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TLR4 triggered complex inflammation in human pancreatic islets

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ABSTRACT

Type 2 Diabetes (T2D) is strongly associated with obesity and inflammation. Toll-like receptor-4 (TLR-4) is the major pro-inflammatory pathway with its ligands and downstream products increased systemically in T2D and in at-risk individuals. Detailed mechanisms of the complex proinflammatory response in pancreatic islets remain unknown.

In isolated human islets LPS induced IL-1β, IL-6, IL-8 and TNF production in a TLR4-dependent manner and severely impaired β-cell survival and function. IL-6 antagonism improved β-cell function. IL-8, which was identified specifically in α-cells, initiated monocyte migration, a process fully blocked by IL-8 neutralization. The TLR4 response was potentiated in obese donors; with higher IL-1β, IL-6 and IL-8 expression than in non-obese donors.

TLR4 activation leads to a complex multi-cellular inflammatory response in human islets, which involves β-cell failure, cytokine production and macrophage recruitment to islets. In obesity, the amplified TLR4 response may potentiate β-cell damage and accelerate diabetes progression.

1. Introduction

Type 2 Diabetes (T2D) is strongly associated with obesity and characterized by chronic insulin resistance, progressive failure of pancreatic β-cells, and finally hyperglycaemia [1]. The association of T2D with inflammatory mediators, such as IL-1β, IL-6, IL-8, TNF as well as circulating acute-phase proteins is highlighted in several prospective and retrospective cohort studies [2–5]. Such measures of chronic, low-grade, sterile inflammation can predict type 2 diabetes progression. Pro-inflammatory cytokines and chemokines can independently cause insulin resistance in peripheral insulin-sensing tissues like fat, liver and muscle [5,6] and progressive β-cell failure. This ultimately shifts metabolism from relative insulin insufficiency - due to the greater insulin demand in obesity - to definite insulin deficiency [7] and on the level of the β-cell from compensation to decompensation [8,9].

Toll-like receptor (TLR)-4 signaling is one of the major pro-inflammatory pathways activated by exogenous pathogen-related or endogenous danger-related molecules. Circulating levels of the classical TLR4 ligand – LPS are elevated in obese and T2D individuals as well as in rodent obesity/diabetes models, recently termed as “metabolic endotoxemia” [10,11]. The implicated mechanism is attributed to the “leaky gut”; associated with alterations in the gut microbiota composition during obesity, causing perturbations of tight junction proteins. This results in an elevation in gut permeability, allowing more gut microbe-derived LPS to enter the circulation [10,12]. Other TLR4 ligands/agonists, including CXCL10, HMGB1, S100A8, hyaluronan, are also elevated in the serum of T2D patients [13–15]. TLR4-deficiency or its pharmacological or genetic inhibition ameliorates obesity- or lipid-induced insulin resistance in mouse models and in humans [16–20]. Saturated fatty acids, particularly palmitate, have also been connected to TLR4 activation in the context of obesity and T2D [16,21–23]. However, palmitate is not a direct agonist for TLR4 [24,25], and indirectly activates TLR4 through the hepato-adipokine fetuin-A [24].

The whole TLR4 complex is not only expressed in cells of the immune system; relatively high expression was found in endocrine cells of pancreatic islets [14,26] and thus, a direct role of TLR4 in islet inflammation was proposed. Indeed, TLR4 activation contributes to β-cell failure; stimulation of isolated rodent islets by TLR4 ligands impairs β-cell function and survival [26–30]. In purified human β-cells and isolated human/rodent islets, LPS treatment reduces insulin gene

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expression [28,29], which confirms a direct TLR4 effect on the level of the β-cell.

The intra- and inter-islet cellular cross talk may accelerate inflammation by paracrine and endocrine effects of inflammatory mediators. Islet resident and infiltrated immune cells can induce mouse islet inflammation and dysfunction in ex vivo conditions [30]. Therefore, research on intact islets, where the islet microenvironment and the crosstalk between different islet cell populations is preserved, will identify the impact of inflammatory stimuli on islet function and cellular viability, rather than working with isolated cell types, which suffer per se from a poor survival due to the loss of their natural integrity and cellular communication [31]. Hence, in this present study, we investigated whether and through which inflammatory mediators TLR4-triggered inflammation and the islet's cellular inflammatory interplay affect β-cell function and viability.

2. Results

2.1. LPS induces inflammatory response, β-cell apoptosis and dysfunction in human islets

24 h treatment with the classical TLR4 ligand LPS induced pro-inflammatory cytokine and chemokine expression in isolated human islets in a dose-dependent manner. IL-1β expression was particularly sensitive to low dose LPS while 20 μg/ml LPS induced maximal expression of all tested cytokines and chemokines IL-1β, IL-6, IL-8, TNF and CCL2 (also named MCP-1; Fig. 1A). Consistently, secreted IL-1β, IL-6, IL-8 and TNF were significantly induced by LPS in human islets (Fig. 1B,C).

Prolonged (but not short-term; data not shown) LPS stimulation of human islets for 72 h induced β-cell apoptosis shown by the TUNEL assay (4.2-fold increase vs. control; Fig. 1D, E) and by caspase-3 cleavage (Fig. 2C). Despite the induction of apoptosis, basal insulin secretion was not affected by prolonged LPS stimulation, while 16.7 mM glucose-stimulated insulin secretion (GSIS) was remarkably reduced (Fig. 1F), which resulted in a 71% decreased stimulatory index (Fig. 1G) and a 72% reduction in insulin content (Fig. 1H). Collectively, these results demonstrate that prolonged LPS treatment not only impairs β-cell survival and insulin secretion in response to glucose stimulation, but also diminishes the total insulin production capacity in β-cells. This is consistent with previous studies in mouse islets [28,29].

2.2. LPS induced pro-inflammatory cytokine and chemokine production and β-cell apoptosis are TLR4 dependent

To verify the TLR4 mediated effect of LPS in human islets, we used a specific TLR4 inhibitor TAK-242, which disrupts the interaction between TLR4 and its adaptor protein TIRAP and TRAM [32–34]. TAK-242 fully blocked LPS-induced IL-1β, IL-6, IL-8 and TNF mRNA expression (Fig. 2A) as well as secretion (Fig. 2B). TAK-242 showed trends to reduced basal IL-1β (insert; Fig. 2A), IL-8 and TNF expression in human islets (Fig. 2A), which was seen in all human islet isolations, however, at a different magnitude. This suggests the existence of endogenous TLR4 ligands in islets, reminiscent of the finding that unstimulated rodent islets in culture also produce pro-inflammatory and pro-oxidant signals through TLR4 [35]. In line with the LPS induced increase in TUNEL positive β-cells (Fig. 1D), it significantly induced Caspase-3 cleavage and phosphorylation of IκBα in human islets (Fig. 2C), which was prevented by TAK-242 treatment (Fig. 2C). There was a similar trend of LPS to induce p46/54 JNK in a TLR4 dependent manner (Fig. 2D).

2.3. IL-6 antagonism improves TLR4-mediated β-cell dysfunction

Among the cytokines and chemokines investigated, IL-6 and IL-8 were particularly highly secreted from LPS-treated human islets (30.0 and 188.3 pg/ml, respectively, Fig. 1B), so we aimed to identify the consequence of such secretion.

IL-6 signaling is blocked by the IL-6 receptor super-antagonist Sant7, an IL-6 variant, which competitively binds to the IL-6 receptor without the ability to bind to the imperative co-receptor Gp130, hereby blocking IL-6 signaling [36]. Already at a dose of 1 μg/ml, Sant7 specifically inhibits IL-6 activity shown in all previously tested cell systems, including human hepatoma, melanoma and myeloma cell lines [36,37] and human primary myeloma cells [38] and lung fibroblasts [39]. Sant7 does not inhibit other cytokines, including the gp130 family cytokines IL-11, CNTF, LIF and OM [40].

In human islets, Sant7 improved LPS-impaired glucose stimulated insulin secretion (Fig. 3A), and insulin content (Fig. 3B) in all human islet isolations, but had no effect on β-cell survival (Fig. 3C) nor on LPS-induced cytokine expression (data not shown). These data suggest, that prolonged TLR4 activation-induced IL-6 alone has a deleterious effect on β-cell function in human islets.

2.4. Islet α-cells produce IL-8, which is responsible for monocyte migration induced by conditioned medium from LPS-treated human islets

A previous study has shown that high glucose in combination with palmitate can induce pancreatic α-cells to produce IL-8 in isolated human islets, which induces monocyte migration [41]. In order to confirm the cellular origin of TLR4 activation induced IL-8 production, we performed immunostaining on paraffin-embedded human islet sections, which exhibited IL-8 induction by LPS in glucagon-producing α-cells (Fig. 4A). The chemokine IL-8 primarily targets neutrophils for chemotaxis, but also targets monocytes to induce migration to inflammatory sites to become recruited macrophages [41,42]. In order to identify whether LPS-treated human islets could recruit monocytes, we isolated monocytes from blood buffy coats of healthy blood donors and then performed a monocyte migration assay using islet-conditioned medium from control and LPS treated islets. Flow cytometry analysis showed a purity of 77.2 ± 2.93% blood monocytes (Suppl. Fig. 1), while the other cells were mainly lymphocytes (data not shown). Medium conditioned by LPS-treated human islets highly increased blood monocyte migration, compared to medium conditioned by untreated human islets (Fig. 4B). Also, LPS-spiked medium had no effect on monocyte migration (Fig. 4B), indicating that the LPS-induced islet-derived humoral chemoattractants rather than LPS itself are responsible for such migration. An IL-8 neutralizing antibody markedly inhibited migration in a dose-dependent manner with no effect of an isotype IgG control (Fig. 4B), exhibiting that TLR4 activation in human islets is able to induce monocyte migration through IL-8. This result provides another evidence that metabolic conditions - previously glucolipotoxicity [41], and now metabolic endotoxemia, induce monocyte migration by islet α-cell-derived IL-8.

2.5. Activated islet-conditioned macrophages contribute to cytokine production

Monocyte-derived macrophages are considered as source of infiltrating macrophages in inflamed islets in T2D [43,44]. However, the low number of such tissue macrophages in islets (~0.5–0.7 macrophages/control islet and 1.4–1.7 macrophages/ islet in patients with T2D [41,45]) restricts the direct investigation of infiltrating macrophages in islets. The local tissue microenvironment determines macrophage profiles in many tissues and organs [46]. Thus, we used an in-vitro macrophage model, in which we cultured monocytes during the M-CSF-mediated monocyte differentiation into macrophages with islet-conditioned medium to mimic islet-infiltrating macrophages. General macrophage markers (CD68, CD11b and CD14) were confirmed with no difference between islet-conditioned (M_{islets}) and classical control M-CSF-induced macrophages (M_0) (Suppl. Fig. 2A) and no obvious morphological difference (data not shown). Similarly to islets, these

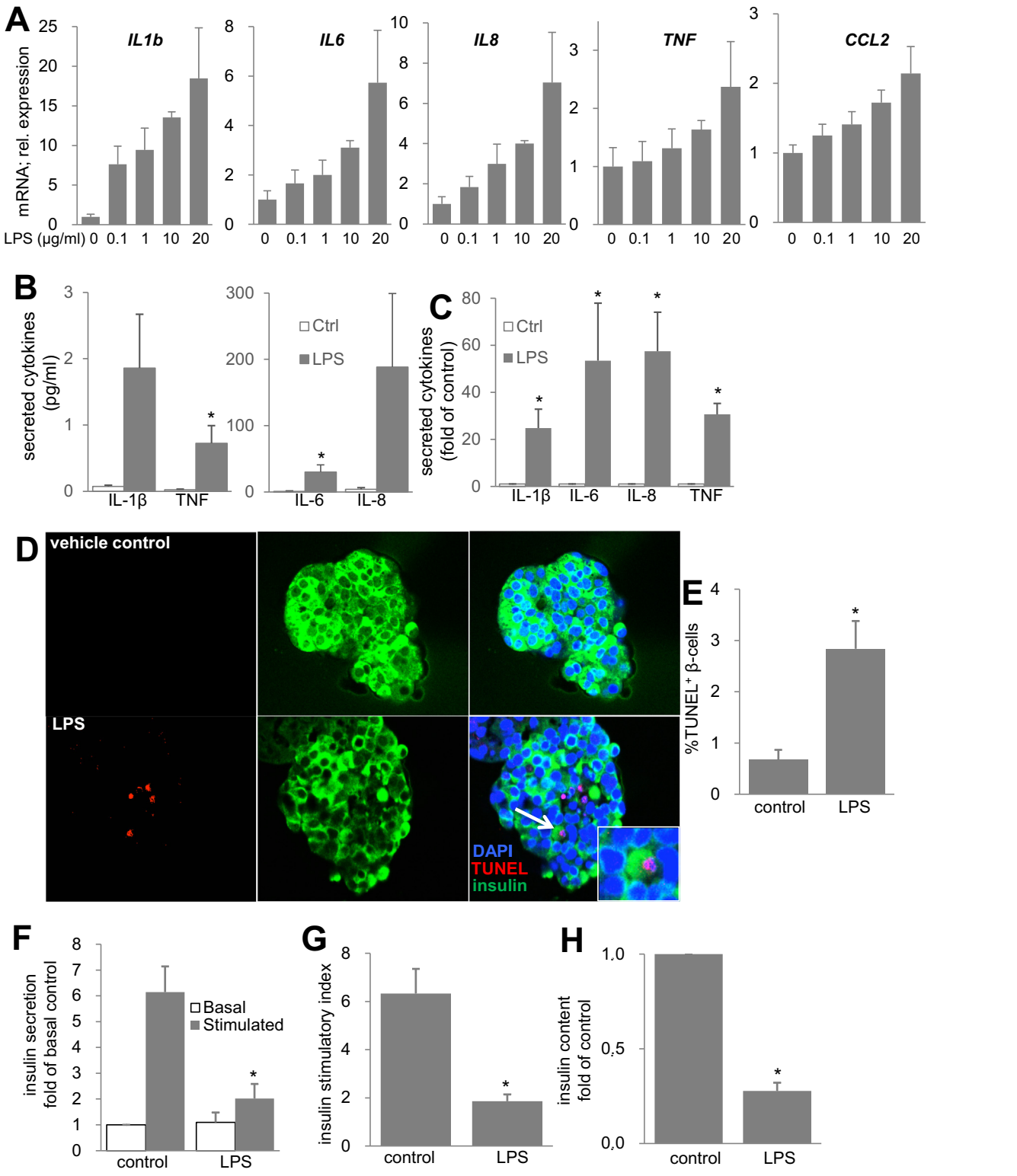

Fig. 1. LPS induces pro-inflammatory cytokine and chemokine production, β-cell apoptosis and dysfunction in isolated human islets.
(A) Human islets were treated with increasing doses of LPS as indicated for 24 h, RNA was isolated and analyzed for cytokine and chemokine expression by realtime RT-PCR. (B, C) Human islets were treated with 20 µg/ml LPS for 24 h, culture media were collected to assay the secreted cytokines and chemokines, showing their absolute concentration from pooled data from 4 isolations (B) and the same data normalized to fold of control (C). (D–H) Cultured human islets were treated with 20 µg/ml LPS for 3 days, followed by (D, E) double staining for TUNEL (red, arrows) and insulin (green) and TUNEL and insulin positive cells (D) were quantified (E). (F) Human islet function was assessed by basal (2.8 mM) and glucose stimulated (16.7 mM) insulin secretion expressed as fold change of basal insulin levels at control. (G) Stimulatory index denotes the amount of glucose stimulated (16.7 mM glucose) insulin secretion divided by the amount of basal insulin secretion and (H) insulin content normalized to total protein. Data are means ± SEM from (A) $n = 2$, (B,C) $n = 4$, (D,E) $n = 5$ and (F–H) $n = 6$ independent experiments from different islet isolations, respectively. *$p < 0.05$ vs. respective control.

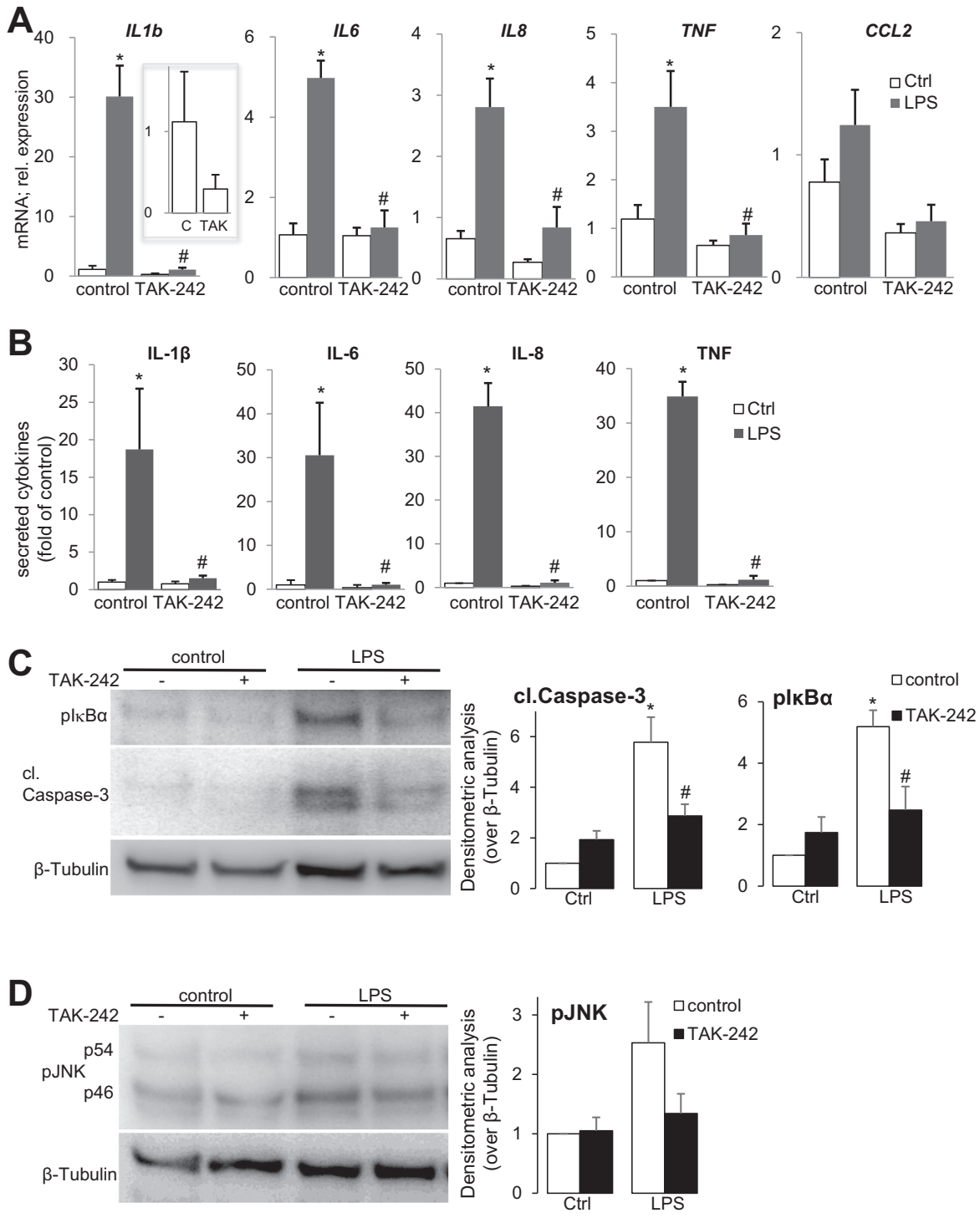

Fig. 2. TLR4 inhibitor blocks LPS induced cytokine and chemokine expression and β-cell apoptosis.
(A–D) Human islets were treated with 20 μg/ml LPS for 24 h with or without 10 μM TLR4 inhibitor TAK-242. (A) RNA was isolated from islets and analyzed for cytokine and chemokine expression by realtime RT-PCR; the insert in the 1st panel visualizes *IL1b* expression at the basal control level with a smaller axis. (B) Secreted cytokines and chemokines from the supernatants was determined by ELISA and shown as fold of control. (C, D) Islet cell lysates were analyzed by Western Blotting for the indicated proteins; β-Tubulin was used as loading control and representative images from one out of three to four independent islet isolations are shown. Densitometric analysis was performed to quantify blots from all independent experiments. Data are means ± SEM from 3 to 5 independent experiments from 3 to 5 different human islet isolations. *p < 0.05 vs. control. #p < 0.05 TAK-242 vs. vehicle control.

macrophages were treated with LPS for 24 h, followed by analysis of pro-inflammatory cytokine expression and secretion. As expected, LPS induced a strong expression of IL-1β, IL-6 and TNF (Fig. 5A).

In order to allow comparison of cytokine levels from islets and infiltrating macrophages, we normalized the cytokine concentration secreted from islet-conditioned macrophages to the estimated number of infiltrating macrophages in islets from T2D donors [41,45]. As shown in Fig. 5B, LPS-treated M_{islets} produced a mean of 1.08 pg/ml IL-1β and of 2.54 pg/ml IL-6, which is similar or less than secreted from islets (1.86 pg/ml and 30 pg/ml, respectively, Fig. 1B). In contrast, there was

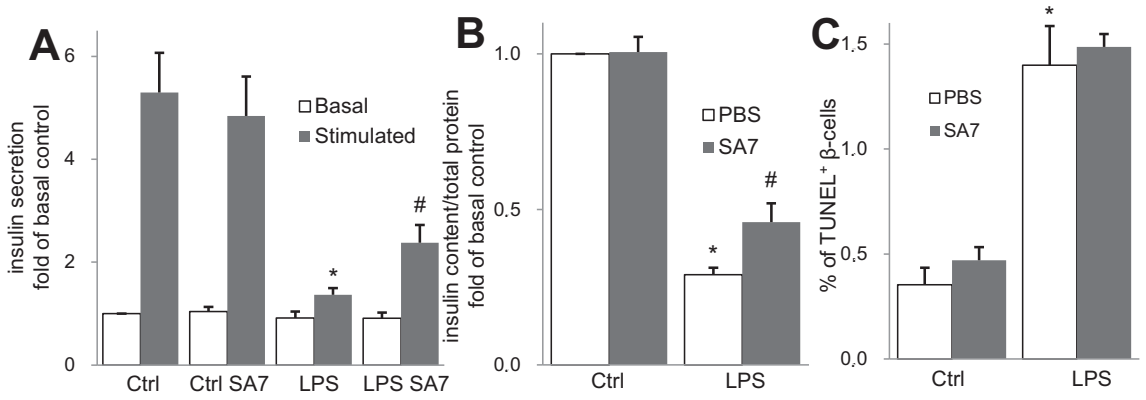

Fig. 3. IL-6 antagonism improves TLR4-mediated β-cell dysfunction.
(A–C) Human islets were treated with LPS for 3 days with or without the IL-6 antagonist Sant7 (SA7), followed by analysis of (A) GSIS (2.8 mM glucose (basal) for 1 h followed by 16.7 mM glucose (stimulation) for 1 h, insulin secretion normalized to total protein) and (B) insulin content normalized to total protein, both expressed as fold of change of basal insulin levels at control. (C) Double staining for TUNEL and insulin and quantification of TUNEL positive β-cells. Data are means ± SEM from 3 to 5 independent experiments from 3 to 5 different human islet isolations. *p < 0.05 vs. respective control, #p < 0.05 Sant7 vs. vehicle treated islets.

much higher TNF secretion from M_{islets} (51.2 pg/ml, Fig. 5B) than from non-diabetic islets (0.72 pg/ml, Fig. 1B).

Unlike islet resident macrophages, which mainly produce IL-1β in response to TLR4 activation [30], islet conditioned activated macrophages secret TNF as the major pro-inflammatory cytokine, and these cells represent the islet infiltrating macrophages. LPS-induced cytokine expression and secretion in M_{islets} from 4 isolations tend to be lower than in classical control macrophages (M_0), which reached significance in IL-6 mRNA expression (Suppl. Fig. 2B,C) and suggests the existence of islet-derived factors to reduce a pro-inflammatory response.

2.6. Elevated TLR4 response in islets from obese donors

Obesity displays a pro-inflammatory, often pre-diabetic stage, which predisposes individuals for the development of the metabolic syndrome, including T2D. TLR4 activation induced α-cells to produce IL-8, which triggered migration of blood monocytes and hereby increased the amount of islet macrophages, which would subsequently amplify TLR4 triggered islet inflammation. To establish the link between obesity and TLR4 response in human islets, we expanded the pool of islet preparations and classified non-obese (BMI < 30; $n = 10$) and obese donors (BMI > 30; $n = 7$; donor details see Suppl. Table 1). In isolated islets, LPS-induced IL-1β, IL-6 and IL-8 mRNA expression was significantly potentiated in obese organ donors, compared to non-obese donors (Fig. 6A). While CCL2 expression was not markedly induced in non-obese donors, it became significant in obese donors (Fig. 6A), also suggestive of a stronger TLR4 response in obesity. Notably, basal IL-1β mRNA had a trend to elevate in islets from obese donors (Fig. 6A; insert), similar to what has been observed in islets from obese rodents [47,48] and from patients with type 2 diabetes [49–51]. As inflammation might be at least in part brought by tissue macrophages, we analyzed expression of the macrophage marker CD68, the total myeloid cell marker CD11b as well as TLR4 itself and its co-receptor CD14 in the same setting of islets from non-obese and obese individuals. Trends to increased expression of all markers were observed in obese donors (Fig. 6B), but didn't reach statistical significance. Therefore, the elevated cytokine expression may come from enhanced TLR4 signaling, rather than from expression of the receptor itself. Taken together, the above results demonstrate that TLR4-triggered islet inflammation is stronger in obese donors. This also implies a further amplified β-cell dysfunction and apoptosis, which accelerate diabetes progression.

3. Discussion

In this study we discovered that LPS-triggered TLR4 activation induces β-cell dysfunction, apoptosis and a pro-inflammatory response through IL-1β, IL-6, TNF and IL-8 production in human islets. While LPS-induced IL-6 was partially responsible for β-cell dysfunction, in confirmation with what we have shown before in rodent islets [30], IL-8 from α-cells was responsible for monocyte migration to islets during TLR4-activation-induced islet inflammation. Such complex cellular TLR4 response in islets is further potentiated in obese individuals, with more IL-1β, IL-6 and IL-8 expression and a tendency to more islet macrophage accumulation, which may be the prerequisite for a higher vulnerability of obese individuals to later development of β-cell damage und diabetes.

3.1. TLR4 as key player in inflammation in metabolic disease

On the basis of multiple mouse studies, which show that TLR4-deficiency improves insulin sensitivity and inhibits obesity-induced tissue inflammation [16,18,19,24,52], TLR4 is regarded as one key factor of the pathogenesis of T2D. TLR4 expression is elevated in insulin-responsive tissues in obese mouse models [16,53,54] and in obese human individuals [55,56] as well as in islets from diabetic mice [57], while there is no significant increase in TLR4 expression in human islets from obese (this study) or T2D individuals [14], which suggests that enhanced TLR4 signaling but not its expression contributes to the islet inflammatory phenotype in obesity and diabetes. Also, some debate exists on the association of diabetes and TLR4 polymorphisms [58–60].

TLR4 signaling is induced by its classical ligand, LPS; its circulating level is increased in obese and T2D individuals, as well as in rodent obesity/diabetes models [10,61]. Obesity is associated with an alteration of the gut microbiota composition, causing perturbations of tight junction proteins that result in an elevation in gut permeability, which induces metabolic endotoxemia [10,12]. Saturated fatty acids, whose circulating levels are significantly increased during obesity, activate TLR4 signaling [16,21–23]. Palmitate indirectly stimulates TLR4 signaling through the hepato-adipokine fetuin-A [24], which itself also activates TLR4 [62]. Palmitate also induces TLR4-independent inflammation in LPS-pre-primed macrophages [25] as part of the metabolic endotoxemia. While palmitate induces impairment in β-cell function and survival in human and rodent islets, which is further potentiated by a combination with high glucose [63,64], our additional data show that such impairment was not TLR4-dependent, as TAK-242 did not significantly induce a reversal of the effects (He & Maedler; unpublished).

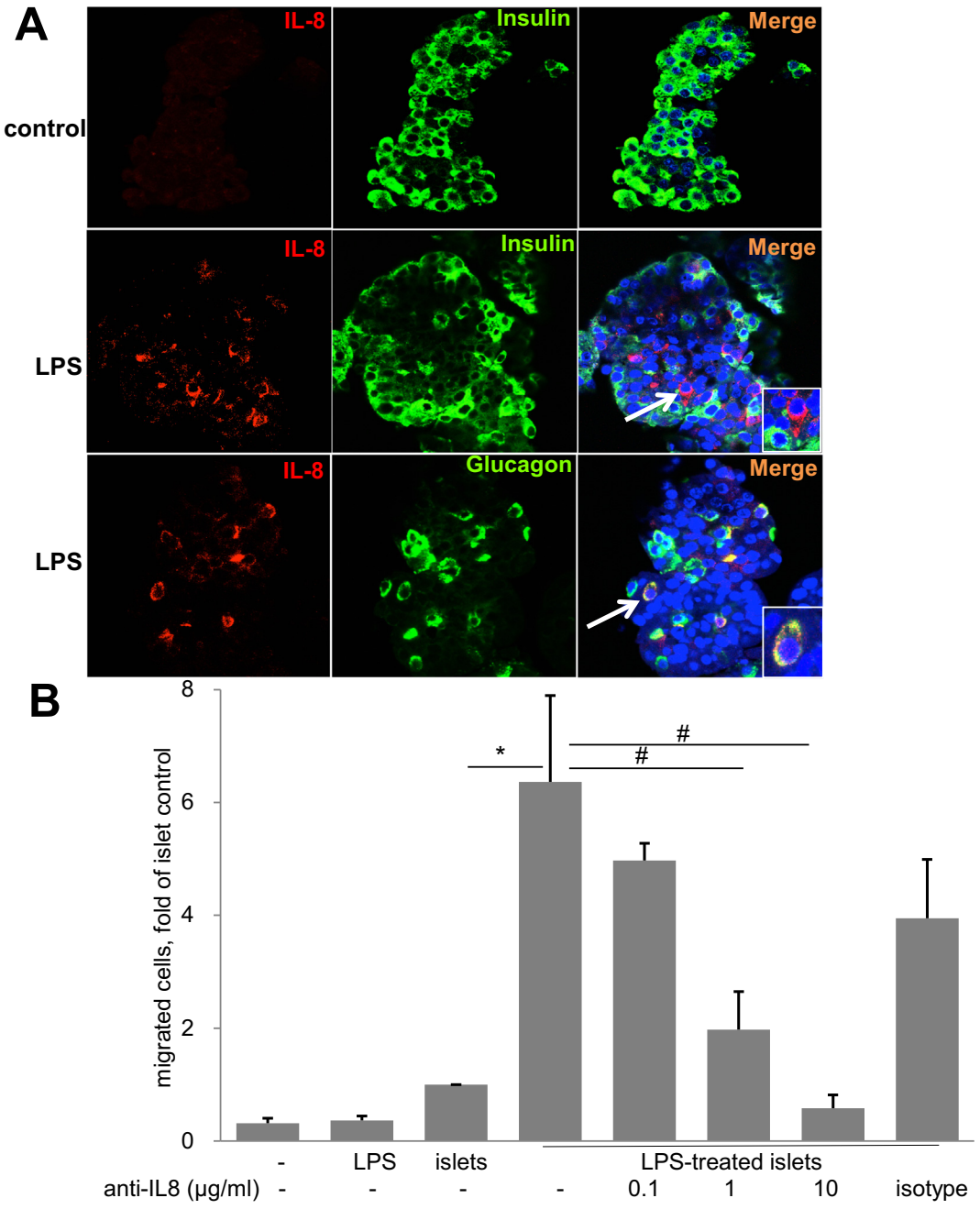

Fig. 4. α-cells produce IL-8, which is responsible for LPS induced monocyte migration to human islets.
(A) Human islets were treated with 20 µg/ml LPS for 24 h, paraffin embedded islet sections were double stained for IL-8 and insulin or glucagon. Representative images from 3 independent experiments were taken with confocal microscope. (B) Monocyte migration was performed in transwell microporous membrane inserts with blood monocytes isolated from blood buffy coats of healthy donors, and culture medium conditioned by LPS-treated human islets treated with or without mouse anti-human IL-8 (0.1, 1 or 10 µg/ml) or isotype mouse IgG control (10 µg/ml). Migrated live cells were stained with fluorescein diacetate and then counted. Islet culture medium itself (without islets) served as blank control ("-"), and the same medium with added LPS was used as LPS-spiked control ("LPS"). Results are shown as fold of untreated islet control ("islets"). Data are means ± SEM pooled from 3 independent experiments from 3 human blood and 3 human islet donors. *p < 0.05 vs. untreated islet control, #p < 0.05 anti-IL-8 vs. w/o anti-IL-8.

3.2. TLR4 and β-cell failure

LPS induces β-cell apoptosis and directly impairs GSIS in rodent islets [26,27,57], while there is no consensus whether insulin content is affected as well [26,28].

Unlike in rodent islets, TLR4 activation by LPS clearly reduces insulin gene expression in purified human β-cells as well as in human islets [28,29], which is in line with our present study, which shows direct impairment of GSIS as well as a severe reduction in insulin content in human islets, probably as a result of both reduction in insulin production and β-cell loss through apoptosis.

Also co-culture with medium conditioned by LPS-treated macrophages impairs GSIS, suggesting that the functional impairment is mediated by a cellular crosstalk between recruited islet macrophages and β-cells within rodent islets [30]. Also palmitate induced mouse β-cell dysfunction through TLR4 depends on recruited macrophages [43]. In support of such cellular crosstalk hypothesis, LPS had no effect on GSIS in a clonal rat β-cell line [65] in the absence of other islet cell

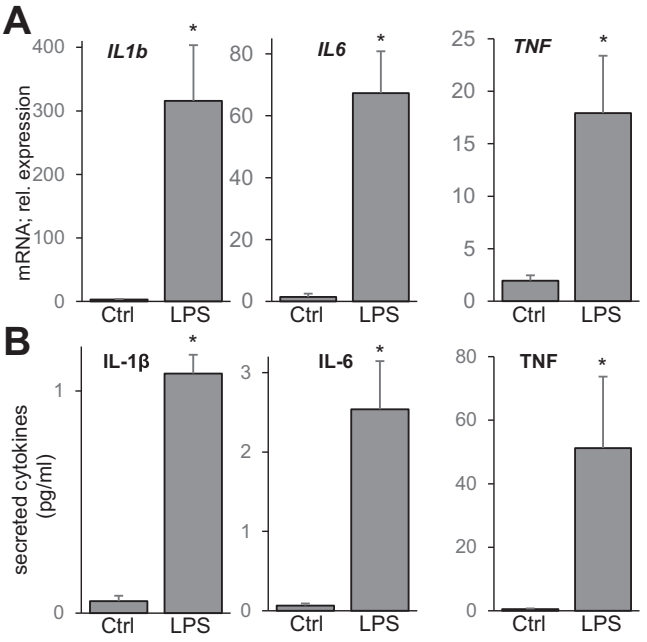

Fig. 5. LPS-induced TLR4 response in Islet-conditioned monocyte-derived macrophages.

Isolated human blood monocytes were induced with M-CSF for macrophage differentiation in the absence (M_0) or presence of islet-conditioned medium (the obtained macrophages are named as M_{islets}), followed by treatment with 100 ng/ml LPS for 24 h. Islet-conditioned monocyte-derived macrophages (M_{islets}) and the control monocyte-derived macrophages (M_0) were analyzed for (A) cytokine gene expression by realtime-PCR and (B) secretion into the supernatant by cytokine multiplex ELISA.

Secreted cytokines were normalized to 140 M_{islets}, equal to the number of infiltrating macrophages in 140 diabetic islets, comparable to the results from Fig. 1B where 140 islets were used for cytokine quantification. Results from M_0 are shown in Suppl. Fig. 2. Data are means ± SEM from $n = 4$ independent experiments from different macrophage preparations. *p < 0.05 LPS vs. control.

types. The intra-islet cellular crosstalk between endocrine cells and myeloid cells not only contributes to the escalation of islet inflammation, but also to β-cell failure [43,66]. Contradictory results exist on the LPS effects on β-cell survival [30,57], which may rather be due to different culture conditions, i.e. LPS origins, doses and culture duration, as we show that β-cell failure was only induced after prolonged LPS exposure for 72 h. Relatively high LPS levels are needed to reveal such effect in human islets. The existence of endogenous TLR4 ligands [67,68], as well as a possible endotoxin contamination of enzymes during islet isolation may account for the basal cytokine expression and enhance the threshold of TLR4 responses.

While in mouse islets, LPS-induced impairment on GSIS relies on recruited macrophages [30], we found here in intact human islets that LPS-triggered TLR4 activation directly induced β-cell dysfunction and apoptosis without the addition of macrophages or conditioned medium. This may imply the inherent differences among species regarding TLR4-elicited islet inflammation, but this also shows that human β-cells are more susceptible to inflammatory injuries triggered by TLR4 ligands than mouse β-cells. Even though recruited macrophages are not indispensable for jeopardizing human β-cell function and survival, we speculate that recruited macrophages will anyway magnify islet inflammation and β-cell impairment, resulting in accelerated diabetes progression in human individuals.

3.3. Cytokines, chemokines and β-cell failure

Physiological concentrations of cytokines, e.g. of IL-1β and IL-6 participate in a balanced metabolic response [69–72], but cytokine excess destroys the β-cells [73]. An anti-IL-1β directed anti-inflammatory therapy was shown to strongly restore β-cell survival and function in human and rodent islets in vitro and in numerous human and rodent studies in vivo [5,74] and also improves insulin sensitivity [75,76]. Also, as a classical pro-inflammatory cytokine, IL-6 contributes to the development of insulin resistance [77], whereas physiologically, IL-6 induces α-cells to produce GLP-1 and hereby promotes insulin secretion [72]. Under LPS treatment, however, impaired β-cell function and insulin content could be both partially reversed by IL-6 antagonism, suggesting that IL-6 at least in part mediates the deleterious effects of prolonged TLR4 activation on β-cell function, which is also reminiscent of a previous study showing that 4-day IL-6 treatment impairs GSIS in human islets [78]. Notably, while IL-6 antagonism showed a consistent but only partial beneficial effect on β-cell function in four human islet isolations in this study, β-cell function was fully restored in two out of six islet isolations, confirming again that human islets from different donors display heterogeneity regarding their cytokine response, as observed before [50]. This can also be assumed from the effects of TLR4 antagonism on basal cytokine reduction in all islet isolations at different magnitudes without reaching statistical significance.

It appears that IL-1β and IL-6 possess both beneficial effects (to increase insulin secretion) and unfavorable effects (to impair β-cell function), while long time and high dose exposure might outweigh the positive effects and therefore weaken β-cell function.

Intriguingly in human islets, the diabetogenic milieu of high glucose/palmitate [41] as well as LPS (this study) induces chemokine IL-8 production from pancreatic α-cells; islet-conditioned medium triggers migration of blood monocytes in an IL-8 dependent manner. This implies that α-cell-derived IL-8 induces macrophage recruitment to magnify islet inflammation as a common mechanism under diabetic conditions.

During TLR4 triggered mouse islet inflammation, β-cell-derived CCL2 (MCP-1) contributes to macrophage accumulation [30,43]. Although we only observed a trend that LPS induced CCL2 expression in human islets from non-obese organ donors, the induction became apparent in islets from obese individuals. In line with our data, the chemokine CCL2 plays a critical role in obesity-induced adipose tissue inflammation by inducing blood monocyte migration and subsequent macrophage accumulation. The disruption of the CCL2-CCR2 axis protects mouse from HFD-induced adipose tissue inflammation and insulin resistance [79].

TLR4 triggers islet-derived chemokines to recruit infiltrating macrophages. Based on monocyte-derived infiltrating pro-inflammatory macrophages in adipose tissue [80], which trigger low-grade inflammation and insulin resistance in obesity and T2D, a similar profile has been shown for islet-infiltrating macrophages in mice [43]. Nevertheless, such direct studies are difficult in humans, where only ~1 single infiltrating macrophage/islet has been estimated [41,45] and in vivo macrophage manipulation is impossible to perform. Thus, we established human islet-conditioned monocyte-derived macrophages (M_{islets}) to mimic the infiltrating islet macrophages. They were highly responsive to LPS treatment and produced abundant pro-inflammatory cytokines like IL-1β, IL-6 and TNF. We normalized the secreted cytokines by M_{islets} to the estimated number of infiltrating macrophages in human diabetic islets. Such normalization revealed that IL-1β and IL-6 were produced at similar levels from islets and M_{islets} in response to LPS stimulation; only TNF was produced at much higher concentrations than from non-diabetic islets.

As inflammatory macrophages are the major source of TNF during inflammation [46], mouse but surprisingly not human islets' resident macrophages produced TNF [30]. Our new data set explains such differences; that in human islets, the activated infiltrating macrophages may be the major source of such TNF production.

An additional phenomenon was observed in this experiment; namely that M_{islets} produced lower cytokines in response to LPS than

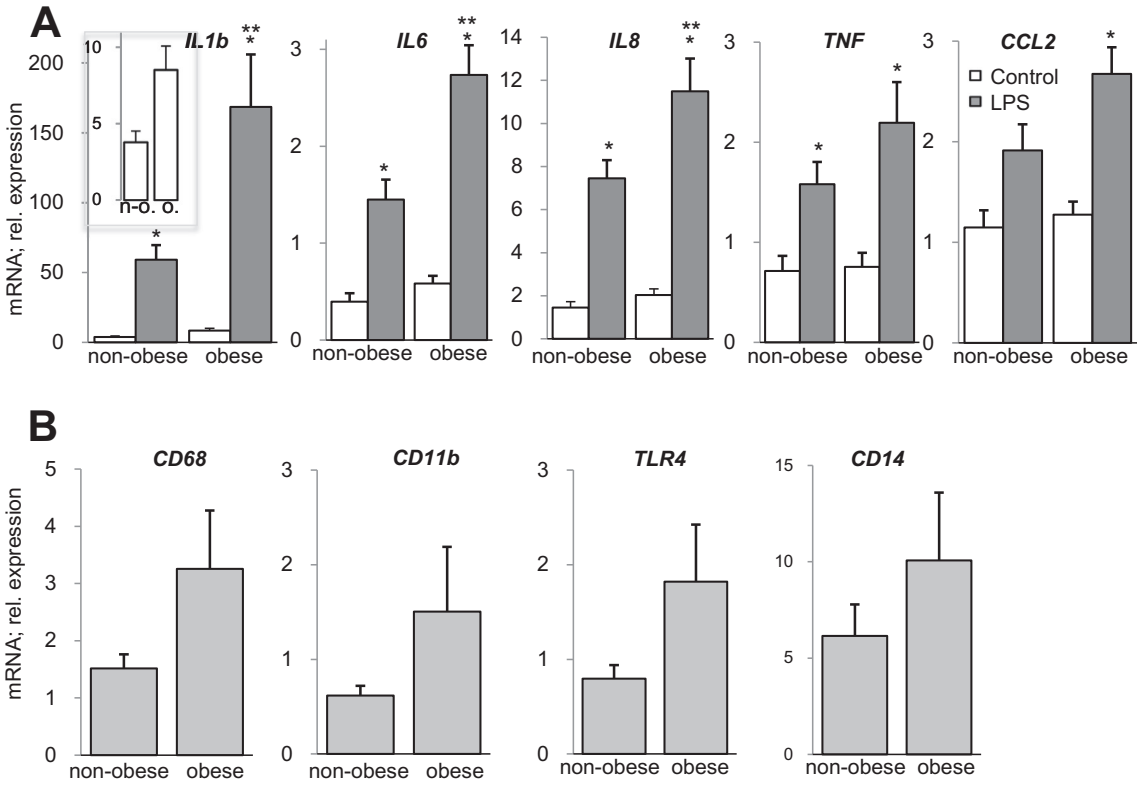

Fig. 6. LPS-induced TLR4 response in islets from obese donors.
(A) Isolated islets from non-obese (BMI $<$ 30, n = 10) and obese (BMI $>$ 30, n = 7) donors were treated with 20 μg/ml LPS for 24 h, RNA was isolated and analyzed for cytokine and chemokine expression by realtime RT-PCR, the insert in the 1st panel visualizes *IL1b* expression at the basal control level with a smaller axis (B) the untreated controls were also analyzed for the expression of macrophage markers CD68 and CD11b, as well as TLR4 and CD14. Data are presented as means ± SEM. *p $<$ 0.05 LPS vs. control, ** p $<$ 0.05, obese (o.) vs. non-obese (n-o.).

classically activated macrophages. This suggests the existence of islet-derived factors to restrict inflammation, which would be worth further investigation.

During T2D progression, TNF is increased in the circulation, in adipose tissue as well as in islets (based on rodent models) [81,82] and has a causative role for insulin resistance [83]. TNF acts synergistically with IL-1β to impair GSIS and promote β-cell apoptosis, while its sole effect was only limited [5], Consistent with our current study, an infiltrating macrophage origin for TNF has been suggested based on previous mouse and rat T2D models [43,66]. Unfortunately, several small clinical studies set to warrant TNF's causative role in the pathophysiology of T2D were underpowered [5], and thus the proof on the TNF effect is still missing.

3.4. Obesity magnifies inflammatory response in islets

Apparent similarities exist between adipose tissue and pancreatic islets in the accumulation of immune cells (particularly macrophages) and pro-inflammatory cytokines during obesity, which thereby contributes to diabetogenesis [43]. Our study reveals a trend of more macrophages in islets from obese individuals, accompanied by an enhanced TLR4 response; however, the small sample size of lean and obese islet donors does not allow a definite conclusion. The elevated IL-1β production may make β-cells more vulnerable to impaired function and survival, while prolonged and increased IL-6 levels will compromise insulin secretion. More IL-8 production will further amplify macrophage accumulation in islets in obesity. Consequently, TLR4 activation is more severe in obese individuals and may concomitantly lead to β-cell deterioration and explains at least in part the increased risk of T2D in obese individuals, when islets do not show any signs of β-cell apoptosis [84], however, a chronically elevated cytokine production may finally lead to impairment of β-cell survival.

Further evidence to support the enhanced TLR4 response in obesity/T2D comes from several recent studies; diet-induced obesity in mice is associated with a mild inflammation in insulin responsive tissues and insulin-producing islets, which is potentiated in older mice in a TLR4-dependent manner [85], leading to a higher inflammatory response and severely impaired glycemia, insulin secretion and β-cell survival., A human study shows that blood monocytes from obese individuals secret significantly more TNF in response to LPS than those from a non-obese group [86]. Similarly, a stronger TLR4 response was observed in blood monocytes from T2D patients, which show elevated IL-1β/6/8 and TNF secretion upon LPS stimulation [61].

Taken together, LPS-triggered TLR4 activation induces cytokine and chemokine production, β-cell dysfunction and apoptosis in human islets, together with blood monocyte recruitment. During obesity, the TLR4 response escalates in pancreatic islets with more IL-1β, IL-6 and IL-8 expression and a subsequent self-augmented vicious inflammation cycle involving the whole islet cell network; α-cells, β-cells, resident macrophages and recruited macrophages, which participate in the potentiation of β-cell dysfunction and accelerates diabetes progression.

4. Experimental procedures

4.1. Human islet isolation, culture and treatment

Human islets were isolated from pancreases of non-diabetic lean and obese organ donors at Prodo Laboratories, Inc. (Aliso Viejo, CA, USA), University of Illinois, Leiden University (Netherlands), Lille University (France) and cultured on extracellular matrix (ECM) coated dishes (Novamed, Jerusalem, Israel) with CMRL-1066 medium (Invitrogen, Carlsbad, CA). Ethical approval for the usage of human

islets was granted by the Ethics Committee of the University of Bremen. To induce maximal TLR4 activation, attached islets were serum-starved for 6 h [87,88], then various concentrations of the classical TLR4 ligand lipopolysaccharide (LPS, from *Escherichia coli* O111:B4, Sigma-Aldrich, Steinheim, Germany) were added for a duration of 24 h or 72 h, with or without 10 μM TLR4 inhibitor TAK-242 (patent by Takeda Pharmaceutical (Osaka, Japan), synthesized by Servier (Suresnes, France) according to the published chemical structure [32–34]), or 1 μg/ml IL-6 receptor antagonist Sant7 [36], respectively. To compare the LPS induced TLR4 response islet isolations were grouped into non-obese (BMI < 30, $n = 10$) and obese (BMI > 30, $n = 7$) donors (donor details in Suppl. Table 1).

4.2. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from cultured human islets with a Trizol extraction system (TriFast, PEQLAB GmbH, Erlangen, Germany), cDNA synthesis and quantitative RT-PCR were performed as previously described [89]. The Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, Forster City, CA) and TaqMan® Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems) were used for analysis. Cyclophilin (PPIA) was used as internal housekeeping controls and the quantitative analysis was performed with the ΔΔCT method. The following TaqMan® Gene Expression Assays (Applied Biosystems) were used: *IL1b* (Hs01555413_m1), *IL6* (Hs99999032_m1), *TNF* (Hs99999043_m1), *CCL2* (Hs00234140_m1), *IL8* (Hs00174103_m1), *CD68* (Hs02836816_g1), *ITGAM* (Hs00355885_m1), *TLR4* (Hs01060206_m1) and *PPIA*(Hs99999904_m1).

4.3. Measurement of secreted cytokines and chemokines

Human islet supernatants were collected and briefly centrifugated to remove floating cells and cell debris. Human IL-1β, IL-6, IL-8 and TNF were assessed by the cytokine multiplex array system using a kit (Human Pro-inflammatory II 4-plex assay) at the Sector Imager 6000® (all from Meso Scale Discovery, Gaithersburg, MD, USA) as per manufacturer's instructions [90].

4.4. Western blot analysis

Cultured human islets were washed twice with ice-cold PBS and lysed with RIPA lysis buffer containing Protease and Phosphatase Inhibitors (Pierce, Rockford, IL). Protein concentrations were measured by BCA assay (Pierce, Rockford, IL). Lysates were fractionated by NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrically transferred into PVDF membranes. Membranes were blocked in 2.5% non-fat dry milk (Cell Signaling Technology, Danvers, MA) and 2.5% BSA (Sigma-Aldrich, Steinheim, Germany) for 1 h at room temperature and then incubated overnight at 4 °C with the following antibodies: rabbit anti–cleaved caspase-3 (#9664), rabbit anti–Phospho-IκBα (#2859), rabbit anti–Phospho-SAPK/JNK (Thr183/Tyr185) (#9251), rabbit anti-β Tubulin (#2146), all from Cell Signaling Technology; followed by incubation at room temperature for 1 h with horseradish-peroxidase–conjugated anti-rabbit IgG secondary antibody (Jackson Immuno Research, West Grove, PA). All primary antibodies were used at 1:1000 dilution in Tris-buffered saline plus Tween-20 (TBS-T) containing 5% BSA. Membranes were developed using a chemiluminescence assay system (Pierce) and analyzed with DocITLS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA). Densitometric analysis was performed with Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD) to quantify blots from all the independent experiments.

4.5. Immunofluorescence

For the detection of apoptosis, isolated human islets cultured on ECM dishes were fixed in 4% paraformaldehyde for 30 min, followed by 0.5% Triton-X-100 for 4 min and blocking buffer (3% BSA + 2% Triton X-100 in TBS-T) for 1 h, before TUNEL labeling as previously described [91]. Briefly, TUNEL reaction mixture was used according to the manufacturer's instruction (In situ Cell Death Detection Kit AP, Roche, Mannheim, Germany) and then double stained for insulin (guinea pig anti-insulin, #A0546, 1:100, Dako, Glostrup, Denmark), followed by incubation with FITC-conjugated donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and mounting with Vectashield with DAPI (Vector Labs, Burlingame, CA).

For IL-8 staining, cultured human islets were fixed in Bouin's solution for 15 min before embedding in paraffin as previously described [91,92]. Human 4-μm sections were deparaffinized, rehydrated and incubated overnight at 4 °C with primary antibodies followed by incubation with FITC or Cy3-conjugated secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA) at room temperature for 1 h. Slides were mounted with Vectashield with DAPI (Vector Labs, Burlingame, Ca). The following primary antibodies were used: mouse anti-IL-8 (#ab18672, 1:1000, Abcam, Cambridge, MA), guinea pig anti-insulin (#A0546, 1:100, Dako, Glostrup, Denmark) and mouse anti-glucagon (#G2654, 1:100, Sigma-Aldrich, Steinheim, Germany). Confocal analyses were performed with an LSM880 ZEISS confocal laser scanning microscope (Zeiss, Jena, Germany).

4.6. Glucose-stimulated insulin secretion (GSIS)

GSIS was performed as described previously [89,91]. Briefly, human islets were pre-incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose for 30 min, followed by KRB buffer containing 2.8 mM glucose for 1 h (as “basal secretion”) and then an additional 1 h in KRB containing 16.7 mM glucose (as “stimulated secretion”). Islets after GSIS were washed with PBS and lysed with RIPA lysis buffer to extract total protein, followed by BCA measurement of protein concentration. Secreted insulin and insulin content from total protein were determined with human insulin ELISA kit (ALPCO Diagnostics, Salem, NH), and normalized to total protein concentration.

4.7. Blood monocyte extraction and migration assay

To evaluate monocyte migration, blood monocytes were isolated from blood buffy coats of anonymous healthy donors (Blutspendedienst Hamburg, Hamburg, Germany) using sequential Ficoll-Paque Plus and Percoll (both from GE Healthcare, Chicago, IL) gradients as described before [93]. Granulocytes and erythrocytes are removed and the final cell suspension is comprised by monocytes as the main part and lymphocytes as the minor part [93]. The purify of monocytes in the final cell suspension was analyzed by flow cytometer CyView Cube 8 (Sysmex Partec, Görlitz, Germany) using the following antibodies: PE mouse anti-human CD3 (#300456), Alexa Fluor® 488 mouse anti-human CD14 (#301817) and APC mouse anti-human CD45 (#368511), all from BioLegend (San Diego, CA) as well as appropriate isotype controls (BioLegend) respectively. Migration was tested using Costar Transwell 5.0 μm microporous membrane inserts (Corning, Corning, NY) by loading 50 thousands of isolated monocytes into the upper chamber and loading human islet-conditioned medium or empty human islet medium (as negative control) or LPS-spiked empty human islet medium (which were all diluted 4 times by CMRL-1066 medium) into the lower chamber. IL-8 was neutralized by addition of 0.1, 1 or 10 μg/ml mouse anti-IL-8 antibody (#ab18672, Abcam, Cambridge, MA) or 10 μg/ml mouse IgG isotype control (Abcam) to the lower chamber and preincubation for 30 min with conditioned media prior to the addition of monocytes to the upper chamber. Migration was allowed to proceed for 4 h at 37 °C before evaluation of total live migrated cells by excising the membrane and staining with Fluorescein diacetate (Sigma-Aldrich, Steinheim, Germany). Fluorescence was analyzed using a Nikon MEA53200 microscope (Nikon GmbH, Dusseldorf, Germany) and NIS-Elements software (Nikon).

4.8. Generation of islet-conditioned monocyte-derived macrophages

Monocytes isolated from human buffy coats were seeded onto tissue culture dishes (1x10e6 cells/ml) in serum-free RPMI 1640 medium. When monocytes became attached, medium was replaced with RPMI 1640 medium with 10% fetal bovine serum and supplemented with 25 ng/ml macrophage colony-stimulating factor (M-CSF; Miltenyi, Bergisch Gladbach, Germany) for 8 days to induce macrophage differentiation. For the last 5 days, media conditioned by islets from non-diabetic donors were added in a 1:4 ratio into the differentiation media. The generated macrophages were named M_{islets}, while macrophages generated without the islet-conditioned media were used as control (M_0). Macrophage maturity was analysis by morphologic observation by microscope, surface marker expression of CD45, CD14 and CD11b by flow cytometry, as well as gene expression of CD68 by realtime-PCR (all as mentioned above). Mature macrophages were then seeded into 24-well plate (10,000 cells/well) in serum-free RPMI 1640 medium for 6 h, followed by addition of 100 ng/ml LPS for 24 h (initially various concentrations of LPS were tested, while 1, 10 and 100 ng/ml have comparable cytokine induction which was stronger than 1 μg/ml LPS). Afterwards, the culture media were collected for cytokine quantification by the cytokine multiplex array and cells were collected for gene expression analysis by realtime RT-PCR.

4.9. Statistical analysis

All values were expressed as means ± SEM with the number of independent individual experiments (biological replicates) presented in the figure legends. The different groups were compared by unpaired two-tail Students *t*-test (for two groups) or ANOVA (one-way or two-way, for multiple groups and more than one condition, respectively) with Bonferroni post-tests. P value < 0.05 was considered statistically significant.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contribution

Designed and performed most of experiments, analyzed the data and wrote the paper: WH.

Performed and analyzed experiments: OR.

Contributed analytic tools and intellectual support: RS, RT, CSK, BG.

Conceived and supervised the project, designed experiments, wrote the paper: KM.

Conflict of interest

CSK, BG are employees of Servier. There is no competing interest in relation to this work. There are no patents, products in development, or other marketed products to declare. All other authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.09.030.

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