was quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corp., New York, NY, USA) as described[40]. Then, viral supernatants were added to fresh medium supplemented with 8 μg/ml Polybrene (Sigma), and the cells were incubated overnight. The next day, the medium was replaced with fresh medium. Transduction efficiency was analyzed 3 to 6 days post transduction.

ERAP1 downregulation

shRNA lentiviral constructs for ERAP1 knockdown were obtained from the Mission shRNA library (Sigma-Aldrich clones TRCN060539; TRCN060540; TRCN060541; TRCN060542). Based on preliminary assays to assess knock-down efficiency (data not shown), we selected the TRCN060542 clone for further use.

The shERAP1 encoding lentivirus was produced as described above.

FACS analysis

Cells were washed in PBS containing 0.1% BSA and subsequently incubated in 100 µl of PBS/0.1% BSA with the appropriate amount of antibody for 60 min at 4°C in the dark. Cells stained with the proper isotype were used as negative control. HLA-A2 and HLA-ABC-FITC were purchased at BD Pharmingen Inc. (San Diego, CA, USA). For flowcytometry, 10,000 events were counted on a FACS LSRII (BD Pharmingen Inc., San Diego, CA). Data analyses were performed using FlowJo software.

RT-PCR / qPCR

Total RNA was extracted from cultured cells using Trizol reagent following manufacturer's instructions. Isolated RNA was quantified using a Nanodrop 1000 spectrophotometer. Approximately 500 ng RNA was reverse transcribed using Superscript RT II kit (Invitrogen, Karlsruhe, Germany). Expression of the genes interest was detected using the following primers: Insulin Fw GCA GCC TTT GTG AAC CAA CA, Insulin Rv CGG GTC TTG GGT GTG TAG AAG; ERAP1 Fw GAA AAC CAT GAT GAA CAC TTG G, ERAP1 Rv CCA CCT CTT CTG GGA GGA TGA G; GAPDH Fw ACA GTC AGC CGC ATC TTC TT, GAPDH Rv AAT GAA GGG GTC ATT GAT GG, XBP1s Fw 5'-CTGAGTCCGCAGCAGGTG-3', XBP1s Rv 5'-GAGATGTTCTGGAGGGGTGA-3'; ATF3 Fw 5'-GTGCCGAAACAAGAAGAAGG-3', ATF3 Rv 5'-TCTGAGCCTTCAGTTCAGCA-3'; CHOP Fw 5'-GACCTGCAAGAGGTCCTGTC-3', CHOP Rv 5'- CTCCTCCTCAGTCAGCCAAG-3'; Polymerase chain reactions were performed on a PTC-200 (Biozym, Landgraaf, The Netherlands) using the following conditions: 94°C for 5 min; 35 cycles of 30" at 94°C, 30" at 60°C, and 1.5 min at 72°C; 10 min at 72°C. Real-time PCR were performed in triplicate using the SybrGreen master mix kit (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands) and an Applied Biosystems Step One Plus. Comparative ΔΔct values were performed using GAPDH gene as reference. Values are represented as mean ± standard error.

For microRNA quantification, total RNA was reverse transcribed using Taqman microRNA Reverse transcription kit (Applied Biosystems) and detected using Hsa-miR17 Taqman microRNA assays (PN4427975, Applied Biosystems) and TaqMan 2x Universal PCR Master Mix, no UNG (applied Biosystems) according to manufacturer's instructions. MicroRNA expression was normalized

to RNU6 using the following primers: RNU6 Fw 5'-CTCGCTTCGGCAGCACA-3'; RNU6 Rv 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot analyses

Cells were lysed in RIPA lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, SDS 0.1%, 0.5% DOC, 1% NP40, with proteases inhibitors). For insulin staining, proteins extracts were loaded on 17% acrylamide/bis acrylamide SDS page gel, and after electrophoresis, transferred to Immobilon-P transfer membrane (PVDF); Millipore, Etten-Leur, The Netherlands and treated with anti-insulin (1:1,000; Santa Cruz SC-9168), and anti-actin (1:5,000, clone C4; ICN Biomedicals, Inc., Zoetermeer, The Netherlands) sera. For ERAP1 analyses, proteins extracts were loaded on 10% acrylamide/bis acrylamide SDS gel. After blotting, the membranes were stained with anti-ERAP1 (Santa Cruz B10; Sc-271823).

CTL killing assay & luciferase assay

2.10^4 to 5.10^4 modified HEK 293T cells were plated in 96 well plate and exposed for 16h to increasing amounts of PPI directed CTLs. After overnight coculture, media was removed and cells were lysed in luciferase lysis buffer [125 mM Tris/HCl, pH 7.8, 10 mM CDTA, 10 mM DTT, 50% (v/v) glycerol, 5% (v/v) Triton X-100]. Light emission was determined using Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity from cells in absence of CTL was used as reference and arbitrary set to 1.

In silico analyses and statistical analysis

Data are presented as mean ± SEM. Calculations were performed using GraphPad Prism 7.

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