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Interleukin-22 reverses human islet dysfunction and apoptosis triggered by hyperglycemia and LIGHT

Shadab Abadpour^{1,2,3}, Bente Halvorsen^{3,4}, Afaf Sahraoui^{1,2,3}, Olle Korsgren⁵, Pål Aukrust^{3,4,6} and Hanne Scholz^{1,2,3}

¹Section for Transplant Surgery, Oslo University Hospital, Oslo, Norway

²Institute for Surgical Research, Oslo University Hospital, Oslo, Norway

³Institute of Clinical Medicine, University of Oslo, Oslo, Norway

⁴Research Institute of Internal Medicine, Oslo University Hospital, Oslo, Norway

⁵Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden

⁶Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Oslo, Norway

Correspondence should be addressed to S Abadpour: Shadab.Abadpour@rr-research.no

Abstract

Interleukin (IL)-22 has recently been suggested as an anti-inflammatory cytokine that could protect the islet cells from inflammation- and glucose-induced toxicity. We have previously shown that the tumor necrosis factor family member, LIGHT, can impair human islet function at least partly via pro-apoptotic effects. Herein, we aimed to investigate the protective role of IL-22 on human islets exposed to the combination of hyperglycemia and LIGHT. First, we found upregulation of LIGHT receptors (LT β R and HVEM) in engrafted human islets exposed to hyperglycemia (>11 mM) for 17 days post transplantation by using a double islet transplantation mouse model as well as in human islets cultured with high glucose (HG) (20 mM glucose) + LIGHT *in vitro*, and this latter effect was attenuated by IL-22. The effect of HG + LIGHT impairing glucose-stimulated insulin secretion was reversed by IL-22. The harmful effect of HG + LIGHT on human islet function seemed to involve enhanced endoplasmic reticulum stress evidenced by upregulation of p-IRE1 α and BiP, elevated secretion of pro-inflammatory cytokines (IL-6, IL-8, IP-10 and MCP-1) and the pro-coagulant mediator tissue factor (TF) release and apoptosis in human islets, whereas all these effects were at least partly reversed by IL-22. Our findings suggest that IL-22 could counteract the harmful effects of LIGHT/hyperglycemia on human islet cells and potentially support the strong protective effect of IL-22 on impaired islet function and survival.

Key Words

- ▶ IL-22
- ▶ hyperglycemia
- ▶ LIGHT
- ▶ human islets
- ▶ apoptosis
- ▶ inflammation

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Introduction

Prolonged and repeated exposure to elevated glucose level, defined as hyperglycemia, contributes to beta cell dysfunction and has been shown to involve in progression of type 1 and type 2 diabetes mellitus (DM) (Biarnes *et al.* 2002, Cnop *et al.* 2005, King 2008, Donath & Shoelson 2011, Bensellam *et al.* 2012, Potter *et al.* 2014).

The mechanisms by which long-term presence of hyperglycemia could reduce beta cell activity are at present not fully understood, but could involve induction of endoplasmic reticulum (ER) and oxidative stress leading to pro-inflammatory responses and apoptosis within an islet (Federici *et al.* 2001).

LIGHT/TNSFS14 (TNF superfamily member 14) is a pro-inflammatory cytokine in the TNF superfamily, which signals through TNF receptor superfamily member 14 (TNFRSF14/Herpes Virus Entry Mediator (HVEM)) and 3 (TNFRSF3/Lymphotoxin β Receptor (LT β R)) (Montgomery *et al.* 1996, Zhai *et al.* 1998, Schneider *et al.* 2004). LIGHT is produced by various cells such as activated T cells, macrophages and platelets (Schneider *et al.* 2004, Otterdal *et al.* 2006, Ware 2009) and plays a pathogenic role in several autoimmune (Otterdal *et al.* 2006, Ware 2009, Lin & Hsieh 2011) and metabolic disorders (Bassols *et al.* 2010, Kim *et al.* 2011). Pancreatic islet cells secrete low level of LIGHT (Halvorsen *et al.* 2016), but we have recently reported elevated level of LIGHT in type 2 DM (T2DM) patients, which could reflect the role of LIGHT in the pathogenesis of T2DM (Halvorsen *et al.* 2016). However, the possible effect of LIGHT on hyperglycemia-induced islet injury has not yet been fully investigated.

Anti-inflammatory cytokines are attractive candidates with the ability to regulate aberrant inflammatory processes leading to islet dysfunction (Russell & Morgan 2014). Interleukin (IL)-22 is a relatively newly discovered anti-inflammatory cytokine within the IL-10 cytokine family (Dumoutier *et al.* 2000). It is mainly produced by activated T cells (e.g., T helper (Th)-22 and Th-17 cells) and innate lymphoid cells (Sabat *et al.* 2014) and binds to a heterodimeric cell surface receptor composed of IL-10 receptor (R) 2 and IL-22R1 subunits (Sabat *et al.* 2014). IL-22 is unusual among most of the interleukins because it does not directly regulate the function of immune cells but rather targets cells in organs such as skin, pancreas, liver and kidney (Wolk *et al.* 2004, Sabat *et al.* 2014). Human islets have been shown to express IL-22R1 and administration of IL-22 to human islets reduced oxidative and ER stress induced by pro-inflammatory cytokines and glucotoxicity in a T2DM mouse model (Hasnain *et al.* 2014). However, the potential role of IL-22 on protecting against hyperglycemia-induced islet dysfunction is still not clear.

We hypothesized that LIGHT could enhance hyperglycemia-induced toxicity in human islets, and this harmful effect could be counteracted by IL-22. This hypothesis was tested by different experimental approaches including the *in vivo* effect(s) of high glucose (HG) on the expression of LIGHT-specific receptors in human islets using a double islet transplantation model in immunodeficient mice (Sahraoui *et al.* 2015), as well as the *in vitro* effect(s) of either HG or LIGHT or a combination thereof with or without IL-22 on human islet function and survival.

Materials and methods

Human islets isolation and culture condition

Human islets were obtained from the JDRF award 31-2008-416 (ECIT Islet for Basic Research program) and isolated according to semi-automated purification system (Friberg *et al.* 2008) from 8 male and 6 female non-diabetic brain-dead donors with mean age 55 years (19–70 years) and mean BMI 25 kg/m² (22–32 kg/m²) provided by the islet isolation facility of the Nordic Network for Clinical Islets Transplantation (Uppsala, Sweden, or Oslo University Hospital, Oslo, Norway) after appropriate informed consent from relatives for multi-organ donation and for use in research. All experiments and methods using human islets were approved by and performed in accordance with the guidelines and regulations made by the regional committee for medical and health research ethics central in Norway (2011/782).

Islet purity was judged by digital imaging analysis (Friberg *et al.* 2011) or dithizone staining and only islets with purity >50% was used in this study. Fresh, free floating isolated islets were cultured in CMRL 1066 (Corning) containing 10% human serum and supplements as previously published (Stahle *et al.* 2011). Cell culture medium was changed the day after isolation and subsequently every 2–3 days until use in experiments. In order to perform *in vitro* experiment, human islets were manually hand-picked and cultured at 37°C (5% CO₂) for 48 h in petri dishes (Sterilin Ltd, New Port, UK) with CMRL 1066 medium supplemented with 2% human AB serum (Milan ANALYTICA AG, Switzerland), 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies AS) without (untreated islets) or with either high glucose (HG; 20 mM), human recombinant (hr) LIGHT (400 ng/mL) (R&D System) or a combination thereof (HG+LIGHT). In parallel experiments, human islets were pre-incubated with or without hrIL-22 (50 ng/mL) (R&D System) 30 min prior to treatment with HG+LIGHT. The final concentration and incubation time for human recombinant LIGHT and IL-22 were chosen based on previously published studies (Hasnain *et al.* 2014, Halvorsen *et al.* 2016). Cells and supernatants were harvested as indicated and stored at –80°C until further analysis.

Mouse islets isolation and culture condition

Mouse islets were isolated from 8 to 10 weeks old male Balb/c mice (Taconic, Denmark) as described previously

(Sahraoui *et al.* 2015). In short, 3 mL of Hank's balanced salt solution supplemented with 0.8 mg/mL Collagenase P from *Clostridium histolyticum* (Roche) was injected into pancreatic duct followed by removal of distended pancreas, incubation at 37°C for 17 min and gradient purification of the pancreatic endocrine tissue. Isolated islets were cultured in petri dishes (Sterilin Ltd, New Port, UK) in RPMI 1640 media (HyClone, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 10 mM Hepes and 1% L-glutamine (Gibco) at 37°C and 5% CO₂. Transplantation of isolated islets was performed the next day as described below.

***In vivo* experimental model**

The experimental protocol was approved by the Norwegian National Animal Research Authority project license no FOTS id 3017 and 4712. The animal experiments were performed in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press). Animals were housed under standard condition in an approved facility with free access to food and water except fasting time where only water was readily accessed. We used a double islet transplantation model described and presented in detail previously (Sahraoui *et al.* 2015). Briefly, male NMRI nu/nu-immunodeficient mice (Taconic, Denmark), 8–10 weeks old, were used as recipients after diabetes was introduced by single intravenous infusion of Alloxan monohydrate (75 mg/kg, Sigma-Aldrich). Before transplantation, all recipients had non-fasting blood glucose levels ≥ 20 mmol/L for 2 consecutive days measured by a glucometer (Accu-Chek Aviva Nano, Roche Diagnostics). 380 mouse islets were transplanted under the left kidney capsule of each mouse followed by a two-week recovery period and transplantation of minimal mass of isolated human islets under the right kidney capsule of the same mice. After 1st islet graft removal, animals were divided into two groups based on their measured non-fasting blood glucose: normoglycemic (NG): $n=9$, mice with non-fasting blood glucose (B-glu) < 11 mM and hyperglycemic (HG): $n=15$, mice with non-fasting B-glu > 20 mM and followed for 17 days. Transplanted human islets response to glucose was analyzed by performing oral glucose tolerance test (OGTT) on day 15 after 1st graft removal (Supplementary Fig. 1, see section on supplementary data given at the end of this article). At termination, blood samples were harvested and plasma stored at -80°C for further

analysis. Transplanted human islet grafts were harvested and fixed in 10% formalin followed by sectioning of the grafts for further immunofluorescent staining.

Immunofluorescent staining of human islets

For immunofluorescent staining of transplanted human islet grafts, paraffin-embedded human islet grafts were deparaffinized using xylene and rehydrated in graded series of ethanol washes (100, 90, 85, 70 and 50%) followed by epitope retrieval in 1× Target Retrieval Solution (DAKO) in a hot water bath (99°C).

For immunofluorescent staining of human islet cells, 60–80 hand-picked equally sized islets were dispersed into single cells using TrypLE Express (Life Technologies AS) and proceed to universal 320 cyto-centrifuges (Hettich Lab Technology, Tuttlingen, Germany).

Islet grafts and dispersed islet cells were fixed and permeabilized by 4% paraformaldehyde (PFA) and 0.5% Triton-X100 in PBS, respectively. Protein Block Serum Free (DAKO) was used to block non-specific staining. Slides were then incubated overnight at 4°C with polyclonal rabbit anti-HVEM 1:100 (Novus Biologicals, Abingdon, UK), polyclonal rabbit anti-LT β R 1:100 (LifeSpan BioSciences, WA, USA), polyclonal guinea pig anti-insulin 1:500 (DAKO) and polyclonal rabbit anti-IL-22R1 1:100 (Abcam). After washing with 1× tris buffered saline plus Tween 20 (TBST), slides were incubated with donkey anti-rabbit Alexafluor 594, goat anti-rabbit Alexafluor 488, donkey-anti-guinea pig Alexafluor 488 and goat anti-guinea pig Alexafluor 594 on concentration 1:300 (Life Technologies AS) for 1 h at room temperature followed by nuclear staining using SlowFade Gold antifade reagent with DAPI (Life Technologies AS). TUNEL analysis was performed on fixed islet graft slides using DeadEnd™ Fluorometric TUNEL system (Promega BiotechAB, Stockholm, Sweden) according to the manufacturer protocol prior to DAPI-nuclear staining. Images were taken by Axio Observer Inverted Microscope (Carl Zeiss AS) operated by the ZEN lite software. Area of LT β R-, HVEM-, insulin-, IL-22R1- and TUNEL-positive cells as well as total number of DAPI-stained nuclei were measured and analyzed using Image J software (National Institute for Health). A minimum of 5 images per slides were taken and minimum 2000 cells were scored.

Western blot analysis

Cell lysis buffer (RIPA buffer supplemented with Halt protease inhibitor (Thermo Scientific)) was added to

human islet pellets (100 islets) before proceeding to mechanical disruption using sonication. Samples were centrifuged and purified using QIAshredder purification column (QIAGEN). Total protein concentration was determined using Pierce BCA protein assay (Life Technologies AS). Equal amount of total protein (20 µg) was separated on mini-PROTEIN GTX precast gels followed by protein bands transfer to PVDF membrane (BioRad). According to antibody datasheets provided by the manufacturer, membranes were blocked with either 5% skimmed milk or 5% BSA in 1× TBST and incubated overnight at 4°C with primary antibodies, LTβR rabbit polyclonal antibody (LifeSpan BioSciences), HVEM rabbit polyclonal antibody (Novus Biologicals), Phosphorylated Inositol Requiring Enzyme 1 (p-IRE1α) rabbit monoclonal antibody, Binding Immunoglobulin Protein (BiP) rabbit monoclonal antibody, (C/EBP homologous protein) CHOP mouse monoclonal antibody (Cell Signaling), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) goat polyclonal antibody (Santa Cruz Biotechnology), all at the concentration of 1:1000. Bound antibodies were labeled with goat anti-rabbit IgG-HPR and donkey anti-goat IgG-HPR (Santa Cruz Biotechnology) 1:10,000. Protein bands were visualized using clarity western ECL chemiluminescence substrate kit (BioRad) or super signal west femto (Thermo Scientific) followed by semi-quantitative measurement of band density using chemiDGC touch imaging system (BioRad).

Glucose stimulation insulin secretion assay

Twenty equally sized islets were hand-picked and transferred into a transwell plate (Corning) containing Krebs-Ringer bicarbonate buffer (11.5 mM NaCl₂, 0.5 mM KCl, 2.4 mM NaHCO₃, 2.2 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 2 mg/L albumin: all Sigma-Aldrich) supplemented with 1.67 mM glucose and incubated for 45 min at 37°C. Transwells were switched to Krebs-Ringer bicarbonate buffer containing 20 mM glucose and incubated for 45 min at 37°C. Supernatants were harvested for analysis of insulin secretion using human insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Stimulation index (SI) was calculated as a ratio of insulin secreted in high concentration of glucose (20 mM) to insulin in low concentration of glucose (1.67 mM).

Insulin content

200 islets were hand-picked and lysed using cell lysis buffer (RIPA buffer supplemented with Halt protease

inhibitor (Thermo Scientific). Acetic-ethanol was added to the lysates and incubated at 4°C for 24 h before insulin content was measured by human insulin ELISA kit (Mercodia AB) and presented as insulin content over total protein concentration of samples.

Viability detection and cell death analysis

Cell death detection

Programmed cell death was analyzed by detection of DNA-histone complex in the cytoplasmic fraction of islet lysates using Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics) according to protocol offered and described by the manufacturer.

Multiplex apoptosis assay

Apoptosis signaling pathways were measured in cytoplasmic and mitochondrial fractions of human islet lysates by Bio-plex Pro RBM apoptosis assay panel 2 (Bd, Bcl-xl, Bim and Mcl-1) and using multiplex technology on a Multiplex Analyzer (BioRad). The cytosolic and mitochondrial fractions of human islets were lysed according to manufacturer protocol.

Caspase 3/7 assay

200 islets were hand-picked and lysed using cell lysis buffer (RIPA buffer supplemented with Halt protease inhibitor (Thermo Scientific)). 20 µL of cell lysates were mixed with 20 µL of Caspase3/7-Glu reagent and proceed to Caspase-Glu 3/7 assay according to the protocol offered and described by the manufacturer (Promega Biotech AB, Nacka, Sweden).

FDA/PI viability assessment

Viability assessment was performed on hand-picked islets using fluorescein diacetate (FDA) 20 µg/mL (Sigma-Aldrich Norway AS) for detection of live cells and propidium iodide (PI) 100 µg/mL (Thermo Fisher Scientific) for evaluating the degree of dead cells. Images were taken using Axio Observer Inverted Microscope (Carl Zeiss AS) operated by ZEN lite software.

Biochemical measurements

Levels of cytokine IL-6 and the chemokines IL-8/CXCL8, interferon γ-induced protein 10 (IP-10)/CXCL10 and monocyte chemoattractant protein (MCP)-1/CCL2 were measured in cell-free supernatants utilizing multiplex

technology on a Multiplex Analyzer (BioRad) following the instructions provided by the manufacturer. Tissue factors (TF, full length; Hyphen BioMed, Neuville-sur-oise, France) were measured in cell-free supernatants with EIA as described by the manufacturers. Human C-peptide levels were measured by using an ultra-sensitive human C-peptide ELISA assay (Mercodia) and mouse pro-inflammatory cytokines release (IL-6, MCP-1, GRO- α , IP-10) were measured in plasma samples from blood drawn from the mice at harvest day by multiplex technology (Meso Scale Diagnostics, MR, USA).

Statistical analysis

Data are presented as means $\pm$ S.E.M. and GraphPad Prism, version 6.0. was used for data analysis. Differences among three groups were evaluated by one-way ANOVA with Bonferroni corrections. Difference between two groups was evaluated using either Mann–Whitney *U* test or Wilcoxon matched pair signed-rank test based on experimental design (unpaired vs paired). Difference in non-fasting B-glu between mice in group NG vs HG was analyzed with two-way ANOVA. Significance was set at $P < 0.05$.

Results

Prolonged hyperglycemia upregulates the expression of LT β R and HVEM receptors in dysfunctional human islet grafts *in vivo*

We have recently reported an increase in the expression of LIGHT receptors LT β R and HVEM in human islets exposed to pro-inflammatory cytokines (i.e., a combination of IL-1 β , TNF and interferon (IFN) γ) *in vitro* (Halvorsen *et al.* 2016). However, the effect of hyperglycemia on expression of the two LIGHT receptors has not been studied. Herein, we investigated the *in vivo* expression of LIGHT receptors in engrafted human islets exposed to normoglycemia (NG; B-glu < 11 mM) and hyperglycemia (HG; B-glu > 20 mM) in an immunodeficient mice model for 17 days (Supplementary Fig. 1A). Functionality tests confirming dysfunctional human islet grafts exposed to hyperglycemic compared to normoglycemic milieu for 17 days in transplanted mice are presented in Supplementary Fig. 1B, C and D. The difference in the pro-inflammatory status of hyperglycemic vs normoglycemic animals was confirmed by showing the significant increase in circulating plasma level of mouse IL-6, MCP-1, GRO- α and IP-10 in hyperglycemic animals,

presented in Supplementary Fig. 1E, F, G and H. Double immunofluorescent staining of the engrafted human islets for LT β R/insulin and HVEM/insulin (Fig. 1A and B) showed an increase in the area of LT β R-positive (Fig. 1D) and HVEM-positive (Fig. 1E) cells in failing human islet grafts exposed to HG compared to the islets exposed to NG. The adverse effect of hyperglycemia on failing human islet function and viability was also confirmed by a reduction in insulin area (Fig. 1F) and an increase in the ratio of TUNEL-positive cells over DAPI-nuclear staining in the human islet grafts in HG compared to NG animals (Fig. 1C and G).

Human islets express IL-22 receptor

The potential of isolated human islet to respond to IL-22 treatment was confirmed by immunofluorescent labeling of human islets for IL-22R1 together with insulin counter-staining (Supplementary Fig. 2A and B). Interestingly, we also found a significant upregulation in the expression of IL-22R1 in human islets treated with HG+LIGHT compared to the untreated islets (Supplementary Fig. 2A and B).

IL-22 reverses the HG + LIGHT-induced upregulation of LIGHT receptors

Confirming the *in vivo* data and our recent *in vitro* data (Halvorsen *et al.* 2016), immunoblotting analysis revealed a 2- and 4-fold increase in protein levels of LT β R (Fig. 2A and B) and HVEM (Fig. 2A, B and C) receptors in human islets exposed to the combination of HG (20 mM) plus LIGHT (400 ng/mL) *in vitro* compared to the untreated islets. In contrast, treatment with either HG or LIGHT alone did not significantly increase HVEM and LT β R protein levels (Fig. 2A, B and C). Notably, adding IL-22 (50 ng/mL) to HG+LIGHT-treated human islets significantly reduced the protein levels of LT β R and HVEM by 2-fold (Fig. 2A, B and C). Immunofluorescent staining of dispersed human islets also confirmed upregulation in the protein levels of LT β R (Fig. 2D and E) and HVEM (Fig. 2F and G) in human islets treated with HG+LIGHT, which were nearly normalized by IL-22 (Fig. 2D, E, D and G).

IL-22 reverses the adverse effect of HG + LIGHT on human islets function

To elucidate the potential functional consequences of IL-22 on HG+LIGHT-treated human islets, we examined

Figure 1

Hyperglycemia upregulates the expression of LTβR and HVEM receptors in transplanted human islets. Representative images showing immunofluorescent double staining for LTβR/insulin (A), HVEM/insulin (B) and TUNEL labeling (C) together with DAPI-nuclear staining in human islets engrafted under normoglycemic (NG) or hyperglycemic (HG) mice kidney capsule. Magnification 40× with scale bars 20 μm. Quantification of the area of LTβR-positive (D) and HVEM-positive cells (E) as well as the area of insulin-positive cells (F) and the ratio of TUNEL-positive cells to DAPI-nuclear staining (G) within transplanted human islet grafts. In all analysis, data is analyzed with Mann Whitney *U* test and presented as mean ± s.e.m. *n* = 9 mice in group NG (b-glu < 11 mM), *n* = 15 mice in group HG (b-glu > 20 mM), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs group NG. HG, hyperglycemic; *n*, number of mice in each group; NG, normoglycemic.

insulin secretion in response to basal (1.67 mM) and stimulated (20 mM) glucose levels in isolated human islets treated with either HG (20 mM), LIGHT (400 ng/mL) and the combination thereof with or without IL-22 (50 ng/mL) for 48 h (Fig. 3A) and calculated the SI (Fig. 3B) as described in the method section. Human islet exposure to HG+LIGHT decreased insulin secretion in response to stimulating glucose concentrations compared to the untreated islets (Fig. 3A) and impaired SI (Fig. 3B). Notably, the adverse effect of HG+LIGHT was reversed by IL-22 as shown by increased insulin secretion in response to glucose exposure resulting in improved SI (Fig. 3A and B). However, there was no significant effect of IL-22 on total insulin content in human islets treated with HG+LIGHT (*P* = 0.05, Fig. 3C).

IL-22 could at least partly protect human islets from ER stress induced by HG + LIGHT

The protective effect of IL-22 on ER stress, induced by pro-inflammatory cytokines has been previously reported in isolated human islets *in vitro* and mouse model for T2DM (Hasnain *et al.* 2014). We therefore investigated the effect of HG and LIGHT on ER stress response, particularly the activation of unfolded protein response (UPR), and explored whether IL-22 could protect human islets from this stress response. The protein level of p-IRE1α, the molecular chaperone BiP and pro-apoptotic CHOP (Eizirik *et al.* 2013) were analyzed in human islets treated with either HG (20 mM) and LIGHT (400 ng/mL) alone or in combination thereof with or without IL-22

Figure 2

IL-22 reverses the effect of HG + LIGHT on expression of LTβR and HVEM receptors. Western blot analysis of LTβR and HVEM in human islets treated with either HG (20mM) or LIGHT (400ng/mL) and HG + hrLIGHT with or without IL-22 (50ng/mL) for 48 h (A). Quantification of LTβR (B) and HVEM (C) band densities normalized to housekeeping gene GAPDH and presented as fold of untr islets. Representative images showing immunofluorescent staining of dispersed human islets treated with HG (20mM) or LIGHT (400ng/mL) and combined HG + LIGHT with or without IL-22 (50ng/mL) for LTβR (D) and HVEM (F) together with insulin and DAPI-nuclear counter-staining. Magnification 10x, scale bar 100μm. Score of LTβR-positive (E) and HVEM-positive cells (G) to the total number of counted cells. For all analysis, data is analyzed by one-way ANOVA with Bonferroni corrections and presented as mean ± s.e.m., $n=3$. * $P<0.05$, *** $P<0.001$ vs untr islets, # $P<0.05$, ### $P<0.001$ vs HG + LIGHT-treated islets. HG, high glucose; n , number of independent donors; Untr, untreated.

(50ng/mL) for 48 h. Immunoblotting analysis of ER stress markers revealed a significant increase in protein levels of p-IRE1α and BiP (4- and 1.6-fold increase, respectively), but not of CHOP, in human islets treated with HG + LIGHT (Fig. 4A, B, C and D). However, these effects were not modulated by IL-22 (Fig. 4A, B, C and D).

IL-22 reduces the expression of pro-inflammatory cytokines triggered by HG + LIGHT in human islets

We have previously shown that inflammatory stimuli increased the expression of HVEM and LTβR in human islets (Halvorsen *et al.* 2016). Herein, we show that treatment with HG + LIGHT, but not HG or LIGHT alone, induced a 2.5-fold increase in protein secretion of IL-6 (Fig. 5A), IL-8 (Fig. 5B), MCP-1 (Fig. 5C), IP-10 (Fig. 5D) and the pro-coagulant mediator TF (Fig. 5E) in human islets compare to the untreated islets. Interestingly, IL-22 significantly attenuated the effect of HG + LIGHT

on the expression of these pro-inflammatory (IL-6, IL-8, IP-10, MCP-1) and pro-coagulant (TF) molecules by approximately 2-fold reduction (Fig. 5).

IL-22 ameliorates apoptosis induced by HG + LIGHT in human islets

We have recently reported that LIGHT promotes apoptosis in human islets (Halvorsen *et al.* 2016). Herein, we show that the pro-apoptotic effect of LIGHT is even stronger when LIGHT is combined with HG as assessed by cell death detection ELISA^{PLUS} and caspase3/7 activity analysis (Fig. 6A and B). As shown in Fig. 6A and B, the pro-apoptotic effect of HG + LIGHT was significantly attenuated by IL-22 in human islets. The anti-apoptotic effect of IL-22 was also demonstrated by reduced PI staining (Fig. 6C) in HG + LIGHT-treated islets. To further elucidate the mechanism involved in the anti-apoptotic effect of IL-22, the protein expression of pro- (Bad, Bim) and

Figure 3

IL-22 recovers the adverse effect of HG+LIGHT on human islets function and mass. Insulin secretion in response to basal (1.67 mM) and stimulated (20 mM) levels of glucose was measured by EIA (A) and calculated as stimulation index (SI) (B) on human islets exposed to either HG (20 mM) or LIGHT (400 ng/mL) and the combination of HG+LIGHT with or without IL-22 (50 ng/mL) for 48 h. Insulin content was measured by EIA in human islets treated with HG or LIGHT and combined HG+LIGHT with or without IL-22 for 48 h and normalized to total protein concentration (C). In A, data is analyzed with Wilcoxon matched paired signed rank test and Mann Whitney U-test. In B and C, data is analyzed by one-way ANOVA with Bonferroni corrections. All data are presented as mean $\pm$ S.E.M., $n=7$. * $P<0.05$ vs untr islets, # $P<0.05$ vs HG+LIGHT-treated islets. § $P<0.05$ vs stimulated glucose level. HG, high glucose; n , number of independent donors; Untr, untreated.

Figure 4

IL-22 might at least partly protect human islets from HG+LIGHT-induced ER stress. Western blot analysis of p-IRE1 α , BiP, CHOP and GAPDH in human islets treated with HG (20 mM) or LIGHT (400 ng/mL) and HG+LIGHT with or without IL-22 (50 ng/mL) for 48 h (A). Quantification of p-IRE1 α (B), BiP (C) and CHOP (D) band densities normalized to GAPDH and presented as fold of untreated islets. For all analysis data is analyzed with one-way ANOVA with Bonferroni corrections and presented as mean $\pm$ S.E.M., $n=3$. * $P<0.05$ vs untr islets. HG, high glucose; n , number of independent donors; Untr, untreated.

anti-apoptotic (Bcl-xl, Mcl-1) mediators were analyzed in human islet cells. Islets treated with HG+LIGHT showed enhanced protein levels of the pro-apoptotic mediators Bim (Fig. 6E) and Bad (Fig. 6D) compared to the untreated islets. This was reversed by treatment of IL-22, although Bad protein level did not reach statistical significance. In addition, IL-22 increased the protein level of anti-apoptotic mediators Bcl-xl (Fig. 6F) and Mcl-1 (Fig. 6G) in human islets treated with HG+LIGHT.

Discussion

IL-22 has recently been suggested as a cytokine that could protect islets against inflammation- and glucose-induced toxicity (Hasnain *et al.* 2014). We have previously reported that LIGHT could impair human islet function at least partly via pro-apoptotic effects (Halvorsen *et al.* 2016). Here, we showed that the deleterious effects of LIGHT in combination with hyperglycemia on insulin secretion were counteracted by IL-22. Human islet cells expressed IL-22 receptor and the protective effects of IL-22 involved downregulation of the LIGHT receptors, reduced induction of pro-inflammatory cytokines and TF and attenuated apoptosis. Therefore, our findings further underscore a role of LIGHT and hyperglycemia in the pathogenesis of islet dysfunction and suggest that these effects could be counteracted by IL-22.

Figure 5

IL-22 protects human islets from inflammatory responses induced by HG + LIGHT. Assessment of pro-inflammatory mediators IL-6 (A), IL-8(B), MCP-1 (C), IP-10 (D) and TF (E) measured by multiplex assay in cell-free supernatant collected after human islets were cultured with either HG (20mM) or LIGHT (400ng/mL) and combination of HG+LIGHT with or without IL-22 (50ng/mL) for 48h. In all analysis, data is presented as a fold changes vs untreated islets with mean $\pm$ s.e.m., $n=8$ and analyzed by one-way ANOVA with Bonferroni corrections. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs untr islets, # $P<0.05$, ## $P<0.01$ vs HG+LIGHT-treated islets. HG, high glucose; n, number of independent donors; TF, tissue factor; Untr, untreated.

We have recently suggested a link between LIGHT and T2DM, as we reported an increase in LIGHT in patients with T2DM (Halvorsen *et al.* 2016). We have also reported upregulation of LIGHT receptors induced by pro-inflammatory cytokines in human islets, mediating LIGHT-induced impairment of islet function in response to high glucose levels (Halvorsen *et al.* 2016). Herein, we report an upregulation of LIGHT receptors (HVEM and LT β R) (i) in failing human islets exposed to prolonged hyperglycemia *in vivo* and (ii) by hyperglycemia in combination with LIGHT in isolated human islets *in vitro*. The adverse synergistic effect of hyperglycemia and inflammation on impaired metabolic control in diabetic patients has previously been reported (Ray *et al.* 2007).

The observed significant decrease in human islet viability and function in this study by exposing islets to the combination of hyperglycemia and LIGHT, but not by each of them alone, support a negative synergistic effect of the combined hyperglycemia and inflammation on human islets and further supports that LIGHT might be an important mediator of these harmful effects.

The imbalance between pro- and anti-inflammatory mediators is one of the main features associated with the pathogenesis of T1 and T2DM (Potter *et al.* 2014, Russell & Morgan 2014). IL-22, which is a newly discovered member of the IL-10 cytokine family (Sabat *et al.* 2014), has been found recently to be a strong and potent suppressor of oxidative and ER stress induced by various pro-inflammatory mediators in MIN6 beta cell line and isolated human pancreatic islets (Hasnain *et al.* 2014). IL-22 has been shown to induce broad protective effect against stress responses in pancreatic islets and hepatocytes (Radaeva *et al.* 2004, Hasnain *et al.* 2014). We showed that IL-22 receptor subunit 1 was expressed in human islets, which confirmed the previously published data (Hasnain *et al.* 2014). In addition, we found an upregulation in the protein level of IL-22R1 after human islets were exposed to hyperglycemia and LIGHT. This could explain the strong observed beneficial effect of IL-22 administration on reversing stress response and improving islet function. In particular, the protective effect of IL-22 on hyperglycemia and LIGHT-induced human islet dysfunction could at least partly involve reduction of ER stress. The presence of ER stress evidenced by an increase in the expression of the ER stress markers p-IRE1 and BiP has been demonstrated in islets of diabetic patients, and in isolated islets prior to, and post transplantation (Lipson *et al.* 2008, Marhfour *et al.* 2012, Negi *et al.* 2012). Moreover, due to low levels of antioxidants, islets are susceptible to oxidative stress, which could disturb ER redox status and protein folding capacity of the ER (Malhotra & Kaufman 2007, Mohseni Salehi Monfared *et al.* 2009). Administration of exogenous mouse IL-22 to the mouse model for T2DM could reverse elevated ER stress activity evidenced by reduction in BiP mRNA and protein levels (Hasnain *et al.* 2014). In the presented study, the effects of IL-22 on downregulation of p-IRE1 and BiP suggest that IL-22 can at least partly prevent ER stress in islets exposed to hyperglycemia and LIGHT. In contrast, a recent published study addressing the effect of IL-22 therapy on beta cell stress in an autoimmune type 1 diabetic mouse model, found no effect on BiP expression and activated ER stress in islets from mice treated with exogenous mouse IL-22 (Borg *et al.* 2017). The IL-22 therapy in this mouse model failed both to prevent onset

Figure 6

IL-22 reverses elevated apoptosis induced by HG + LIGHT in human islets. Apoptosis evaluated in cytoplasmic fraction of human islets treated with either HG (20 mM) or LIGHT (400 ng/mL) and the combination of HG + LIGHT with or without IL-22 (50 ng/mL) for 48 h by Cell death ELISA^{PLUS} (A) and Caspase 3/7 activity measurement (B) ($n=7$). Fluorescein diacetate (FDA)/propidium iodide (PI) staining followed by islets visualization with fluorescent microscope plus the bright-field images taken from the same islets showing islets integrity (C). Magnification 10 $\times$, scale bar 200 μ m. Protein expression assessment of Bad (D), Bim (E), Bcl-xl (F) and Mcl-1 (G) by multiplex assay in cytoplasmic and mitochondrial fractions of human islets treated with either HG or LIGHT and combined HG + LIGHT with or without IL-22. Data is presented as fold of untreated islets, mean $\pm$ S.E.M. and analyzed by one-way ANOVA with Bonferroni corrections, $n=4$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs untr islets, # $P<0.05$, ## $P<0.01$ vs HG + LIGHT-treated islets. HG, high glucose; n , number of independent donors; Untr, untreated.

of diabetes and to improve beta cell function when was given as a treatment for overt type 1 diabetes. There might be several explanations for the different results observed in that study compare to ours. We focus our study on a direct effect of IL-22 in human islets after induced harsh stressed milieu by a combination of hyperglycemia and LIGHT. Our data suggested that the elevated protein level of IL-22 receptor and short-term administration of IL-22 significantly protected human islets from stressed environment. In addition, the contrasting results could also be due to a difference in islets, as we focused on the effect of isolated human islets whereas the study by Borg and coworkers was focused on the effect of IL-22 on mouse islets in a diabetic mouse pancreas. Lastly, the concentration and frequency of IL-22 administration required for overcoming autoimmunity-induced ER stress lack optimizations in this autoimmune mouse model.

The combination of hyperglycemia and LIGHT led to a significant increase in secretion of the pro-inflammatory cytokine IL-6 and the chemokines IL-8, IP-10 and MCP-1,

which all are known to play a role in pathogenesis of diabetes as well as islets loss post transplantation (Kristiansen & Mandrup-Poulsen 2005, Kaminitz *et al.* 2007, Corrado *et al.* 2014, Montane *et al.* 2014). Our data showed the suppressive role of IL-22 on the secretion of these pro-inflammatory mediators, which was consistent with previously published data (Hasnain *et al.* 2014) and further support an anti-inflammatory role of IL-22 in human islet cells. Moreover, IL-22 also down-regulated TF, a potent upstream molecule in the coagulation cascade, in human islets exposed to hyperglycemia and LIGHT. TF has not only been shown to increase in diabetic patients (Eden *et al.* 2015), but also been reported to involve in activation of the instant blood-mediated inflammatory reaction (IBMIR) and consequently, the induction of islet loss post transplantation (Moberg *et al.* 2005, Hardstedt *et al.* 2016). Therefore, the ability of IL-22 to attenuate secretion of pro-inflammatory cytokines and TF illustrates its potential as an inhibitor for IBMIR and islet cell dysfunction post transplantation (Cabric *et al.* 2007).

We have previously reported that LIGHT induces apoptosis in human islets (Halvorsen *et al.* 2016) and here we show that this effect was more pronounced when LIGHT was combined with hyperglycemia. We also observed that this pro-apoptotic effect in human islets treated with a combination of hyperglycemia and LIGHT was accompanied by a marked imbalance between pro- and anti-apoptotic proteins. Apoptosis induced by hyperglycemia or pro-inflammatory cytokines occurs when the concentration of pro-apoptotic elements exceeds that of anti-apoptotic proteins in the mitochondrial intrinsic pathway (Grunnet *et al.* 2009, Tomita 2016). Interestingly, IL-22 reversed the apoptotic effect of hyperglycemia and LIGHT through an increase in the anti-apoptotic proteins Mcl-1 and Bcl-xl over pro-apoptotic proteins Bad and Bim, which could explain the mechanism involved in anti-apoptotic effect of IL-22. CHOP is known as one of the main mediators for beta cell death during ER stress (Eizirik *et al.* 2013). CHOP knockdown in INS-cell line as well as mouse and human islets reported a partial and transitory protective effect against pro-inflammatory cytokines induced apoptosis (Shao *et al.* 2010, Allagnat *et al.* 2012). It has been suggested that ER stress triggers apoptosis in islets via activation of intrinsic mitochondrial pathway in CHOP-dependent and -independent ways (Eizirik *et al.* 2013). In our study, we did not observe a clear upregulation of CHOP protein expression in human islets exposed to hyperglycemia and LIGHT. We therefore cannot conclude that IL-22 is directly inhibiting the apoptosis in islets via reduction of ER stress in a CHOP-dependent pathway, and this will need further investigation.

Although, glucose is required for beta cell function and insulin secretion, prolonged islets exposure to hyperglycemia has been shown to induce an adverse effect on insulin secretion and islet function (Bensellam *et al.* 2012). As we have shown previously, LIGHT could also impair insulin release when human islets were exposed to high glucose level (Halvorsen *et al.* 2016). Here, we also observed that the combination of hyperglycemia and LIGHT attenuated islet cells response to high glucose concentration and consequently, induced the observed reduction in stimulation index. Interestingly, improvements in insulin secretion and human islet response to glucose induced by IL-22 could be a result of reduced apoptosis, stress and inflammatory responses induced by hyperglycemia and LIGHT in human islets.

Another advantage of modulating the IL-22/IL-22 receptor system is that this cytokine does not directly regulate the function of immune cells as IL-10 does (Moore *et al.* 2001, Wolk *et al.* 2004). Therefore,

therapeutic interventions targeting the IL-22/IL-22 receptor system might not result in some of the immune-related side effects that occur when other classic cytokines like TNF- α or INF- γ are modulated (Sabat *et al.* 2014). Interestingly, IL-22 gene expression has been found to increase in the islets of patients with new-onset of type 1 DM (Kuric *et al.* 2017), which could support a novel modulatory effect of IL-22 on islet dysfunction induced by inflammatory responses in T1DM. However, IL-22 also represents a broad and sometimes paradoxical protective and pro-inflammatory functions depending on the tissue surrounding (Gimeno Brias *et al.* 2016). Administration of mouse IL-22 has recently been reported to have no effect on islet cells area, glycemic control, beta cell function, ER stress and macrophages and neutrophils infiltration in mouse model for T1DM (Borg *et al.* 2017). However, most of these studies are restricted to rodent islets and more information regarding the IL-22 regulation and function on human islets as well as the therapeutic concentration and frequency of IL-22 administration are required before manipulation of this cytokine as a treatment option for T1DM.

Various attempts have been done over the years to improve isolate human islet function and viability in pre-culture phase prior to transplantation (Moberg *et al.* 2003, Ichii *et al.* 2006, Johansson *et al.* 2006, Sahraoui *et al.* 2014). Our findings showed the potential protective effects of IL-22 on reversing activated stress responses, pro-inflammatory and pro-coagulant mediators and apoptosis induced by hyperglycemia and LIGHT in isolated human islets. Therefore, IL-22 could induce a beneficial effect on isolated human islets in the pre-culture phase prior to transplantation.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0182>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conception and design of the experiments: S A, B H and H S. Experimentation and data analysis: S A, A S and H S. Human islets isolation: O K, H S. Drafting of manuscript: S A and H S. Editing the manuscript: S A, H S, B H, P A, O K, S A and H S are the guarantors of this work and, as such, have full access to all the data in the study and take the responsibility for the integrity of the data and the accuracy of the analysis.

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